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Isolation, identification and characterization of vasoactive substances from endothelial cells, platelets and mononuclear leukocytes

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Thesis submitted in fulfillment of the requirements for the degree of
“Doctor in de medische wetenschappen”

I. CONTENT

II. Abbreviations

ACH	acetylcholine
ADP	adenosine diphosphate
Ang II	angiotensin II
AN69	acrylonitrile-sodium-methalyl-sulfonate copolymer 69
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AT1 / AT2	angiotensin receptor-1; angiotensin receptor-2
Ap_nA	diadenosine polyphosphates
Ap_2A	diadenosine pyrophosphate
Ap_3A	diadenosine triphosphate
Ap_4A	diadenosine tetraphosphate
Ap_5A	diadenosine pentaphosphate
Ap_6A	diadenosine hexaphosphate
Ap_7A	diadenosine heptaphosphate
Ap_2G	adenosine guanosine pyrophosphate
Ap_3G	adenosine guanosine triphosphate
Ap_4G	adenosine guanosine tetraphosphate
Ap_5G	adenosine guanosine pentaphosphate
Ap_6G	adenosine guanosine hexaphosphate
ACN	acetonitrile
ACTH	adrenocorticotropic hormone
BP	blood pressure
BQ123	endothelin receptor antagonist (D-P-V-L-W)
$CaCl_2$	calcium chloride
CD	cluster of differentiation
CD8	T-cell associated leukocytes
CD19	B-cell associated leukocytes
CDI	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
CI	chemical ionisation
CID	collision-induced dissociation
C.V.	coefficients of variation
EDCF	endothelial-derived vasoconstricting factor
EDTA	ethylenediaminetetraacetic acid
EI	electron impact
ESI-MS	electrospray mass-spectrometry
ET	endothelin
FT-ICR-MS	fourier-transform ion cyclotron resonance mass spectrometry
GC/MS	gaschromatography / mass-spectrometry
Gp_2G	diguanosine pyrophosphate
Gp_3G	diguanosine triphosphate
Gp_4G	diguanosine tetraphosphate
Gp_5G	diguanosine pentaphosphate
Gp_6G	diguanosine hexaphosphate
GC/MS	gas-chromatography / mass-spectrometry
HEK	human embryonic kidney cells
HEPES	N-[2-hydroxyethyl]-piperazine-N´-2-ethanesulfonic acid

HMEC	human microvessel endothelial cells
HPLC	high-performance liquid chromatography
HPA	3-hydroxy-picolinic acid
ICR	ion cyclotron resonance
Ip_5I	diinosine pentaphosphate
KCl	potassium chloride
K_M	michaelis constant
K_2HPO_4	dipotassium hydrogenphosphate
KH_2PO_4	potassium dihydrogenphosphate
$KCLO_4$	potassium perchlorate
LC	liquid chromatography
LPS	lipopolysaccharide
MALDI-MS	matrix-assisted laser desorption/ionisation mass-spectrometry
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
$MgSO_4$	magnesium sulfate
MS	mechanical stress
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
NF023	8,8´-[carbonylbis(imino-3,1-phenylene-carbonyl-imino)]-bis(1,3,5-naphthalene-trisulfonic acid)
NO	nitric oxide
NOS	nitric oxide synthetase
min	minutes
MS	mass-spectrometry
mU	milli-unit
m/z	mass/charge ratio of an ion
NE	norepinephrine
PGI_2	prostacyclin
PSD-MALDI-MS	post-source-decay MALDI- mass-spectrometry
PKC	protein kinase C
RAS	renin-angiotensin system
PPADS	pyridoxal-phosphate-6-azophenyl-2,4-disulphonic acid
pH	pondus hydrogenii
RP	reversed-phase
RETOF	reflectron type time-of-flight
qTOF MS	orthogonal-acceleration time-of-flight mass-spectrometry
SEM	standard error mean
TEAA	triethylammonium acetate
TBA	tetrabutylammonium hydrogensulfate
TFA	trifluoracid acid
TNP-ATP	2´,3´-O-(2,4,6-trinitrophenyl) adenosine triphosphate
TOF	time-of-flight mass analyzer
TOF-TOF	of two combination time-of-flight mass analyzer
UTP	uridine triphosphate
Up_4A	uridine adenosine tetraphosphate
UV	ultraviolet
VEGF	vascular endothelial growth factor

A. Introduction and aim of the thesis

A.1. Clinical conditions related to this thesis: hypertension and chronic renal failure

A.1.1. Hypertension

Almost one fourth of the global population and more than one half of the population over 50 years of age in industrialized countries develop hypertension[1]. Whereas normal blood pressure is defined as a blood pressure below 120/80 mmHg, and pre-hypertension as 120-139/80-89 mmHg, stage-1 hypertension refers to values between 140-159/90-99 mmHg and stage-2 hypertension is defined as values greater than 160/100 mmHg[2,3]. In ninety-five percent of the cases the cause of hypertension is unknown[4]. Elevated blood pressure is a major risk factor for myocardial infarction, cerebrovascular accidents and end-stage renal failure and is one of the major factors causing death. In most cases, medical drug therapy of hypertension sufficiently decreases blood pressure and, therefore, adequate treatment results in acceptable survival rate. However, in most cases, the cause of hypertension is unknown and remains untreated.

A.1.1.1. Essential hypertension

If no identifiable disorder can be held responsible, hypertension is classified as *essential*. Extensive studies have been carried out to elucidate the complex pathogenesis of this form of hypertension. Arterial hypertension occurs when changes develop that alter the relationship between blood volume and total peripheral resistance. Essential hypertension may result from an interaction of genetic and environmental factors that affect cardiac output, peripheral resistance, or both[5, 6].

Genetic factors definitely play a role in the development of high blood pressure. Essential hypertension probably is a polygenetic and heterogeneous disorder in which the combined effect of mutations or polymorphisms at several gene loci influences blood pressure[7]. Single-gene disorders are known to cause relatively rare forms of hypertension, e.g., gene defects in enzymes involved in aldosterone metabolism and mutations in proteins that affect sodium reabsorption[8]. Essential hypertension has been associated with heterogeneity of the genes encoding for the renin-angiotensin system: there is an association of hypertension with polymorphisms in both the angiotensinogen locus and the angiotensin-II type I receptor[9].

Environmental factors are thought to contribute to expression of the genetic determinants of increased blood pressure. Stress, obesity, smoking, physical inactivity and heavy consumption of salt have all been implicated as exogenous factors in hypertension[6]. One further hypothesis implicates increased peripheral resistance as the primary cause of hypertension. Increased resistance is a causative factor due either to the induction of functional vasoconstriction or to structural changes in the vessel wall (e.g., hypertrophic remodelling or hyperplasia of smooth muscle cells), leading to a thickened wall and narrowed lumen or both[6].

The regulation of systemic blood pressure is, in general, a complex process involving *multiple neural* and *endocrine mechanisms*. Especially *circulating vasoconstrictive factors* are important for the genesis of essential hypertension. For example, Dahl et al.[10] and Greenberg et al.[11] suggested several circulating factors that induced hypertension in normotensive rats during parabiotic experiments. Similar results were provided from cross-circulation experiments by Zidek et al.[12]. Furthermore, hypertension is induced in normotensive animals by chronic administration of serum or plasma from hypertensive animals[13,14]. After cross-incubation of cells from normotensive donors with plasma from hypertensive donors, the cells from the normotensive donors showed physiological abnormalities, which were characteristic for cells from hypertensive donors[15,16].

Abnormalities in the functions of one or more of the relaxing and constricting factors at the vessel wall may be involved in the development of hypertension. The maintenance of hypertension clearly depends on the development of vascular hypertrophy that increases peripheral resistance. Folkow[17] first demonstrated the role of vascular smooth muscle cell proliferation in the pathogenesis of hypertension and proposed a mechanism to explain its action in hypertension (Figure 1). Hypertension is initiated by a minor over-activity of a specific pressor mechanism (Figure 1; arrow A) that raises blood pressure (BP) slightly: this initiates a feedback mechanism (Figure 1; arrows B and C) inducing vascular hypertrophy resulting in the maintenance of hypertension. Lever hypothesized that both genetic (Figure 1; arrow D) and trophic mechanisms contribute to that hypertrophy (Figure 1; arrow E)[18].

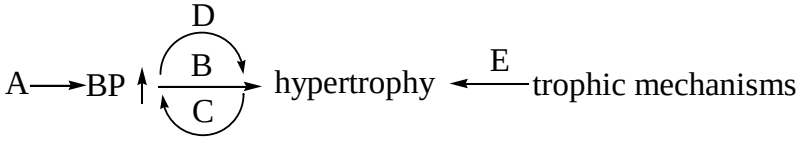

Figure 1: Hypothesis for the initiation and maintenance of hypertension[18](A = overactivity of specific pressor mechanism; B = inducing vascular hypertrophy; C = feedback mechanism; D = hypertrophic by genetic mechanism; E = trophic mechanisms).

A.1.1.2. Secondary hypertension

The remaining five percent of hypertensive adult patients have an identifiable or "secondary" disorder responsible for the hypertension[4]. The most common causes of secondary hypertension are renal parenchymal diseases[19], endocrine disorders[4], cardiovascular disorders and other identifiable causes, such as Cushing's syndrome, drugs, diet and excess erythropoietin[20].

A.1.2. Cells and organs involved in the regulation of vascular tone

Since the pathogenesis of primary hypertension remains unresolved, there is continued interest in the identification of *novel endogenous compounds* with strong vasoconstrictive properties. In the last decades, several organs and cells have been described which release substances with those properties. In the following paragraphs, these organs and cells will be summarized.

A.1.2.1. Vascular endothelium

The vascular endothelium is a complex and dynamic organ and one of the largest secretory tissues of the human organism[21, 22]. The vascular endothelium, once thought to be only a mechanical barrier between the blood and the vessel wall, is now recognized to be an endocrine organ showing an amazing variety of regulatory functions. The vascular endothelium is one of the most important humoral regulators of vasomotor tone[22].

Endothelium-derived mediators have essential functions in vascular regulation[22]. Endothelium dysfunction involves an imbalance between vasoregulating substances together with a disturbance of hemostasis and vessel structure resulting in the development of cardiovascular diseases, such as hypertension, atherosclerosis, and heart failure[21]. The understanding of cellular and molecular biology of the vascular endothelium is essential for the development of new approaches for both the prevention and therapy of cardiovascular diseases[22].

The first clue to the endocrine functions of the endothelium came from the seminal observations of Furchgott and Zawadzki[23]. Some years after they had demonstrated the release of an *endothelium-derived relaxing factor* by stimulated endothelium cells, this factor was identified as nitric oxide (NO)[24-26]. Moreover, the endothelium releases a further factor with strong vasodilative properties. This effect is initiated by hyperpolarization of vascular smooth muscle. Therefore, this factor is named “endothelium-derived hyperpolarizing factor” (EDHF)[27].

Vasoconstrictive substances mediate vascular tone, structure and function, influencing vascular smooth muscle cell (VSMC) proliferation, apoptosis[28], platelet aggregation[29], monocyte and leukocyte adhesion[30] as well as thrombosis[31]. An increase in the proliferation rate of vascular smooth muscle cells sets off medial hypertrophy, which results in an increase in peripheral resistance[32].

Whereas the discovery of NO as a central messenger in the cardiovascular system revolutionized our understanding of vascular regulation, subsequent work on *endothelium-derived vasoconstricting factors* (EDCFs) have had less impact on currently accepted concepts regarding vasoregulation. The only EDCF identified so far, the peptide endothelin[33], may play a role in mediating target-organ damage in cardiovascular disease, but its direct contribution to vascular tone in hypertensive diseases is still under debate. In addition to endothelin release[33], vascular endothelium may regulate vasomotor tone by releasing reactive oxygen species[34], arachidonate derivatives such as thromboxane or prostacyclin[35] and nucleosides such as ATP[36].

Given that EDCFs belonging to several classes of substances are still unidentified, *one of the main topics of this thesis is the isolation, identification and characterization of a yet unknown EDCF*. Since a first logical step to direct any further identification procedures is to classify potential novel EDCFs according to the receptors mediating their vasoconstricting actions, *we sought confirmation of their identity by defining their receptors*.

A.1.2.2. Adrenal glands

The adrenal glands are fundamentally involved in vasoregulation. The adrenal glands are two small organs situated on top of each kidney. Both in anatomy and in function they consist of two distinct regions: the inner medulla and the cortex. The medulla is the source of the catecholamines, epinephrine and norepinephrine. The chromaffin cells are the principal cell type of the inner medulla.

The medulla is innervated by preganglionic sympathetic fibers and is, in essence, an extension of the sympathetic nervous system. The cortex secretes several classes of steroid hormones such as glucocorticoids[37] and mineralocorticoids[38], which have a direct effect on the vascular tone[39].

Steroid hormones and catecholamines[40,41] are well-known substances with strong vasoconstrictive properties. In the last decade it has been shown, however, that the adrenal glands release at least two further potent vasoconstrictive mediators, the diadenosine polyphosphates diadenosine pentaphosphate (Ap_5A)[42] and diadenosine hexaphosphate (Ap_6A)[43]. Because of these reports, we assumed that the adrenal gland might generate further mediators with vasoregulatory properties. *Therefore, the adrenal glands were screened within the framework of this thesis for further mediators with direct effects on vascular physiology.*

A.1.2.3. Platelets

Platelets are essential for blood coagulation[44]. Therefore, platelet activation is central to the pathogenesis of disturbed coagulatory hemostasis and arterial thrombosis[45]. Platelets interact with coagulation factors; conversely, the coagulation factor thrombin is a potent platelet-activating agonist[46]. During thrombin-induced aggregation almost the entire content of blood platelets is released[47,48] and these compounds contribute to local vascular control mechanisms. Previously unidentified vasopressive agents have been found recently in platelets and were isolated and characterized as diadenosine polyphosphates[29,49]. Diadenosine polyphosphates have multiple biological and pharmacological activities. Besides their mainly potent vasoconstrictive or vasodilatory properties, depending on their structure, diadenosine polyphosphates may also play a role in platelet aggregation. It has already been described that the amount of diadenosine polyphosphate in platelets is increased in pathophysiologic disturbances such as chronic renal failure[50], therefore, may contribute to the blood pressure increase of these patients. *Within the framework of this thesis it was investigated whether different hemofiltration membranes have different effects on the diadenosine polyphosphate content in platelets and thereby indirectly affecting the blood pressure of these patients. Moreover, dinucleoside polyphosphates of platelets were used as a model system for establishing a chromatographic assay for isolation and quantification of dinucleoside polyphosphates from tissues, cells and body fluids.*

A.1.2.4. Heart tissue

The human heart is not only a muscle, but an endocrine organ[51]. For example, the identification of renin-angiotensin system components and angiotensin-II receptors in cardiac tissue suggests the existence of autocrine and/or paracrine systems. This system is independent from angiotensin-II derived from the circulatory system[52,53]. Angiotensin-II, the effector peptide of the renin-angiotensin system, regulates vascular tone and cellular growth in response to de-

velopmental, physiological, and pathological processes. To be functional, a local renin-angiotensin system should produce sufficient amounts of autocrine and/or paracrine factors to elicit biological responses, contain the final effector (angiotensin-II receptor), and respond to humoral, neural, and/or mechanical stimuli[54]. A recent study demonstrates the presence of the diadenosine polyphosphates Ap_2A and Ap_3A in cardiac-specific granules and the effects of these substances on the myocardium and coronary vessels, indicating their role as endogenous modulators of myocardial functions and coronary perfusion[55]. Ap_2A and Ap_3A influence cardiac output and hereby the blood pressure[56]. An increased cardiac output is the most typical hemodynamic change due to high stroke volume and increased heart rate[57]. In general, an increase in cardiac output of the left ventricle is frequently the consequence of an increased release of neurotransmitters, hormones, and vasoconstrictive substances that have direct effects on the growth of cardiomyocytes, cardiac interstitium and vasoconstriction[58,59].

In human platelets not only Ap_2A[60] and Ap_3A[61], but also Ap_4A[62], Ap_5A[63] and Ap_6A[63] are present. Therefore, the hypothesis was raised that besides Ap_2A and Ap_3A, also Ap_4A, Ap_5A and Ap_6A are mediators secreted by the human heart tissue. *Consequently, another topic of this thesis is to test the hypothesis that besides Ap_2A and Ap_3A, also Ap_4A, Ap_5A and Ap_6A are released by human heart tissue.*

A.1.2.5. Mononuclear leukocytes

Leukocytes are essential for inflammatory regulation[44]. Leukocytes include granular leukocytes (basophils, eosinophils, and neutrophils) as well as non-granular leukocytes (lymphocytes and monocytes)[6]. The peripheral blood leukocyte count is influenced by several factors, including the size of the myeloid (for granulocytes and monocytes) and lymphoid precursor and storage cell pools. Mature lymphocytes and monocytes are transported by the blood and lymph to the body's extravascular space. They are morphologically distinguishable from mature granulocytic leukocytes by their large, non-lobed nuclei and lack of coarse, heavily stained cytoplasmic granules. Lymphocytes are classified into five broad categories, based on immunophenotype[64]: (1) precursor B-cells (immature B-cells); (2) peripheral B-cells (mature B-cells); (3) precursor T-cells (immature T-cells); (4) peripheral T-cells (mature T-cells); (5) peripheral natural killer (NK) cells (mature NK cells).

Antigen receptor genes rearrange during B-cell and T-cell differentiation through a mechanism that ensures that each mononuclear leukocyte generates a single, unique antigen recep-

tor; it is known that primarily T-cell associated (CD1-CD8) as well as is B-cell associated (CD10, CD19-CD23) antigens exist[65-67]. Yet not all of the substances released from mononuclear leukocytes within the scope of inflammatory processes are known[30]. As recent evidence shows that there is a close relation between inflammation and atherosclerosis[68], knowledge of the identity of the substances released from the leukocytes is important. *Pilot experiments of this thesis showed that leukocytes released yet unknown substances with direct vasoconstrictive effects, and some of these vasoconstrictors were identified in the framework of this thesis.*

A.1.2.6. Parathyroid glands

The parathyroid glands are four small glands located adjacent to the two thyroid gland lobes in the neck. The parathyroid gland has been causally associated with some forms of hypertension[69]. The activity of the parathyroid glands is controlled by the level of free calcium in the bloodstream; a decreasing level of free calcium[70], low active vitamin D analogues and high phosphate concentration[71] stimulate the synthesis and secretion of vasoconstrictive parathyroid hormone (PTH). A further hypertensive factor, isolated from plasma of spontaneously hypertensive rats, is the yet unidentified parathyroid hypertensive factor (PHF)[72]. Elevated PHF has been suggested to play a causal role in the pathogenesis of hypertension[73]. *Extensive studies have not yet led to an identification of PHF.*

A.1.3. Vasoregulatory mediators

The cells and organs mentioned in **section A.1.2** release several factors with strong vasoregulatory properties, for example:

- the peptide *endothelin* released from the endothelium (see **paragraph A.1.3.1**)[33],
- a yet unidentified vasoconstrictive factor (called *parathyroid hypertensive factor* (PHF)) released by the parathyroid glands (see chapter **paragraph A.1.3.2**)[74],
- the octapeptide *angiotensin-II,* (see **paragraph A.1.3.3**)[75],
- *nitric oxide* (NO) e.g. released from the endothelium (see **paragraph A.1.3.4)**[76],
- the mineralocorticoid *aldosterone* (see **paragraph A.1.3.5**)[77],
- the *catecholamines* (see **paragraph A.1.3.6**)[78], and
- *dinucleoside polyphosphates* with adenosine and/or guanosine as purinergic base e. g. isolated from platelets (see **paragraph A.1.3.7**)[79,80].

A.1.3.1. Endothelin

Endothelin is a 21-amino acid peptide with strong vasoconstrictive[33] and growth stimulating properties[81]. Endothelin is secreted by vascular endothelium cells[82,83] and is therefore one of the applicants to be named endothelium-derived contractile factor (EDCF). Cloning and sequencing of proendothelin shows that mature endothelin is generated through an unusual proteolytic processing, and regional homologies to a group of neurotoxins suggest that endothelin is an endogenous modulator of voltage-dependent ion channels. Expression of the endothelin gene is regulated by several vasoregulatory agents, indicating the existence of a further cardiovascular control system[33]. These results raise the possibility that the endothelium of microvessels regulate the local blood flow through the production of endothelin[83]. In general, this peptide may participate through different mechanisms in the elevation of blood pressure and/or in the maintenance of hypertension.

A.1.3.2. Parathyroid hypertensive factor

Some years ago, a hypertensive factor had been demonstrated in the plasma of spontaneously hypertensive rats (SHR), but not in that of normotensive rats[84]. This factor is derived from parathyroid glands and therefore called *parathyroid hypertensive factor* (PHF)[74]. It has been shown that it produces a delayed increase in blood pressure. This increase is coupled to an increase of calcium uptake in the rat-tail artery, which had a similar time-course[74]. The evidence that calcium is involved in the mechanism of action is supported by the inhibitory effect of calcium antagonists on the vascular action of PHF[84]. Parathyroidectomy and parathyroid transplant experiments indicated that PHF originated in the parathyroid gland[69]. Cultured parathyroid glands from spontaneously hypertensive rats (SHR) but not from normotensive rats produced a factor in the medium with the same biological property and retention time on high performance liquid chromatography as plasma PHF. In both animal models and human studies, PHF seemed to be associated with low or normal levels of plasma renin and the salt-sensitive type of hypertension[84].

A.1.3.3. Angiotensin-II

Angiotensin-II is a well-known vasopressive as well as growth stimulating octapeptide that is the principal end product of the renin-angiotensin system (RAS-system)[85]. Angiotensin-II is converted from angiotensin-I by the angiotensin-converting enzyme (ACE) located in the luminal surface of the vascular endothelium. Angiotensin-I in turn is converted from liver-derived angiotensinogen by renin[86,87]. The physiologic effects of angiotensin-II are mainly

mediated by the AT1-receptor. The stimulation of this receptor initiates different intracellular reactions, such as an increase of the intracellular Ca^{2+}-concentration[88,89]. The RAS is a regulatory cascade that plays an essential role in the regulation of blood pressure, and of electrolyte and volume homeostasis[90]. The first and rate-limiting component of this endocrine cascade is renin, a protease synthesized and secreted predominantly by the juxtaglomerular apparatus in the nephron[90]. Patients with high levels of plasma renin activity have had a higher risk of developing stroke or myocardial infarction than those with low plasma renin activity[91-93]. Subsequent to these studies, the development of pharmacological probes blocking the RAS has helped to define the contribution of this system to blood pressure control and to the pathogenesis of diseases such as hypertension, congestive heart failure and chronic renal failure[94].

Besides its vasoconstrictive properties, angiotensin-II also has growth-stimulating effects on vascular smooth muscle cells (VSMC*) in vitro*. Adding angiotensin-II to quiescent cultures of rat aortic smooth muscle cells results in rapid stimulation of the proto-oncogene c-fos, which may be a key control step in the initiation of cell growth[95]. In quiescent rat aortic smooth muscle cell cultures, angiotensin-II induces a 20% increase in cellular protein content and a 50% increase in the fraction of cells with cDNA content with the virtual absence of cells in the S-phase of the cell cycle, consistent with either arrest of cells in the G2 phase of the cell cycle or development of tetraploidy[96].

The classic concept that RAS is especially regulated by circulating hormones has undergone a number of changes since the last decade. Several lines of clinical and experimental evidence indicate that components of the RAS are synthesized *in situ* by several tissues and that angiotensin-II is regulated independent of the circulating renin-angiotensin system. Angiotensinogen, renin, ACE, and angiotensin-II receptors are present in the myocardium[97]. Most of the *angiotensin-I* found in cardiac tissue is synthesized *in situ*[97,98]; moreover, angiotensin-II is produced locally in many other tissues, such as endothelium[99], blood vessels[100], heart[101] and brain[102]. This local angiotensin-II production depending on tissue RAS has recently attracted growing interest[103-105], but has yet to be fully characterized.

A.1.3.4. Nitric oxide

Nitric oxide (NO) is implicated in neuronal transmission, immune response and vasoregulation, besides acting as a platelet function modulator. A number of recent studies in the experimental model of renal mass-reduction in rats have proposed the hypothesis that abnor-

malities of the NO synthetic pathway could play a key role in mediating the complex hemodynamic and hemostatic disorders associated with the progression of hypertension and renal disease[106]. Nitric oxide is synthesized by NO-synthase (NOS). Three distinct isoenzymes of NOS are known.

Two calcium/calmodulin–dependent constitutive NOS isoenzymes dominantly expressed in the brain and endothelium, and a calcium-independent NOS induced by cytokines, have been identified so far. NO inhibits vascular smooth muscle cell proliferation, cytokine-induced endothelium expression of adhesion molecules, and the production of proinflammatory cytokines[107-109].

Sufficient production of NO in the vascular endothelium seems to be essential for the maintenance of normal blood pressure[110], and defects either in the production or action of NO are likely to be associated with essential hypertension[111]. In experimental models of vascular disease, increased superoxide production (and the subsequent inactivation of NO) seems to be critically involved in reduced NO bioactivity and endothelium dysfunction[112].

A.1.3.5. Aldosterone

Aldosterone has often been neglected in the pathophysiologic consequences of the activated RAS in arterial hypertension and chronic heart failure[113]. The steroid hormone aldosterone is secreted by the glomerulosa cells of the adrenal cortex and controls the sodium and the potassium balance of vertebrates. Under physiological conditions, the control of secretion is probably confined to the stimulatory factors corticotropin (ACTH), Ang II, and K^+ and the inhibitory factor atrial natriuretic hormone (ANP). Adrenal glomerulosa cells are a cell type in which Ca^{2+} and cAMP are equally significant in stimulation secretion coupling. The effect of ACTH is mediated by cAMP, the effect of Ang II by Ca^{2+} and diacylglycerol (DAG), and that of K^+ by Ca^{2+}. ANP attenuates agonist-induced Ca^{2+}-influx.. Under physiological conditions, aldosterone secretion may be attributable to increased activity of the renin-angiotensin system and/or increased plasma levels of K^+ [77].

A.1.3.6. Catecholamines

The catecholamines norepinephrine and epinephrine are released from the sympathetic nerves and the adrenal medulla, and play a central role in circulatory homeostasis via their cardiac and vascular effects. They influence renal function, and adrenergic innervation has been iden-

tified in the renal vasculature and in the proximal tubule, loop of Henle, and distal tubule[114-116]. Renal sympathetic activity tends to be increased in states of effective circulating volume depletion. In this setting, norepinephrine is a potent vasoconstrictor, acting to reduce renal blood flow and therefore to preserve perfusion to the critical coronary and cerebral circulations[114,115,117].

Dopamine is another catecholamine, which is primarily synthesized in the basal ganglia and in the proximal tubule from circulating L-dihydroxyphenylalanine (L-dopa), via the enzyme L-amino acid decarboxylase[118-120]; dopaminergic nerves are also present in the kidney, but their physiologic significance is unclear[121]. Dopamine generally has opposite renal effects to norepinephrine and epinephrine. It is, at lower concentrations, a renal vasodilator that acts at the interlobular arteries and both the afferent and efferent arterioles[121,122]. The direct effects of dopamine may contribute to a decrease in vascular resistance[123].

A.1.3.7. Dinucleoside polyphosphates

In recent years, dinucleoside polyphosphates have been described as a group of substances that are involved as extra-cellular and intracellular mediators in the direct regulation of vascular tone as well as growth of vascular smooth muscle[124] and mesangial cells[125]. These molecules comprise two nucleotides (ribosylated nucleic acids) linked by a polyphosphate chain through phosphoester bonds at the 5´-position of the two ribose moieties. Dinucleoside polyphosphates containing an adenine and/or guanine base have previously been identified[29,48,49,126]. The molecular structure of dinucleoside polyphosphates is given in Figure 2 for diadenosine polyphosphates (Ap_nA). Diadenosine polyphosphates, with a backbone of two to seven phosphates, are known to occur naturally[79].

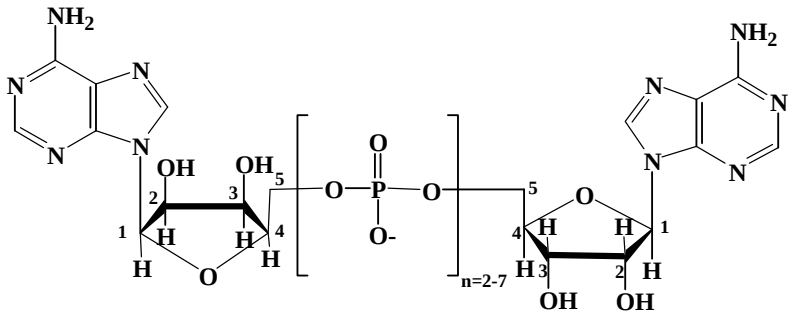

Figure 2: Molecular structure of diadenosine polyphosphates (Ap_nA with n=2-7)

Diadenosine tetraphosphate (Ap_4A) was the first member of this group to be found in mammalian tissues, in hepatocytes[127], and subsequently both Ap_4A and Ap_3A were detected in human tissues and in platelets[47,48]. Also diadenosine pentaphosphate (Ap_5A) and diadenosine hexaphosphate (Ap_6A) were isolated from human platelets and characterized as potent vasoconstrictors[49]. Several years later, diadenosine heptaphosphate (Ap_7A) was isolated from human platelets as well[29]. The most recently discovered member of this homologous series is diadenosine pyrophosphate, Ap_2A, which was found in human myocardial tissue[55] as well as platelets[49]. The monophosphate compound, Ap_1A, is not known to exist naturally, while the phosphate-free adenyladenosine has 5´- and 3´-linkages and, therefore, does not really belong to this group. To the best of our knowledge, there is no description in the literature showing a relationship between the Ap_nA amount and phosphate amount in different tissues and body fluids. A direct relationship is unlikely because Ap_nA are probably synthesized from the corresponding mononucleotides, and not by nucleosides and phosphates.

Diadenosine polyphosphates have a direct effect on the vascular tone. For example, in the vasculature of isolated perfused rat kidney, Ap_5A and Ap_6A were active at a concentration of low nanomolar range. Intra-aortic injection in the rat caused a prolonged increase in blood pressure[49]. The vasoconstrictive effect of Ap_7A on the vasculature of the isolated perfused rat kidney is slightly lower than that of Ap_6A[29]. A comparison of the homologous series of Ap_nA compounds with phosphate chain lengths from two to six shows that Ap_5A is the most potent inhibitor of ADP-induced platelet aggregation, and that Ap_6A and Ap_4A are more potent than Ap_3A and Ap_2A.

Some dinucleoside polyphosphates have a vasodilatory effect, for example, Ap_2A in the isolated mesenteric arterial bed of rats[128]. When arteries with an intact endothelium are perfused with Ap_3A and Ap_4A, both induce vasodilatation, whereas Ap_4A causes vasoconstriction in arteries from which the endothelium has been removed[129]. Arterial infusion of Ap_4A produced a dose-dependent decrease of systemic blood pressure and coronary vascular resistance[130].

Dinucleoside polyphosphates do not only directly influence the vascular physiology, but also increase the proliferation rate of vascular smooth muscle cells. Growth-stimulating effects of nucleoside polyphosphates have been demonstrated in numerous types of vascular beds[131],

involving the subsequent activation of protein kinase C (PKC), *Raf-1*, and mitogen-activated protein kinase (MAPK)[132,133].

The physiologic effects of dinucleoside polyphosphates are mediated by the P2 purinoceptor system[134]. The purinoceptor system controlling vascular homeostasis displays a high degree of complexity. This is exemplified by the large number of agonists involved (adenosine, ADP, ATP, UDP, UTP, dinucleoside polyphosphates) and the diversity of purinoceptors (*P2 receptors*).

P2 receptors are divided into two main classes based on whether they are ligand-gated ion channels (P2X receptors) or are coupled to G proteins (P2Y receptors)[135,136]. The P2X/P2Y nomenclature was adopted from the one originally used in a subdivision of P2 receptors proposed in 1985 by Burnstock and Kennedy, who described "P2X-" and "P2Y-purinoceptors" with distinct pharmacological profiles and tissue distributions: the "P2X purinoceptor" was shown to be most potently activated by the stable analogs of ATP, α,β-methylene-ATP (α,β-meATP), and β,γ-meATP. At the "P2Y-purinoceptor" 2-methylthio-ATP (2MeSATP) was the most potent agonist and α, β-meATP and β,γ-meATP were weak or inactive.

There is no evidence for a specific or selective dinucleoside polyphosphate receptor, and the effects of the dinucleoside polyphosphate are blocked by P2X receptor antagonists such as PPADS or suramin, or by desensitisation of P2X receptors with α,β-methylene-ATP subtype[137-141] or blockade by Ip_5I[142].

The calcium-permeable P2X1 receptor is considered the principal mediator of vasoconstriction[141], with P2X1 protein clusters on the adventitial surface of blood vessels immediately adjacent to sympathetic nerve varicosities[142]. However, P2X1 transcripts colocalise with mRNA for P2X2, P2X4, and P2X5 in muscle cells of a number of blood vessels, and this points to the added presence of heteromeric P2X receptors[143-147]. For example, heteromeric P2X1/5 receptors have been implicated in vasoconstriction of submucosal arterioles in the guinea pig[148]. UTP- and ATP-induced vasoconstriction in intrapulmonary artery is consistent with activation of the P2Y4 receptor subtype[149], which is sensitive to Ap_4A. It has been proposed that dinucleoside polyphosphate vasoconstriction is also mediated by the adenosine A1 receptor[150-154]. Distinct tissue distributions and functions reinforced this subdivision: P2X-

purinoceptors were shown to be present in vas deferens, urinary bladder, and vascular smooth muscle, and to mediate contraction.

P2Y receptor stimulation increases the expression of *c-fos* mRNA in cultured aortic smooth muscle cells[155]. In its effect to stimulate proliferation of vascular tissue, Ap_4A is equipotent to ATP[156]. Ap_3A, Ap_4A, Ap_5A[126] as well as Ap_2A, Ap_2G and Gp_2G[49] induce cell proliferation in vascular smooth muscle cells and furthermore stimulate c-fos proto-oncogene expression. The proliferative effect of the diguanosine polyphosphates Gp_nG (with n=3-6) is significantly stronger than that of ATP in vascular tissues[157]. Ap_3A, Ap_4A, Ap_5A, and Ap_6A also stimulate growth in rat glomerular mesangial cells in micromolar concentrations[125,158]. Moreover, they potentiate the growth response to platelet-derived growth factor, but not to insulin-like growth factor-1[125].

The dinucleoside polyphosphates are also potent antagonists of ADP-induced platelet aggregation[29]. The interaction with ADP occurs at the P2T receptor and appears to be a competitive inhibition, with Ap_4A having a K_i of approximately 0.7 mmol L^{-1} [138]. A comparison of the homologous series of Ap_nA compounds with phosphate chain lengths from two to six shows that Ap_5A is the most potent inhibitor of ADP-induced platelet aggregation, and that Ap_6A and Ap_4A are more potent than Ap_3A and Ap_2A. These dinucleoside polyphosphates inhibit the release of ADP from platelets, with a potency that decreases with decreasing chain length. Thus, dinucleoside polyphosphates in platelets may fulfil an anti-aggregatory role. Ap_3A, Ap_4A, Ap_5A, and Ap_6A all produce an increase in intracellular free calcium via a G-protein coupled receptor[159].

Dinucleoside polyphosphates are metabolised by ectohydrolases, which are present in a broad variety of cell types, including aortic endothelium cells[160] and mesangial cells[161]. Human phosphohydrolase shows a clear preference for Ap_5A and Ap_6A as substrates[162]. The enzymatic breakdown of dinucleotides leads to the generation of mononucleotides that, in turn, are biologically active in vascular tissues. This enzymatic breakdown not only inactivates agonists but also transforms these agonists into other, occasionally more potent compounds[79].

A.1.3.8. Endothelium-derived hyperpolarizing factor

In addition to nitric oxide (chapter A.1.3.4.), the endothelium generates a second factor with vasodilatory properties. This factor leads to a hyperpolarization of vascular smooth muscle

cells, and therefore this factor is named “endothelium-derived hyperpolarizing factor” (EDHF)[27]. This factor modulates endothelium-dependent vascular relaxation, particularly in smaller coronary and peripheral vessels. The hyperpolarizing effect of the EDHF occurs via the opening of vascular smooth muscle potassium channels. When vascular smooth muscle is hyperpolarized, voltage-sensitive calcium channels are closed, leading to a reduction in intracellular calcium and relaxation[163]. The EDHF has a major function in the control of blood pressure during the physiologic conditions[164]. This factor makes a significant contribution to vascular tone. In coronary circulation, the importance of the hyperpolarizing factor in modulating endothelium-dependent vascular relaxation seems to increase as vessel size decreases[165]. The EDHF responses are diminished in hypertension and preeclampsia[166].

A.1.4. Calcium metabolism

The relationships between the cytosolic free Ca^{2+} (Ca_i), protein kinase C (PKC), and the Na^+/H^+ antiport may hold the key to unravelling the causes and origin of essential hypertension[167]. Once Ca^{2+} was recognized as a carrier of signals, it became important to understand how its concentration within cells was regulated[168]. The control of Ca^{2+} concentration in the cytoplasm and organelles is the sole function of certain proteins that, as a rule, are intrinsic to the plasma membrane and to the membranes of organelles and transport Ca^{2+} across them. These proteins belong to various classes: Ca^{2+} channels in the plasma membrane are gated by voltage, by ligands, or by the emptying of internal Ca^{2+} stores. In the endosarcoplasmic reticulum, they are activated by the second messengers, inositol triphosphate and cyclic ADP ribose. Cyclic ADP ribose is assumed to act on channels that are also called ryanodine receptors and that are sensitive to the agonist caffeine. Accessory protein factors, among them calmodulin, may be required for the Ca^{2+}-releasing effect of cyclic ADP ribose. ATPases are found in the plasma membrane, in the endosarcoplasmic reticulum Ca^{2+} pump (SERCA), in the Golgi apparatus, and in the nuclear envelope. These proteins have no direct role in the processing of the Ca^{2+} signal, but may be targets of Ca^{2+} regulation. The existence of different Ca^{2+} transporters is justified by their different properties, which satisfy all demands of cells in terms of Ca^{2+} homeostasis; e.g. some pumps have high Ca^{2+} affinity but limited transport capacity[169]. Eukaryotic plasma membranes contain three Ca^{2+}-transporting systems: a Ca^{2+}-channel, an ATPase, and a Na^+/Ca^{2+}-exchanger[170]. The ATPase is a high-affinity, low-capacity system, which continuously pumps Ca^{2+} out of cells. The Na^+/Ca^{2+}-exchanger is a low-affinity, high-capacity system, which is particularly active in excitable cells. The exchanger probably functions in both the Ca^{2+}-efflux and influx directions[171]. The vasoconstric-

tion and vasomotion of vascular smooth muscle is associated with Ca^{2+} variations[172]. Abnormally high Ca^{2+} has been found in blood cells, cultured aortic and mesenteric arterial smooth muscle cells, and in intact aortas and renal arteries of hypertensive animals[173-177].

In essential hypertension[178] and in patients with end-stage renal failure[179], disturbances of Ca^{2+} metabolism are common[180]. The disturbed Ca^{2+} metabolism leads to an increase of the intracellular Ca^{2+} concentration, which is one reason for the secondary hypertension of chronic renal failure[181,182].

In this condition, the Ca^{2+} permeability of the membrane seems to be increased[183,184], and simultaneously the Ca^{2+}-ATPase activity decreases. Based on a decreased Ca^{2+}-ATPase activity in renal failure, a study suggested a circulating Ca^{2+}-ATPase inhibitor in the plasma[185], which accumulates in chronic renal failure and may be an important factor for disturbed cellular Ca^{2+} metabolism. Ca^{2+}-ATPase inhibitors may play a role in the pathophysiology of the uremic syndrome characterizing end-stage renal disease (stage 5) and, potentially, in inducing toxic effects on cellular Ca^{2+} metabolism in renal failure[186].

A.1.5. End-stage renal failure

When renal function deteriorates, a gradual accumulation occurs in the body of a host of compounds, which under normal conditions are secreted into the urine by the healthy kidneys[187]. This process goes along with functional disturbances in almost all organ and cell systems, related to modification in biological and biochemical function, attributed to the retention mentioned above. The retention process is named uremia, referring to the most abundant but probably inert molecule: urea. As uremic retention solutes exert biological activity, they are named uremic toxins[187].

Cardiovascular disorders, as described above, are the leading causes of mortality in patients with end-stage renal disease[188]. The high risk for cardiovascular diseases results from the additive effect of multiple factors, including hemodynamic overload and several metabolic and endocrine abnormalities more or less specific to uremia[189].

Chronic progressive renal disease is becoming increasingly prevalent as the population ages. With improved treatment and longer patient survivals, the number of patients requiring care for end-stage renal disease is increasing and shows no signs of reaching a plateau in the near

future. While renal transplantation is considered the treatment of choice for all suitable patients, the number of patients requiring dialysis increases progressively. Dialysis is a life-saving therapy that can provide patients with an acceptable quality of life for prolonged periods of time. The interest in dialysis is due mainly to its potential advantages in reducing morbidity and mortality, although mortality remains markedly higher and accelerated compared to matched populations without renal failure.

There are two types of dialysis, each with its advantages and disadvantages: hemodialysis (HD) and peritoneal dialysis (PD). In hemodialysis, blood of the patient is guided over an extracorporeal semipermeable membrane[190]. In peritoneal dialysis, the peritoneal membrane of the patient is used as semipermeable membrane. In classical hemodialysis, the main concept of transport is diffusion. This implies that the method is highly efficient for removal of small solutes, but that there is far less or even no clearance for larger solutes. To improve clearance of larger solutes more permeable (high flux) membranes can be used for hemofiltration[191]. In this setting the main concept of transport is convection, which enhances the clearance of larger solutes but not of protein-bound toxins. In peritoneal dialysis, the main concept of transport is diffusion. The efficiency of this technique is rather low, which is however tackled by the continuous nature of the treatment. In addition, there is substantial clearance of larger and even protein-bound toxins through the larger pores of the peritoneal membrane[192].
In view of the different physicochemical transport principles of the different dialysis modalities, characterization and understanding of the kinetic behaviour of the different uremic retention products is crucial[193,194].

Until now, removal of uremic toxins has been performed by dialysis in an empirical and resource-based way. Although the above-mentioned technological developments have been designed especially to improve the delivery of therapy, they have not yet satisfactorily decreased mortality and morbidity.

An additional risk factor of dialysis is that not only toxic substances but also essential solutes, such as vitamins or trace elements, are eliminated, although until now such disadvantages of high-efficacy dialytic removal have not been convincingly demonstrated. The ideal removal technique should as much as possible reflect the capacities of the native kidney, though current technical possibilities are too limited to reach this aim[195]. *Whereas in the first part of this*

thesis we will focus on the production of vasoactive compounds by normal cells, the second part will be devoted to vasoactive compounds retained in renal failure.

A.1.6. References

1. Bonow RO, Smaha LA, Smith SC, Jr., Mensah GA, Lenfant C: World Heart Day 2002: the international burden of cardiovascular disease: responding to the emerging global epidemic. *Circulation* 106:1602-1605, 2002
2. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. Guidelines Subcommittee. *J Hypertens* 17:151-183, 1999
3. De Backer G, Ambrosioni E, Borch-Johnsen K, Brotons C, Cifkova R, Dallongeville J, Ebrahim S, Faergeman O, Graham I, Mancia G, Manger Cats V, Orth-Gomer K, Perk J, Pyorala K, Rodicio JL, Sans S, Sansoy V, Sechtem U, Silber S, Thomsen T, Wood D: European Guidelines on Cardiovascular Disease Prevention in the Clinical Practice. Third work group of the european societies and other societies on cardiovascular disease prevention in the clinical practice. *Neurologia* 19:440-450, 2004
4. Wofford MR, King DS, Wyatt SB, Jones DW: Secondary Hypertension: Detection and Management for the Primary Care Provider. *J Clin Hypertens* 2:124-131, 2000
5. Kaplan NM: Treatment of hypertension: insights from the JNC-VI report. *Am Fam Physician* 58:1323-1330, 1998
6. Schoen: Blood Vessel, in: *Robbins Pathologic basis of disease*, 6th edition. Eds: W.B. Saunders Company, Philadelphia, USA, 1999, pp 493-541
7. Hingorani AD, Brown MJ: Identifying the genes for human hypertension. *Nephrol Dial Transplant* 11:575-586, 1996
8. Lifton RP: Molecular genetics of human blood pressure variation. *Science* 272:676-680, 1996
9. Thibonnier M, Schork NJ: The genetics of hypertension. *Curr Opin Genet Dev* 5:362-370, 1995
10. Dahl LK, Knudsen KD, Iwai J: Humoral transmission of hypertension: evidence from parabiosis. *Circ Res* 24:Suppl:21-33, 1969
11. Greenberg S, Gaines K, Sweatt D: Evidence for circulating factors as a cause of venous hypertrophy in spontaneously hypertensive rats. *Am J Physiol* 241:H421-430, 1981
12. Zidek W, Heckmann U, Losse H, Vetter H: Effects on blood pressure of cross circulation between spontaneously hypertensive and normotensive rats. *Clin Exp Hypertens* 8:347-354, 1986
13. Hirata Y, Tobian L, Simon G, Iwai J: Hypertension-producing factor in serum of hypertensive Dahl salt-sensitive rats. *Hypertension* 6:709-716, 1984
14. Wright GL, Bookout C: A hypertensive substance in erythrocytes of spontaneously hypertensive rats. *Can J Physiol Pharmacol* 60:622-627, 1982
15. Lindner A, Kenny M, Meacham AJ: Effects of a circulating factor in patients with essential hypertension on intracellular free calcium in normal platelets. *N Engl J Med* 316:509-513, 1987
16. Zidek W, Sachinidis A, Spieker C, Storkebaum W: Effect of plasma from hypertensive subjects on Ca^{2+} transport in permeabilized human neutrophils. *Clin Sci* 74:53-56, 1988
17. Folkow B: Physiological aspects of primary hypertension. *Physiol Rev* 62:347-504, 1982

18. Lever AF: Slow pressor mechanisms in hypertension: a role for hypertrophy of resistance vessels? *J Hypertens* 4:515-524, 1986
19. Preston RA: Renoprotective effects of antihypertensive drugs. *Am J Hypertens* 12:19S-32S, 1999
20. Onusko E: Diagnosing secondary hypertension. *Am Fam Physician* 67:67-74, 2003
21. Gibbons GH: Endothelial function as a determinant of vascular function and structure: a new therapeutic target. *Am J Cardiol* 79:3-8, 1997
22. Bachetti T, Morbidelli L: Endothelial cells in culture: a model for studying vascular functions. *Pharmacol Res* 42:9-19, 2000
23. Furchgott RF, Zawadzki JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373-376, 1980
24. Feletou M, Vanhoutte PM: Endothelium-dependent hyperpolarization of vascular smooth muscle cells. *Acta Pharmacol Sin* 21:1-18, 2000
25. Fleming I, Busse R: NO: the primary EDRF. *J Mol Cell Cardiol* 31:5-14, 1999
26. Palmer RM, Ferrige AG, Moncada S: Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327:524-526, 1987
27 Feletou M, Vanhoutte PM: The alternative: EDHF. *J Mol Cell Cardiol* 31:15-22; 1999
28. Bennett MR, Boyle JJ: Apoptosis of vascular smooth muscle cells in atherosclerosis. *Atherosclerosis* 138:3-9, 1998
29. Jankowski J, Tepel M, van der Giet M, Tente IM, Henning L, Junker R, Zidek W, Schlüter H: Identification and characterization of P_1,P_7 Di(adenosine-5')-heptaphosphate from human platelets. *J Biol Chem* 274:23926-23931, 1999
30. Østerud B, Bjørklid E: Role of Monocytes in Atherogenesis. *Physiol Rev* 83:1069–1112, 2003
31. Heitzer T, Schlinzig T, Krohn K, Meinertz T, Munzel T: Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation* 104:2673-2678, 2001
32. Touyz RM: Molecular and cellular mechanisms regulating vascular function and structure-implications in the pathogenesis of hypertension. *Can J Cardiol* 16:1137-1146, 2000
33. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332:411-415, 1988
34. Miyauchi H, Minamino T, Tateno K, Kunieda T, Toko H, Komuro I: Akt negatively regulates the *in vitro* lifespan of human endothelial cells via a p53/p21-dependent pathway. *Embo J* 23:212-220, 2004
35. Hollenberg SM, Tong W, Shelhamer JH, Lawrence M, Cunnion RE: Eicosanoid production by human aortic endothelial cells in response to endothelin. *Am J Physiol* 267:H2290-2296, 1994
36. Li Q, Puro DG: Adenosine activates ATP-sensitive K^+ currents in pericytes of rat retinal microvessels: role of A1 and A2a receptors. *Brain Res* 907:93-99, 2001
37. Axelrod L: Perioperative management of patients treated with glucocorticoids. *Endocrinol Metab Clin North Am* 32:367-383, 2003
38. Munakata M, Imai Y, Hashimoto J, Omata K, Nakao M, Yamamoto M, Abe K: Normal sympathetic vasomotor and cardiac parasympathetic activities in patients with primary aldosteronism: assessment by spectral analysis. *J Auton Nerv Syst* 52:213-223, 1995
39. DeLano FA, Schmid-Schonbein GW: Enhancement of glucocorticoid and mineralocorticoid receptor density in the microcirculation of the spontaneously hypertensive rat. *Microcirculation* 11:69-78, 2004

40. Li Q, Grimelius L, Zhang X, Lukinius A, Grondal S, Hoog A, Johansson H: Ultrastructural localization of endothelin-1 in nonneoplastic, hyperplastic, and neoplastic adrenal gland. *Ultrastruct Pathol* 19:489-494, 1995
41. Kim SY, Kim JH, Kim CH, Nam SW, Kim YJ, Kim JI, Park SH, Han JY, Kim JK, Chung KW, Sun HS: A case of pheochromocytoma with hyperamylasemia. *Korean J Gastroenterol* 42:172-175, 2003
42. Pintor J, Torres M, Miras-Portugal MT: Carbachol induced release of diadenosine polyphosphates-Ap_4A and Ap_5A- from perfused bovine adrenal medulla and isolated chromaffin cells. *Life Sci* 48:2317-2324, 1991
43. Pintor J, Rotllan P, Torres M, Miras-Portugal MT: Characterization and quantification of diadenosine hexaphosphate in chromaffin cells: granular storage and secretagogue-induced release. *Anal Biochem* 200:296-300, 1992
44. Tagliasacchi D, Carboni G: Blood cells, in Wheater´s Functional Histology 4th edition. Eds: Young B, Heath JW, Churchill Livingstone, London, UK, 1997, pp 46-64
45. Puri RN, Colman RW: ADP-induced platelet activation. *Crit Rev Biochem Mol Biol* 32:437-502, 1997
46. Heemskerk JW, Bevers EM, Lindhout T: Platelet activation and blood coagulation. *Thromb Haemost* 88:186-193, 2002
47. Flodgaard H, Klenow H: Abundant amounts of diadenosine 5',5'''-P_1,P_4-tetraphosphate are present and releasable, but metabolically inactive, in human platelets. *Biochem J* 208:737-742, 1982
48. Lüthje J, Ogilvie A: The presence of diadenosine 5',5'''-P_1,P_3-triphosphate (Ap_3A) in human platelets. *Biochem Biophys Res Commun* 115: 253-260, 1983
49. Schlüter H, Offers E, Brüggemann G, van der Giet M, Tepel M, Nordhoff E, Karas M, Spieker C, Witzel H, Zidek W: Diadenosine phosphates and the physiological control of blood pressure. *Nature* 367:186-188, 1994
50. Jankowski J, Hagemann J, Yoon M S, von der Giet M, Stephan N, Zidek W, Schlüter H, Tepel M: Increased vascular growth in hemodialysis patients induced by platelet-derived diadenosine polyphosphates. *Kidney Int* 59:1134-1141, 2001
51. Currie MG, Geller DM, Cole BR, Siegel NR, Fok KF, Adams SP, Eubanks SR, Galluppi GR, Needleman P: Purification and sequence analysis of bioactive atrial peptides (atriopeptins). *Science* 223:67-69, 1984
52. Thone-Reineke C, Zimmermann M, Neumann C, Krikov M, Li J, Gerova N, Unger T: Are angiotensin receptor blockers neuroprotective? *Curr Hypertens Rep* 6:257-266, 2004
53. Suzuki J, Horiuchi M: Renin-angiotensin system and heart failure. *Nippon Rinsho* 61:751-755, 2003
54. Dostal DE, Baker KM: The cardiac renin-angiotensin system: conceptual, or a regulator of cardiac function? *Circ Res* 85:643-650, 1999
55. Luo J, Jankowski J, Knobloch M, van der Giet M, Gardanis K, Russ T, Vahlensieck U, Neumann J, Schmitz W, Tepel M, Deng MC, Zidek W, Schlüter H: Identification and characterization of diadenosine 5',5'''-P_1,P_2 -diphosphate and diadenosine 5',5'''-P_1,P_3-triphosphate in human myocardial tissue. *Faseb J* 13:695-705, 1999
56. Gabriels G, Rahn KH, Schlatter E, Steinmetz M: Mesenteric and renal vascular effects of diadenosine polyphosphates (Ap_nA). *Cardiovasc Res* 56:22-32, 2002
57. Rosenthal, Braunwald: Hematological-oncological disorders and heart disease, in *Heart Disease: Textbook of CardiovascularMedicine*, edited by Braunwald E, 4th ed, Philadelphia, USA, WB Saunders, 1992, pp 1786-1808
58. Dzau VJ: The role of mechanical and humoral factors in growth regulation of vascular smooth muscle and cardiac myocytes. *Curr Opin Nephrol Hypertens* 2:27-32, 1993

59. Katz AM: The cardiomyopathy of overload: an unnatural growth response in the hypertrophied heart. *Ann Intern Med* 121:363-371, 1994
60. Jankowski J, Hagemann J, Tepel M, van der Giet M, Stephan N, Henning L, Gouni-Berthold I, Sachinidis A, Zidek W, Schlüter H: Dinucleotides as growth promoting extracellulary mediators: Presence of dinucleoside diphosphates Ap_2A, Ap_2G and Gp_2G in releasable granlues of platelets. *J Biol Chem* 276:8904-8909, 2001
61. Lüthje J, Ogilvie A: Diadenosine triphosphate (Ap_3A) mediates human platelet aggregation by liberation of ADP. *Biochem Biophys Res Commun* 118:704-709, 1984
62. Lüthje J, Ogilvie A: Catabolism of Ap_4A and Ap_3A in whole blood. The dinucleotides are long- lived signal molecules in the blood ending up as intracellular ATP in the erythrocytes. *Eur J Biochem* 173:241-245, 1988
63. Jankowski J, Potthoff W, van der Giet M, Tepel M, Zidek W, Schlüter H: High-Performance Liquid Chromatographic Assay of the Diadenosine Polyphosphates in Human Platelets. *Anal Biochem* 269:72-78, 1999
64. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC: A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. *Blood* 84:1361-1392, 1994
65. Otero DC, Rickert RC: CD19 function in early and late B cell development. II. CD19 facilitates the pro-B/pre-B transition. *J Immunol* 171:5921-5930, 2003
66. Crow MK, Kushner B, Jover JA, Friedman SM, Mechanic SE, Stohl W: Human peripheral blood T helper cell-induced B cell activation results in B cell surface expression of the CD23 (BLAST-2) antigen. *Cell Immunol* 121:99-112, 1989
67. Berardi AC, Meffre E, Pflumio F, Katz A, Vainchenker W, Schiff C, Coulombel L: Individual CD34+CD38lowCD19-CD10- progenitor cells from human cord blood generate B lymphocytes and granulocytes. *Blood* 89:3554-3564, 1997
68. Galle J: Atherosclerosis and arteriitis: implications for therapy of cardiovascular disease. *Herz* 29:4-11, 2004
69. Pang PK, Lewanczuk RZ: Parathyroid origin of a new circulating hypertensive factor in spontaneously hypertensive rats. *Am J Hypertens* 2:898-902, 1989
70. Robbins P: The endocrine system, in: *Pathologic basis of disease*. W. B. Saunders Company, Philadelphia, USA, 1999, pp 1121-1169
71. Rodriguez M, Canalejo A, Garfia B, Aguilera E, Almaden Y: Pathogenesis of refractory secondary hyperparathyroidism. *Kidney Int Suppl*:155-160, 2002
72. Benishin CG, Labedz T, Guo DD, Lewanczuk RZ, Pang PK: Identification and purification of parathyroid hypertensive factor from organ culture of parathyroid glands from spontaneously hypertensive rats. *Am J Hypertens* 6:134-140, 1993
73. Sutherland SK, Benishin CG: Regulation of parathyroid hypertensive factor secretion by Ca^{2+} in spontaneously hypertensive rat parathyroid cells. *Am J Hypertens* 17:266-272, 2004
74. Pang PK, Benishin CG, Lewanczuk RZ: Parathyroid hypertensive factor, a circulating factor in animal and human hypertension. *Am J Hypertens* 4:472-477, 1991
75. Leung PS: The peptide hormone angiotensin II: its new functions in tissues and organs. *Curr Protein Pept Sci* 5:267-273, 2004
76. Alderton WK, Cooper CE, Knowles RG: Nitric oxide synthases: structure, function and inhibition. *Biochem J* 357:593-615, 2001
77. Spat A, Hunyady L: Control of aldosterone secretion: a model for convergence in cellular signaling pathways. *Physiol Rev* 84:489-539, 2004

78. Esler M, Lambert G, Brunner-La Rocca HP, Vaddadi G, Kaye D: Sympathetic nerve activity and neurotransmitter release in humans: translation from pathophysiology into clinical practice. *Acta Physiol Scand* 177:275-284, 2003
79. Hoyle CHV, Hilderman RH, Pintor JJ, Schlüter H, King BF: Diadenosine polyphosphates as extracellular signal molecules. *Drug Develop Res* 52:260-273, 2001
80. McLennan AG: Dinucleoside polyphosphates-friend or foe? *Pharmacol Ther* 87:73-89, 2000
81. Bobik A, Grooms A, Millar JA, Mitchell A, Grinpukel S: Growth factor activity of endothelin on vascular smooth muscle. *Am J Physiol* 258:C408-415, 1990
82. Durieu-Trautmann O, Federici C, Creminon C, Foignant-Chaverot N, Roux F, Claire M, Strosberg AD, Couraud PO: Nitric oxide and endothelin secretion by brain microvessel endothelial cells: regulation by cyclic nucleotides. *J Cell Physiol* 155:104-111, 1993
83. Yoshimoto S, Ishizaki Y, Kurihara H, Sasaki T, Yoshizumi M, Yanagisawa M, Yazaki Y, Masaki T, Takakura K, Murota S: Cerebral microvessel endothelium is producing endothelin. *Brain Res* 508:283-285, 1990
84. Pang PK, Lewanczuk RZ, Benishin CG: Parathyroid hypertensive factor. *J Hypertens Suppl* 8:S155-159, 1990
85. Geisterfer AA, Peach MJ, Owens GK: Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ Res* 62:749-756, 1988
86. Riordan JF: Angiotensin II: biosynthesis, molecular recognition, and signal transduction. *Cell Mol Neurobiol* 15:637-651, 1995
87. Wright JW, Krebs LT, Stobb JW, Harding JW: The angiotensin IV system: functional implications. *Front Neuroendocrinol* 16:23-52, 1995
88. Haller H, Lindschau C, Quass P, Distler A, Luft FC: Nuclear calcium signaling is initiated by cytosolic calcium surges in vascular smooth muscle cells. *Kidney Int* 46:1653-1662, 1994
89. Haller H, Quass P, Lindschau C, Luft FC, Distler A: Platelet-derived growth factor and angiotensin II induce different spatial distribution of protein kinase C-alpha and -beta in vascular smooth muscle cells. *Hypertension* 23:848-852, 1994
90. Li YC, Kong J, Wei M, Chen ZF, Liu SQ, Cao LP: 1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system. *J Clin Invest* 110:229-238, 2002
91. New MI: Hypertension in congenital adrenal hyperplasia and apparent mineralocorticoid excess. *Ann N Y Acad Sci* 970:145-154, 2002
92. Gavras H, Lever AF, Brown JJ, Macadam RF, Robertson JI: Acute renal failure, tubular necrosis, and myocardial infarction induced in the rabbit by intravenous angiotensin II. *Lancet* 2:19-22, 1971
93. Brunner HR, Laragh JH, Baer L, Newton MA, Goodwin FT, Krakoff LR, Bard RH, Buhler FR: Essential hypertension: renin and aldosterone, heart attack and stroke. *N Engl J Med* 286:441-449, 1972
94. Burnier M: Angiotensin II type 1 receptor blockers. *Circulation* 103:904-912, 2001
95. Naftilan AJ, Pratt RE, Eldridge CS, Lin HL, Dzau VJ: Angiotensin II induces c-fos expression in smooth muscle via transcriptional control. *Hypertension* 13:706-711, 1989
96. Geisterfer AA, Owens GK: Arginine vasopressin-induced hypertrophy of cultured rat aortic smooth muscle cells. *Hypertension* 14:413-420, 1989
97. Neri Serneri GG, Boddi M, Coppo M, Chechi T, Zarone N, Moira M, Poggesi L, Margheri M, Simonetti I: Evidence for the existence of a functional cardiac renin-angiotensin system in humans. *Circulation* 94:1886-1893, 1996

98. van Kats JP, Danser AH, van Meegen JR, Sassen LM, Verdouw PD, Schalekamp MA: Angiotensin production by the heart: a quantitative study in pigs with the use of radiolabeled angiotensin infusions. *Circulation* 98:73-81, 1998
99. Xiao HD, Fuchs S, Frenzel K, Teng L, Bernstein KE: Circulating versus local angiotensin II in blood pressure control: lessons from tissue-specific expression of angiotensin-converting enzyme (ACE). *Crit Rev Eukaryot Gene Expr* 14:137-145, 2004
100. Müller DN, Bohlender J, Hilgers KF, Dragun D, Costerousse O, Menard J, Luft FC: Vascular angiotensin-converting enzyme expression regulates local angiotensin II. *Hypertension* 29:98-104, 1997
101. Dell'Italia LJ, Meng QC, Balcells E, Wei CC, Palmer R, Hageman GR, Durand J, Hankes GH, Oparil S: Compartmentalization of angiotensin II generation in the dog heart. Evidence for independent mechanisms in intravascular and interstitial spaces. *J Clin Invest* 100:253-258, 1997
102. Lind RW, Swanson LW, Ganten D: Organization of angiotensin II immunoreactive cells and fibers in the rat central nervous system. An immunohistochemical study. *Neuroendocrinology* 40:2-24, 1985
103. Engeli S, Negrel R, Sharma AM: Physiology and pathophysiology of the adipose tissue renin-angiotensin system. *Hypertension* 35:1270-1277, 2000
104. Navar LG, Harrison-Bernard LM, Nishiyama A, Kobori H: Regulation of intrarenal angiotensin II in hypertension. *Hypertension* 39:316-322, 2002
105. Dostal DE: The cardiac renin-angiotensin system: novel signaling mechanisms related to cardiac growth and function. *Regul Pept* 91:1-11, 2000
106. Aiello S, Remuzzi G, Noris M: Nitric oxide/endothelin balance after nephron reduction. *Kidney Int Suppl* 65:S63-67, 1998
107. Katsuyama K, Shichiri M, Marumo F, Hirata Y: NO inhibits cytokine-induced iNOS expression and NF-χB activation by interfering with phosphorylation and degradation of Iχ B-α. *Arterioscler Thromb Vasc Biol* 18:1796-1802, 1998
108. Cornwell TL, Arnold E, Boerth NJ, Lincoln TM: Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol* 267:C1405-1413, 1994
109. De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA, Jr., Shin WS, Liao JK: Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* 96:60-68, 1995
110. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC: Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 377:239-242, 1995
111. Moncada S, Higgs EA: Molecular mechanisms and therapeutic strategies related to nitric oxide. *Faseb J* 9:1319-1330, 1995
112. Bitar MS, Wahid S, Mustafa S, Al-Saleh E, Dhaunsi GS, Al-Mulla F: Nitric oxide ics and endothelial dysfunction in type II model of genetic diabetes. *Eur J Pharmacol* 511:53-64, 2005
113. Duprez D, De Buyzere M, Rietzschel ER, Clement DL: Aldosterone and vascular damage. *Curr Hypertens Rep* 2:327-334, 2000
114. Moss NG: Renal function and renal afferent and efferent nerve activity. *Am J Physiol* 243:F425-433, 1982
115. Di Bona GF: Neural control of renal function: cardiovascular implications. *Hypertension* 13:539-548, 1989

116. Barajas L, Powers K, Wang P: Innervation of the renal cortical tubules: a quantitative study. *Am J Physiol* 247:F50-60, 1984
117. Tucker BJ, Mundy CA, Blantz RC: Adrenergic and angiotensin II influences on renal vascular tone in chronic sodium depletion. *Am J Physiol* 252:F811-817, 1987
118. Jose PA, Raymond JR, Bates MD, Aperia A, Felder RA, Carey RM: The renal dopamine receptors. *J Am Soc Nephrol* 2:1265-1278, 1992
119. Seri I, Kone BC, Gullans SR, Aperia A, Brenner BM, Ballermann BJ: Influence of Na^+ intake on dopamine-induced inhibition of renal cortical Na^+-K^+-ATPase. *Am J Physiol* 258:F52-60, 1990
120. Denton MD, Chertow GM, Brady HR: "Renal-dose" dopamine for the treatment of acute renal failure: scientific rationale, experimental studies and clinical trials. *Kidney Int* 50:4-14, 1996
121. DiBona GF: Renal dopamine containing nerves. What is their functional significance? *Am J Hypertens* 3:64S-67S, 1990
122. Edwards RM: Response of isolated renal arterioles to acetylcholine, dopamine, and bradykinin. *Am J Physiol* 248:F183-189, 1985
123. Bughi S, Horton R, Antonipillai I, Manoogian C, Ehrlich L, Nadler J: Comparison of dopamine and fenoldopam effects on renal blood flow and prostacyclin excretion in normal and essential hypertensive subjects. *J Clin Endocrinol Metab* 69:1116-1121, 1989
124. Ogilvie A. Extracellular functions for ApnA, in: McLennan AG: *Ap_4A and Other Dinucleoside Polyphosphates*. Eds: McLennan AG. CRC Press Inc, London, UK, 1992, pp 229-273.
125. Heidenreich S, Tepel M, Schlüter H, Harrach B, Zidek W: Regulation of rat mesangial cell growth by diadenosine phosphates. *J Clin Invest* 95:2862-2867, 1995
126. Jankowski J, Schlüter H, Zidek W, Spieker C: Isolation and identification of Ap_3A, Ap_4A and Ap_5A as growth stimulating factor in human platelets. *Biological Chemistry* 377:193, 1996
127. Rapaport E, Zamecnik PC: Presence of diadenosine 5',5'''-P_1,P_4-tetraphosphate (Ap_4A) in mamalian cells in levels varying widely with proliferative activity of the tissue: a possible positive "pleiotypic activator". *Proc Natl Acad Sci U S A* 73:3984-3988, 1976
128. Ralevic V, Burnstock G: Discrimination by PPADS between endothelial P2Y- and P2U-purinoceptors in the rat isolated mesenteric arterial bed. *Br J Pharmacol* 118:428-434, 1996
129. Busse R, Ogilvie A, Pohl U: Vasomotor activity of diadenosine triphosphate and diadenosine tetraphosphate in isolated arteries. *Am J Physiol* 254:H828-832, 1988
130. Nakae I, Takahashi M, Takaoka A, Liu Q, Matsumoto T, Amano M, Sekine A, Nakajima H, Kinoshita M: Coronary effects of diadenosine tetraphosphate resemble those of adenosine in anesthetized pigs: involvement of ATP-sensitive potassium channels. *J Cardiovasc Pharmacol* 28:124-133, 1996
131. Erlinge D: Extracellular ATP: a growth factor for vascular smooth muscle cells. *Gen Pharmacol* 31:1-8, 1998
132. Yu SM, Chen SF, Lau YT, Yang CM, Chen JC: Mechanism of extracellular ATP-induced proliferation of vascular smooth muscle cells. *Mol Pharmacol* 50:1000-1009, 1996
133. Wilden PA, Agazie YM, Kaufman R, Halenda SP: ATP-stimulated smooth muscle cell proliferation requires independent ERK and PI3K signaling pathways. *Am J Physiol* 275:H1209-1215, 1998
134. Ralevic V, Burnstock G: Receptors for purines and pyrimidines. *Pharmacol Rev* 50:413-492, 1998

135. Abbracchio MP, Burnstock G: Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol Ther* 64:445-475, 1994
136. Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams M: Nomenclature and classification of purinoceptors. *Pharmacol Rev* 46:143-156, 1994
137. Bo X, Sexton A, Xiang Z, Nori SL, Burnstock G: Pharmacological and histochemical evidence for P2X receptors in human umbilical vessels. *Eur J Pharmacol* 353:59-65, 1998
138. Kunapuli SP: Multiple P2 receptor subtypes on platelets: a new interpretation of their function. *Trends Pharmacol Sci* 19:391-394, 1998
139. Wang L, Karlsson L, Moses S, Hultgardh-Nilsson A, Andersson M, Borna C, Gudbjartsson T, Jern S, Erlinge D: P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. *J Cardiovasc Pharmacol* 40:841-853, 2002
140. Hoyle CH, Pintor J, Gualix J, Miras-Portugal MT: Antagonism of P2X receptors in guinea-pig vas deferens by diinosine pentaphosphate. *Eur J Pharmacol* 333:R1-2, 1997
141. Kennedy C: ATP as a cotransmitter in perivascular sympathetic nerves. *J Auton Pharmacol* 16:337-340, 1996
142. Hansen MA, Dutton JL, Balcar VJ, Barden JA, Bennett MR: P2X (purinergic) receptor distributions in rat blood vessels. *J Auton Nerv Syst* 75:147-155, 1999
143. Lewis CJ, Evans RJ: P2X receptor immunoreactivity in different arteries from the femoral, pulmonary, cerebral, coronary and renal circulations. *J Vasc Res* 38:332-340, 2001
144. Pulvirenti TJ, Yin JL, Chaufour X, McLachlan C, Hambly BD, Bennett MR, Barden JA: P2X (purinergic) receptor redistribution in rabbit aorta following injury to endothelial cells and cholesterol feeding. *J Neurocytol* 29:623-631, 2000
145. Lewis CJ, Evans RJ: Lack of run-down of smooth muscle P2X receptor currents recorded with the amphotericin permeabilized patch technique, physiological and pharmacological characterization of the properties of mesenteric artery P2X receptor ion channels. *Br J Pharmacol* 131:1659-1666, 2000
146. Turner CM, Vonend O, Chan C, Burnstock G, Unwin RJ: The pattern of distribution of selected ATP-sensitive P2 receptor subtypes in normal rat kidney: an immunohistological study. *Cells Tissues Organs* 175:105-117, 2003
147. Yamamoto K, Korenaga R, Kamiya A, Qi Z, Sokabe M, Ando J: $P2X_4$ receptors mediate ATP-induced calcium influx in human vascular endothelial cells. *Am J Physiol Heart Circ Physiol* 279:H285-292, 2000
148. Surprenant A, Schneider DA, Wilson HL, Galligan JJ, North RA: Functional properties of heteromeric $P2X_{1/5}$ receptors expressed in HEK cells and excitatory junction potentials in guinea-pig submucosal arterioles. *J Auton Nerv Syst* 81:249-263, 2000
149. McMillan MR, Burnstock G, Haworth SG: Vasoconstriction of intrapulmonary arteries to P2-receptor nucleotides in normal and pulmonary hypertensive newborn piglets. *Br J Pharmacol* 128:549-555, 1999
150. Gabriels G, Endlich K, Rahn KH, Schlatter E, Steinhausen M: *In vivo* effects of diadenosine polyphosphates on rat renal microcirculation. *Kidney Int* 57:2476-2484, 2000
151. Vahlensieck U, Boknik P, Knapp J, Linck B, Muller FU, Neumann J, Herzig S, Schlüter H, Zidek W, Deng MC, Scheld HH, Schmitz W: Negative chronotropic and inotropic effects exerted by diadenosine hexaphosphate (Ap_6A) via A1-adenosine receptors. *Br J Pharmacol* 119:835-844, 1996

152. van der Giet M, Khattab M, Börgel J, Schlüter H, Zidek W: Differential effects of diadenosine phosphates on purinoceptors in the rat isolated perfused kidney. *Br J Pharmacol* 120:1453-1460, 1997
153. van der Giet M, Jankowski J, Schlüter H, Zidek W, Tepel M: Mediation of the vasoactive properties of diadenosine tetraphosphate via various purinoceptors. *J Hypertens* 16:1939-1943, 1998
154. van der Giet M, Cinkilic O, Jankowski J, Tepel M, Zidek W, Schlüter H: Evidence for two different P2X-receptors mediating vasoconstriction of Ap_5A and Ap_6A in the isolated perfused rat kidney. *Br J Pharmacol* 127:1463-1469, 1999
155. Malam-Souley R, Seye C, Gadeau AP, Loirand G, Pillois X, Campan M, Pacaud P, Desgranges C: Nucleotide receptor P2u partially mediates ATP-induced cell cycle progression of aortic smooth muscle cells. *J Cell Physiol* 166:57-65, 1996
156. Erlinge D, You J, Wahlestedt C, Edvinsson L: Characterisation of an ATP receptor mediating mitogenesis in vascular smooth muscle cells. *Eur J Pharmacol* 289:135-149, 1995
157. Schlüter H, Gross I, Bachmann J, Kaufmann R, van der Giet M, Tepel M, Nofer JR, Assmann G, Karas M, Jankowski J, Zidek W: Adenosine(5') oligophospho-(5') guanosines and guanosine(5') oligophospho-(5') guanosines in human platelets. *J Clin Invest* 101:682-688, 1998
158. Schulze-Lohoff E, Zanner S, Ogilvie A, Sterzel RB: Vasoactive diadenosine polyphosphates promote growth of cultured renal mesangial cells. *Hypertension* 26:899-904, 1995
159. Pintor J, Gualix J, Miras-Portugal MT: Dinucleotide receptor modulation by protein kinases (protein kinases A and C) and protein phosphatases in rat brain synaptic terminals. *J Neurochem* 68:2552-2557, 1997
160. Mateo J, Miras-Portugal MT, Rotllan P: Ecto-enzymatic hydrolysis of diadenosine polyphosphates by cultured adrenomedullary vascular endothelial cells. *Am J Physiol* 273:C918-927, 1997
161. von Drygalski A, Ogilvie A: Ecto-diadenosine 5',5'''-P_1,P_4-tetraphosphate (Ap_4A)-hydrolase is expressed as an ectoenzyme in a variety of mammalian and human cells and adds new aspects to the turnover of Ap_4A. *Biofactors* 11, 2000
162. Safrany ST, Ingram SW, Cartwright JL, Falck JR, McLennan AG, Barnes LD, Shears SB: The diadenosine hexaphosphate hydrolases from Schizosaccharomyces pombe and Saccharomyces cerevisiae are homologues of the human diphosphoinositol polyphosphate phosphohydrolase. Overlapping substrate specificities in a MutT-type protein. *J Biol Chem* 274:21735-21740, 1999
163. Petersson J, Zygmunt PM, Hogestatt ED: Characterization of the potassium channels involved in EDHF-mediated relaxation in cerebral arteries. *Br J Pharmacol* 120:1344-1350, 1997
164. Bryan RMJr, You J, Golding EM, Marrelli SP: Endothelium-derived hyperpolarizing factor: a cousin to nitric oxide and prostacyclin. *Anesthesiology* 102:1261-1277,2005
165. Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, Nagao T, Egashira K, Fujishima M, Takeshita A: The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J Cardiovasc Pharmacol* 80:703-711, 1996
166. Coleman HA, Tare M, Parkington HC: Endothelial potassium channels, endothelium-dependent hyperpolarization and the regulation of vascular tone in health and disease. *Clin Exp Pharmacol Physiol* 31:641-649;2004

167. Yao X, Kwan HY, Dora KA, Garland CJ, Huang Y: A mechanosensitive cation channel in endothelial cells and its role in vasoregulation. *Biorheology* 40:23-30, 2003
168. Carafoli E: Intracellular calcium homeostasis. *Annu Rev Biochem* 56:395-433, 1987
169. Carafoli E: Calcium signaling: a tale for all seasons. *Proc Natl Acad Sci USA* 99:1115-1122, 2002
170. Carafoli E: Calcium Pump of the Plasma Membrane. *The American Physiological Society* 71:129-146, 1991
171. Carafoli E, Longoni S: The plasma membrane in the control of the signaling function of calcium. *Soc Gen Physiol Ser* 42:21-29, 1987
172. Lee CH, Poburko D, Kuo KH, Seow CY, van Breemen C: Ca^{2+} oscillations, gradients, and homeostasis in vascular smooth muscle. *Am J Physiol Heart Circ Physiol* 282:H1571-1583, 2002
173. Jelicks LA, Gupta RK: NMR measurement of cytosolic free calcium, free magnesium, and intracellular sodium in the aorta of the normal and spontaneously hypertensive rat. *J Biol Chem* 265:1394-1400, 1990
174. Sada T, Koike H, Ikeda M, Sato K, Ozaki H, Karaki H: Cytosolic free calcium of aorta in hypertensive rats. Chronic inhibition of angiotensin converting enzyme. *Hypertension* 16:245-251, 1990
175. Oshima T, Young EW, McCarron DA: Abnormal platelet and lymphocyte calcium handling in prehypertensive rats. *Hypertension* 18:111-115, 1991
176. Bendhack LM, Sharma RV, Bhalla RC: Altered signal transduction in vascular smooth muscle cells of spontaneously hypertensive rats. *Hypertension* 19:II142-148, 1992
177. Arvola P, Ruskoaho H, Wuorela H, Pekki A, Vapaatalo H, Porsti I: Quinapril treatment and arterial smooth muscle responses in spontaneously hypertensive rats. *Br J Pharmacol* 108:980-990, 1993
178. Laville M: Hypertension in patients with chronic renal disease. *Rev Prat* 54:641-645, 2004
179. Salusky IB, Goodman WG: Cardiovascular calcification in end-stage renal disease. *Nephrol Dial Transplant* 17:336-339, 2002
180. Boero R, Guarena C: Pathogensis of arterial hypertension in chronic uraemia: The role of reduced Na^+/K^+-ATPase activity. *Hypertension Suppl* 6:363-365, 1988
181. Shalev O: Decreased erythrocyte Ca^{2+}/Mg^{2+}-ATPase activity in hemodialyzed uremic patients. Renal Failure 13:27-30, 1991
182. Erne P, Bolli P, Burgisser E, Buhler FR: Correlation of platelet calcium with blood pressure. Effect of antihypertensive therapy. *N Engl J Med* 26:1084-1088, 1984
183. Lamb FS, Moreland RS, Webb RC: Calcium and contractile responses to ouabain and potassium-free solution in aortae from spontaneously hypertensive rats. *J Hypertens* 6:821-828, 1988
184. Dominiczak AF, Bohr DF: Cell membrane abnormalities and the regulation of intracellular calcium concentration in hypertension. *Clin Sci* 79:415-423, 1990
185. Zidek W, Rustemeyer T, Schlüter W, Karas M, Kisters K, Graefe U: Isolation of an ultrafilterable Ca^{2+}-ATPase inhibitor from the plasma of uraemic patients. *Clin Sci* 82:659-665, 1992
186. Jankowski J, Luftmann H, Tepel M, Leibfritz D, Zidek W, Schlüter H: Characterization of dimethylguanosine, phenylethylamine, and phenylacetic acid as inhibitors of Ca^{2+} ATPase in end-stage renal failure. *J Am Soc Nephrol* 9:1249-1257, 1998
187. Vanholder R, Glorieux G, De Smet R, Lameire N: New insights in uremic toxins. *Kidney Int Suppl*:S6-10, 2003

188. Salusky IB, Goodman WG: Cardiovascular calcification in end-stage renal disease. *Nephrol Dial Transplant* 17:336-339, 2002
189 London GM: Cardiovascular disease in chronic renal failure: pathophysiologic aspects. *Semin Dial* 16:85-94, 2003
190. Barenbrock M, Schäfer RM: Hämodialyse, Hämofiltration und Hämodiafiltration, in *Dialyseverfahren in Klinik und Praxis.* Eds: Hörl W, Wanner C, Stuttgart, Thieme Verlag, Stuttgart, Germany, 2004, pp 37-49
191. Vienken J, Diamantoglou M, Henne W, Nederlof B: Artificial dialysis membranes: from concept to large scale production. *Am J Nephrol* 19:355-362, 1999
192. Vychytil A, Haag-Weger M: Kontinuierliche ambulante und automatisierte Peritonealdialyse, in *Dialyseverfahren in Klinik und Praxis.* Eds: Hörl W, Wanner C, Stuttgart, Thieme Verlag, 2004, pp 69-106
193. Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, Clark W, Cohen G, De Deyn PP, Deppisch R, Descamps-Latscha B, Henle T, Jorres A, Lemke HD, Massy ZA, Passlick-Deetjen J, Rodriguez M, Stegmayr B, Stenvinkel P, Tetta C, Wanner C, Zidek W: Review on uremic toxins: classification, concentration, and interindividual variability. *Kidney Int* 63:1934-1943, 2003
194. Weissinger EM, Kaiser T, Meert N, De Smet R, Walden M, Mischak H, Vanholder RC: Proteomics: a novel tool to unravel the patho-physiology of uraemia. *Nephrol Dial Transplant* 19:3068-3077, 2004
195. Locatelli F, Di Filippo S, Manzoni C: Removal of small and middle molecules by convective techniques. *Nephrol Dial Transplant* 15 Suppl 2:37-44, 2000

A.2. Isolation of biomolecules by chromatographic methods

For purification of biomolecules (such as proteins and peptides), traditionally a combination of different types of chromatographic methods have been used, such as reversed phase chromatography, ion-exchange chromatography, affinity chromatography, size-exclusion chromatography and displacement chromatography[1]. Recently, a great deal of effort has been made to improve and optimise surface properties of particles used as chromatographic matrices. This led to the development of the different chromatographic modes of ion-exchange chromatography, reversed-phase chromatography, and affinity chromatography based on non-porous media[2].

A.2.1. Reversed-phase chromatography

Reversed-phase chromatography is a separation of molecules based on their hydrophobicity. Hydrophilic molecules elute first from a reversed-phase column, whereas strongly hydrophobic molecules elute last. The most common chromatographic mode used for reversed-phase separations of biomolecules is a gradient mode, using two different mobile phases. In this operational mode the sample, dissolved in a hydrophilic and typically aqueous eluent, will be injected onto a column, which is equilibrated with the same hydrophilic eluent. Gradient elution allows the separation of a complex mixture of components that exhibit a broad range of retention in a single run. A hydrophobic molecule will bind to the column, because the affinity of the molecule to the hydrophobic stationary phase is larger than to the hydrophilic mobile phase.

After the sample is loaded onto the stationary phase, the concentration of a hydrophobic eluent is continuously increased. This continuous increase of the eluent is named “gradient elution”. The molecule elutes when the affinity of the molecule to the liquid phase is stronger than that to the stationary phase. When ionic solutes are to be separated, the addition of a counter-ion (ion pair reagent) will improve the retention of the ionic species. All types of molecules with hydrophobic moieties can be chromatographed with a reversed-phase gel.

Full knowledge of the processes underlying biomolecule separation is most desirable but in practice rarely attainable. Molecules are separated in reversed-phase chromatography by partitioning in the mobile phase and stationary phase. The partitioning is governed by equilibrium, specific for the solute interacting with the mobile phase and stationary phase. As the mobile phase moves the solute down the column, there is a constant movement of the solute

from the mobile phase to the stationary phase and vice versa. The more hydrophobic the solute, the higher its affinity to the stationary phase, the more time it spends in this stationary phase and the later it leaves the column. As a result, molecules with different equilibrium constants elute at different times and are separated.

The reason for the almost exclusive use of gradient elution in the separation of biomolecules is that the retention mechanism of large biomolecules is different from that of small molecules, due to the amphiphilic nature of the larger. In contrast to small molecules, biomolecules interact with the stationary phase via only a hydrophobic part of the molecular structure, whereas the more hydrophilic remainder of the molecule is in contact with the mobile phase. These large biomolecules desorb from the stationary phase when the concentration of the organic eluent reaches a particular concentration. Before this particular concentration of the organic eluent component is reached, the large biomolecules are adsorbed nearly completely by the stationary phase. After this concentration, the equilibrium is modified towards a complete shift of the biomolecules with the mobile phase.

Ionic hydrophobic substances exhibit an increase in retention time with an increase in the counter-ion hydrophobicity. The higher the number of positively charged groups present in a molecule, the longer will be the retention time[3]. Very hydrophilic substances may only be bound to the sorbent with ion-pair reagents, such as trifluoracid acid (TFA) or tetrabutylammonium salts. Furthermore, selectivity varies with different buffers or ion-pairing agents, and the resolution of critical sample components may be better with alternative buffers instead of the more commonly used TFA.

Acidic ion-pair reagents are used at low concentrations because low levels help to prolong column life through decreased acidity in the mobile phase. With increasing concentrations of the ion-pairs, the retention time of molecules increases[4]. Triethylammonium acetate as ion-pairing reagent is useful to increase the retention of anionic molecules[5]. In addition, triethylammonium acetate is volatile and therefore an alternative to TFA.

A.2.2. Ion exchange chromatography

Ion exchange matrices were originally designed for the separation of small, charged molecules[6]. Nowadays, *ion exchange gradient chromatography* is probably the most widely used liquid chromatographic method for the separation of biomolecules[7]. Generally, adsorption

and desorption processes in ion exchange chromatography are determined by the properties of the three interacting entities: the stationary phase, the constituents of the mobile phase and the solute whereby the former two predominantly contribute to the great variability in the design of ion-exchange-chromatography experiments. On the one hand, numerous different stationary phases are available, and on the other hand, infinite variations in the composition of the mobile phase are possible. However, although constant in its composition, the biomolecule itself can also to a limited degree be considered as a variable parameter because the pH-value and additives of the mobile phase can alter the surface properties of a biomolecule, such as the charge density and the accessibility or relative location of charged residues.

A.2.3. Affinity chromatography

Affinity chromatography separates elements on the basis of a reversible interaction between a molecule and a specific ligand coupled to a chromatographic matrix. Biological interactions between ligand and target molecule can be the result of electrostatic or hydrophobic interactions, van der Waals forces and/or hydrogen bonding. To elute the target molecule from the affinity medium the interaction can be reversed, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. In a single step, affinity purification can offer immense time-saving over less selective multi-step procedures. For higher degrees of purification, or when there is no suitable ligand for affinity purification available, an efficient multi-step process has to be developed, however, using the purification strategy of capture, intermediate purification and polishing[8].

A.2.4. Size exclusion chromatography

For *size exclusion chromatography* porous stationary phases with defined pore diameters are required. Separation depends on differences in size of the biomolecules in a sample. In size exclusion chromatography, biomolecules are separated according to their molecular size in solution. The main applications of size exclusion chromatography are: separation of biomolecules differing in size, determination of average molecular weights, determination of hydrodynamic diameters, and separation of biomolecules from small molecules, e.g. for desalting. Size exclusion chromatography is especially useful as a preliminary isolation procedure to separate a vast amount of impurities from the components of interest and/or as a final step in the separation of homogeneous biomolecules from their aggregates. The larger the size of a biomolecule, the smaller the amount of accessible pore volume and the earlier the biomolecule is eluted. The separation is finished when the smallest molecule, usually the eluent

molecule, is eluted. Molecules that are larger than the largest pore diameter cannot penetrate into the pores and pass through the column first. They are eluted with the interstitial volume of the column, i.e. the dead or void volume in size exclusion chromatography, while the smallest molecule is eluted with the total volume of the mobile phase of the column[9].

A.2.5. Displacement chromatography

Displacement chromatography can be used with every type of chromatographic medium that utilises adsorption processes for the separation. In displacement chromatography the separation is driven by the competition of the solutes for the binding sites of the sorbent. The sample components are forced to move down and at last elute from the column by the displacer, which ideally binds more strongly to the sorbent than any sample component.

During the separation process, the sample components, driven by the displacer into adjacent homogeneous zones, move at the same velocity as the displacer front. The displacement chromatography is finished when the displacer has saturated the sorbent completely. The order of elution corresponds to the affinity of each sample component to the column. In frontal chromatography the purification of a component, which has no or only a low affinity to a sorbent, is achieved by adsorbing the other sample components to the column. The component of interest elutes first, whereas all other components elute later. All molecules separable by any kind of adsorption chromatography can also be chromatographed by displacement chromatography. Displacement chromatography of biomolecules has been performed with cation exchange, anion exchange, immobilized metal ion affinity chromatography and reversed-phase chromatography[10].

A.2.6. References

1. Janson J, Jönsson J: Introduction to chromatography. In: *Protein Purification: Principles, high resolution methods, and applications*, Eds: Janson, Rydén, Wiley-VCH, New York, 1998, pp 45-60
2. Kruip J: Chromatography on porous matrices. In: *Protein Liquid Chromatogaphy*, Eds: Kastner M, Elsevier, Amsterdam, Netherlands, 2000, pp 785-824
3. Dass C, Mahalakshmi P, Grandberry D: Manipulation of ion-pairing reagents for reversed-phase high-performance liquid chromatographic separation of phosphorylated opioid peptides from their non-phosphorylated analogues. *J Chromatogr A* 678:249-257, 1994
4. Guo DC, Mant CT, Hodges RS: Effects of ion-pairing reagents on the prediction of peptide retention in reversed-phase high-performance liquid chromatography. *J Chromatogr* 386:205-222, 1987

5. Winkler, Briza, Kunz: Spectral properties of some ion-pairing reagents commonly used in reversed-phase high-performance liquid chromatography of peptides and proteins in acetonitrile gradient systems. *J Chromatogr A* 361:191-198, 1986
6. Yamamoto S, *Ion-exchange chromatography of proteins near the isoelectric points.* J Chromatogr A 852: 31-6, 1999
7. Karlsson E, Rydén L, Brewer J: Ion exchange chromatography, In: *Protein Purification. Principles, high resolution methods, and applications*. Eds: Janson, Rydén, Wiley-VCH, New York, 1998, pp 145-205
8. Gagnon P: Protein A affinity purification, In: *Purification Tools of mononuclear Antibodies*. Eds: Gagnon P, *Validated Biosystem*, Tucson 1996, pp 158-165
9. Rögner M: Size Exclusion Chromatography, In: *Protein Liquid Chromatogaphy*, Eds: Kastner M, Elsevier, Amsterdam, Netherlands, 2000, pp 89-143
10. Schlüter H, Jankowski J: Displacement chromatography, In: *Protein Liquid Chromatogaphy*, Eds: Kastner M, Elsevier, Amsterdam, Netherlands, 2000, pp 505-522

A.3. Identification of biomolecules by mass-spectrometric methods

During the two past decades, important achievements in bioorganic mass-spectrometry have been made by the development of new ionisation techniques for the analysis of biomolecules. Traditional mass-spectrometric methods, which proved useful for analysing compounds with low molecular mass, were of little use for measuring underivatised compounds with high molecular mass. The general problem to be solved was to convert polar, non-volatile biomolecules into intact, isolated ionised molecules in the gas-phase. The new, innovative mass-spectrometric methods overcome these problems.

A.3.1. Gas-chromatography / mass-spectrometry (GC/MS)

The *GC/MS instrument* (schematic drawing shown in Figure 1) represents a device that separates chemical mixtures and detects mass-signals by a mass-detector[1-4]. Once the sample solution is introduced into the inlet of the gas-chromatograph (GC), it is vaporized immediately because of the high temperature (250 °C) and swept onto the column by the carrier gas. The sample compounds are separated while flowing through the column and undergoing the separation processes, which are specific for GC.

As the sample components emerge from the column opening, they enter the ionisation chamber of the mass-spectrometer (MS) through a capillary. In general, the ions are produced by electron impact (EI). For the EI ionisation a collimated beam of electrons impact the sample molecules causing the loss of an electron from the molecule. A molecule with one missing electron is represented by M^+ and is called the molecular ion or parent ion. When the resulting mass signal from this ion is detected by the mass-spectrum, it gives the molecular weight of the compound.

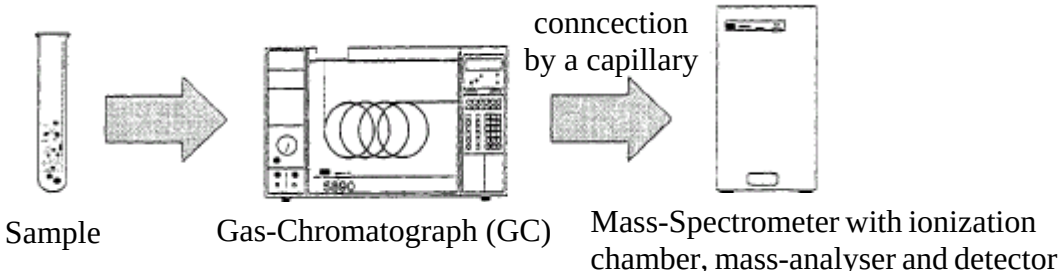

Figure 1: Schematic drawing of a gas-chromatography / mass-spectrometry

Some of the molecular ions fragment into smaller daughter ions and neutral fragments. Both positive and negative ions are formed, but only positively charged species will be detected.

The fragmentation of the ions yields information about the detailed structure of a molecule. The next component is a mass analyser, which separates the positively charged particles according to their mass. Several types of separating techniques exist; the most common are quadrupoles and ion traps. After the ions have been separated according to their masses, they enter a detector and then move onto an amplifier to boost the signal. The detector sends information to a computer. The latter records all the data produced, converts the electrical impulses into visual and hard-copy displays, and also drives the mass-spectrometer. Identification of a compound based on its mass spectrum relies on the fact that every compound has a unique fragmentation pattern. Even isomers can be differentiated if the operator is experienced. Generally, more information is generated than could possibly be used. A library of known mass spectra, which may contain several thousands of compounds, is stored in the computer and may be searched using computer algorithms to assist the analyst in identifying the unknown compound.

A.3.2. Matrix-assisted laser desorption/ionisation mass-spectrometry (MALDI)

The *matrix-assisted laser desorption/ionisation (MALDI)*[5] makes use of short, intense pulses of laser light to induce the formation of intact gaseous ions. The MALDI mass-spectrometer (schematic drawing shown in Figure 2) has been demonstrated to be capable of mass spectrometric analysis of biomolecules in the molecular mass range between 500 Da and a few hundred thousand daltons.

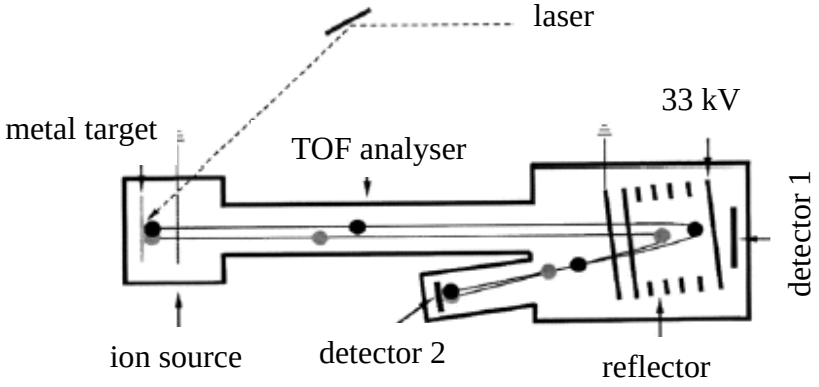

Figure 2: Schematic drawing of a laser desorption/ionisation mass-spectrometer

In MALDI mass-spectrometry ions are generated in the ion source by using a laser. For the required irradiances in the range of 10^6 to 10^7 W/cm^2, the laser beams are focussed to values between 30 and 500 µm by suitable optical lenses. The substances to be analysed and the ma-

trices are co-crystallized on a metal target. The matrix serves two major functions: (1) absorption of energy from the laser light and transfer into excitation energy of the solid system; thereby an instantaneous phase transition of a small volume of the sample to gaseous species is induced; in this way, the analyte molecules are desorbed together with matrix molecules; (2) ionisation of the analyte biomolecules by photoexcitation or photoionisation of matrix molecules, followed by proton transfer to the analyte molecule. The latter process has not been proven unequivocally to date, however.

Conventional MALDI-spectrometers mainly use a time-of-flight-(TOF)- analyser. In the TOF analyser, the mass to charge ratio is determined by measuring their flight time. After ions are accelerated in the ion source to a fixed kinetic energy, they pass the field free drift tube with a velocity proportional to their molecular mass. Due to their mass-dependent velocity, ions are separated during their flight. The detector at the end of the flight tube (labelled as detector 1 in Figure 2) produces a signal for each ion species. The molecular mass is calculated from the time-of-flight-signal. The differences in velocity of ions with identical molecular masses are minimized using a reflector unit. The molecular masses of the reflected ions are detected by detector-2 (Figure 2).

Moreover, MS/MS data can be accumulated by the MALDI-TOF. To accumulate MS/MS data, first of all the ions of interest are separated in the ion source. These ions fragment during the flight in the TOF analyser. This process is called post-source decay (PSD). The reflector separates these fragment ions regarding their molecular masses and the detector at the end of the flight tube (labelled as detector 2 in Figure 2) produces a signal for each fragment ion. The molecular mass of the fragment ions is calculated from the time needed for the flight from the reflector to detector 2.

A.3.3. TOF-TOF mass-spectrometry

TOF-TOF mass-spectrometry is based on a new mass spectrometer design (schematic drawing shown in Figure 3) that overcomes many of the limitations of post-source decay (PSD) MALDI-TOF mass-spectrometry[6]. The TOF-TOF instrument combines the advantages of high sensitivity for biomolecule analysis associated with MALDI and comprehensive fragmentation information provided by collision-induced dissociation. Unlike the post-source decay technique that is widely used with MALDI-TOF instruments and typically combines 10-

14 separate spectra of different mass regions for one MS/MS spectrum, the TOF-TOF instrument allows complete fragment ion spectra to be obtained in a single acquisition.

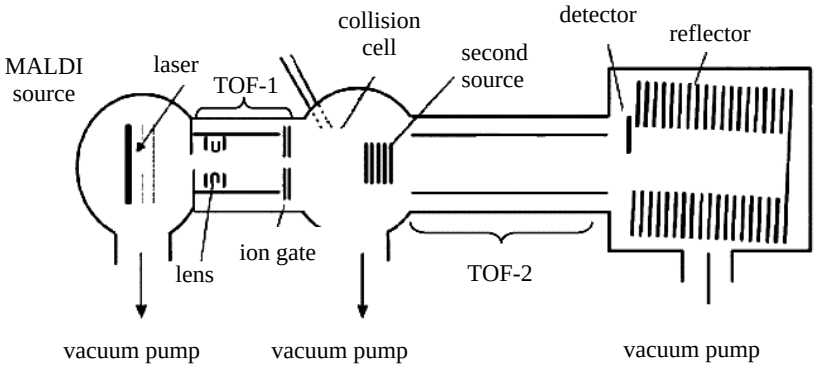

Figure 3: Schematic drawing of a TOF-TOF mass-spectrometer

The MALDI TOF-TOF is similar in geometry to a conventional high quality reflectron MALDI TOF[7] when operating the instrument in both MS and MS/MS modes; however, there are some differences in the ion optics between the TOF-TOF and the MALDI TOF reflectron instrument. The substantial difference between the conventional reflectron MALDI TOF and the TOF-TOF is the use of a high-resolution reflector that permits better focusing across the broad energy range of fragments in the MS/MS mode without negative consequences in the MS mode. The MS/MS capabilities of the instrument are facilitated through the addition of the optical elements of the second source region of Figure 3: the lens, ion gate and collision cell. When the instrument is operated in MS/MS mode, the first source is operated as a linear TOF-MS. The ion gate is operated as a double-sided deflection gate, with the first gate acting as the low mass gate and the second gate as the high mass gate.

When the laser fires, the low mass gate is “on,” and the high mass gate is “off;” all ions, which enter the region of the timed ion selectors, are deflected from the optical axis of the instrument. Using the precursor ion mass, the instrument geometry, and the operating voltages, the arrival time of the precursor ion to each of the gates is calculated. When the precursor enters the plane of the low mass gate, the voltage applied to the deflecting electrodes is rapidly switched off and the precursor trajectory is unaffected.

After the precursor has passed through both electrodes, the high mass gate is switched on and the remaining ions are deflected from the axis of the spectrometer. The width of the precursor mass window typically functions to include the desired precursor mass and its isotope cluster. Collision energy in the TOF-TOF is defined by the potential difference between the source acceleration voltage and the floating collision cell. All fragments formed from the ion of interest in this region move with essentially the same velocity as the precursor, and thus enter the second source at the same time as the precursor ion. When the collection of precursor and fragment ions has entered the second source, a high voltage pulse is applied to the source and the ions are accelerated towards the detector. The firing of the second source serves as the starting point (t= 0) for the recording of the fragment mass spectrum of the substance of interest. The molecular masses of the fragment ions are calculated from the time of flight through the second TOF tube to the detector.

A.3.4. Fourier-Transform Ion Cyclotron Resonance mass-spectrometry (FT-MS)

Fourier-Transform Ion Cyclotron Resonance mass-spectrometry (FT-MS) [8] (schematic drawing shown in Figure 4) has received attention for its ability to perform mass measurements with a very high resolution and accuracy. Interest in FT-MS for biomolecules arose since MALDI has been used for ionization[9]. The FT-ICR mass spectrometer consists of three main sections. The first section is the sample source, which can be any of the available techniques, although MALDI is the most common. The second section is the ion transfer region, where the molecules of interest are focused, extracted by a hexapole filter and guided by a quadrupole ion guide into the third part of the FT-ICR instrument.

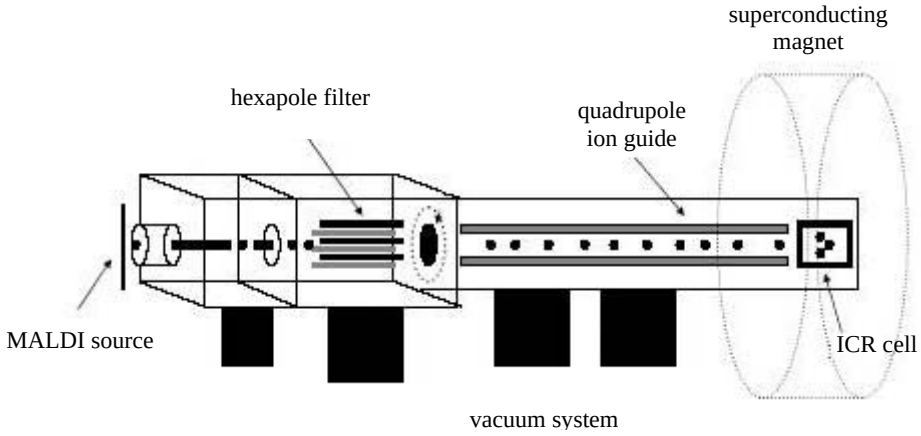

Figure 4: Schematic drawing of a Fourier-Transform Ion Cyclotron Resonance mass-spectrometer

This third part is the main section of the FT-ICR mass-spectrometer and consists of three components: a vacuum system, a superconducting magnet and an ICR cell. After ionisation, the ions are trapped in the ion cyclotron resonance cell (ICR cell), which is situated in the homogeneous region of a superconducting magnet. The FT mass-spectrometry is based on the ion cyclotron resonance principle. In the magnetic field (B) the ions with a charge (e) and of a velocity (v) are constrained to move in circular orbits. They are submitted to the Lorentz force $F_L=ev \times B$. This force is directed towards the centre of the cyclotron orbit, which is counter-balanced by an outward directed centrifugal force. The cyclotron frequency can be calculated from the angular velocity. The cyclotron frequency increases with increasing magnetic field strength but decreases with increasing mass. The ICR frequency is independent of the velocity of the ions, which is one of the fundamental reasons why FT-ICR mass spectrometers are able to achieve ultra-high resolution. Ions moving parallel to the magnetic field are not influenced by the field. The ions are trapped in an ICR cell. For excitation an oscillating electric field is applied, which is transmitted by a sine wave signal generator. If the frequency of the oscillating field equals the cyclotron frequency, all ions with a particular mass-to-charge ratio (m/z) are steadily and coherently accelerated to a larger orbit radius. After excitation, these ions move as a single ion packet on an orbit with a radius, which is independent of the original velocity of the ions. In FT-MS usually all ions of different m/z are excited and detected simultaneously. A composite transit signal is obtained that represents a time-domain spectrum, i.e. the signal intensity is recorded versus time. In order to get the frequency components representing each mass-to-charge ratio (m/z) of the ion in the ICR cell from this spectrum, it is converted into a frequence-domain spectrum by Fourier-transformation (FT).

A.3.5. Advantages and disadvantages of the mass-spectrometric methods

Table.1 gives an overview of the advantages and disadvantages of the mass-spectrometric methods described above. In general, one single mass-spectrometric method is not sufficient for the identification of a biomolecule. Therefore, the combination of different mass-spectrometric methods is recommended.

Table 1: Advantages and disadvantages of above-mentioned mass-spectrometric methods

mass-spectrometric method	advantages / disadvantages
GC/MS	**Advantage:** robust method **Disadvantage:** only for gaseous or evaporated substances
MALDI	**Advantages**: mass range: 300,000 Da Sensitivity: low femtomoles soft ionization: low fragmentation tolerance of low salt concentration analysis of inhomogenous samples possible sample vaporisation not necessary **Disadvantages**: less resolution matrix background actinic degradation
TOF-TOF	**Advantages**: high sensitivity: low femtomoles soft ionisation: low fragmentation analysis of inhomogeneous samples Sensibility higher than MALDI *de novo* sequencing possible high mass accuracy **Disadvantages**: high investment costs large size not for continuous ionisation source
FT-ICR	**Advantages**: highest recorded mass resolution of all mass spectrometers accurate mass measurement accumulation time unlimited MS/MS analysis possible **Disadvantages**: high investment costs large size not a high throughput technique

A.3.6. References

1. Wilkins CL: Hyphenated techniques for analysis of complex organic mixtures. *Science* 222:291-296, 1983
2. Wilkins CL: Linked gas chromatography infrared mass-spectrometry. *Anal Chem* 59:571A-581A, 1987
3. Yamakawa K, Nishitani K: Historical study of separation technology innovation in the field of pharmaceutical science. *Yakushigaku Zasshi* 38:28-41, 2003
4. Message G, M: Gaschromatography methods and techniques relevant to GC/MS in: *Practical aspects of gas-chromatography/mass-spectropmetry*. Wiley New York, 1984, pp 167-183
5. Hillenkamp F, Karas M, Beavis RC, Chait BT: Matrix-assisted Laser Desorption/Ionization Mass-spectrometry. *Anal Chem* 63:1196A-1203A, 1991
6. Medzihradszky KF, Campbell JM, Baldwin MA, Falick AM, Juhasz P, Vestal ML, Burlingame AL: The characteristics of peptide collision-induced dissociation using a high-performance MALDI-TOF/TOF tandem mass spectrometer. *Anal Chem* 72:552-558, 2000
7. Juhasz P, Roskey MT, Smirnov IP, Haff LA, Vestal ML, Martin SA: Applications of delayed extraction matrix-assisted laser desorption ionization time-of-flight mass-spectrometry to oligonucleotide analysis. *Anal Chem* 68:941-946, 1996
8. Marshall AG, Hendrickson CL, Jackson GS: Fourier transform ion cyclotron resonance mass-spectrometry: a primer. *Mass Spectrom Rev* 17:1-35, 1998
9. McIver J, Robert T, Li Y, Hunter RL: High-resolution laser desorption mass-spectrometry of peptides and small proteins. *Proc Natl Acad Sci* 91:4801-4805, 1994

A.4. Aim of the thesis

In summary, many *factors* and *pathways* influencing and regulating vascular tone and/or proliferation may be involved in the pathogenesis of hypertension in general and essential hypertension in a more specific way. Until now, none of the known factors seems to have a significant or predominant role. Moreover, the physiologic and pathophysiologic pathways remain in part not clarified.

One model where hypertension and vascular damage are preponderant in the large majority of effected patients is chronic renal failure. This thesis attempts to reveal novel mechanisms possibly at play in hypertension and vascular damage in general and in chronic renal failure.

In recent years, new and highly effective chromatographic methods for separation of biomolecules were developed and innovative mass-spectrometric methods have become available to identify the molecular structure of yet unknown compounds. Taking into account this methodological background, the aim of this thesis is (a) the identification of unknown substances with strong vasoconstrictive properties and (b) the clarification of yet unexplored physiologic and pathophysiologic pathways in hypertension.

A.5. Overview of the thesis

The isolation, identification and characterization of new biomolecules related to vascular regulation in general are described in Chapters **B.1.-B.6.**, and those with specific relevance to uraemia are described in Chapters **C.1.-C.4.**.

In **Chapter B.1.** a new chromatographic method for isolation and quantification of dinucleoside polyphosphates is described. This method was the basis for the isolation of diadenosine polyphosphates from human plasma (**Chapter B.2.**), uridine adenosine tetraphosphate (Up_4A) from endothelial cells (**Chapter B.3.**) and dinucleoside polyphosphates from adrenal glands (**ChapterB.4.**). **Chapter B.5.** describes the isolation of dinucleoside polyphosphates from human heart, emphasizing the endocrine function of heart tissue. **Chapter B.6.** and **C.1.** depict the isolation of angiotensin-II and a new angiotensin-like peptide (two peptides with strong vasoconstrictive properties) from supernatants of stimulated mononuclear leukocytes. For these studies, new chromatographic methods and MALDI-TOF-TOF-mass spectrometric analysis were applied. In **Chapter C.1.** we also describe how the newly detected angiotensin-

like peptide shows a relatively more pronounced elevation of concentration in renal failure than regular angiotensin.

By using chromatographic methods as well as MALDI-reflectron-mass spectrometry and conventional gas-chromatography/mass-spectrometry it became possible to identify as well two other substances which accumulate in chronic renal failure; these compounds are insufficiently removed by current dialytic strategies. The biochemical and pathophysiologic characteristics of these two substances are described in **Chapter C.2.** and **C.3.**. **Chapter C.4.** depicts the isolation of a potent inhibitor of iNOS using conventional gas-chromatography/ mass-spectrometry as well as new chromatographic methods. In **Chapter D.** we summarize the results of the thesis and evaluate their impact on future approaches for research in the fields of hypertension and chronic renal failure.

B. Original publications relating to the regulation of the vascular tone

B.1. Isolation and quantification of dinucleoside polyphosphates by using monolithic reversed-phase chromatography columns

Vera Jankowski, Raymond Vanholder, Lars Henning, Sevil Karadogan, Walter Zidek, Hartmut Schlüter, Joachim Jankowski

B.1.1. Abstract

In former studies, dinucleoside polyphosphates were quantified using ion-pair reversed-phase perfusion chromatography columns, which allows a detection limit in the µmolar range. The aim of this study was both to describe a chromatographic assay with an increased efficiency of the dinucleoside separation, which enables the reduction of analytical run times, and to establish a chromatographic assay using conditions, which allow MALDI-mass spectrometric analysis of the resulting fractions.

We compared the performance of conventional silica reversed-phase chromatography columns, a perfusion chromatography column and a monolithic reversed-phase C18 chromatography column. The effects of different ion-pair reagents, flow-rates and gradients on the separation of synthetic diadenosine polyphosphates as well as of diadenosine polyphosphates isolated from human platelets were analysed.

Sensitivity and resolution of the monolithic reversed-phase chromatography column were both higher than that of the perfusion chromatography and the conventional reversed-phase chromatography columns. Using a monolithic reversed-phase C18 chromatography column, diadenosine polyphosphates were separable at baseline not only in the presence of tetrabutylammonium hydrogensulfate (TBA) but also in the presence of triethylammonium acetate (TEAA) as ion-pair reagent. The later reagent is useful because, in contrast to TBA, it is compatible with MALDI mass-spectrometric methods. This makes TEAA particularly suitable for identification of unknown nucleoside polyphosphates. Furthermore, because of the lower backpressure of monolithic reversed-phase chromatography columns, we were able to significantly increase the flow rate, decreasing the amount of time for the analysis close to 50%, especially using TBA as ion-pair reagent.

J Chromatogr B 819 (1):131-139, 2005

In summary, monolithic reversed-phase C18 columns markedly increase the sensitivity and resolution of dinucleoside polyphosphate analysis in a time-efficient manner compared to reversed-phase perfusion chromatography columns or conventional reversed-phase columns. Therefore, further dinucleoside polyphosphate analytic assays should be based on monolithic silica C18 columns instead of perfusion chromatography or conventional silica reversed-phase chromatography columns.

B.1.2. Introduction

Diadenosine polyphosphates have previously been isolated from human tissues and cells such as platelets[1-6], erythrocytes[7], heart[8-10], placenta[11], and human plasma[12]. Diadenosine polyphosphates are involved as intra- and extracellular mediators in the regulation of numerous physiological functions, e.g. growth of vascular smooth muscle cells and control of vascular tone[3,5,12-14]. The book entitled "Ap_4A and other dinucleoside polyphosphates" edited by McLennan gives an excellent overview on the biology and physiology of dinucleoside polyphosphates[15]. Reviews about the role of diadenosine polyphosphates in the cardiovascular system have been published recently[16,17]. Vascular effects of Ap_nA vary with the number of phosphate groups linking the adenosine molecules[3,18,19].

Perfusion chromatography is generally used for purification of biomacromolecules[20,21]. Nevertheless, diadenosine polyphosphates are also quantified in speed-vac-dried eluates from reversed-phase chromatographies by ion-pair reversed-phase perfusion chromatography[22]. Reversed-phase gradient systems are used in order to ensure the simultaneous separation of molecules with a broad range of hydrophobicity. The chromatographic quantitation of the diadenosine polyphosphates is in general based on the method described by Brüggemann et al.[23] with tetrabutylammonium sulfate as ion-pair reagent and perfusion reversed-phase chromatography columns[21]. In contrast to the method of Brüggemann et al.[23], the flow-rate used for quantification was reduced from 1 ml min^{-1} to 300 µl min^{-1} in recent studies[12,24]. Although high flow rates are typical of perfusion chromatography, the reduction of the flow-rate increases the intensity of the peaks and thus decreases the detection limit. This approach is appropriate for the quantification of diadenosine polyphosphates if the concentration is sufficiently high as described in several publications[12,22,24,25], but fails, if the concentrations are low.

Therefore we developed based on a monolithic silica HPLC reversed-phase column two strategies: one with an increased efficiency of the dinucleoside separation for dinucleoside

polyphosphate quantification, and one for dinucleoside polyphosphate identification using conditions which allow MALDI-mass spectrometric analysis of the resulting fractions. For a broad variation of the stationary phase characteristics, we used two conventional silica reversed-phase columns, a perfusion chromatography column, and a monolithic reversed-phase chromatography column. We investigated the effects of different ion-pair reagents, gradients and flow rates on the chromatographic resolution. In particular, our attention was turned to the compatibility of the chromatographic strategy with mass spectrometry methods like MALDI-MS.

B.1.3. Materials and Methods

HPLC water (gradient grade) and acetonitrile were purchased from Merck (Darmstadt, Germany), and all other substances from Sigma-Aldrich (Taufkirchen, Germany). A porous reversed-phase column ("Poros R2/H" (50 x 4.6 mm I.D., Perseptive Biosystems, Freiburg, Germany)), a monolithic reversed-phase column ("Chromolith^{TM} SpeedROD" (50 x 4.6 mm I.D., Merck, Darmstadt, Germany)), and two conventional reversed-phase columns ((a) LiChrospher 100 RP-18e (55 x 4 mm I.D., Merck, Darmstadt, Germany); (b) Superspher 100 RP-18e (55 x 4 mm I.D., Merck, Darmstadt, Germany)) were compared.

REVERSED-PHASE CHROMATOGRAPHY WITH TRIETHYLAMMONIUM ACETATE (TEAA) AS ION-PAIR REAGENT

Diadenosine polyphosphates (Ap_nA with n=2-6; each 3µg) were separated by gradient elution on each of the four above mentioned reversed-phase columns in the presence of the ion-pair reagent triethylammonium acetate (TEAA; 40 mmol L^{-1} (final concentration)) as eluent A and water-acetonitrile (80:20, v-%/v-%) as eluent B. The column temperature was ambient (22 ± 1 °C). The mobile phase was pumped at a flow-rate of 1 ml min^{-1} by a high-pressure gradient pump system (Merck, Darmstadt, Germany). The column eluate was monitored with a variable wavelength UV detector (759 A, Absorbance Detector, Applied Biosystems, Darmstadt, Germany). The diadenosine polyphosphate mixture was dissolved in eluent A. The diadenosine polyphosphates were eluted with the following gradient: 0-2 min: 0 % eluent B, 2-62 min: 0-60 % B, 62-63 min: 60-100 % eluent B. The concentration of eluent B of 60 % corresponds to an acetonitrile concentration of 12 % in the total eluate volume. UV absorption was measured at 254 nm. Data were recorded and processed with the Chromeleon Lab System 6.0 (Dionex, Idstein, Germany).

Low backpressure is one of the important characteristic features of perfusion and monolithic reversed-phase chromatography columns in comparison to conventional silica reversed-phase chromatography columns, allowing high flow rates. To investigate the effect of increasing flow rate on the resolution, the flow rate was increased in the range between 1 ml min^{-1} up to 6 ml min^{-1}, in a separate set of experiments.

REVERSED-PHASE CHROMATOGRAPHY WITH TETRABUTYLAMMONIUM HYDROGENSULFATE (TBA) AS ION-PAIR REAGENT

To evaluate whether the ion-pair reagent would have an impact on the performance of the reversed-phase columns, the ion-pair reagent tetrabutylammonium hydrogensulfate (TBA) was used instead of TEAA. Diadenosine polyphosphates Ap_nA (with n=2-6; each 3 µg) were separated by gradient elution on each of the four above mentioned reversed-phase columns in the presence of ion-pair reagent 2 mmol L^{-1} tetrabutylammonium hydrogensulfate in a phosphate buffer (10 mmol L^{-1} K_2HPO_4, final concentration; pH 6.8) as eluent A and water-acetonitrile (20:80, v-%/v-%) as eluent B. The phosphate buffer was necessary to adjust the pH of the ion-pair reagent tetrabutylammonium hydrogensulfate solution to a value of 6.8. The column temperature was ambient (22 ± 1 °C). The diadenosine polyphosphates were eluted with the following gradient: 0 min: 100 % eluent A, 0-30 min: 0- 45 % B; 30-33 min: 45-100 % eluent B; 33-36 min: 100 % B. The concentration of eluent B of 45 % corresponds to an acetonitrile concentration of 36 %. All other experimental conditions were identical as described above. To investigate again the effect of the flow rate on the resolution, the flow rate was increased in the range between 1 ml min^{-1} up to 6 ml min^{-1}.

ISOLATION OF DINUCLEOSIDE POLYPHOSPHATES FROM HUMAN PLATELETS

Dinucleoside polyphosphates were isolated from human platelets as described elsewhere[2,5]. Briefly, human platelets were washed with an isotonic solution of NaCl and centrifuged (4,000 rpm, 4°C, 10 min) twice. The supernatant was aspirated and the pellets frozen to -30°C. The platelet pellets were rethawed in double distilled water (10 ml). The resulting suspension as well as the washing solution was deproteinized with 0.6 mol L^{-1} (final concentration) perchloric acid and centrifuged (4,000 rpm, 4°C, 5 min). After adjusting pH to 7.0 with 5 mol L^{-1} KOH the precipitated proteins and $KClO_4$ were removed by centrifugation (4,000 rpm, 4°C, 5 min). 1 mol L^{-1} triethylammonium acetate (TEAA) was added to the supernatant up to a final concentration of 40 mmol L^{-1}. Supernatant was concentrated on a preparative reversed-phase column (LiChroprep RP-18 B, Merck, Darmstadt, Germany) in the presence of

the ion-pair reagent triethylammonium acetate (TEAA; 40 mmol L^{-1} (final concentration)) as eluent A and water-acetonitrile (80:20, V/V) as eluent B. The column temperature was ambient (22 ± 1 °C). The mobile phase was pumped at a flow-rate of 5 ml min^{-1} by a high-pressure gradient pump system (Merck, Darmstadt, Germany). The diadenosine polyphosphates were eluted with a stepwise gradient. The lyophilized eluate of the reversed-phase chromatography dissolved in 1 mol L^{-1} ammonium acetate (pH 9.5) was loaded to a phenyl boronic acid resin. The resin was prepared according to Barnes et al.[26]. The adsorbed substances were eluted with 1 mmol L^{-1} HCl (flow rate: 1 ml min^{-1}). The eluate from the phenyl boronic acid resin to which 1 mol L^{-1} TEAA was added to a final concentration of 40 mmol L^{-1} was desalted by a reversed-phase chromatography (LiChroprep RP-18 B, Merck, Darmstadt, Germany; equilibration and sample buffer: 40 mmol L^{-1} TEAA in water; flow rate: 5 ml min^{-1}). The lyophilized eluate was used for reversed-phase chromatography using the four different reversed-phase columns.

MATRIX ASSISTED LASER DESORPTION/IONISATION MASS SPECTROMETRY (MALDI-MS) and POST-SOURCE DECAY (PSD)-MALDI-MS

The identity of the diadenosine polyphosphates was confirmed by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS)[27] and post-source decay (PSD)-MALDI-MS[28]. A reflectron-type time-of-flight (RETOF) mass spectrometer (Reflex III, Bruker-Daltronic, Bremen Germany) was used according to Hillenkamp et al.[27]. The sample was mounted on x, y, z movable stage allowing for irradiation of selected sample areas. In this study, a nitrogen laser (Laser Science Inc., Franklin, MA, USA) with an emission wavelength of 337 nm and 3 ns pulse duration was used. Typically, the laser beam was focused to a diameter of 50 µm at an angle of 45° to the surface of the target. Microscopic sample observation was possible via a diachronic mirror in the beam path. 10-20 single spectra were accumulated for a better signal-to-noise ratio. In MALDI-MS large fractions of the desorbed analyte ions undergo post-source decay (PSD) during flight in the field free drift path[28]. Using a RETOF set-up, sequence information from PSD fragment ions of precursors produced by MALDI were obtained. Sample preparations for MALDI- and PSD-MALDI experiments were identical. The concentrations of the analysed dinucleoside polyphosphates were 1-10 µmol L^{-1} in bidistilled water. 1 µl of the analyte solution was mixed with 1 µl of the matrix solution (50 mg ml^{-1} 3-hydroxy-picolinic acid in water). The mixture was gently dried on an inert metal surface before introduction into the mass spectrometer. For calibration of the mass

spectra, synthetic diadenosine hexaphosphate (Ap_6A) was used as external standard. The mass accuracy was in the range of 0.05 %.

B.1.4. Results and Discussion

Besides the broad variation of the experimental chromatographic conditions like flow rate, ion-pair reagent, this study focused on the broad variation of the stationary phase characteristics. The chromatographic conditions used for testing the different chromatography columns were identical –within the limits of the column backpressure.

We compared the performance of conventional silica reversed-phase columns with the performance of a perfusion chromatography column and a monolithic reversed-phase chromatography column. As examples for conventional reversed-phase chromatography columns, we used a Lichrospher and Superspher of Merck (Darmstadt, Germany). These reversed-phase chromatography columns are characterized by the composition of spherical particles of silica with endcapped octadecyl derivative. The particle size of Lichrospher vs. Superspher amounts to 5 µm vs. 4 µm, resulting in a different number of theoretical plates (55,000 vs < 100,000 N/m).

In recent studies, the low molecular weight and highly charged dinucleoside polyphosphates, which also contain hydrophobic fragments, were fractionated by perfusion chromatography (e.g. [24,29,30]), although the latter technique is generally only used for purification of high molecular weight biomacromolecules[20,21]. The main advantage of perfusion chromatography is that the resolution does virtually not depend on flow rate, whereas conventional materials exhibit a marked reduction in resolution with increased flow rates. In general, synthetic polymers such as polystyrenedivenylbenzene are used as matrix building blocks due to their excellent physical stability (allowing pressures up to 200 bar) and chemical stability compared to most other substances used[21]. This makes column cleaning easy because even aggressive chemicals such as acids and bases can be used. This in turn enables longer lifetimes of the chromatography columns. In addition, the amount of time needed for column cleaning is minimal. A high resolution and unchanged binding capacity are characteristic for perfusion reversed-phase columns. In addition, costs are generally lower for perfusion chromatography, compared to conventional reversed-phase chromatography, either due to intrinsic financial advantages, or to a gain time.

The previous reports mentioned above indicate that diadenosine polyphosphates are quantifiable by perfusion chromatography with tetrabutylammonium hydrogensulfate (TBA) as ion-pair reagent (e.g. [1,24]). An exemplary reversed-phase chromatographic separation of synthetic diadenosine polyphosphates Ap_nA (with n=2-6) is shown in Figure 1 using TBA as ion-pair reagent and a monolithic silica reversed-phase column (Figure 1A), a perfusion reversed-phase column (Figure 1B), and two conventional silica reversed-phase columns, a LiChrospher (Figure 1C) and a Superspher (Figure 1D). The peaks labelled in the figure represent the UV absorption of synthetic diadenosine polyphosphates Ap_nA (with n=2-6). Using TBA and the monolithic silica reversed-phase column, the differences in retention time of the dinucleoside polyphosphates can markedly be increased in comparison to perfusion reversed-phase as well as conventional silica reversed-phase columns. Moreover, the peak widths of the dinucleoside polyphosphates decrease using TBA as an ion-pair reagent. As a consequence, less concentrated dinucleoside polyphosphates are quantifiable by using monolithic silica reversed-phase columns with TBA as ion-pair reagent. The monolithic silica reversed-phase columns are based on the “sol-gel" technology, which employs highly porous monolithic rods of silica with a bimodal pore structure. The column consists of both a macroporous and mesoporous structure. The macropores are on average 2 µm in diameter and together form a dense network of pores through which the eluent can rapidly flow to reduce the separation time.

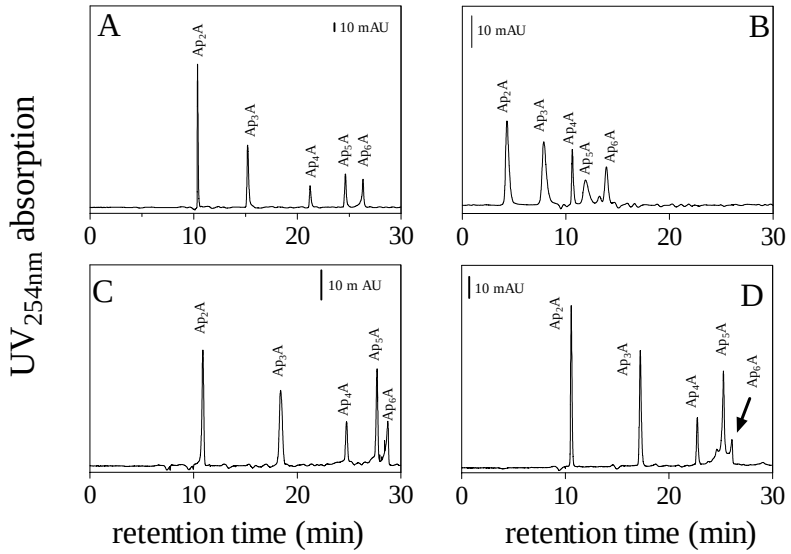

Figure 1: Reversed-phase chromatography of synthetic diadenosine polyphosphates (Ap_nA; with n=2-6) on

(A) an analytical monolithic reversed-phase high performance liquid chromatographic column (Chromolith^{TM} SpeedROD (50 x 4.6 mm I.D., Merck, Darmstadt, Germany)); eluent A: 2 mmol L^{-1} tetrabutylammonium hydrogensulfate and 10 mmol L^{-1} K_2HPO_4 in water (pH 6.8); eluent B: water-acetonitrile (20:80 %, v/v); gradient: 0 min: 0 % eluent B, 0-30 min: 0-45 % B, 30-33 min: 45- 100 % eluent B; 33-36 min: 100 % B; flow rate: 1.0 ml min^{-1}).

(B) an analytical reversed-phase high performance liquid chromatographic column (Poros R2/H (50 x 4.6 mm I.D., Perseptive Biosystems, Freiburg, Germany)). The conditions were identical to the conditions in Figure 1A.

(B) an analytical reversed-phase high performance liquid chromatographic column (LiChrospher 100 RP-18e (55 x 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in Figure 1A.

(D) an analytical reversed-phase high performance liquid chromatographic column (Superspher 100 RP-18e (55 x 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in Figure 1A. The figure shows characteristic chromatograms of three, independent chromatographies using identical conditions (N=3).

This approach, however, fails in combination with mass-spectrometric analysis and in the case of low concentrations of dinucleoside polyphosphates. For identification, dinucleoside polyphosphates are generally analysed by MALDI-mass spectrometry (e.g. [30-33]). Due to the strong ionic bonding of TBA with the phosphates of the dinucleoside polyphosphates and because of the low steam pressure of TBA, this ion-pair reagent is not removable by lyophilisation in the presence of dinucleoside polyphosphates. For that reason, the use of TBA as ion-pair reagent precludes the identification of the diadenosine polyphosphates by MALDI-mass spectrometry. But for the separation of ionic solutes such as dinucleoside polyphosphates, the

addition of an ion-pair reagent as a counterion is essential to ensure the retention of the ionic species. This indicates that an alternative to the ion-pair reagent TBA is necessary.

Therefore, the identities of the diadenosine polyphosphates as showed in Figure 1 were confirmed by comparing the respective retention times with those of single synthetic dinucleoside polyphosphates. Because of the use of TBA as ion-pair reagent and perfusion reversed-phase columns, in former studies, two strategies were necessary, one for the identification and one for the quantification of dinucleoside polyphosphates. TBA was used as ion-pair reagent for the chromatographic quantification of the dinucleoside polyphosphates; for the chromatographic isolation and identification, triethylammonium acetate (TEAA) was used as an alternative cationic ion-pair reagent. In contrast to TBA, TEAA is completely removable from dinucleoside polyphosphates by lyophilisation and therefore subsequent MALDI mass analysis of dinucleoside polyphosphates is possible.

Again, in contrast to monolithic reversed-phase chromatography, reversed-phase perfusion chromatography and chromatography with conventional silica reversed-phase columns with TEAA as the ion-pair reagent do not lead to sufficient separation of dinucleoside polyphosphates. Figure 2 presents characteristic reversed-phase chromatograms showing the separation of synthetic diadenosine polyphosphates Ap_nA (with n=2-6) in the presence of triethylammonium acetate (TEAA) as ion-pair reagent using the four columns under study (Figure 2A-D). The resolution of perfusion chromatography as well as of conventional reversed-phase chromatography is lower than that of the monolith reversed-phase chromatography. Dinucleoside polyphosphates are obviously only separable in the presence of the cationic ion-pair reagent TEAA if monolithic silica reversed-phase columns are used for the chromatography.

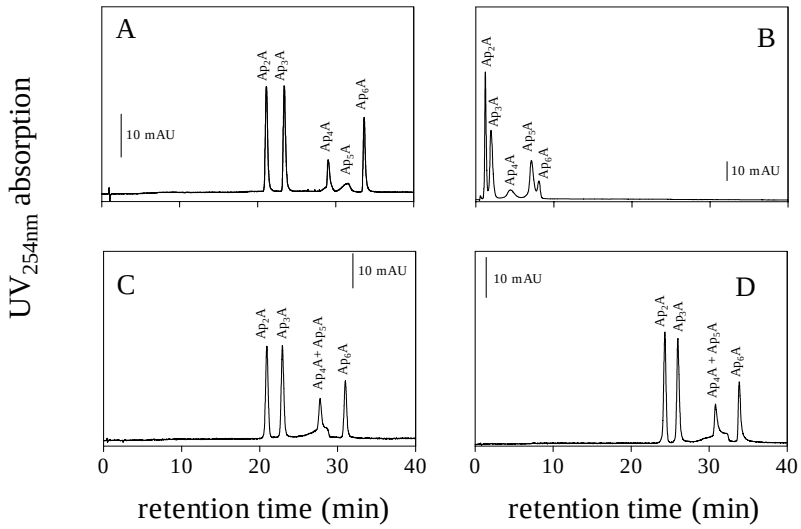

Figure 2: Reversed-phase chromatography of synthetic diadenosine polyphosphates (Ap_nA; with n=2-6) with triethylammonium acetate (TEAA) as ion-pair reagent on:

(A) an analytical monolithic reversed-phase high performance liquid chromatographic column (Chromolith^{TM} SpeedROD (50 x 4.6 mm I.D., Merck, Darmstadt, Germany)); eluent A: 40 mmol L-1 TEAA in water; eluent B: water-acetonitrile (80:20 %, v/v); gradient: 0-2 min: 0 % eluent B, 2-62 min: 0-60, respectively % B, 62-63 min: 60-100 % eluent B. The concentration of eluent B of 60 % corresponds to an acetonitrile concentration of 12 %.

(B) an analytical reversed-phase high performance liquid chromatographic column (Poros R2/H (50 x 4.6 mm I.D., Perseptive Biosystems, Freiburg, Germany)). The conditions were identical to the conditions in Figure 2A.

(C) an analytical reversed-phase high performance liquid chromatographic column (LiChrospher 100 RP-18e (55 x 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in Figure 2A.

(D) an analytical reversed-phase high performance liquid chromatographic column (Superspher 100 RP-18e (55 x 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in Figure 2A. The figure shows characteristic chromatograms of three, independent chromatographies using identical conditions (N=3).

In contrast to reversed-phase chromatography with the ion-pair reagent TEAA, in the case of reversed-phase chromatography with TBA as ion-pair reagent a buffer system is essential to adjust the pH at 6.8 (Figure 3A). Diadenosine polyphosphates are insufficiently retained and fractionated by the ion-pair reversed-phase chromatography with TBA in the absence of a buffer system (Figure 3B). In addition, chromatography of diadenosine polyphosphates at acid pH values bears the risk of hydrolysis of diadenosine polyphosphates.

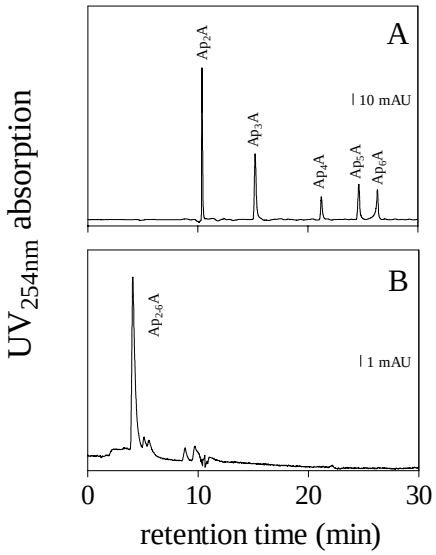

Figure 3: Reversed-phase chromatography of synthetic diadenosine polyphosphates (Ap_nA; with n=2-6) on an analytical monolithic reversed-phase high performance liquid chromatographic column (Chromolith™ SpeedROD (50 x 4.6 mm I.D., Merck, Darmstadt, Germany) using the ion-pair reagent tetrabutylammonium hydrogensulfate in the presence (A) and in the absence the buffer system K_2HPO_4 (B). The figure shows characteristic chromatograms of three, independent chromatographies using identical conditions (N=3). Conditions:

(A) eluent A: 2 mmol L^{-1} tetrabutylammonium hydrogensulfate and 10 mmol L^{-1} K_2HPO_4 in water (pH 6.8); eluent B: water-acetonitrile (20:80 %, v/v); gradient: 0 min: 0 % eluent B, 0-30 min: 0-45 % B, 30-33 min: 45- 100 % eluent B; 33-36 min: 100 % B; flow rate: 1.0 ml min^{-1}).

(B) eluent A: 2 mmol L^{-1} tetrabutylammonium hydrogensulfate without K_2HPO_4 in water (pH 6.8); eluent B: water-acetonitrile (20:80 %, v/v). The gradient and the flow rate were identical to the conditions in Figure 3A.

The chemical stability of conventional silica reversed-phase columns is generally limited. Silica-based reversed-phase sorbents operate within the pH limits of 2 < pH < 8, because at pH > 8 silica slowly dissolves[34] and at pH < 2 the covalently bound silane ligands are hydrolyzed[35]. Due to this limited chemical stability only restricted purification procedures can be performed with limited operating life times as a result. This is another reason to prefer monolith reversed-phase chromatography columns for separation of dinucleoside polyphosphates.

Figure 4 shows characteristic reversed-phase chromatograms of a homogenate of human platelets using TEAA as ion-pair reagent and with the four columns under study (Figures 4A-D). In contrast to the other tested reversed-phase chromatography columns, the resulting chromatogram of the monolithic silica reversed-phase column shows no interference with individual dinucleoside polyphosphates.

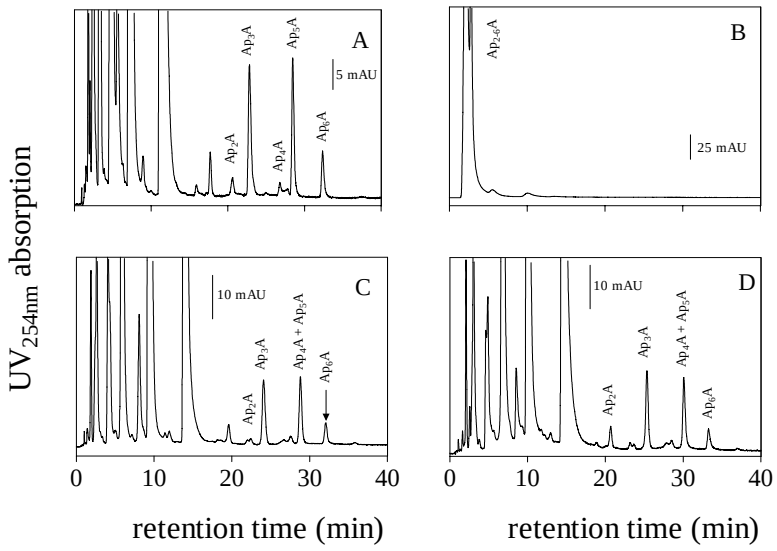

Figure 4: Reversed-phase chromatography of a homogenate of human platelets after reversed-phase and affinity-chromatography on:

(A) an analytical monolithic reversed-phase high performance liquid chromatographic column (Chromolith^{TM} SpeedROD (50 x 4.6 mm I.D., Merck, Darmstadt, Germany)); eluent A: 40 mmol L^{-1} triethylammonium acetate (TEAA) in water; eluent B: water-acetonitrile (80:20, v/v); gradient: 0-2 min: 0 % eluent B, 2-62 min: 0-25 % B, 62-63 min: 25- 100 % eluent B; flow rate: 1.0 ml min^{-1}).

(B) an analytical reversed-phase high performance liquid chromatographic column (Poros R2/H (50 x 4.6 1 mm I.D., Perseptive Biosystems, Freiburg, Germany)). The conditions were identical to the conditions in Figure 4A.

(C) an analytical reversed-phase high performance liquid chromatographic column (LiChrospher 100 RP-18e (55 x 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in Figure 4A.

(D) an analytical reversed-phase high performance liquid chromatographic column (Superspher 100 RP-18e (55 x 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in Figure 4A. The figure shows characteristic chromatograms of three, independentd chromatographies using identical conditions (N=3).

The chromatographies of the wash solutions using the identical chromatographic conditions showed no significant UV absorption at 254 nm, indicating the absence of dinucleoside polyphosphates in these solutions (data not shown).

While TEAA is superior as an ion-pair reagent if subsequent mass spectrometry is needed for identification of individual compounds, for all other chromatographic conditions, TBA seems to be superior regarding the analysis time. Figure 5 depicts analogous separations with TBA as the ion-pair reagent. The resolution of the separation is further increased compared to the corresponding resolution of the chromatography with TEAA as the cationic ion-pair reagent. The distinct elution of the dinucleoside polyphosphates using the latter chromatographic con-

ditions, allows quantification of very low concentrated derivatives. Using TBA instead of TEAA as ion-pair reagent, the analysis time can obviously be decreased without a decrease of the resolution of the chromatography.

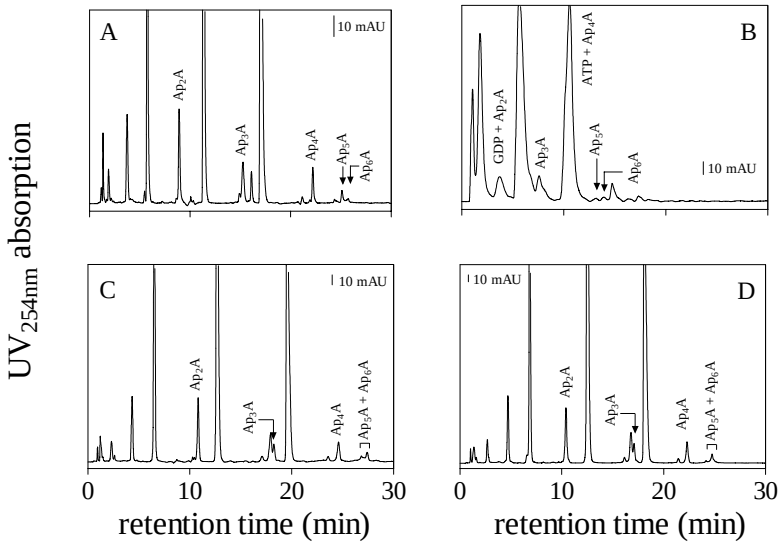

Figure 5: Reversed-phase chromatography of a homogenate of human platelets after reversed-phase and affinity-chromatography on:

(A) an analytical monolithic reversed-phase high performance liquid chromatographic column (Chromolith^{TM} SpeedROD (50 x 4.6 mm I.D., Merck, Darmstadt, Germany)); eluent A: 2 mmol L^{-1} tetrabutylammonium hydrogensulfate and 10 mmol L^{-1} K_2HPO_4 in water (pH 6.8); eluent B: water-acetonitrile (20:80 %, v/v); gradient: 0 min: 0 % eluent B, 0-30 min: 0-45 % B, 30-33 min: 45- 100 % eluent B; 33-36 min: 100 % B; flow rate: 1.0 ml min^{-1}).

(B) an analytical reversed-phase high performance liquid chromatographic column (Poros R2/H (50 x 4.6 mm I.D., Perseptive Biosystems, Freiburg, Germany)). The conditions were identical to the conditions in Figure 5A.

(C) an analytical reversed-phase high performance liquid chromatographic column (LiChrospher 100 RP-18e" (55 x 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in Figure 5A.

(D) an analytical reversed-phase high performance liquid chromatographic column (Superspher 100 RP-18e (55 x 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in Figure 5A. The figure shows characteristic chromatograms of three, independent chromatographies using identical conditions (N=3).

The combination of TBA as the cationic ion-pair reagent with monolithic silica reversed-phase columns yields a chromatogram with baseline separated and sharp UV-absorption peaks. Using this combination of chromatographic conditions, not only the abundant and known dinucleoside polyphosphates like Ap_2A, Ap_3A, Ap_4A, Ap_5A and Ap_6A, are baseline separated, but also less concentrated, yet unknown nucleoside polyphosphates may be in all probability separated with high resolution and small peak width.

Next, the effect of increasing the flow rate on the resolution of the monolithic reversed-phase column was analyzed. Due to the greater back-pressure of the reversed-phase columns compared to the monolith reversed-phase and the perfusion reversed-phase chromatography columns, the flow rates used in chromatography with conventional silica reversed-phase chromatography columns are in general lower than those used in chromatography with monolithic or perfusion chromatography columns. Higher flow rates result in a decrease in the analysis time. Figure 6 shows characteristic reversed-phase chromatograms of synthetic diadenosine polyphosphates using the monolithic reversed-phase column in the presence of the ion-pair-reagent TBA (Figure 6A-D) and in the presence of the ion-pair-reagent TEAA (Figure 6F-I) using a flow rate of 1 - 6 ml min^{-1}. Analysis time can be reduced by close to one magnitude without a significant decrease in the resolution. The separation of a platelet extract in the presence of TBA and TEAA using a flow rate of 6 ml min^{-1} is shown Figure 6E and 6J, respectively. In the presence of TBA, the differences of retention times of diadenosine polyphosphates increase.

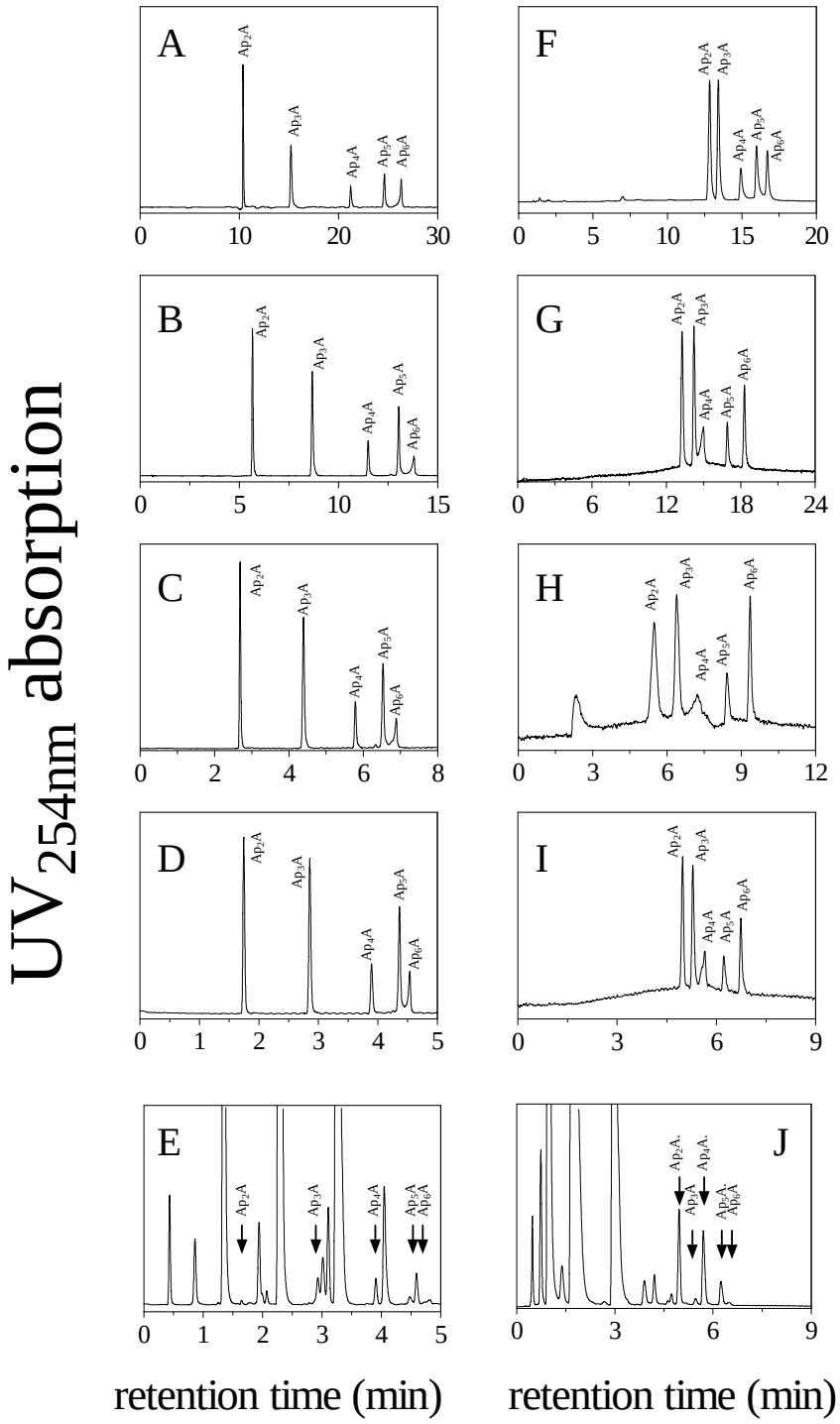

Figure 6: Reversed-phase chromatography of synthetic diadenosine polyphosphates (Ap_nA; with n=2-6) on an analytical monolithic reversed-phase high performance liquid chromatographic column (Chromolith™ SpeedROD (50 x 4.6 mm I.D., Merck, Darmstadt, Germany) using TBA (Figure 6A-D) respectively TEAA (Figure 6F-I) as ion-pair reagent and flow rate in the range of 1 ml min^{-1} up to 6 ml min^{-1}. Figure 6E and 6J show the chromatography of a platelet extract using TBA and TEAA as ion-pair reagent. The figure shows characteristic chromatograms of three, independent chromatographies using identical conditions (N=3).

(A) eluent A: 2 mmol L^{-1} TBA and 10 mmol L^{-1} K_2HPO_4 in water (pH 6.8); eluent B: water-acetonitrile (20:80 %, v/v); gradient: 0 min: 0 % eluent B, 0-30 min: 0-45 % B, 30-33 min: 45- 100 % eluent B; 33-36 min: 100 % B; flow rate: 1 ml min^{-1}.

(B) eluent A: 2 mmol L^{-1} TBA and 10 mmol L^{-1} K_2HPO_4 in water (pH 6.8); eluent B: water-acetonitrile (20:80 %, v/v); gradient: 0 min: % B, 0-15 min: 0-40 % B, 15-16 min: 40-100 % eluent B; 16-17 min: 100 % B; flow rate: 2 ml min^{-1}.

(C) eluent A: 2 mmol L^{-1} TBA and 10 mmol L^{-1} K_2HPO_4 in water (pH 6.8); eluent B: water-acetonitrile (20:80 %, v/v); gradient: 0 min: % B, 0-7.5 min: 0-40 % B, 7.5-8.2 min: 40-100 % eluent B; 8.2-9.2min: 100 % B; flow rate: 4 ml min^{-1}.

(D) eluent A: 2 mmol L^{-1} TBA and 10 mmol L^{-1} K_2HPO_4 in water (pH 6.8); eluent B: water-acetonitrile (20:80 %, v/v); gradient: 0 min: 0 % B, 0-5 min: 0-40 % B, 5-5.5 min: 40-100 % eluent B; 5.5-6.5 min: 100 % B; flow rate: 6 ml min^{-1}.

(E) reversed-phase chromatography of a platelet extract using the conditions as described in (D).

(F) eluent A: 40 mmol L^{-1} TEAA in water; eluent B: water-acetonitrile (80:20 %, v/v); gradient: 0-2 min: 0 % B, 2-62 min: 0-60 % B, 62-63 min: 60-100 % eluent B; The concentration of eluent B of 60 % corresponds to an acetonitrile concentration of 12 %; flow rate: 1 ml min^{-1}.

(G) eluent A: 40 mmol L^{-1} TEAA in water; eluent B: water-acetonitrile (80:20 %, v/v); gradient: 0 min: 0 % B, 0-32 min: 0-35 % B, 32-33 min: 35-100 % B; 33-34 min: 100 % B; flow rate: 2 ml min^{-1}.

(H) eluent A: 40 mmol L^{-1} TEAA in water; eluent B: water-acetonitrile (80:20 %, v/v); gradient: 0-1 min: 0 % B, 1-16 min: 0-35 % B, 16-17 min: 35-100 % eluent B. flow rate: 4 ml min^{-1}.

(I) eluent A: 40 mmol L^{-1} TEAA in water; eluent B: water-acetonitrile (80:20 %, v/v); gradient: 0-1 min: 0 % B, 1-11 min: 0-35 % B, 11-12 min: 35-100 % eluent B. flow rate: 6 ml min^{-1}.

(J) reversed-phase chromatography of a platelet extract using the conditions as described in Figure 6I.

Characteristic MALDI mass-spectra of diadenosine polyphosphates fractionated using the conditions as described in Figure 6H are shown in Figure 7. As mentioned above, the use of TBA as ion reagent prevents the analysis by MALDI mass-spectrometry (data not shown).

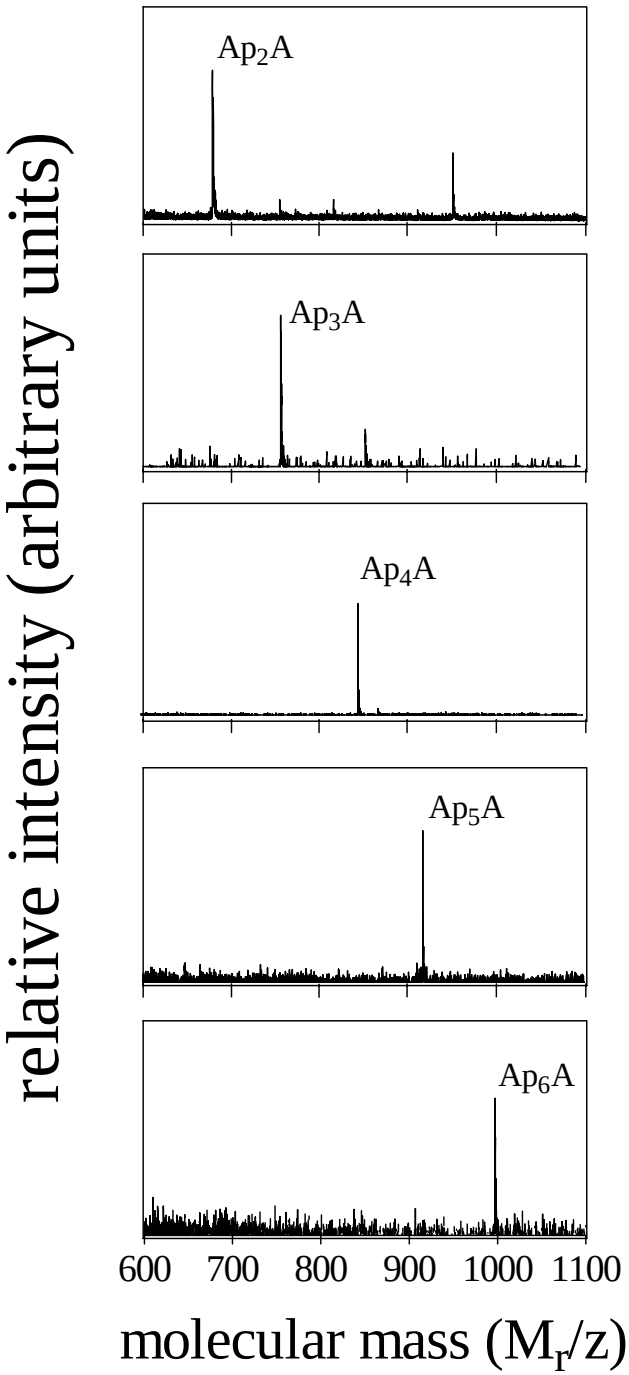

Figure 7: MALDI mass spectra of the substances underlying the UV absorption-peaks (labelled as Ap_2A, Ap_3A, Ap_4A, Ap_5A and Ap_6A in Figure 6H). The figure shows characteristic MALDI mass spectra of three, independent MALDI mass spectra using identical conditions (N=3).

In summary, isolation and quantification of dinucleoside polyphosphates by using monolithic silica C18 columns has obviously essential advantages compared to chromatography with

perfusion reversed-phase media or conventional silica reversed-phase media. In the future, the usage of monolithic silica reversed-phase columns will lead to isolation and quantification of yet unknown dinucleoside polyphosphates.

B.1.5. References

1. Schlüter H, Offers E, Brüggemann G, van der Giet M, Tepel M, Nordhoff E, Karas M, Spieker C, Witzel H, Zidek W: Diadenosine phosphates and the physiological control of blood pressure. *Nature* 367:186-188, 1994
2. Jankowski J, Tepel M, van der Giet M, Tente IM, Henning L, Junker R, Zidek W, Schlüter H: Identification and characterization of P_1, P_7-Diadenosine-5'- heptaphosphate from human platelets. *J Biol Chem* 274:23926-23931, 1999
3. Gabriels G, Rahn KH, Schlatter E, Steinmetz M: Mesenteric and renal vascular effects of diadenosine polyphosphates (Ap_nA). *Cardiovasc Res* 56:22-32, 2002
4. Davies G, MacAllister RJ, Bogle RG, Vallance P: Effect of diadenosine phosphates on human umbilical vessels: novel platelet-derived vasoconstrictors. *Br J Clin Pharmacol* 40:170-172, 1995
5. Jankowski J, Hagemann J, Tepel M, van der Giet M, Stephan N, Henning L, Gouni-Berthold I, Sachinidis A, Zidek W, Schlüter H: Dinucleotides as growth promoting extracellulary mediators: Presence of dinucleoside diphosphates Ap_2A, Ap_2G and Gp_2G in releasable granlues of platelets. *J Biol Chem* 276:8904-8909, 2001
6. Ogilvie A, Lüthje J, Pohl U, Busse R: Identification and partial characterization of an adenosine(5')tetraphospho(5')adenosine hydrolase on intact bovine aortic endothelial cells. *Biochem J* 259:97-103, 1989
7. Luo J, Jankowski J, Tepel M, von der Giet M, Zidek W, Schlüter H: Identification of diadenosine hexaphosphate in human erythrocytes. *Hypertension* 34:872-875, 1999
8. Westhoff T, Jankowski J, Schmidt S, Luo J, Giebing G, Schlüter H, Tepel M, Zidek W, van der Giet M: Identification and characterization of adenosine 5'-tetraphosphate in human myocardial tissue. *J Biol Chem* 27:27, 2003
9. Luo J, Jankowski J, Knobloch M, van der Giet M, Gardanis K, Russ T, Vahlensieck U, Neumann J, Schmitz W, Tepel M, Deng MC, Zidek W, Schlüter H: Identification and characterization of diadenosine 5',5'''-P_1,P_2-diphosphate and diadenosine 5',5'''-P_1,P_3-triphosphate in human myocardial tissue. *Faseb J* 13:695-705, 1999
10. Luo J, Jankowski V, Gungar N, Neumann J, Schmitz W, Zidek W, Schlüter H, Jankowski J: Endogenous diadenosine tetraphosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate in human myocardial tissue. *Hypertension* 43:1055-1059, 2004
11. Jankowski J, Yoon MS, Stephan N, Zidek W, Schlüter H: Vasoactive diadenosine polyphosphates in human placenta: possible candidates in the pathophysiology of preeclampsia? *J Hypertens* 19:567-573, 2001
12. Jankowski J, Jankowski V, Laufer U, van der Giet M, Henning L, Tepel M, Zidek W, Schlüter H: Identification and quantification of diadenosine polyphosphate concentrations in human plasma. *Arterioscler Thromb Vasc Biol* 23:1231-1238, 2003
13. Jankowski J, Jankowski V, Seibt B, Henning L, Zidek W, Schlüter H: Identification of dinucleoside polyphosphates in adrenal glands. *Biochem Biophys Res Commun* 304:365-370, 2003
14. Gabriels G, Endlich K, Rahn KH, Schlatter E, Steinhausen M: In vivo effects of diadenosine polyphosphates on rat renal microcirculation. *Kidney Int* 57:2476-2484, 2000
15. McLennan, AG: *Ap_4A and other dinucleoside polyphosphates*. McLennan AG, CRC Press Inc., Boca Raton, 1992, pp 29-61

16. Hoyle CHV, Hilderman RH, Pintor JJ, Schlüter H, King BF: Diadenosine polyphosphates as extracellular signal molecules. *Drug Develop Res* 52:260-273, 2001
17. McLennan AG: Dinucleoside polyphosphates-friend or foe? *Pharmacol Ther* 87:73-89, 2000
18. Flores NA, Stavrou BM, Sheridan DJ: The effects of diadenosine polyphosphates on the cardiovascular system. *Cardiovasc Res* 42:15-26, 1999
19. Schlüter H, Tepel M, Zidek W: Vascular actions of diadenosine phosphates. *J Auton Pharmacol* 16:357-362, 1996
20. Afeyan NB, Gordon NF, Mazsaroff I, Varady L, Fulton SP, Yang YB, Regnier FE: Flow-through particles for the high-performance liquid chromatographic separation of biomolecules: perfusion chromatography. *J Chromatogr* 519:1-29, 1990
21. Regnier FE: Perfusion chromatography. *Nature* 350:634-635, 1991
22. Jankowski J, Potthoff W, van der Giet M, Tepel M, Zidek W, Schlüter H: High-Performance Liquid Chromatographic Assay of the Diadenosine Polyphosphates in Human Platelets. *Anal Biochem* 269:72-78, 1999
23. Brüggemann B, Schlüter H, Verspohl E, Zidek W: *Fresenius Z Anal Chem* 350:719-721, 1994
24. Jankowski J, Schlüter H, Henning L, van der Giet M, Jankowski V, Zidek W, Tepel M: The AN69 hemofiltration membrane has a decreasing effect on the intracellular diadenosine pentaphosphate concentration of platelets. *Kidney Blood Press Res* 26:50-54, 2003
25. Jankowski J, Hagemann J, Yoon MS, van der Giet M, Stephan N, Zidek W, Schlüter H, Tepel M: Increased vascular growth in hemodialysis patients induced by platelet-derived diadenosine polyphosphates. *Kidney Int* 59:1134-1141, 2001
26. Barnes LD, Robinson AK, Mumford CH, Garrison PN: Assay of diadenosine tetraphosphate hydrolytic enzymes by boronate chromatography. *Anal Biochem* 144:296-304, 1985
27. Hillenkamp F, Karas M: Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. *Methods Enzymol* 193:280-295, 1990
28. Kaufmann R, Kirsch D, Spengler B: Sequencing of peptides in a time-of-flight mass spectrometer: evaluation of postsource decay following matrix-assisted laser desorption ionisation (MALDI). *Int J Mass Spectrom Ion Proc* 131:355-385, 1994
29. Schlüter H, Gross I, Bachmann J, Kaufmann R, van der Giet M, Tepel M, Nofer JR, Assmann G, Karas M, Jankowski J, Zidek W: Adenosine(5') oligophospho-(5') guanosines and guanosine(5') oligophospho-(5') guanosines in human platelets. *J Clin Invest* 101:682-688, 1998
30. Hollah P, Hausberg M, Kosch M, Barenbrock M, Letzel M, Schlatter E, Rahn KH: A novel assay for determination of diadenosine polyphosphates in human platelets: studies in normotensive subjects and in patients with essential hypertension. *J Hypertens* 19:237-245, 2001
31. Vartanian AA: Gelsolin and plasminogen activator inhibitor-1 are Ap_3A-binding proteins. *Ital J Biochem* 52:9-16, 2003
32. Dieckmann R, Pavela-Vrancic M, von Dohren H: Synthesis of diadenosine polyphosphates by non-ribosomal peptide synthetases (NRPS). *Biochim Biophys Acta* 1546:234-241, 2001
33. Maksel D, Gooley PR, Swarbrick JD, Guranowski A, Gange C, Blackburn GM, Gayler KR: Characterization of active-site residues in diadenosine tetraphosphate hydrolase from Lupinus angustifolius. *Biochem J* 357:399-405, 2001
34. Kirkland JJ, van Straten MA, Claessens HA: High pH mobile phase effects on silica-based reversed-phase high-performance liquid chromatographic columns. *J Chromatogr A* 691:3-19, 1995

35. Glajch JL, Kirkland JJ, Kohler J: Effect of column degradation on the reversed-phase high-performance liquid chromatographic separation of peptides and proteins. *J Chromatogr* 384:81-90, 1987

B.2. Identification and quantification of diadenosine polyphosphate concentrations in human plasma

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B.2.1. Abstract

Diadenosine polyphosphates have been demonstrated to be involved in the control of vascular tone as well as the growth of vascular smooth muscle cells and hence, possibly, in atherogenesis. In this study we investigated the question, whether diadenosine polyphosphates are present in human plasma and whether a potential source can be identified, which may release diadenosine polyphosphates into the circulation.

Plasma diadenosine polyphosphates (Ap_nA with n=3-6) were purified to homogeneity by affinity-, anion exchange- and reversed-phase-chromatography from deproteinized human plasma. Analysis of the homogeneous fractions with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) revealed molecular masses ($[M+H]^+$) of 757, 837, 917 and 997 Da. Comparison of the post-source-decay (PSD)-MALDI mass spectra of these fractions with those of synthetic diadenosine polyphosphates revealed that these isolated substances were identical to Ap_3A, Ap_4A, Ap_5A and Ap_6A. Enzymatic analysis showed an interconnection of the phosphate groups with the adenosines in the 5´-positions of the ribose moieties.

The mean total plasma diadenosine polyphosphate concentrations (µmol L^{-1}; mean ± SEM) in cubital veins of normotensive subjects amounted to 0.89 ± 0.59 for Ap_3A, 0.72 ± 0.72 for Ap_4A, 0.33 ± 0.24 for Ap_5A and 0.18 ± 0.18 for Ap_6A. Cubital venous plasma diadenosine polyphosphate concentrations from normotensives did not differ significantly from those in the hypertensive patients studied. There was no significant difference between arterial and venous diadenosine polyphosphate plasma concentrations in five hemodialysis patients, making a significant degradation by capillary endothelial cells unlikely. Free plasma diadenosine polyphosphate concentrations are considerably lower than total plasma concentrations, as about 95 % of the plasma diadenosine polyphosphates were found to be protein-bound. There were no differences in the diadenosine polyphosphate plasma concentrations depending on

the method of blood sampling and anticoagulation, suggesting that platelet aggregation does not artificially contribute to plasma diadenosine polyphosphate levels in significant amounts.

The Ap_nA (with n=3-6) total plasma concentrations in adrenal veins were significantly higher than the plasma concentrations in both infrarenal and suprarenal vena cava: adrenal veins: Ap_3A: 4.05 ± 1.63; Ap_4A: 6.18 ± 2.08; Ap_5A: 0.53 ± 0.28; Ap_6A: 0.59 ± 0.31; infrarenal vena cava: Ap_3A: 1.25 ± 0.66; Ap_4A: 0.91 ± 0.54; Ap_5A: 0.25 ± 0.12; Ap_6A: 0.11 ± 0.06; suprarenal vena cava: Ap_3A: 1.40 ± 0.91; Ap_4A: 1.84 ± 1.20; Ap_5A: 0.33 ± 0.13; Ap_6A: 0.11 ± 0.07 (µmol L^1; mean ± SEM; each $p<0.05$ (concentration of adrenal veins vs. infrarenal or suprarenal veins respectively)).

The presence of diadenosine polyphosphates in physiologically relevant concentrations in human plasma was demonstrated. Because in adrenal venous plasma significantly higher diadenosine polyphosphate concentrations were measured than in plasma from the infrarenal and suprarenal vena cava, it can be assumed that, beside platelets, the adrenal medulla may be a source of plasma diadenosine polyphosphates in humans.

B.2.2. Introduction

In the circulation diadenosine polyphosphates have been shown to be important extracellular mediators affecting vascular tone, growth of vascular cells (for review see ref. [1]) and platelet aggregation[2,3]. Diadenosine tri- and tetraphosphate (Ap_3A, Ap_4A) were the first diadenosine polyphosphates to be identified in human platelets[4,5], followed by diadenosine penta- and hexaphosphate (Ap_5A, Ap_6A)[6] and diadenosine heptaphosphate (Ap_7A)[7]. In 1999, diadenosine diphosphate (Ap_2A) and Ap_3A were shown to be present in human myocardial tissue[8]. Dinucleoside diphosphates, Ap_2A, Ap_2G and Gp_2G are described as a new class of growth promoting extracellular mediators, which are released from granules after activation of platelets[9].

Dinucleoside polyphosphates can be released into the circulation from activated platelets[4-7,10], from chromaffin cells of the adrenal glands[11-15] or from synaptic vesicles[16]. After their release local concentrations in the range of 10^{-5} mol L^{-1} or even higher can be assumed[17]. Like ATP, the dinucleoside polyphosphates may be coreleased with catecholamines on sympathetic nerve stimulation and may thus significantly modify the cardiovascular actions of the sympathoadrenergic system[11].

Diadenosine polyphosphates can act as vasoconstrictors or vasodilators in rat mesenteric arteries[18] and in the vasculature of the rat kidney[19]. Ap_5A is the most potent vasoconstricting diadenosine polyphosphate, followed by Ap_6A and Ap_4A. The ionotropic P2X1 receptor is considered the principal mediator of vasoconstriction[20]. The P2X1 receptors cluster on the adventitial surface of vascular smooth muscle cells immediately adjacent to sympathetic nerve varicosities[21]. The P2X1 receptor is coexpressed with P2X2, P2X4, and P2X5 receptors in muscle cells of a number of blood vessels, suggesting that also heteromeric P2X receptors occur[22-24]. Not only P2X-receptors but also metabotropic P2Y receptors have been reported to mediate vasoconstriction[25-28].

Besides these purinergic receptors, a specific dinucleotide receptor was described in rat midbrain synaptosomes[29]. The dinucleotide receptor is preferentially stimulated by diadenosine polyphosphates and is insensitive to ATP, UTP, adenosine, and their respective analogues[29].
Beside the vasoactive actions growth-stimulating effects of nucleoside polyphosphates have been shown in numerous blood vessels, involving the subsequent activation of protein kinase C (PKC), *Raf-1,* and mitogen-activated protein kinase (MAPK)[30,31]. Activation of the P2Y2 receptor increases the expression of *c-fos* mRNA in cultured aortic smooth muscle cells[32]. Dinucleoside polyphosphates also stimulate proliferation in vascular tissue where Ap_4A was shown to be equipotent as ATP[33].

In the present study an assay for isolation, identification and quantification of plasma diadenosine polyphosphates (Ap_nA with n=3-6) was established. Moreover, the present study was aimed to gain data on the secretion of diadenosine polyphosphates into plasma in order to define the sources of plasma diadenosine polyphosphates in humans. Therefore, plasma levels in adrenal veins were compared with those in vena cava in order to examine whether human adrenal glands release diadenosine polyphosphates.

B.2.3. Material and methods

CHEMICALS

High-performance liquid chromatography water (gradient grade) and acetonitrile were purchased from Merck (Germany), all other substances from Sigma-Aldrich (Germany).

ISOLATION AND IDENTIFICATION OF DIADENOSINE POLYPHOSPHATES IN HUMAN PLASMA

The study for the isolation and identification of diadenosine polyphosphates in human plasma was approved by the local ethical committee. Peripheral blood (4 ml) was obtained by catheterization of the cubital vein and was collected in tubes containing K_2-EDTA (7.2 mg). Moreover, adrenal venous blood (4 ml) was obtained by catheterization of adrenal veins, which was performed to further evaluate primary hyperaldosteronism in six hypertensive patients with clinical and biochemical evidence of that disease, and were collected in tubes containing K_2-EDTA (7.2 mg). Samples were also obtained from inferior vena cava and from cubital veins. All patients had an adrenal vein catheterization because of primary hyperaldosteronism with unremarkable adrenal CT scan. The clinical and biochemical characteristics of the patients are given in Table 1. In none of the patients the diagnosis of an adrenal adenoma was made, in all six patients bilateral hyperplasia was assumed. For comparison, six plasma samples of cubital veins from normotensive age-and sex-matched patients (blood pressure 119 ± 4/81 ± 2 mm Hg) being treated for acute minor illness such as back pain or dyspepsia were collected. In order to test whether intravascular degration of diadenosine polyphosphates by capillary endothelial cells affected diadenosine polyphosphate concentrations, the arteriovenous gradient of diadenosine polyphosphates was determined in five patients on regular hemodialysis treatment. Arterial blood was taken from the Cimino-Brescia fistulas and venous blood from the cubital vein.

Table 1: Clinical and biochemical characteristics of the patients (values are mean ± SEM) and control subjects

	hyperaldostero-nism patients (N = 6)	hemodialysis patients (N=5)	control subjects (N = 6)
age (years)	59.6 ± 10.4	60.4 ± 12.9	54.5 ± 11.2
sex (m / f)	3/3	3/2	3/3
blood pressure (mm Hg)	163 ± 7 / 98 ± 4	145 ± 6 / 79 ± 4	119 ± 4 / 81 ± 2
red blood cells (10^6/µl)	4.9 ± 0.4	3.3 ± 0.5	5.2 ± 0.3
white blood cells (10^3/µl)	7.7 ± 2.5	8.4 ± 2.7	6.8 ± 2.6
platelets (10^3/µl)	253 ± 75	204 ± 72	234 ± 68

The blood samples were centrifuged at 2,100 g for 10 min at 4°C for isolation of plasma (step 1). All blood samples were centrifuged after a standardized interval of 15 min after sampling. The plasma was deproteinized with 0.6 mol L^{-1} (final concentration) perchloric acid and centrifuged (2,100 g, 4°C; 5 min) (step 2). After adjusting pH to 7.0 with 5 mol L^{-1} KOH the precipitated proteins and $KClO_4$ were removed by centrifugation (2,100 g, 4°C, 5 min).

To test whether the method of blood sampling and anticoagulation may artificially affect plasma diadenosine polyphosphate concentrations due to platelet aggregation, the above method of blood sampling for limiting platelet activation[34,35]. To this purpose, blood (4 ml) of 6 healthy control subjects was collected from the cubital vein using a 19G winged infusion set (Myco-Medical, US) and tubes containing citrate 0.11 mol L^{-1} at a pH of 8.1. Citrated blood was centrifuged at 120 g for 15 min to obtain platelet-rich plasma, which was centrifuged at 300 g for 20 min at room temperature. The supernatant was used for diadenosine polyphosphate determinations.

DETERMINATION OF THE AMOUNT OF PROTEIN-BOUND DIADENOSINE POLYPHOSPHATES IN HUMAN PLASMA

To test the percentage of protein-bound diadenosine polyphosphates, plasma was isolated as described above and divided into two parts. One part was ultrafiltrated with a centrifuge filter (size exclusion limit: 10 kDa; 3,400 g; 10 min; 25°C), the other was left untreated. Then, in both portions the diadenosine polyphosphate concentrations were determined as described below.

EXTRACTION OF HUMAN PLASMA FOR QUANTIFICATION OF DIADENOSINE POLYPHOSPHATES

After deproteinization diadenosine octaphosphate (5 µg) was added to the sample as internal standard. Triethylammonium acetate (TEAA) in water was added to the deproteinated plasma to a final concentration of 40 mmol L^{-1}. The mixture was loaded to a preparative reversed-phase column (step 3, LiChroprep RP-18, 240 x 10 mm, Merck, Germany; equilibration and sample buffer: 40 mmol L^{-1} TEAA in water; flow rate: 2.5 ml min^{-1}). Diadenosine polyphosphates were eluted by 30 % acetonitrile in water and lyophilized.

Next, the eluate was dissolved in 1 mol L^{-1} ammonium acetate at pH 9.5 and concentrated on a phenyl boronic acid resin, prepared according to Barnes et al.[36] (step 4). The substances were eluted from the phenyl boronic acid resin by 1 mmol L^{-1} HCl in water (flow rate: 0.2 ml min^{-1}). The eluate from the phenyl boronic acid resin with 1 mol L^{-1} TEAA to a final concentration of 40 mmol L^{-1} added was desalted by a reversed-phase HPLC chromatography (step 5) (Superspher RP-18 endcapped, 250 x 4 mm, Merck, Germany; eluent A: 40 mmol L^{-1} TEAA in water; eluent B: acetonitrile; flow rate: 0.5 ml min^{-1}). The desalted and lyophilized eluate of the phenyl boronic acid resin was chromatographed by anion-exchange chromatography (UnoQ-1, 7 x 35 mm, BioRad, USA; eluent A: 20 mmol L^{-1} K_2HPO_4, pH 8; eluent B: 20 mmol L^{-1} K_2HPO_4, pH 8 with 1 mol L^{-1} NaCl; gradient: 0-2 min 0 % B, 2-100 min 0-40 % B, 100-105 min 40-100 % B, 105-116 min 100 % B; flow rate: 0.5 ml min^{-1}). Fractions were collected according to UV absorbance at 254 nm (peak fractionation).

To the eluate of the anion-exchange chromatography 1 mol L^{-1} TEAA was added (final concentration of 40 mmol L^{-1}) and was loaded to an analytic reversed-phase column (Superspher 100 RP-18 endcapped, 250×4 mm, Merck, Germany). Nucleoside polyphosphates were eluted by 20 % acetonitrile in water. The isolated diadenosine polyphosphates were identified by

MALDI-MS, PSD-MALDI as well as enzymatic cleavage experiments. Moreover, to validate the identification of the diadenosine polyphosphates by their retention time synthetic Ap_3A, Ap_4A, Ap_5A and Ap_6A (each 1 µg) were added to aliquots of the samples. For quantification of diadenosine polyphosphate peak areas were determined by an integrator. The concentrations of diadenosine polyphosphates were calculated by using calibration curves obtained with synthetic diadenosine polyphosphates.

ENZYMATIC CLEAVAGE EXPERIMENTS

Aliquots of the desalted fractions of anion-exchange chromatography were incubated with enzymes as follows. The samples were dissolved 1) in 20 µl 200 mmol L^{-1} Tris buffer (pH 8.9) and incubated with 5'-nucleotide hydrolase (3 mU, EC 3.1.15.1; from *Crotalus durissus*, from Boehringer Mannheim, Germany), purified according to Sulkowski and Laskowski[37] 9 min at 37 °C); 2) in 20 µl 200 mmol L^{-1} Tris and 20 mmol L^{-1} EDTA buffer (pH 7.4) and incubated with 3'-nucleotide hydrolase (1 mU; EC 3.1.16.1,from *Calf spleen*, from Boehringer Mannheim, Germany) 1 hour at 37 °C and 3) in 20 µl 10 mmol L^{-1} Tris, 1 mmol L^{-1} $ZnCl_2$ and 1 mmol L^{-1} $MgCl_2$ buffer (pH 8) and incubated with alkaline phosphatase (1 mU; EC 3.1.3.1, from C*alf intestinal mucosa*, from Boehringer Mannheim, Germany) 1 h at 37 °C. The reaction was terminated by an ultrafiltration with a centrifuge filter (exclusion limit 10 kDa). After filtration of the enzymatic cleavage products the filtrate, dissolved in 80 µl eluent A, was subjected to reversed-phase chromatography (Chromolith SpeedROD^{TM}, 4.6 x 50 mm, Merck, Germany; eluent A: 2 mmol L^{-1} tetrabutylammonium hydrogensulfate in 10 mmol L^{-1} K_2HPO_4, pH 6.8; eluent B: 80 % ACN in water; gradient: 0-30 min: 0-40 % B; 30-33 min: 40-100 % B; 33-36 min: 100 % B; flow: 1 ml min^{-1}).

MATRIX ASSISTED LASER DESORPTION/ IONIZATION MASS SPECTROMETRY (MALDI-MS)

Aliquots of the desalted fractions of anion-exchange chromatography were examined by MALDI-MS and post-source decay (PSD)-MALDI-MS. A reflectron type time-of-flight (RETOF) mass spectrometer (Reflex III, Bruker, Germany) was used according to Hillenkamp and Karas[38]. The sample was mounted on an x, y, z movable stage allowing irradiation of selected sample areas. In this study, a nitrogen laser (VSL-337 ND, Laser Science) with an emission wavelength of 337 nm and 3 ns pulse duration was used. The laser beam was focused to a diameter of 50 µm at an angle of 45° to the surface of a target. Microscopic sample observation was possible. 10-20 single spectra were accumulated for a better signal-to-noise

ratio. In MALDI-MS large fractions of the desorbed analyte ions undergo post-source decay (PSD) during flight in the field free drift path. Using a RETOF set-up, sequence information from PSD fragment ions of precursors produced by MALDI was obtained[39]. Sample preparations for MALDI- and PSD-MALDI experiments were identical.

Lyophilized aliquots of the desalted fractions of the anion-exchange chromatography were dissolved in bidistilled water, up to a concentration of 1-10 µmol L^{-1}. The amount of the underlying substances was estimated by the UV absorption of the anion-exchange chromatography fraction. 1 µl of the analyte solution was mixed with 1 µl of matrix solution (50 mg ml^{-1} 3-hydroxy-picolinic acid in water). To this mixture cation exchange beads (AG 50 W-X12, 200-400 mesh, Bio-Rad, USA) equilibrated with NH_4^+ as counterion were added to remove Na^+ and K^+ ions. The mixture was gently dried on an inert metal surface before introduction into the mass spectrometer. The mass accuracy was in the range of approximately 0.01 %.

SYNTHESIS AND CHROMATOGRAPHY OF DIADENOSINE OCTAPHOSPHATE AS INTERNAL STANDARD

Diadenosine octaphosphate was synthesized and chromatographed as described elsewhere[40]. Briefly, Ap_8A was synthesized by mixing adenosine tetraphosphate (50 mmol L^{-1}) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.5 mol L^{-1}), N-[2-hydroxy-ethyl]-piperazine-N´-[2-ethanesulfonic acid] (HEPES; 2 mol L^{-1}) and magnesium chloride (125 mmol L^{-1}). The substances were dissolved in water, thoroughly mixed with a vortex mixer and incubated at 37°C at pH 6.5 for 48 h. The reaction mixture was concentrated on a preparative C18 reversed-phase column (LiChroprep, 310 x 65 mm, 65-40 µm, Merck, Germany) using 40 mmol L^{-1} aqueous triethylammonium acetate (TEAA) in water (eluent A; flow rate: 2 ml min^{-1}). After removing substances not binding to the gel with aqueous 40 mmol L^{-1} TEAA (flow rate: 2 ml min^{-1}) nucleoside containing fractions were eluted with 30 % acetonitrile in water (eluent B; flow rate: 2 ml min^{-1}). By this procedure, the recovery of Ap_8A was about 10 % of the diadenosine octaphosphate used. The concentrate was displaced on two reversed-phase columns (columns: Superspher RP-18 endcapped, 300 x 8 mm, Merck, Germany; carrier: 40 mmol L^{-1} TEAA in water; displacer: 160 mmol L^{-1} n-butanol; flow 100 µl min^{-1}). As a result of displacement chromatography anion-exchange chromatography yielded baseline separated diadenosine polyphosphate. The identity of the diadenosine polyphosphate was ascertained using MALDI-MS (matrix assisted laser desorption/ionization mass spectrometry) as described above.

VALIDITY OF THE ASSAY

Stock solutions of diadenosine polyphosphates (100 µmol L^{-1}) were prepared in water. Stock solutions were stored at -30°C. Solutions with various diadenosine polyphosphate concentrations were obtained by dilution of the stock solutions with water. For validation of the assay, standard diadenosine polyphosphate solutions of different concentrations were added to plasma directly prior to thawing. The precision of the assay for diadenosine polyphosphates was determined using a plasma sample and synthetic diadenosine polyphosphate solutions. The intra- (n=4) and inter-assay (n=4) variabilities were assessed and expressed as coefficients of variation (C.V.).

STATISTICS

Results are presented as means ± SEM. Two-sided p values <0.05 were considered significant. All values reported are mean ± SEM

B.2.4. Results

Figure 1 shows a characteristic anion-exchange chromatogram of an extract from human plasma after precipitation of proteins and affinity chromatography. The peaks labelled in Figure 1 represent the diadenosine polyphosphates Ap_3A, Ap_4A, Ap_5A and Ap_6A.

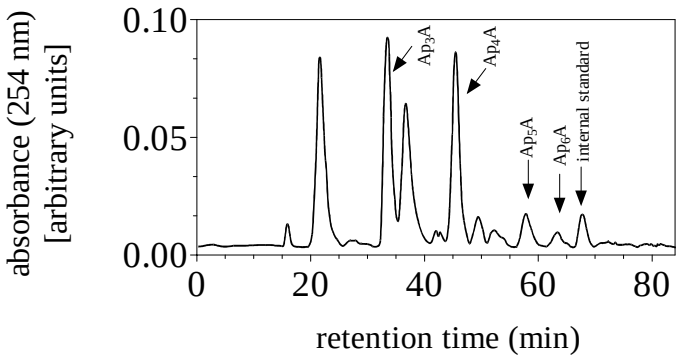

Figure 1: Typical anion-exchange chromatogram of a plasma extract of adrenal veins.

The identity of the diadenosine polyphosphates was confirmed by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) as well as post-source decay (PSD) matrix assisted laser desorption/ionization mass spectrometry. A characteristic post-source decay (PSD)-MALDI- mass spectrum of Ap_5A is given in Figure 2, and the interpretation of the PSD-MALDI data is given in Table 2.

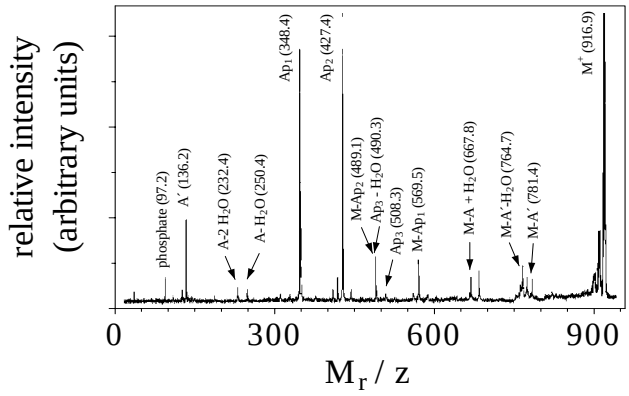

Figure 2: Positive-ion PSD-MALDI mass spectrum of the fraction labelled as Ap_5A in Figure 1 (abbreviations: A´ = adenine; A = adenosine; M = protonated parent ion; p = phosphate group e.g. Ap_4 adenosine tetraphosphate) (abscissa: relative mass/charge, m/z, z=1; ordinate: relative intensity, arbitrary units).

Table 2: Molecular masses of characteristic fragments from the PSD-MALDI-MS spectra of the desalted fractions of the anion-exchange chromatography (Figure 1) labelled Ap_3A, Ap_4A, Ap_5A and Ap_6A. M = protonated parent ion,-: minus, A' = adenine, A = adenosine, p = phosphate group, e.g. Ap_3 = ATP.

Interpretation of the fragment ions measured by PSD-MALDI-MS	Ap_3A	Ap_4A	Ap_5A	Ap_6A
A´	136	136	136	136
A-2 H_2O	232	232	232	232
A-H_2O	250	250	250	250
Ap_1	348	348	348	348
Ap_2-H_2O	409	410		410
Ap_2	428	427	427	428
Ap_3-H_2O		490	490	490
Ap_3	508	508	508	508
M – Ap_2	329		489	569
M-Ap_1	409	489	569	649
M – A		571		
M – A + H_2O			668	
M-A´-H_2O	605	685	765	845
M-A´	622	701	781	861
M	757	837	917	997

To exclude that the diadenosine polyphosphates found are isomers with other than 5´-5´ bonds between the ribose and the phosphate moieties, the isolated substances were incubated with 3´- and 5´-nucleotide hydrolase as well as with alkaline phosphatase. Figure 3 shows

representative chromatograms of the enzymatic cleavage experiments with diadenosine pentaphosphate before (Figure 3.A) and after incubation with 5´-nucleotide hydrolase (Figure 3.B), 3´-nucleotide hydrolase (Figure 3.C) and alkaline phosphatase (Figure 3.D).When the fractions containing the diadenosine polyphosphate (Figure 3.A) were treated with 5´-nucleotide hydrolase (*Crotalus durissus*), the UV-peak of the intact diadenosine pentaphosphate and UV-peaks of the hydrolysis products AMP, and adenosine tetraphosphate (Ap_4) appeared (Figure 3.B). Incubation of the fractions containing diadenosine polyphosphates with 3´-nucleotide hydrolase (Figure 3.C) or alkaline phosphatase (Figure 3.D) yielded no cleavage products. The results of the enzymatic cleavage experiments show that the polyphosphate chain interconnects the two adenosines via phosphoester bonds to the 5´-positions of the riboses.

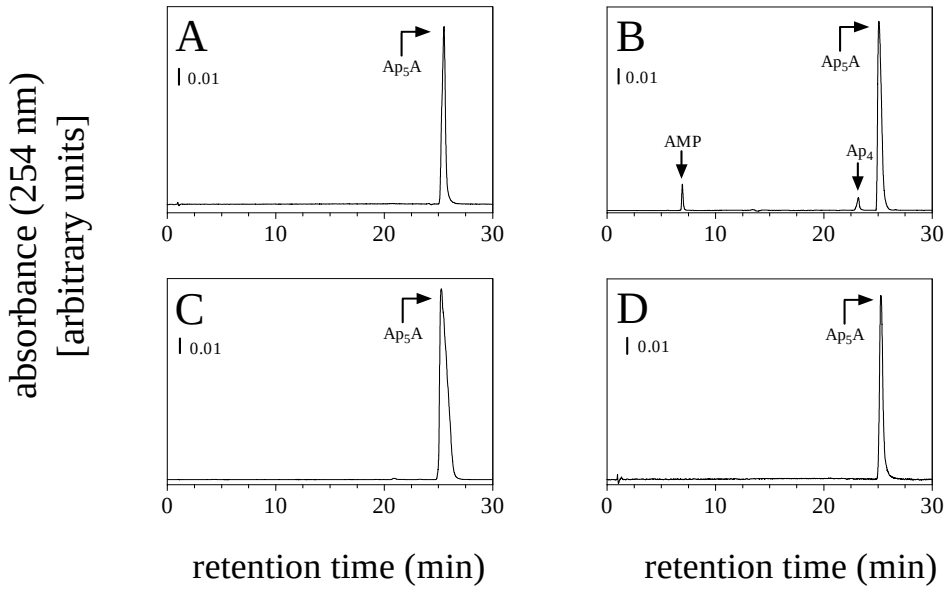

Figure 3: Chromatograms of the desalted fraction of the anion-exchange chromatography labelled as Ap_5A in Figure 1 (after anion-exchange- and reversed-phase chromatography) before (A) and after (B) incubation with 5'-nucleotidase, with 3'-nucleotidase (C) and with alkaline phosphatase (D).

The mean diadenosine polyphosphate concentrations (µmol L^{-1}; mean ± SEM) of cubital veins of normotensive patients amounted to 0.89 ± 0.59 for Ap_3A, 0.72 ± 0.72 for Ap_4A, 0.33 ± 0.24 for Ap_5A and 0.18 ± 0.18 for Ap_6A. There was no significant difference between cubital venous diadenosine polyphosphate concentrations in the patients with primary hyperaldosteronism and in normotensive subjects (Table 3). Table 3 furthermore shows that there were no significant differences between central and peripheral venous diadenosine polyphosphate plasma concentrations. In order to test whether the capillary endothelium significantly con-

tributes to intravascular degradation of diadenosine polyphosphates, arterial and venous plasma diadenosine polyphosphate concentrations were compared in five hemodialysis patients. The measurements revealed no significant arteriovenous difference in any of the diadenosine polyphosphate concentrations (Table 3).

Table 3: Plasma diadenosine polyphosphate concentrations (mean ± SEM) in µmol L^{-1} obtained from patients with primary hyperaldosteronism subjected to infrarenal vena cava, adrenal vein, suprarenal vena cava and cubital vein catheterization, normotensive patients and hemodialysis patients (arterial and venous plasma).

	infrarenal vena cava primary hyperaldosteronism	adrenal veins primary hyperaldosteronism	suprarenal vena cava primary hyperaldosteronism	cubital vein primary hyperaldosteronism	cubital vein normotensive patients	arterial plasma hemodialysis patients	venous plasma hemodialysis patients	significance / adrenal veins vs infrarenal and suprarenal vena cava
Ap_3A	1.2 ± 0.7	4.1 ± 1.6	1.4 ± 0.9	0.8 ± 0.3	0.9 ± 0.6	0.9 ± 0.3	0.7 ± 0.3	$p < 0.05$
Ap_4A	0.9 ± 0.5	6.2 ± 2.1	1.8 ± 1.2	0.6 ± 0.2	0.7 ± 0.7	0.5 ± 0.3	0.7 ± 0.2	$p < 0.05$
Ap_5A	0.2 ± 0.1	0.5 ± 0.3	0.3 ± 0.13	0.5 ± 0.2	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.2	$p < 0.05$
Ap_6A	0.1 ± 0.1	0.6 ± 0.3	0.1 ± 0.1	0.3 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	$p < 0.05$

In a further series of experiments, in six healthy probands the influence of blood sampling on plasma diadenosine polyphosphate concentrations was examined, comparing the above sampling method with best practice conditions. There were no significant differences in the diadenosine polyphosphate plasma concentrations with either sampling method (0.51 ± 0.09 vs. 0.59 ± 0.26 Ap_3A, 0.38 ± 0.23 vs. 0.32 ± 0.12 for Ap_4A, 0.35 ± 0.27 vs. 0.42 ± 0.09 for Ap_5A, 0.29 ± 0.23 vs. 0.25 ± 0.09 for Ap_6A (µmol L^{-1}); $p>0.05$; n=6, each EDTA vs. citrate). The concentrations of Ap_3A, Ap_4A, Ap_5A and Ap_6A in the plasma of infrarenal vena cava, adrenal veins and the suprarenal vena cava are given in Table 3. All patients had an adrenal vein catheter because of primary hyperaldosteronism with unremarkable adrenal CT scan.

Diagnosis of an adrenal adenoma was made in none of the patients, and in all six patients bilateral hyperplasia was assumed. Furthermore, there was no significant difference between cubital venous diadenosine polyphosphate concentrations in the patients with primary hyperaldosteronism and in normotensive subjects (Table 3). Filtration experiments of human plasma using a 10 kDa cut-off filtration membrane revealed that 5.7 ± 4.5 % of the diadenosine polyphosphates isolated from human plasma were not-protein-bound, with no significant differences depending on the number of phosphate groups. The plasma concentrations of Ap_3A, Ap_4A, Ap_5A and Ap_6A in infrarenal vena cava, adrenal veins and suprarenal vena cava are given in Table 3.

The absolute recovery of diadenosine-5´-5´-octaphosphate, 5 µg of which were added as internal standard to the plasma sample, was found to be 43.3 ± 18.6 %. The calibration graphs showed good linearity for concentrations of the diadenosine polyphosphates (Ap_3A, Ap_4A, Ap_5A, Ap_6A) ranging from 0.05 to 15 µmol L^{-1} (r=0.999). The peak area ratios of the diadenosine polyphosphates were linear in the concentration ranges investigated. Based on a signal-to-noise ratio of three, the detection limit for the diadenosine polyphosphates was 16 nmol L^{-1}. The precision of the assay for diadenosine polyphosphates was determined using a plasma sample and synthetic diadenosine polyphosphates. To evaluate the quantification of diadenosine polyphosphates in human plasma, the intra- (n=4) and inter-assay (n=4) variabilities were assessed. The intra-assay variability for a human plasma sample and standard solutions, assessed on four consecutive days was 8.2 %. The inter-assay coefficient of variation (C. V.) for a plasma sample and a standard solution, assessed on four consecutive days was 11.1 %.

B.2.5. Discussion

Quantification of diadenosine polyphosphates from human plasma requires several sample preparation steps. First, the large amount of proteins and peptides has to be removed. These substances were denatured by perchloric acid and removed by centrifugation. Second, the large number of small hydrophobic and hydrophilic substances has to be separated. Third, carbohydrates have to be removed from the sample before affinity-chromatography because of the characteristic of carbohydrates to bind to the affinity-gel. These aims were achieved by reversed-phase chromatography.

Next, a highly selective concentration step with a boronate derivative of a cation exchange gel was used which selectively retains nucleoside polyphosphates containing two or more sets of 1,2 cis-diol groups. Whereas mononucleoside polyphosphates like ATP with less than two cis-diol groups do not bind to the boronate in ammonium acetate at buffer concentrations of 1 mol L^{-1} due to charge repulsions between the negative phosphate groups and the carboxyl groups of the cation-exchange gel, the boryl ester formation of the two cis-diol groups of diadenosine polyphosphates is sufficient to overcome charge repulsion[36]. The eluate from the boronate gel contained salts, which prevent diadenosine polyphosphates to bind to the anion-exchanger. Therefore the eluate was desalted by concentration on a reversed-phase gel. For quantification of the diadenosine polyphosphates the plasma extract from the reversed-phase chromatography was subjected to anion-exchange chromatography.

The diadenosine polyphosphate concentrations in human plasma were found to be in the µmolar range. From the concentration-response curves published it appears that diadenosine polyphosphate plasma concentrations quantified in the present study are sufficient to affect vascular tone. In concentrations > 10^{-8} mol L^{-1} diadenosine polyphosphates have a growth stimulating effect on vascular smooth muscle cells[9,41,42]. Given the EC_{50} value for Ap_5A in the 10^{-8} molar range, the plasma levels reported here seem surprisingly high. As it is highly unlikely that the plasma diadenosine phosphate concentrations exceed the EC_{50} by two orders of magnitude, the question arises, whether or not a fraction of the circulating diadenosine polyphosphates exists in a bound form. Indeed, the results show that a considerable portion of plasma diadenosine polyphosphates is protein-bound, as they are retained by a 10 kDa filter. Therefore, it may be assumed that only a small portion of total plasma diadenosine polyphosphates directly affect vascular tone.

Further studies have to show whether there is a gender difference in the diadenosine polyphosphate concentration. Different diadenosine polyphosphate concentration may be a reason for the higher blood pressure of men. Because of the limited number of patients and healthy subjects of this study, this point cannot be clarified with data of the present study.

The findings show that diadenosine polyphosphate concentrations vary within the venous system . The adrenal venous diadenosine polyphosphate concentrations higher than those in other veins leave two alternative explanations: either diadenosine polyphosphates are produced by the adrenal medulla, or adrenal vascular endothelium is less effective than other

endothelial cells in degrading diadenosine polyphosphates by its ectonucleases. Between both hypotheses a decision can be made on the basis of arterial diadenosine polyphosphate concentrations. The measurements revealed that there is no significant gradient between non-adrenal venous and arterial diadenosine polyphosphate concentrations.

In general, uremia leads to endothelial dysfunction. This endothelial dysfunction may be a limiting effect to investigate the endothelial metabolism of dinucleoside polyphosphates by determination of an arteriovenous gradient of diadenosine polyphosphates in chronic renal failure patients. Since there was no significant difference in the dinucleoside polyphosphate concentration in venous plasma of CRF patients and in venous plasma of healthy control subjects, an effect of the endothelial dysfunction on the dinucleoside polyphosphate metabolism is unlikely, but has to be considered.

This finding implies that the ectonucleases located on vascular endothelial cells do not degrade sufficient amounts of diadenosine polyphosphates to lower circulating venous diadenosine polyphosphate concentrations significantly. Therefore the increased adrenal venous plasma diadenosine polyphosphate concentrations compared with nonadrenal venous plasma cannot be due to a decreased endothelial degradation restricted to adrenal vascular endothelium. These findings were obtained by comparison of diadenosine polyphosphate concentrations of arterial and venous plasma of chronic renal failure patients. The diadenosine polyphosphate concentrations in the arterial and venous plasma of these patients, and the concentrations in cubital veins of normotensive patients, are not significantly different. The endothelial dysfunction of chronic renal failure patients has no strong effect on the diadenosine polyphosphate concentrations and can be neglected.

The concentrations of the various diadenosine polyphosphates differ from each other. Obviously, those diadenosine polyphosphates with a higher number of phosphate groups show lower concentrations than those with a lower number of phosphates. This pattern may either be due to an increased degradation of diadenosine polyphosphates by circulating enzymes or by a decreased rate of synthesis with increasing number of phosphate groups. Although this question cannot be solved on the basis of the present data, findings reported in the literature may give an answer: concerning platelets, several studies revealed that the diadenosine polyphosphate contents decreased with an increasing number of phosphate groups[41,43]. Since the intraplatelet diadenosine polyphosphates are not accessible by the extracellular degrading

enzymes, it appears more likely that the synthetic pathway is less effective with increasing number of phosphate moieties to be incorporated.

In earlier studies, platelet diadenosine polyphosphates had been quantified referring the platelet content to the pertinent whole blood volume. In the platelets contained in 1 L whole blood the following amounts of diadenosine polyphosphates were found[42]: Ap_3A: 192.5 ± 14.7 nmol; Ap_4A: 223.8 ± 16.8 nmol; Ap_5A: 100.2 ± 7.9 nmol; Ap_6A: 32.0 ± 1.9 nmol (mean ± SEM). Conceivably, even if 100 % of the platelet diadenosine polyphosphates are assumed to be released and hence to be distributed within the pertinent volume, the resulting plasma concentrations would be far less than those reported here. Therefore, a significant artefactual contribution to plasma diadenosine polyphosphates by platelet aggregation appears to be unlikely. This conclusion is further supported by the fact that the method of blood sampling and anticoagulation does not significantly affect plasma diadenosine polyphosphate concentrations, when best practice conditions are compared with those initially applied.

Therefore human plasma diadenosine polyphosphates cannot solely stem from platelets. Thus we tested the hypothesis that diadenosine polyphosphates in human plasma are, at least partially, derived from the adrenal glands. Adrenal venous plasma diadenosine polyphosphate concentrations are significantly higher than those in the vena cava, both infrarenal and suprarenal (Table 3). This result suggests that human adrenal glands release Ap_3A, Ap_4A, Ap_5A and Ap_6A.

These results are in accordance with results from animal experiments, which showed that the adrenal medulla contains diadenosine polyphosphates[14,15]. They are released from perfused bovine adrenal glands and also from isolated chromaffin cells activated with carbachol. The ratio of the released diadenosine polyphosphates to released ATP and catecholamines is in the same order as that found in isolated chromaffin granules[13]. With regard to these results, it can be inferred that diadenosine polyphosphates are also released by the chromaffin granules of human adrenal glands.

To what extent may these findings be relevant for human physiology and pathophysiology? If the adrenal medulla secretes not only adrenaline and noradrenaline into the circulation, but also diadenosine polyphosphates, this seems to be of minor clinical significance, since substitution of adrenal steroids is generally sufficient to restore well-being and normal hemodynam-

ics after bilateral adrenalectomy. Moreover, the clinical picture of Addison's disease due to autoimmune adrenalitis, leaving the adrenal medulla unaffected, and the one due to adrenal tuberculosis, destroying both medulla and cortex, do not show significant differences. On the other hand, the role of adrenal medulla in vascular and metabolic regulation has not been ultimately defined, and a potential role of either catecholamines or other secretory products such as diadenosine polyphosphates has not yet been examined in detail. Furthermore, over the last decades it has been repeatedly documented that plasma catecholamines are elevated in essential hypertensive patients[44,45.] From this finding, the sympathetic nervous system can be excluded as the sole source of increased plasma catecholamines, since chromaffin tissue, but not the sympathetic nervous system is capable of synthesizing adrenaline. Since diadenosine polyphosphates and catecholamines are generally co-released by adrenal or sympathetic nervous tissue, diadenosine polyphosphates may also be secreted in increased amounts in essential hypertension. Indeed, there is one report showing increased platelet diadenosine polyphosphate contents in essential hypertension[46]. Therefore the present findings suggest that an increased diadenosine polyphosphate secretion by the adrenal medulla may be one potential mechanism underlying increased platelet diadenosine polyphosphates in essential hypertension[46]. Plasma diadenosine polyphosphate levels in essential hypertension have not been determined in this study, since the patients studied had all exhibited a primary hyperaldosteronism. In these patients, peripheral venous plasma diadenosine polyphosphate concentrations were similar to those seen in normotensive controls. Therefore, it is unlikely that adrenal diadenosine polyphosphate release is specifically related to hyperaldosteronism.

In summary, plasma concentrations of diadenosine polyphosphates in the range of 10^{-6} mol L^{-1} are compatible with systemic effects of these agents. By this finding, our view on diadenosine polyphosphates as hormones may be modified and extended.

B.2.6. References

1. Flores NA, Stavrou BM, Sheridan DJ: The effects of diadenosine polyphosphates on the cardiovascular system. *Cardiovasc Res* 42:15-26, 1999
2. Lüthje J, Baringer J, Ogilvie A: Highly efficient induction of human platelet aggregation in heparinized platelet-rich plasma by diadenosine triphosphate (Ap_3A). *Thromb Haemost* 54:469-471, 1985
3. Lüthje J, Ogilvie A: Diadenosine triphosphate (Ap_3A) mediates human platelet aggregation by liberation of ADP. *Biochem Biophys Res Commun* 118:704-709, 1984
4. Lüthje J, Ogilvie A: The presence of diadenosine 5',5'''-P_1,P_3-triphosphate (Ap_3A) in human platelets. *Biochem Biophys Res Commun* 115:253-260, 1983

5. Flodgaard H, Klenow H: Abundant amounts of diadenosine 5',5'''-P_1,P_4-tetraphosphate are present and releasable, but metabolically inactive, in human platelets. *Biochem J* 208:737-742, 1982
6. Schlüter H, Offers E, Brüggemann G, van der Giet M, Tepel M, Nordhoff E, Karas M, Spieker C, Witzel H, Zidek W: Diadenosine phosphates and the physiological control of blood pressure. *Nature* 367:186-188, 1994
7. Jankowski J, Tepel M, van der Giet M, Tente IM, Henning L, Junker R, Zidek W, Schlüter H: Identification and characterization of P_1, P_7-diadenosine-5'- heptaphosphate from human platelets. *J Biol Chem* 274:23926-23931, 1999
8. Luo J, Jankowski J, Knobloch M, van der Giet M, Gardanis K, Russ T, Vahlensieck U, Neumann J, Schmitz W, Tepel M, Deng MC, Zidek W, Schlüter H: Identification and characterization of diadenosine 5',5'''-P_1,P_2 - diphosphate and diadenosine 5',5'''-P_1,P_3-triphosphate in human myocardial tissue. *Faseb J* 13:695-705, 1999
9. Jankowski J, Hagemann J, Tepel M, van der Giet M, Stephan N, Henning L, Gouni-Berthold I, Sachinidis A, Zidek W, Schlüter H: Dinucleotides as growth promoting extracellulary mediators: Presence of dinucleoside diphosphates Ap_2A, Ap_2G and Gp_2G in releasable granlues of platelets. *J Biol Chem* 276:8904-8909, 2001
10. Schlüter H, Gross I, Bachmann J, Kaufmann R, van der Giet M, Tepel M, Nofer JR, Assmann G, Karas M, Jankowski J, Zidek W: Adenosine(5') oligophospho-(5') guanosines and guanosine(5') oligophospho-(5') guanosines in human platelets. *J Clin Invest* 101:682-688, 1998
11. Castillo CJ, Moro MA, Del Valle M, Sillero A, Garcia AG, Sillero MA: Diadenosine tetraphosphate is co-released with ATP and catecholamines from bovine adrenal medulla. *J Neurochem* 59:723-732, 1992
12. Castro E, Pintor J, Miras-Portugal MT: Ca^{2+}-stores mobilization by diadenosine tetraphosphate, Ap_4A, through a putative P2Y purinoceptor in adrenal chromaffin cells. *Br J Pharmacol* 106:833-837, 1992
13. Pintor J, Torres M, Miras-Portugal MT: Carbachol induced release of diadenosine polyphosphates-Ap_4A and Ap_5A- from perfused bovine adrenal medulla and isolated chromaffin cells. *Life Sci* 48:2317-2324, 1991
14. Pintor J, Rotllan P, Torres M, Miras-Portugal MT: Characterization and quantification of diadenosine hexaphosphate in chromaffin cells: granular storage and secretagogue-induced release. *Anal Biochem* 200:296-300, 1992
15. Rodriguez del Castillo A, Torres M, Delicado EG, Miras-Portugal MT: Subcellular distribution studies of diadenosine polyphosphates-Ap_4A and Ap_5A-in bovine adrenal medulla: presence in chromaffin granules. *J Neurochem* 51:1696-1703, 1988
16. Zimmermann H, Volknandt W, Wittich B, Hausinger A: Synaptic vesicle life cycle and synaptic turnover. *J Physiol Paris* 87:159-170, 1993
17. Ogilvie A: Extracellular functions of Ap_n A, in *Ap_4A and other dinucleoside polyphosphates.* Eds: McLennan AG, Boca Raton, Florida, CRC Press, 1992, pp 229-273
18. Ralevic V, Hoyle CH, Burnstock G: Pivotal role of phosphate chain length in vasoconstrictor versus vasodilator actions of adenine dinucleotides in rat mesenteric arteries. *J Physiol* 483:703-713, 1995
19. van der Giet M, Khattab M, Borgel J, Schlüter H, Zidek W: Differential effects of diadenosine phosphates on purinoceptors in the rat isolated perfused kidney. *Br J Pharmacol* 120:1453-1460, 1997
20. Kennedy C: ATP as a cotransmitter in perivascular sympathetic nerves. *J Auton Pharmacol* 16:337-340, 1996
21. Hansen MA, Dutton JL, Balcar VJ, Barden JA, Bennett MR: P2X (purinergic) receptor distributions in rat blood vessels. *J Auton Nerv Syst* 75:147-155, 1999

22. Nori S, Fumagalli L, Bo X, Bogdanov Y, Burnstock G: Coexpression of mRNAs for $P2X_1$, $P2X_2$ and $P2X_4$ receptors in rat vascular smooth muscle: an in situ hybridization and RT-PCR study. *J Vasc Res* 35:179-185, 1998
23. Phillips JK, McLean AJ, Hill CE: Receptors involved in nerve-mediated vasoconstriction in small arteries of the rat hepatic mesentery. *Br J Pharmacol* 124:1403-1412, 1998
24. Yamamoto K, Korenaga R, Kamiya A, Qi Z, Sokabe M, Ando J: $P2X_4$ receptors mediate ATP-induced calcium influx in human vascular endothelial cells. *Am J Physiol Heart Circ Physiol* 279:H285-292, 2000
25. Fukumitsu A, Takano Y, Iki A, Honda K, Saito R, Katsuragi T, Kamiya H: Endogenous ATP released by electrical field stimulation causes contraction via P2X- and P2Y-purinoceptors in the isolated tail artery of rats. *Jpn J Pharmacol* 81:375-380, 1999
26. Hillaire-Buys D, Dietz S, Chapal J, Petit P, Loubatieres-Mariani MM: Involvement of P2X and P2U receptors in the constrictor effect of ATP on the pancreatic vascular bed *J Soc Biol* 193:57-61, 1999
27. Inscho EW, Cook AK, Mui V, Miller J: Direct assessment of renal microvascular responses to P2-purinoceptor agonists. *Am J Physiol* 274:F718-727, 1998
28. McMillan MR, Burnstock G, Haworth SG: Vasoconstriction of intrapulmonary arteries to P2-receptor nucleotides in normal and pulmonary hypertensive newborn piglets. *Br J Pharmacol* 128:549-555, 1999
29. Pintor J, Miras-Portugal MT: P2 purinergic receptors for diadenosine polyphosphates in the nervous system. *Gen Pharmacol* 26:229-235, 1995
30. Yu SM, Chen SF, Lau YT, Yang CM, Chen JC: Mechanism of extracellular ATP-induced proliferation of vascular smooth muscle cells *Mol Pharmacol* 50:1000-1009, 1996
31. Wilden PA, Agazie YM, Kaufman R, Halenda SP: ATP-stimulated smooth muscle cell proliferation requires independent ERK and PI3K signaling pathways. *Am J Physiol* 275:H1209-1215, 1998
32. Malam-Souley R, Seye C, Gadeau AP, Loirand G, Pillois X, Campan M, Pacaud P, Desgranges C: Nucleotide receptor P2U partially mediates ATP-induced cell cycle progression of aortic smooth muscle cells. *J Cell Physiol* 166:57-65, 1996
33. Erlinge D, You J, Wahlestedt C, Edvinsson L: Characterisation of an ATP receptor mediating mitogenesis in vascular smooth muscle cells. *Eur J Pharmacol* 289:135-149, 1995
34. Bowry SK, Müller-Berghaus G: Aggregation of washed platelets from non-anticoagulated human blood is not reversible. *Thromb Haemost* 56:172-177, 1986
35. Bowry SK, Selmayr E, Müller-Berghaus G: Evidence of increased association of fibrinogen with platelets caused by sodium citrate. *J Lab Clin Med* 111:315-325, 1988
36. Barnes LD, Robinson AK, Mumford CH, Garrison PN: Assay of diadenosine tetraphosphate hydrolytic enzymes by boronate chromatography. *Anal Biochem* 144:296-304, 1985
37. Sulkowski E, Laskowski S: Venom exonuclease (phosphodiesterase) immobilized on concanavalin-A-sepharose. *Biochem Biophys Res Commun* 57:463-468, 1974
38. Hillenkamp F, Karas M: Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. *Methods Enzymol* 193:280-295, 1990
39. Kaufmann R, Kirsch D, Spengler B: Sequencing of peptides in a time-of-flight mass spectrometer: evaluation of postsource decay following matrix-assisted laser desorption ionisation (MALDI). *Int J Mass Spectrom Ion Proc* 131:355-385, 1994

40. Jankowski J, Potthoff W, Zidek W, Schlüter H: Purification of chemically synthesized dinucleoside(5',5') polyphosphates by displacement chromatography. *J Chromatogr B Biomed Sci Appl* 719:63-70, 1998
41. Heidenreich S, Tepel M, Schlüter H, Harrach B, Zidek W: Regulation of rat mesangial cell growth by diadenosine phosphates. *J Clin Invest* 95:2862-2867, 1995
42. Jankowski J, Potthoff W, van der Giet M, Tepel M, Zidek W, Schlüter H: High-Performance Liquid Chromatographic Assay of the Diadenosine Polyphosphates in Human Platelets. *Anal Biochem* 269:72-78, 1999
43. Jankowski J, Hagemann J, Yoon MS, van der Giet M, Stephan N, Zidek W, Schlüter H, Tepel M: Increased vascular growth in hemodialysis patients induced by platelet-derived diadenosine polyphosphates. *Kidney Int* 59:1134-1141, 2001
44. Jindra A, Jachymova M, Horky K, Peleska J, Umnerova V, Bultas J, Savlikova J: Association analysis of two tyrosine hydroxylase gene polymorphisms in normotensive offspring from hypertensive families. *Blood Press* 9:250-254, 2000
45. Lindqvist M, Kahan T, Melcher A, Bie P, Hjemdahl P: Forearm vasodilator mechanisms during mental stress: possible roles for epinephrine and ANP. *Am J Physiol* 270:E393-399, 1996
46. Hollah P, Hausberg M, Kosch M, Barenbrock M, Letzel M, Schlatter E, Rahn KH: A novel assay for determination of diadenosine polyphosphates in human platelets: studies in normotensive subjects and in patients with essential hypertension. *J Hypertens* 19:237-245, 2001

B.3. Uridine adenosine tetraphosphate: a novel endothelium derived vasoconstrictive factor

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B.3.1. Abstract and Introduction

Beyond serving as a mechanical barrier, the endothelium shows important regulatory functions. The discovery of nitric oxide (NO)[1] revolutionized our understanding of vasoregulation. In contrast, the identity of endothelium-derived vasoconstrictive factors (EDCFs) remains unclear. The supernatant obtained from mechanically stimulated human endothelial cells obtained from dermal vessels elicited a vasoconstrictive response in an isolated perfused rat kidney. A purinoceptor blocker had a greater effect than an endothelin receptor blocker in decreasing endothelium derived vasoconstriction in the isolated perfused rat kidney. The nucleotide uridine adenosine tetraphosphate (Up_4A) was isolated from the supernatant of stimulated human endothelium and identified by mass spectrometry. Up_4A most likely exerts vasoconstriction predominantly via P2X1 receptors, and probably also through P2Y2 and P2Y4 receptors. Plasma concentrations of Up_4A that cause vasoconstriction are found in healthy subjects. Stimulation with adenosine 5´-triphosphate (ATP), uridine 5´-triphosphate (UTP), acetylcholine, endothelin, A23187 and mechanical stress releases Up_4A from endothelium, suggesting that Up_4A contributes to vascular autoregulation. To our knowledge, Up_4A is the first dinucleotide isolated from living organism that contains both purine and pyrimidine moieties. We conclude that Up_4A is a novel potent non-peptidic EDCF. Its vasoactive effects, plasma concentrations and its release upon endothelial stimulation strongly suggest that Up_4A has a functional vasoregulatory role.

B.3.2. Results and Discussion

The supernatant obtained from human endothelial cells stimulated by mechanical stress elicited a vasoconstrictive response when injected into an isolated perfused rat kidney (Figure 1.A curve 1). To determine the contribution of endothelin to endothelium derived vasoconstriction we first applied the endothelin receptor antagonist BQ123 (Figure 1.A; curve 2). The contribution of endothelin was 9.3 ± 9.1% of the total vasoconstrictive response (Figure 1.B, n = 12). To determine the contribution of vasoactive mononucleotides, we added alkaline phosphatase to the supernatant to degrade these mononucleotides, thereby further diminishing the vasoconstrictive response to the endothelial cell supernatant by 42.5 ± 8.6% (Figure 1.A,

curve 3; Figure 1.B, n = 12). Matrix assisted laser desorption/ionisation mass spectrometry (MALDI) mass spectra before and after addition of alkaline phosphatase shows the effective degradation of ATP and UTP by alkaline phosphatase (Figure 1.C). To verify whether the remaining vasoconstrictive response was attributable to nucleotides, we applied the unselective purinergic antagonists pyridoxal-phosphate-6-azophenyl-2,4-disulphonic acid (PPADS) and suramin. In the presence of PPADS, the vasoconstrictive response elicited by supernatants from endothelial cells after incubation with alkaline phosphatase and BQ123 was diminished (Figure 1.A; curve 4, n = 7), suggesting that vasoconstrictive nucleotides resistant to alkaline phosphatase may contribute 28.8 ± 6.5% of total EDCF actions under these conditions. Suramin similarly reduced the vasoconstrictive response by 32.4 ± 8.6% of total EDCF effects (Figure 1.A, curve 5; Figure 1.B, n = 5). The data showed that endothelin contributes to EDCF effects in our experimental setting considerably less than other EDCFs, especially nucleotides. These experiments helped us to choose the further purification steps applied to endothelial cell supernatants (Figure 1.D). First, we deproteinized supernatants of stimulated endothelial cells to select the fractions that were likely to contain endothelium derived nucleotides. After deproteination, we desalted the supernatants by a preparative reversed-phase chromatography. Then we subjected the 20% acetonitrile (ACN) eluates of the reversed phase chromatography to a purification step designed to separate dinucleoside polyphosphates from mononucleoside polyphosphates using a phenylboronate affinity column. Using this column, we separated mononucleotides (Figure 1.E, arrow 1) from nucleotides containing at least two pairs of neighbouring cis-diol groups which are present in ribose moieties (Figure 1.E; arrow 2).

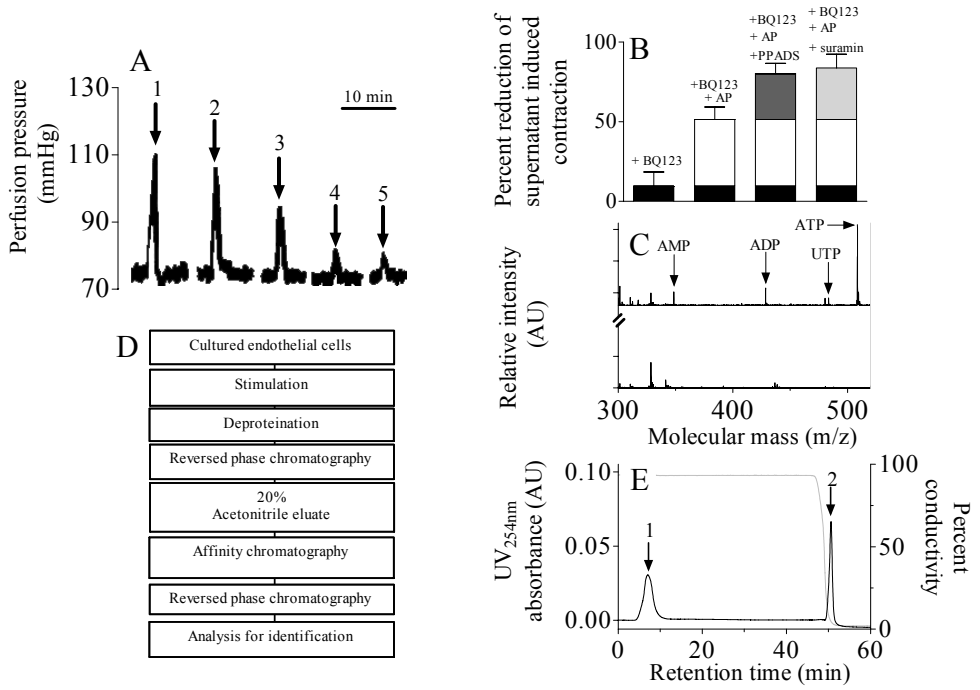

Figure 1: Vasoconstrictive effects of supernatants from stimulated endothelial cells and isolation of uridine adenosine tetraphosphate.

(A) Effect of aliquots of supernatants from endothelial cells stimulated by mechanical stress on the perfusion pressure in the isolated rat kidney: Curve 1: control; curve 2: in the presence of the endothelin inhibitor BQ123; curve 3: after incubation with immobilised alkaline phosphatase and in the presence of the endothelin inhibitor BQ123; curve 4: after incubation with immobilised alkaline phosphatase and in the presence of the endothelin inhibitor BQ123 and the purinergic antagonist PPADS; curve 5: after incubation with immobilised alkaline phosphatase and in the presence of the endothelin inhibitor BQ123 and the purinergic antagonist suramin.

(B) Effect of aliquots of supernatants from endothelial cells stimulated by mechanical stress on the perfusion pressure in the isolated rat kidney; experiments as shown in Figure 1.A. The columns represent the inhibitory effects attributable to the single inhibitors, which have been added sequentially to the medium (■ = BQ123, □ = alkaline phosphatase (AP), ■ = PPADS, ■ = suramin).

(C) MALDI mass spectrum of the aliquot of supernatants from endothelial cells stimulated by mechanical stress before (upper spectrum) and after (lower spectrum) incubation with immobilised alkaline phosphatase.

(D) Scheme for isolation of Up_4A from supernatants of stimulated endothelial cells.

(E) Affinity chromatography of supernatant of stimulated endothelial cells. The fraction containing mononucleotides and the one containing nucleotides with at least two pairs of neighbouring cis-diol groups are labelled in the Figure (arrow 1 and 2).

Next, we further fractionated the remaining nucleotides by analytical reversed-phase chromatography (Figure 2.A). We selected those fractions showing vasoconstrictive properties by testing their vasoactivity in the isolated perfused rat kidney. The fraction showing the strong-

est vasoconstriction (arrow in Figure 2.A) underwent further analysis. The Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrum of this fraction showed a molecular mass of 814 Da. Figure 2.B shows the FT-ICR-fragmentation mass-spectrum of the underlying substance. Each signal was attributable to a fragment of Up_4A (Table 1), suggesting that Up_4A was the substance under investigation.

Table 1: Molecular masses of Up_4A fragments obtained by MS/MS-FT-ICR mass spectrometry**.** The mass spectrum was obtained from the peak labelled in Figure 2.A. The left column: fragment masses measured by MS/MS-FT-ICR mass spectrometry; right column: fragment masses expected theoretically from their respective structures. M^+ = protonated parent ion; U´= uracil; A = adenosine; U = uridine; p = phosphate group, e.g. Ap_3 = ATP; w/o=without

Interpretation of the FT-ICR mass signals	FT-ICR mass signal [m/z] measured	FT-ICR mass signal [m/z] calculated
Up_4A w/o U´	255.33	254.18
Ap_1 w/o 2 H_2O	316.24	315.21
Ap_2 w/o 2 H_2O	395.35	392.20
Up_3 w/o 2 H_2O	450.02	448.13
Up_3 w/o H_2O	466.13	466.20
Ap_3 w/o H_2O	490.00	490.00
M w/o Up_1	490.00	490.23
M w/o U and w/o 2 H_2O	533.96	533.84
M w/o U + H_2O	585.03	587.84
Up_4 + 3 H_2O	617.95	618.11
Not interpreted	629.02	
M w/o U´ and w/o H_2O	682.95	682.93
Not interpreted	722.94	
M^+	814.03	814.10

Next, we assessed the type of ester bonds between the phosphate and ribose moieties within the isolated substance. To differentiate between a 3' and a 5' bond, we used enzymes selectively splitting one of these bonds (i.e., 5' nucleotide hydrolase and 3' nucleotide hydrolase, respectively). We used alkaline phosphatase to cleave free phosphate groups. The intact nu-

cleotide was split by 5′ phosphodiesterase (Figure 2.C), whereas 3′ phosphodiesterase and alkaline phosphatase had no effect (data not shown), suggesting that the phosphate moieties are bound to the ribose by a 5′ bond.

To further substantiate the hypothesized structure, we compared the fragment mass spectrum to that obtained from synthetic Up_4A. Therefore we synthesized Up_4A using a modification of a technique described earlier[2]. The comparison of the mass spectra obtained from authentic Up_4A and from the fraction under investigation showed their identity.

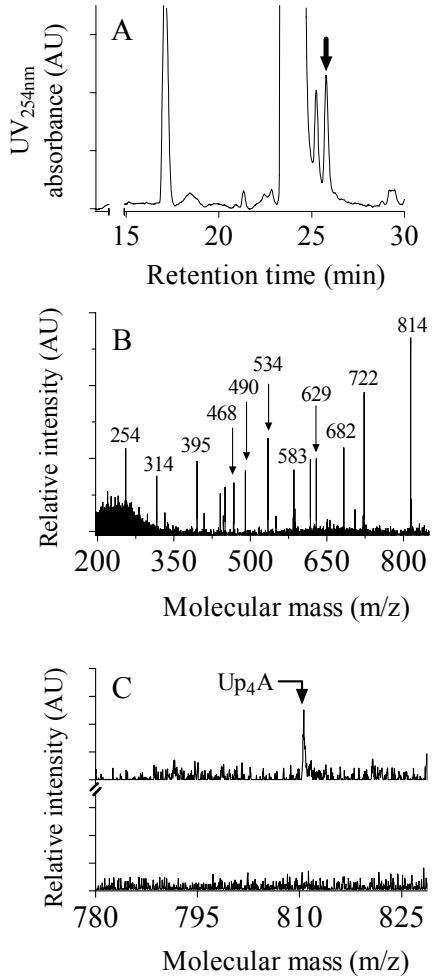

Figure 2: Final purification step and identification of uridine adenosine tetraphosphate from supernatants of stimulated endothelial cells.

(A) Analytical reversed phase high performance liquid chromatography of the fraction of supernatant of stimulated endothelial cells containing the remaining nucleotides after exclusion of mononucleotides. Arrow: the fraction with the strongest vasoconstrictive properties.

(B) FT-ICR-fragmentation mass-spectrum of the fraction labelled in Figure 2.A by an arrow.

(C) Enzymatic cleavage analysis of the vasoconstrictive fraction of reversed-phase chromatography in Figure 2.A. Figure 2.C shows typical MALDI mass spectra before (upper spectrum) and after incubation with 5'-nucleotidase (lower spectrum) out of 5 similar experiments.

To exclude the idea that Up_4A may form spontaneously from mononucleotides at a pH that is not in the physiologic range, due to the addition of perchloric acid, we monitored the reaction products generated after addition of perchloric acid during the purification procedure. MALDI mass spectrometry did not show the spontaneous formation of Up_4A under these conditions (data not shown). Next, we tested vasoconstrictive properties of synthesized Up_4A

using the isolated perfused rat kidney. In this model, Up_4A was a potent vasoconstrictor (Figure 3.A).

Which purinoceptors mediate the effects of Up_4A? The selective P2X1 and P2X3 desensitizer, α,β-methylene ATP[2] markedly inhibited Up_4A-induced vasoconstriction (Figure 3.A). In vascular smooth muscle cells, P2X1 is the dominant P2X subtype[3-5], although P2X2, P2X3, P2X4 and P2X5 have also been found[6-8]. The strong effects of α,β-methylene ATP render major contributions from other P2X receptors than P2X1 or P2X3 unlikely. Although currently no ideal inhibitor discriminating between P2X1 and P2X3 receptors is known[9], we applied 8,8′-[carbonylbis(imino-3,1-phenylene-carbonyl-imino)]bis(1, 3, 5-naphthalenetrisulfonic acid) (NF023) and diinosine pentaphosphate (Ip_5I) , which show a P2X1:P2X3 receptor affinity ratio of about 20 and 1,000, respectively[9,10]. Furthermore, 2′,3′-O-(2,4,6-trinitrophenyl) adenosine triphosphate (TNP-ATP) also inhibits P2X1 and P2X3 receptors[11,12]. NF023, TNP-ATP and Ip_5I, the latter being applied in a concentration inhibitory for P2X1, but far below the IC50 reported for the P2X3 receptor, markedly inhibited the Up_4A response, supporting the view that P2X1 receptors contribute the largest part to the vasoconstrictive Up_4A response (Figure 3.B, each n = 3). This conclusion is in accordance with findings obtained in P2X1 receptor knockout mice showing that vasoconstrictive responses to ATP are abolished in arteries lacking this P2X subtype[13].

Part of the Up_4A response was resistant to α,β-methylene ATP. Because uridine-containing nucleotides affect vascular tone through P2Y2 and P2Y4 receptors[14], this finding allows us to consider the possibility that Up_4A might also activate on these P2Y receptor subtypes. Thus, Up_4A may turn out to be not only a modulator of acute vascular processes like vasoconstriction, but also of other long-term effects on vascular wall structure, because P2Y2 and P2Y4 receptors mediate numerous other effects including cell proliferation and differentiation[14].

By blocking nitric oxide synthase, the vasoconstrictive effect of Up_4A was increased (Figure 3.A, c, $P < 0.05$, n = 3). The effects of ATP and UTP also tended to be increased after inhibition of nitric oxide synthase, but these differences did not reach statistical significance (each n = 3). To rule out the idea that the vasoconstrictive response of Up_4A is caused by its degradation products, we compared the concentration-response curves of ATP and UTP to that of Up_4A (Figure 3.C). ATP and UTP affected vascular tone much less than Up_4A, indicating that these degradation products can only account for a small part, if any, of the Up_4A effects. But because endothelial cells are known to release ATP upon stimulation with several sub-

stances[15], Up_4A may also be a stimulator of endothelial ATP release, and this indirect effect may be a further component of vascular Up_4A actions, in addition to its direct actions.

Last, we confirmed that pharmacological inhibition of Up_4A, ATP and UTP actions did not modify the endothelin response and that, inversely, the endothelin inhibitor did not affect the response to nucleotides (Figure 3.D). Furthermore, the question arose whether Up_4A is also present in human plasma. With the purification procedure indicated in Figure 1.D, we obtained a chromatogram from plasma of healthy individuals (n=6), which showed Up_4A as an isolated peak (Figure 3.E), as evidenced by FT-ICR mass spectrometry. The quantification of these ultraviolet absorption peaks showed an Up_4A plasma concentration of 55.5 ± 15.2 nmol L^{-1} in these six healthy persons. The recovery rate for Up_4A was 13.1 ± 3.7% in human plasma and 12.0 ± 1.8% in culture medium (each n = 5). As we used a P(1),P(2):P(2),P(3)-bis-methylene diadenosine triphosphate as an internal standard, we excluded losses during purification as a source of error. The recovery rate of ATP and UTP in either plasma or culture medium was below the detection limit of 10 pmol L^{-1} with the purification procedure described for Up_4A, indicating that the separation of mononucleotides from dinucleotides was effective. The concentration-response curve of Up_4A (Figure 3.A) shows that these Up_4A plasma concentrations are sufficient to elicit relevant vasoconstrictive effects. Nevertheless, the *in vivo* effects of this Up_4A concentration may not correspond to those shown by *in vitro* experiments, because a multitude of additional factors including the activation of counter-regulatory systems determine systemic actions. Up_4A also affected vascular tone in the intact animal. Moreover, its vasoactive potency was comparable to that of noradrenaline. Both Up_4A and noradrenaline increased mean arterial blood pressure when injected intra-aortically in the anaesthetized rat (Figure 3.F). Whereas noradrenaline elicited a sharp, short-lasting increase in blood pressure, the same amount of Up_4A showed a more prolonged effect on blood pressure.

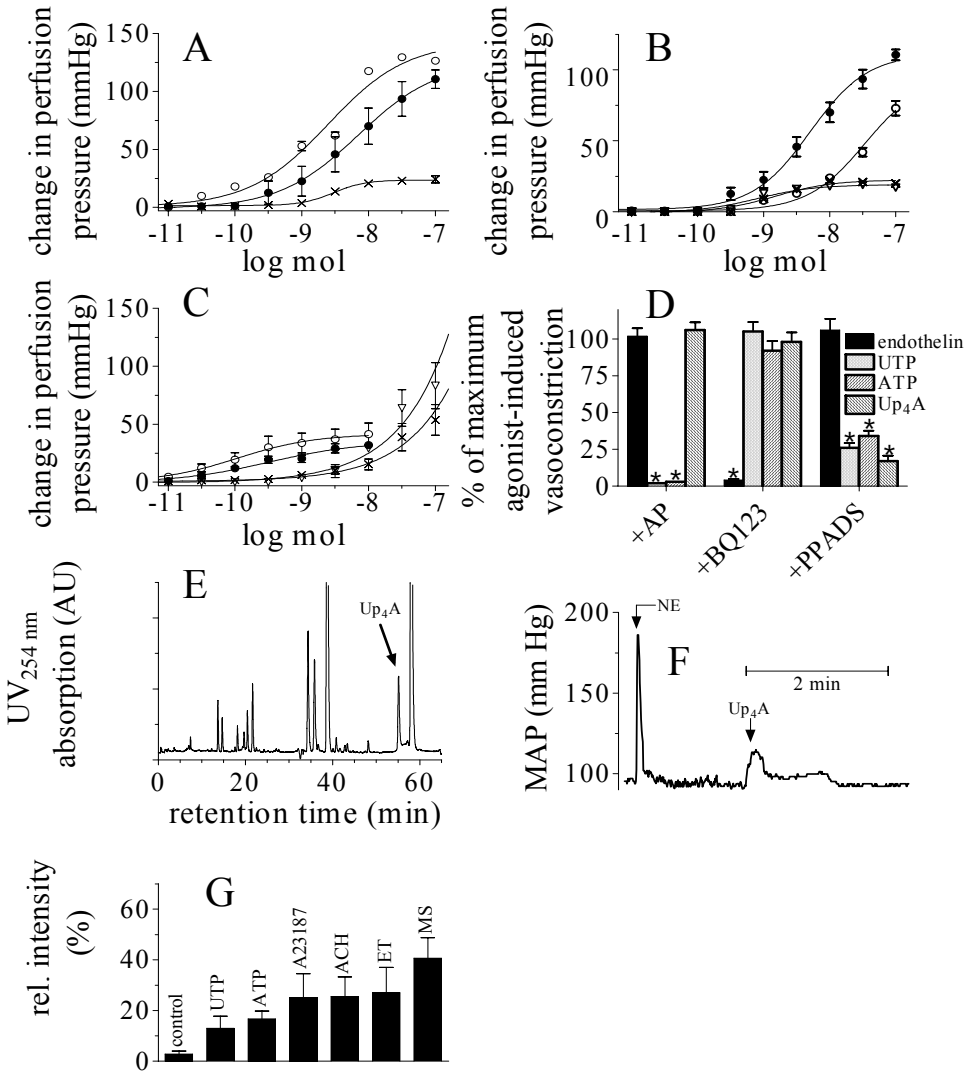

Figure 3: Vascular effects and quantification of uridine adenosine tetraphosphate release after stimulation of endothelial cells and in human plasma:

(A) Change in perfusion pressure in the isolated perfused rat kidney induced by Up_4A alone (●), with α,β-methylene ATP (×), and with L-NAME (○).

(B) Change in perfusion pressure in the isolated perfused rat kidney induced by Up_4A alone (●), and with NF023 (×), Ip_5I (○), and TNP-ATP (▽).

(C) Change in perfusion pressure in the isolated perfused rat kidney induced by ATP in the absence (●) and presence of L-NAME (○) or by UTP in the absence (×) and presence of L-NAME (▽).

(D) Influence of immobilised alkaline phosphatase (AP), BQ123 and PPADS on the increase in perfusion pressure in the isolated perfused rat kidney induced by Up_4A, endothelin, ATP and UTP.

(E) Typical reversed phase chromatogram of a plasma extract from a healthy subject isolated by the purification procedure indicated in Figure 1.D.

(F) Mean arterial pressure (MAP) in an anaesthetized rat (typical tracing out of 5 similar experiments) after intraaortic injection (arrows) of norepinephrine (NE) and Up_4A.

(G) Increased Up_4A concentrations in supernatants after stimulating endothelial cells with UTP, ATP, A23187, acetylcholine (ACH), endothelin (ET) or mechanical stress (MS).

Given that other purinergic agonists are also present in human plasma, what vasoconstrictive potency does plasma Up_4A contribute compared to these mononucleotides? Plasma ATP concentrations are in the range of 80 nmol L^{-1} (e.g. [16]), which are similar to the Up_4A concentration found in this study. There is no valid assay for plasma UTP determinations[17], but generally an ATP:UTP ratio of 10:1 is assumed[18], suggesting that any vascular effects of plasma UTP may be lower than those of ATP. With regard to these findings in literature, vascular effects of plasma Up_4A may exceed those of plasma mononucleotides, because in this concentration range Up_4A shows stronger vasoconstrictive actions than either ATP or UTP (Figure 3.A, C).To assess the relative contribution of the mononucleotides, ATP and UTP versus the dinucleotide Up_4A to endothelium derived vasoconstriction, we determined ATP and UTP concentrations in endothelial cell supernatants before and after mechanical stimulation. Before stimulation both mononucleotides were undetectable; after stimulation we found 34.8 ± 20.3 and 22.0 ± 10.2 nmol L^{-1} ATP and UTP, respectively (each n = 3), suggesting a substantial contribution of these nucleotides to endothelium derived vasoconstriction (Figure 3.C).

Next, we studied endothelial Up_4A release (Figure 3.G). Acetylcholine, endothelin, A23187, mechanical stress, and ATP and UTP stimulated the release of Up_4A from endothelial cells. These results show that several physiologic stimuli can release Up_4A from human endothelial cells. Because endothelin is a stimulator of Up_4A release, Up_4A release may affect part of the endothelin-mediated vasoconstriction. Taken together, the experiments showed (i) that human endothelial cells secrete Up_4A, (ii) that Up_4A is a potent vasoconstrictor, (iii) that in human plasma Up_4A occurs in concentrations effecting vasoconstriction, and (iv) that several physiologic stimuli and an increase in cytosolic free Ca^{2+} concentration stimulate the release of Up_4A.

In conclusion, these findings are of interest for several reasons: first, Up_4A appears to be a new, potent nonpeptidic endothelium derived vasoconstrictor. Second, to our knowledge, Up_4A is the first dinucleotide found in living organisms that contains both a pyrimidine and a purine moiety. Dinucleotides containing two purine moieties are known, and their role in vasomotor regulation is increasingly recognized. Our data show that the activity of Up_4A differs from those of dinucleotides exclusively containing purines[19]. The uridine moiety may confer an affinity towards P2Y receptor subtypes, as is known from uridine-containing mononucleotides. Because Up_4A is secreted by human endothelial cells and is present in effective

concentrations in human plasma, a role of Up_4A in the regulation of vascular tone and in cardiovascular disease seems to be likely.

B.3.3. Methods

The local ethical committee approved the experiments involving rats and humans. Informed consent was obtained from all human subjects.

CULTURE OF ENDOTHELIAL CELLS

We cultured human endothelial cells from dermal microvessels (HMEC-1) in MCDB131 medium supplemented with 100 U ml^{-1} penicillin/streptomycin, 1% (v/v) L-glutamine and 7.5% (v/v) fetal bovine serum (see Supplementary Methods).

STIMULATION OF CULTURED ENDOTHELIAL CELLS

We washed cell-culture flasks of endothelial cells (n = 30) three times with a physiological salt solution. Then we exposed six cell-culture flasks of endothelial cells to mechanical stress for 10 min by using a horizontal shaking machine after addition of 15 ml physiologic salt solution. To stimulate endothelial cells we added ATP, UTP, Ca-ionophore A23187, acetylcholine or endothelin (final concentration 1 μmol L^{-1}) to eight cell-culture flasks each. After 10 min we collected and pooled the supernatant and incubated aliquots of the resulting supernatants with immobilized alkaline phosphatase (see Supplementary Methods).

CHROMATOGRAPHIC ANALYSIS OF THE SUPERNATANTS OF ENDOTHELIAL CELLS

We added triethylammonium acetate (final concentration 40 mmol L^{-1}) to the supernatants and titrated pH to 6.5. Next, we used a C18 reversed-phase column (LiChroprep, 310 × 65 mm, 65–40 μm, Merck) to concentrate the supernatant of stimulated and unstimulated endothelial cells. We removed nonbinding substances with triethylammonium acetate. Then we eluted binding substances stepwise with 20% ACN, in water at a flow rate of 1.0 ml min^{-1}. We monitored the elution by measuring the ultraviolet absorption at 254 nm. Last we froze the eluate at –80 °C and lyophilized it.

Then we further purified the eluate of the preparative reversed-phase chromatography column with affinity chromatography. We synthesized the affinity chromatography gel by coupling phenyl boronic acid to a cation exchange resin (Biorex 70, Bio-Rad), according to previous

studies[20]. We packed the affinity resin into a glass column and equilibrated it with 1 mol L^{-1} ammonium acetate (pH 9.5). We adjusted the pH of the eluate from the preparative reversed-phase chromatography to pH 9.5 and loaded it to the affinity column. We washed the column with an ammonium acetate solution with a flow rate of 1.0 ml $min^{-1}$and eluted binding substances with 1 mmol L^{-1} HCl solution. We monitored the elution by measuring the UV absorption at 254 nm. Then we froze eluate at –80 °C and lyophilized it. We added 1 mol L^{-1} triethylammonium acetate to the eluate of the affinity chromatography (final concentration, 40 mmol L^{-1}). We injected the eluate of the affinity chromatography into a reversed phase high performance liquid column (Chromolith RP-18e 100–4.6, Merck) for desalting (see Supplementary Methods). Then we dissolved the lyophilized eluate in 40 mmol L^{-1} triethylammonium acetate (eluent A) and injected the eluate in two reversed phase columns (Chromolith RP-18e 100–4.6, Merck) connected in series. We used 80% ACN (eluent B) and the following gradient for the elution: 0–10% B for 40 min, 10–100% B for 1 min, 100% B for 2 min. We collected 18 1 ml-fractions at a flow rate of 1.0 ml min^{-1}, monitoring UV absorption at 254 nm. To quantify the concentration of ATP and UTP, we fractionated medium (30 ml) as described above. In this case, we used α,β-methylene ATP (10 μg) as internal standard and fractionated the non-binding substances rather than the substances binding to the affinity column. The analysis of the isolated substances by FT-ICR and MALDI mass spectrometry, as well as enzymatic cleavage experiments are presented as supplementary information (see Supplementary Methods).

MEASUREMENTS OF PERFUSION PRESSURE IN THE ISOLATED PERFUSED RAT KIDNEY

We evaluated the effects of aliquots of supernatants of stimulated endothelial cells, of supernatants of stimulated endothelial cells after incubation with alkaline phosphatase, of fractions of the reversed phase chromatography and of ATP and UTP (each 10 pmol L^{-1} to 0.1 μmol L^{-1}) on vascular tone in an isolated perfused rat kidney with a constant flow of 8 ml min^{-1} (see Supplementary Methods).

In some experiments, we added the P_2-purinoceptor antagonists PPADS (10 μmol L^{-1}) and suramin (50 μmol L^{-1}), TNP-ATP (100 μmol L^{-1})[11], Ip_5I (100 nmol L^{-1}), NF023 (10 μmol L^{-1}), or the specific ET_A receptor antagonist cyclo-D-P-V-L-W (BQ123; Sigma-Aldrich) (1 μmol L^{-1}) to the perfusate 30 min before challenge with potential vasoconstrictors. Fur-

thermore, in some experiments, we specifically desensitized P2X1 and P2X3 receptors by continuous perfusion of the kidney with 10 μmol L^{-1} α,β-methylene ATP (n = 3).

MEASUREMENT OF MEAN ARTERIAL BLOOD PRESSURE AFTER INTRAARTERIAL ADMINISTRATION OF UP_4A

We measured intra-arterial blood pressure as previously published[21] (see Supplementary Methods).

ISOLATION AND IDENTIFICATION OF UP_4A IN HUMAN PLASMA

We obtained peripheral blood (50 ml) by catheterisation of the cubital vein in six healthy individuals, collecting the blood in tubes containing K_2-EDTA (7.2 mg). The mean age of the subjects (4 males, 2 females) was 32.7 ± 2.3 years, systolic blood pressure 114.2 ± 3.3 mmHg, diastolic blood pressure 77.2 ± 2.1 mmHg (each mean ± S.E.M.) (see Supplementary Methods). The isolation steps and identification steps of uridine adenosine tetraphosphate from human plasma were the same as described above for the identification of uridine adenosine tetraphosphate from the supernatants of stimulated endothelial cells and as demonstrated in Figure 1.D.

ACKNOWLEDGEMENTS

We thank M. Witt for accumulation of the FT-ICR mass spectra. We thank L. Lebeau for gift of the internal standard. This study was supported by a grant of the Deutsche Forschungsgemeinschaft (Zi 315/15–1) and by a grant of the Hospal Research Funding™ (J. Jankowski).

B.3.4. References

1. Palmer RM, Ferrige AG, Moncada S: Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327:524-526, 1987
2. Kasakov L, Burnstock G: The use of the slowly degradable analog, alpha, beta-methylene ATP, to produce desensitisation of the P2-purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea-pig urinary bladder. *Eur J Pharmacol* 86:291-294, 1982
3. Bo X, Sexton A, Xiang Z, Nori SL, Burnstock G: Pharmacological and histochemical evidence for P2X receptors in human umbilical vessels. *Eur J Pharmacol* 353:59-65, 1998
4. Kunapuli SP, Daniel JL: P2 receptor subtypes in the cardiovascular system. *Biochem J* 336:513-523, 1998
5. Wang L, Karlsson L, Moses S, Hultgardh-Nilsson A, Andersson M, Borna C, Gudbjartsson T, Jern S, Erlinge D: P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. *J Cardiovasc Pharmacol* 40:841-853, 2002

6. Pulvirenti TJ, Yin JL, Chaufour X, McLachlan C, Hambly BD, Bennett MR, Barden JA: P2X (purinergic) receptor redistribution in rabbit aorta following injury to endothelial cells and cholesterol feeding. *J Neurocytol* 29:623-631, 2000
7. Nori S, Fumagalli L, Bo X, Bogdanov Y, Burnstock G: Coexpression of mRNAs for P2X1, P2X2 and P2X4 receptors in rat vascular smooth muscle: an in situ hybridization and RT-PCR study. *J Vasc Res* 35:179-185, 1998
8. Turner CM, Vonend O, Chan C, Burnstock G, Unwin RJ: The pattern of distribution of selected ATP-sensitive P2 receptor subtypes in normal rat kidney: an immunohistological study. *Cells Tissues Organs* 175:105-117, 2003
9. North RA: Molecular physiology of P2X receptors. *Physiol Rev* 82:1013-1067, 2002
10. King BF, Liu M, Pintor J, Gualix J, Miras-Portugal MT, Burnstock G: Diinosine pentaphosphate (Ip_5I) is a potent antagonist at recombinant rat $P2X_1$ receptors. *Br J Pharmacol* 128:981-988, 1999
11. Lewis CJ, Surprenant A, Evans RJ: 2',3'-O-(2,4,6- trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP) - a nanomolar affinity antagonist at rat mesenteric artery P2X receptor ion channels. *Br J Pharmacol* 124:1463-1466, 1998
12. Honore P, Mikusa J, Bianchi B, McDonald H, Cartmell J, Faltynek C, Jarvis MF: TNP-ATP, a potent $P2X_3$ receptor antagonist, blocks acetic acid-induced abdominal constriction in mice: comparison with reference analgesics. *Pain* 96:99-105, 2002
13. Vial C, Evans RJ: $P2X_1$ receptor-deficient mice establish the native P2X receptor and a P2Y6-like receptor in arteries. *Mol Pharmacol* 62:1438-1445, 2002
14. Burnstock G: Purinergic signaling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol* 22:364-373, 2002
15. Burnstock G: Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *J Anat* 194:335-342, 1999
16. Gorman MW, Marble DR, Ogimoto K, Feigl EO: Measurement of adenine nucleotides in plasma. *Luminescence* 18:173-181, 2003
17. Lazarowski ER, Boucher RC: UTP as an extracellular signaling molecule. *News Physiol Sci* 16:1-5, 2001
18. Traut TW: Physiological concentrations of purines and pyrimidines. *Mol Cell Biochem* 140:1-22, 1994
19. van der Giet M, Westhoff T, Cinkilic O, Jankowski J, Schlüter H, Zidek W, Tepel M: The critical role of adenosine and guanosine in the affinity of dinucleoside polyphosphates to P2X-receptors in the isolated perfused rat kidney. *Br J Pharmacol* 132:467-474, 2001
20. Barnes LD, Robinson AK, Mumford CH, Garrison PN: Assay of diadenosine tetraphosphate hydrolytic enzymes by boronate chromatography. *Anal Biochem* 144:296-304, 1985
21. Nofer JR, van der Giet M, Tölle M, Wolinska I, von Wnuck Lipinski K, Baba HA, Tietge UJ, Godecke A, Ishii I, Kleuser B, Schäfers M, Fobker M, Zidek W, Assmann G, Chun J, Levkau B: HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest* 113:569-581, 2004

B.3.5. Supplementary Methods

Since not all methods were described in the publication, in this section a more extensive description of the methods is given.

CHEMICALS

HPLC water (gradient grade) and acetonitrile (ACN) were from Merck, all other substances from Sigma Aldrich.

CULTURE OF ENDOTHELIAL CELLS

Human endothelial cells from dermal microvessels (HMEC-1) is the first immortalized human microvascular endothelial cell line that retains the morphologic, phenotypic, and functional characteristics of normal human microvascular endothelial cells[1]. Experiments comparing the phenotypic characteristics of HMEC-1 with human dermal microvascular endothelial cells or human umbilical vein endothelial cells revealed that HMEC-1 have features of both small- and large-vessel endothelial cells[1]. On day 0 we placed the cells into 175 cm^2 cell-culture flasks (Nunc Inc.) and stimulated the cells on day 2 at approximately 70% confluency. Confluent cultures of HMEC-1 showed typical cobblestone appearance and showed the characteristic expression of von Willebrand factor, endothelial nitric oxide synthase, VEGF receptor 1 (FLT-1) and absence of smooth muscle α-actin staining. The authors are grateful for the HMEC-1 cells provided by Prof. G. Schoenfelder (Charité-, Institute for Clinical Pharmacology and Toxicology, Berlin, Germany)

STIMULATION OF CULTURED ENDOTHELIAL CELLS

The pooled supernatant underwent deproteinization with perchloric acid (final concentration 0.6 mol L^{-1}), centrifugation at 3,500 U min^{-1} for 5 min at 4 ^{o}C and neutralization with KOH to pH 9.5. We removed the precipitated proteins and the insoluble reaction product $KClO_4$ by centrifugation (3,500 U min^{-1}; 4 °C; 5 min). For control reactions, we washed 30 cell-culture flasks of endothelial cells three times with 15 ml of a physiological salt solution. To avoid mechanical stress, we added salt solution extremely slowly. After the washing step, we added 15 ml physiological salt solution to the endothelial cells. 10 min later, we collected and pooled the supernatant.

CHROMAOTGRAPHIC ANALYSIS OF THE SUPERNATANTS OF ENDOTHELIAL CELLS

AFFINITY CHROMATOGRAPHY

We synthesized the affinity chromatography gel, by coupling phenyl boronic acid to a cation exchange resin (Biorex 70, Bio-Rad), according to Barnes *et al.*[2]. We packed the affinity resin into a glass column and equilibrated it with 1 mol L^{-1} ammonium acetate (pH 9.5). We adjusted the pH of the eluate from the preparative reversed-phase chromatography to pH 9.5 and loaded it to the affinity column. We washed the column with an ammonium acetate solution with a flow rate of 1.0 ml $min^{-1}$and eluted binding substances with 1 mmol L^{-1} HCl solution. We monitored the elution by measuring the UV absorption at 254 nm. Then we froze eluate at –80 °C and lyophilized it.

REVERSED-PHASE CHROMATOGRAPHY

After removing substances not binding to the column with aqueous 40 mmol L^{-1} triethylammonium acetate, we eluted the absorbed substances with 20% ACN in water at a flow rate of 1.0 ml min^{-1}. We monitored the elution by measuring UV absorption at 254 nm. We froze each eluate at –80 °C and lyophilized it.

DETERMINATION OF RECOVERY RATES

To calculate the recovery rate for ATP, UTP and Up_4A, in a control experiment, we spiked either culture medium or plasma (40 ml) with ATP (5 µg), UTP (5 µg) and Up_4A (5 µg). We fractionated these samples as described above.

FOURIER TRANSFORM ION CYCLOTRON RESONANCE (FT-ICR) MASS SPECTROMETRY

We examined the lyophilized fractions from the reversed-phase chromatography by FT-ICR mass spectrometry. We performed all experiments using an Apex III FT-ICR mass spectrometer (Bruker Daltronic) with a 7 Tesla superconducting magnet. We produced positive ions in an external Apollo electrospray ion source (Bruker Daltronic) with a flow rate of 2 µl min^{-1} for direct infusion experiments and 200 nl min^{-1} for liquid chromatography (LC) experiments. We performed the reversed-phase LC experiments using the LC Packings Ultimate nano-LC system with FAMOS autosampler (LC Packings). We used external accumulation of the electrospray ions on a hexapole ion trap prior to injection into the ICR trap in order to increase the sensitivity and duty cycle of the mass spectrometer. In direct

increase the sensitivity and duty cycle of the mass spectrometer. In direct injection experiments we used an accumulation time of 1 s. During liquid separation experiments we reduced the external accumulation time to 0.1 s by avoiding hexapole-quenching events. We captured ions in the ICR trap using the sidekick technique[3], whereby we applied a voltage to ions entering the ICR trap that shifts them radially from the axis. Preliminary results showed the optimum value for the sidekick potential to be –6V. We used this value throughout all experiments.

MATRIX ASSISTED LASER DESORPTION/ IONISATION MASS SPECTROMETRY (MALDI-MS)

We equally examined the lyophilized fractions from the reversed-phase chromatography by MALDI-MS and post-source decay (PSD)-MALDI-MS. We used a reflectron type time-of-flight (RETOF) mass spectrometer (Reflex III, Bruker-Daltronic). The concentrations of the analysed substances were 1-10 µmol L^{-1} in double distilled water. We mixed 1 µl of the analyte solution with 1 µl of matrix solution (50 mg ml^{-1} 3-hydroxy-picolinic acid in water). To this mixture, we added cation exchange beads (AG 50 W-X12, 200–400 mesh, Bio-Rad) equilibrated with NH_4^+ as a counterion to remove Na^+ and K^+ ions. We dried the mixture gently on an inert metal surface before introduction into the mass spectrometer. The mass accuracy was in the range of 0.01%.

ENZYMATIC CLEAVAGE EXPERIMENTS

We performed enzymatic cleavage experiments as described elsewhere[4]. Briefly, we mixed 5´-nucleotide hydrolase (3 mU) from Crotalus durissus, EC 3.1.15.1 (Roche), 3`-nucleotide hydrolase (1 mU) from calf spleen, EC 3.1.16.1 (Roche) and alkaline phosphatase (1 mU) from calf intestinal mucosa, EC 3.1.3.1 (Roche), respectively with 50 µl $NaHCO_3$ and activated CNBr-Sepharose 6 MB beads (Amersham-Pharmacia Biotech). We incubated the mixture for 2 h at room temperature. After incubation we washed the beads 3 times with double distilled water. We incubated aliquots of the fractions from the reversed-phase chromatography with these enzyme-beads for 3 h at room temperature and examined these aliquots by MALDI-MS. We accumulated 40–50 single spectra to improve the signal-to-noise ratio. We determined the identity of the reaction products by post-source decay (PSD)-MALDI-MS[5, 6]. For sample preparation and measurements we used the same conditions as for the original samples.

SYNTHESIS OF URIDINE (5')-ADENOSINE (5') TETRAPHOSPHATE

We synthesized uridine adenosine tetraphosphate according to Ng and Orgel[7]. We dissolved ATP (0.25 mmol L^{-1}), uridine 5´-monophosphate (0.25 mmol L^{-1}), (N-[2-hydroxyethyl]-piperazine-N´-[2-ethanesulfonic acid]) (2 mol L^{-1}, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (2.5 mol L^{-1}) and magnesium chloride ($MgCl_2$; 125 mmol L^{-1}) in water. Then we thoroughly mixed the solution with a vortex mixer and incubated it at 37 °C at pH 6.5 for 24 h. We purified the chemically synthesized dinucleoside (5´,5´) polyphosphates as described elsewhere[8]. Briefly, we concentrated the synthesized dinucleoside polyphosphates on a C18 reversed-phase column (LiChroprep, 310 ×25 mm, 65–40 µm, Merck) using 40 mmol L^{-1} aqueous in water (eluent A; flow rate: 2 ml min^{-1}). After removing non-binding substances with eluent A (flow rate: 2 ml min^{-1}), we eluted nucleotides with 20% ACN in water (eluent B; flow rate: 2 ml min^{-1}). We monitored the elution by UV absorption at 254 nm, then lyophilized and stored the eluate at –80 °C. We dissolved the lyophilized eluate of the preparative reversed-phase chromatography in aqueous 40 mmol L^{-1} triethylammonium acetate solution and injected it on two C18 reversed-phase columns connected in series (Supersphere, 300 ×8 mm, 4 µm, Merck) which we had equilibrated with aqueous 40 mmol L^{-1} triethylammonium acetate (carrier). We pumped the carrier through the system with a flow rate of 100 µl min^{-1} during injection of the sample. After the injection, we used n-butanol (100 mmol L^{-1}) in 40 mmol L^{-1} triethylammonium acetate as displacer (flow rate: 100 µl min^{-1}). We monitored the displacement chromatography by UV absorption at 254 nm. The fraction size was 1 ml. We lyophilized each fraction of the displacement-chromatography possibly containing dinucleoside polyphosphates , dissolved it in 1 ml 20 mmol L^{-1} K_2HPO_4 in water, pH 8, (eluent A) and chromatographed it by using an anion-exchanger (column: UNO Q-12, BioRad)(eluent B: 20 mmol L^{-1} K_2HPO_4 and 1 mol L^{-1} NaCl (pH 8) in water; gradient: 0–10 min: 0–5% B; 10–115 min: 5–40% B; 115–120 min: 40–100% B; flow rate: 1.0 ml min^{-1}; UV absorption wavelength: 254 nm). Then we desalted the fractions of the anion-exchange chromatography by HPLC reversed-phase C18 chromatography. We equilibrated the reversed-phase column (Chromolith™ Performance RP-18e 100–4.6, Merck) with eluent 40 mmol L^{-1} triethylammonium acetate. We pumped each sample dissolved in 40 mmol L^{-1} triethylammonium acetate with a flow rate of 1.0 ml min^{-1} onto the column. After washing the column with 15 ml eluent A, we eluted the substances with 35% ACN in water (eluent B). We lyophilized the resulting fractions and stored them at – 80 °C. Then we examined the lyophilized fractions from the HPLC reversed-phase C18 chromatography by MALDI-MS.

MEASUREMENTS OF PERFUSION PRESSURE IN THE ISOLATED PERFUSED RAT KIDNEY

We continuously monitored perfusion pressure by a transducer (Statham P23 GB, Siemens) connected to a bridge amplifier (Hugo Sachs). We excised and immediately mounted the kidney into the perfusion system. The perfusion procedure generally followed the description given by van der Giet et. al.[9]. Briefly, we perfused the isolated rat kidney by a peristaltic pump in a single-pass system with a solution containing 115 mmol L^{-1} NaCl, 4.6 mmol L^{-1} KCl, 1 mmol L^{-1} $CaCl_2$, 1.2 mmol L^{-1} $MgSO_4$, 1.2 mmol L^{-1} NaH_2PO_4, 22 mmol L^{-1} $NaHCO_3$, 49 mmol L^{-1} glucose and 35 g of gelatine L^{-1} (Haemaccel; Behringwerke), and equilibrated with 95% O_2 / 5% CO_2.

We assessed vasoconstrictive responses of the isolated perfused rat kidney at basal tone after an equilibration period of 30 min. To construct concentration-response curves, we allowed 5 min to elapse between consecutive doses.

MEASUREMENT OF MEAN ARTERIAL BLOOD PRESSURE AFTER INTRAARTERIAL ADMINISTRATION OF Up_4A

In 5 anaesthetized rats (1.5 g kg^{-1} b.w. urethane) we recorded intra-arterial blood pressure through a polyethylene catheter inserted into the femoral artery and connected to a pressure transducer (Statham P23 GB, Siemens). We injected noradrenaline and Up_4A (100 nmol each) intra-aortically via a polyethylene catheter inserted into the right carotid artery.

ISOLATION AND IDENTIFICATION OF Up_4A IN HUMAN PLASMA

We centrifuged the blood samples at 2,100 g for 10 min at 4 °C for isolation of plasma, after a standardized interval of 15 min after sampling. We added 10 µg of P(1),P(2):P(2),P(3)-Bis-methylene diadenosine triphosphate as internal standard. We deproteinized the plasma with 0.6 mol L^{-1} (final concentration) perchloric acid and centrifuged (2,100 g, 4 °C, 5 min). After adjusting pH to 7.0 with 5 mol L^{-1} KOH, we removed the precipitated proteins and $KClO_4$ by centrifugation (2,100 g, 4 °C, 5 min).

References

1. Xu Y, Swerlick RA, Sepp N, Bosse D, Ades EW, Lawley TJ: Characterization of expression and modulation of cell adhesion molecules on an immortalized human dermal microvascular endothelial cell line (HMEC-1). *J Invest Dermatol* 102:833-837, 1994

2. Barnes LD, Robinson AK, Mumford CH, Garrison PN: Assay of diadenosine tetraphosphate hydrolytic enzymes by boronate chromatography. *Anal Biochem* 144:296-304, 1985
3. Rakov VS, Futrell JH, Denisov EV, Nikolaev EN: Instrumentation of kinetic energy-resolved surface-induced dissociation in Fourier transform mass spectrometry. *European Journal of Mass Spectrometry* 6:299-317, 2000
4. Jankowski J, Stephan N, Knobloch M, Fischer S, Schmaltz D, Zidek W, Schlüter H: Mass-spectrometry-linked screening of protein fractions for enzymatic activities-a tool for functional genomics. *Anal Biochem* 290:324-329, 2001
5. Kaufmann R, Kirsch D, Spengler B: Sequencing of peptides in a time-of-flight mass spectrometer: evaluation of postsource decay following matrix-assisted laser desorption ionisation (MALDI). *Int J Mass Spectrom Ion Proc* 131:355-385, 1994
6. Jankowski J, Grosse-Hüttmann P, Zidek W, Schlüter H: Identification of dinucleoside polyphosphates by matrix-assisted laser desorption/ionisation post-source decay mass spectrometry. *Rapid Commun Mass Spectrom* 17:1189-1194, 2003
7. Ng KE, Orgel LE: The action of water-soluble carbodiimide on adenosine-5'-polyphosphates. *Nucleic Acids Res* 15:3573-3580, 1987
8. Jankowski J, Potthoff W, Zidek W, Schlüter H: Purification of chemically synthesised dinucleoside(5',5') polyphosphates by displacement chromatography. *J Chromatogr B Biomed Sci Appl* 719:63-70, 1998
9. van der Giet M, Khattab M, Börgel J, Schlüter H, Zidek W: Differential effects of diadenosine phosphates on purinoceptors in the rat isolated perfused kidney. *Br J Pharmacol* 120:1453-1460, 1997

B.4. Identification of dinucleoside polyphosphates in adrenal glands

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B.4.1. Abstract

Dinucleoside polyphosphates have been characterized as extracellular mediators controlling numerous physiological functions like vascular tone or cell proliferation. Here we describe the isolation and identification of dinucleoside polyphosphates Ap_nA (with n=2-3), Ap_nG (with n=2-6) as well as Gp_nG (with n=2-6) from adrenal glands. These dinucleoside polyphosphates are localized in granules of the adrenal glands. The dinucleoside polyphosphates diadenosine diphosphate (Ap_2A), diadenosine triphosphate (Ap_3A), the adenosine guanosine polyphosphates (Ap_nG) and diguanosine polyphosphates (Gp_nG), both with phosphate group (p) numbers (n) ranging from 2 to 6, were identified by fractionating them to homogeneity with preparative size-exclusion- and affinity-chromatography as well as analytical anion-exchange and reversed-phase-chromatography from deproteinized adrenal glands and by analysis of the homogenous dinucleoside polyphosphates containing fractions with post-source-decay (PSD) matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS). The identity of the dinucleoside polyphosphates was confirmed by retention time comparison with synthetic dinucleoside polyphosphates. Enzymatic analysis demonstrated an interconnection of the phosphate groups with the adenosines in the 5´-positions of the riboses in all dinucleoside polyphosphates purified from adrenal glands. In conclusion, the identification of these dinucleoside polyphosphates in adrenal gland granules emphasizes that these dinucleoside polyphosphates can be released from the adrenal glands into the circulation.

B.4.2. Introduction

Since the first description of diadenosine triphosphate (Ap_3A) in human platelets[1], a growing number of analogues have been described in mammalians and humans. First, it appeared that human platelets contain also diadenosine polyphosphates with 4, 5 and 6 phosphate groups[1,2], and subsequently Ap_2A and Ap_7A were identified in human cells[3,4]. A further principal step in the discovery of dinucleoside polyphosphates in human tissue was the identification of Ap_nGs and Gp_nGs with n ranging from 2 to 6 in human platelets[3,5].

Whereas the Ap_nAs and Ap_nGs exert both vasoconstrictive and growth stimulating effects on vascular smooth muscle cells, the Gp_nGs are only active as growth stimulating factors. The

effects of dinucleoside polyphosphates on vascular tone critically depend on their number of phosphate groups and the existence of at least one adenine group within the molecule[6,7]. Ap_nGs and Gp_nGs, (with n = 2 - 6), were shown to be released upon stimulation from platelets[3,5]. Yet, no further source for the storage and release of dinucleoside polyphosphates is known. Since Castillo, Castro and Pintor demonstrated that diadenosine polyphosphates, Ap_nA (with n=4-6), are stored in granules of adrenal glands[8-11], this tissue may be a candidate for the storage of Ap_nGs and Gp_nGs, (with n = 2 - 6), too. Therefore in the present study the presence of Ap_nGs and Gp_nGs in the adrenal glands and their granules was investigated.

B.4.3. Material and methods

CHEMICALS

HPLC water (gradient grade) and acetonitrile were purchased from Merck (Germany), all other substances from Sigma-Aldrich (Germany).

PURIFICATION PROCEDURES

Bovine adrenal glands were obtained on ice from a local slaughterhouse, cleaned of lipid matter, and cut into small pieces of 1 cm^3. Tissue was frozen by liquid nitrogen, and stored at -80°C for 12 h, lyophilized and powdered. 20 g tissue (dry weight) was suspended in 20 ml perchloric acid (final concentration 0.6 mol L^{-1}). The extract was centrifuged at 100,000 g for 30 min at 4°C and the supernatant was neutralized with KOH to pH 9.5. After a second centrifugation at 6,000 g for 10 min at 4°C triethylammonium acetate (TEAA, 40 mmol L^{-1} final concentration) was added to the supernatant, and the pH was titrated to 6.5 with HCl. The supernatant was pumped through a preparative reversed-phase column (Lichro-prep, 310 x 65 mm, 40-65 µm, Merck, Germany). After removing substances not binding to the column with aqueous 40 mmol L^{-1} TEAA the adsorbed molecules were eluted with 30 % acetonitrile (ACN) in water at a flow rate of 1.0 mL min^{-1}. The elution was detected by measuring the UV absorption at 254 nm. The eluate was frozen at –80°C and lyophilised. Size-exclusion-chromatography was performed using a size-exclusion gel Sephacryl S-100 High Resolution (1000 x 16 mm, S100 HR, Pharmacia BioTech, Sweden), which was equilibrated with water. The lyophilized samples from the preparative reversed-phase column were resolved in 5 mL water and ethylenediaminetetraacetic acid (EDTA; 5 mmol L^{-1}) and adenosine 5'-triphosphate (ATP; 5 mmol L^{-1}) was added. The sample was loaded onto the column. The eluent (water) was pumped with a flow rate of 1 mL min^{-1} onto the column. The eluate was monitored with a UV-detector at 254 nm.

The eluate of the size-exclusion chromatography was purified further with affinity chromatography. The affinity chromatography gel, phenyl boronic acid coupled to a cation exchange resin (BioRex 70, Bio-Rad, USA), was synthesized according to Barnes et al.[13]. The affinity resin was packed into a glass column and equilibrated with 1 mol L^{-1} ammonium acetate (pH 9.5). The eluate from the size-exclusion chromatography was adjusted to pH 9.5 and loaded onto the affinity column. The column was washed with 1 mol L^{-1} ammonium acetate (pH 9.5) with a flow rate of 1 mL min^{-1}. Binding substances were eluted with 1 mmol L^{-1} HCl. The eluate was frozen and lyophilised. Fractions were monitored with a UV detector at 254 nm.

Fractions from affinity chromatography were desalted by reversed-phase high-performance liquid chromatography (Superspher 100 C18 endcapped, 100 x 2,1 mm, 4 µm, 10 nm, Merck, Germany). The fractions dissolved in 40 mmol L^{-1} TEAA were injected on the reversed-phase column. After a washing period of 10 min with eluent 40 mmol L^{-1} TEAA, the nucleotide-containing fraction was eluted with 30 % acetonitrile in water. The $UV_{\lambda 254\ nm}$ absorbing fraction was collected. The eluate was frozen and lyophilised.

The desalted and lyophilised eluate of the affinity-chromatography was dissolved in aqueous 40 mmol L^{-1} TEAA solution and injected on two C18 reversed-phase columns connected in series (Superspher, 300 x 8 mm, 4 µm, Merck, Germany) used in the displacement modus[14]. The columns were equilibrated with aqueous 40 mmol L^{-1} TEAA before. The carrier was pumped through the system with a flow rate of 100 µL min^{-1} during injection of the sample. After the injection was finished n-butanol (100 mmol L^{-1} in 40 mmol L^{-1} TEAA) was used as displacer (flow rate: 100 µL min^{-1}). The displacement chromatography was monitored by UV-absorption at 254 nm. The fraction size was 1 mL.

Each lyophilised fraction from displacement-chromatography was fractionated by anion-exchange chromatography. The anion exchange column (50 x 5 mmol L^{-1}, Mono-Q HR 5/5; Pharmacia Biotech, Sweden) was equilibrated with eluent 10 mmol L^{-1} K_2HPO_4 (eluent A). The sample dissolved in 10 mmol L^{-1} K_2HPO_4 was injected on the column at a flow rate of the mobile phase of 0.5 mL min^{-1}. Binding substances were eluted using a linear gradient with increasing concentration of 50 mmol L^{-1} K_2HPO_4 and 1 mol L^{-1} NaCl (eluent B). The time program of the gradient was 0-10 min 0-5 % B, 10-100 min 5-35 % B, 100-105 min 35-40 %

B, 105-110 min 40-100 % B. The wavelength of the UV detector was fixed to 254 nm. Fractions were collected every 1 min. The fractions from anion exchange-chromatography were further separated by reversed-phase chromatography (Superspher 100 RP C18 end-capped, 250 x 4 mmol L^{-1}, Merck, Germany). The fractions dissolved in 40 mmol L^{-1} TEAA (eluent A) were injected on the column. Acetonitrile (eluent B) and the following gradient were used for the elution: 0-4 min 0-2 % B, 4-79 min 2-7 % B, 79-85 min 7-60 % B, 86-90 min 60-80 % B. The flow rate was 0.5 mL min^{-1}. The wavelength of the UV detector was 254 nm. 1 mL fractions were collected. Fractions with a significant $UV_{\lambda 254nm}$-absorption were rechromatographed using the conditions as described.

MATRIX ASSISTED LASER DESORPTION/ IONISATION (MALDI-) AND POST SOURCE DECAY (PSD-) - MASS SPECTROMETRY

The lyophilised fractions from the reversed-phase chromatography were examined by MALDI-MS and post-source decay (PSD)-MALDI-MS. A reflectron type time-of-flight (RETOF) mass spectrometer (Reflex III, Bruker, Germany) was used according to Hillenkamp and Karas[15]. The sample was mounted on an x, y, z movable stage allowing irradiation of selected sample areas. In this study, a nitrogen laser (VSL-337 ND, Laser Science) with an emission wavelength of 337 nm and 3 ns pulse duration was used. The laser beam was focused to a diameter of typical 50 µm at an angle of 45° to the surface of a target. Microscopic sample observation was possible. 10-20 single spectra were accumulated to improve the signal-to-noise ratio. In MALDI-MS large fractions of the desorbed analyte ions undergo post-source decay (PSD) during flight in the field free drift path. Using a RETOF set-up, sequence information from PSD fragment ions of precursors produced by MALDI was obtained[16]. Sample preparation for MALDI-MS and MALDI-PSD-MS experiments was identical. The concentrations of the analysed substances were 1-10 µmol L^{-1} in double distilled water. 1 µL of the analyte solution was mixed with 1 µL of matrix solution (50 mg mL^{-1} 3-hydroxypicolinic acid in water). To this mixture cation exchange beads (AG 50 W-X12, 200-400 mesh, Bio-Rad, USA) equilibrated with NH_4^+ as counterion were added to remove Na^+ and K^+ ions. The mixture was gently dried on an inert metal surface before introduction into the mass spectrometer. The mass accuracy was in the range of 0.01 %.

RETENTION TIME COMPARISON

The individual components were further identified on the basis of their retention times compared with the synthetic molecules. The lyophilised fractions from reversed-phase chromatog-

raphy with TEAA as ion-pair reagent were therefore separated by reversed-phase chromatography using tetrabutylammonium hydrogensulfate (TBA) as ion-pair reagent. The fractions dissolved in 2 mmol L^{-1} TBA in 10 mmol L^{-1} K_2HPO_4 (pH 6.8) were injected on the column (RP-18e Chromolith SpeedROD^{TM}; 50-4.6 mm; macropore size 2 µm; Merck, Germany). Acetonitrile (80 % (v/v) in water; eluent B) and the following gradient was used for the elution: 0-30 min: 0-40 % B; 30-33 min: 40-100 % B; 33-36 min: 100 % B; flow: 1 mL min^{-1}. UV-absorption was detected at 254 nm.

ENZYMATIC CLEAVAGE EXPERIMENTS

Aliquots of the fraction from the reversed-phase column were incubated with enzymes as follows. The samples were dissolved in 1) 20 µL 200 mmol L^{-1} Tris buffer (pH 8.9) incubated with 5-nucleotide hydrolase 3 mU from Crotalus durissus, EC 3.1.15.1 (Roche, Germany), and purified according to Sulkowski and Laskowski[17] for 9 min at 37°C; 2) 20 µL 200 mmol L^{-1} Tris and 20 mmol L^{-1} EDTA buffer (pH 7.4) and incubated with 3`-nucleotide hydrolase (1 mU) from calf spleen, EC 3.1.16.1 (Roche, Germany) for 1 h at 37°C; and 3) 20 µL 10 mmol L^{-1} Tris, 1 mmol L^{-1} $ZnCl_2$ and 1 mmol L^{-1} $MgCl_2$ buffer (pH 8) and incubated with alkaline phosphatase (1 mU) from calf intestinal mucosa, EC 3.1.3.1, (Roche, Germany) 1 h at 37 °C. The reaction was terminated by ultrafiltration with a centrifuge filter (exclusion limit 10 kDa, Millipore, USA). After filtration of the enzymatic cleavage products, the filtrate, dissolved in 80 µL eluent A (10 mmol L^{-1} K_2HPO_4, pH 7), was subjected to anion-exchange chromatography (MiniQ PC 3.2/3, Pharmacia, Sweden). The gradient corresponded to: 0-3 min: 0 % B (50 mmol L^{-1} K_2HPO_4, pH 7 with 1 mol L^{-1} NaCl); 3-20 min: 0-50 % B; 20-21 min: 50-100 % B. The flow rate was 100 µL min^{-1}.

ISOLATION OF GRANULES FROM ADRENAL GLANDS AND EXTRACTION OF DINUCLEOSIDE POLYPHOSPHATES

Fresh bovine adrenal glands were obtained on ice from a local slaughterhouse, cleaned of lipid matter and the adrenal glands were dissected. The granules were obtained from the adrenal glands according to established techniques[18,19]. Briefly, 50 ml 0.32 mol L^{-1} sucrose were added to the adrenal glands. The suspension was homogenised with an ultra-turrax at 4°C (750 rpm) and the homogenate was centrifuged at 800 g at 4°C for 10 min. The supernatant was centrifuged again at 10,000 g at 4°C for 20 min. To the supernatant 50 ml 1.6 mol L^{-1} sucrose was added and the suspension was centrifuged at 100,000 g at 4 °C for 45 min. The pellet was resuspended in 10 mL water and divided into three aliquots. For extraction of dinu-

cleoside polyphosphates from the granules into the aliquots 2 mL acetonitrile, n-butanol and 1-propanol were added respectively. The suspension was thoroughly mixed with a vortex mixer. The dinucleoside polyphosphates were chromatographed to homogeneity by preparative reversed-phase-, analytical anion-exchange- and analytical reversed-phase chromatography using the conditions as described above. The identification steps were identical as described above.

SYNTHESIS AND CHROMATOGRAPHY OF DINUCLEOSIDE POLYPHOSPHATES

In contrast to diadenosine polyphosphates and diguanosine polyphosphates, adenosine guanosine polyphosphates are not commercially available. Therefore synthesis of adenosine guanosine polyphosphates was necessary in order to control the authenticity of the isolated substances. Adenosine guanosine polyphosphates were synthesised according to Ng and Orgel[20] and chromatographed to homogeneity according to Jankowski et al.[21]. Briefly, adenosine 5´-polyphosphates, guanosine 5´-polyhosphates, N-[2-hydroxyethyl]-piperazine-N´-2-ethanesulfonic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and magnesium chloride were dissolved in water, thoroughly mixed with a vortex mixer and incubated at 37°C at pH 6.5 for 48 h. Dinucleoside polyphosphates were concentrated on a C18 reversed-phase column using 40 mmol L^{-1} aqueous TEAA and were eluted with 30 % acetonitrile in water. The lyophilized concentrate of the reversed-phase column was injected on two C18 reversed-phase columns connected in series (Supersphere, 300 x 8 mm, 4 µm, Merck, Germany) and was chromatographed in the displacement mode by use of n-butanol (100 mmol L^{-1}). The fractions of the displacement-chromatography were lyophilised and each fraction chromatographed with an anion-exchange column (column: Mono Q, 100 x 10 mm, 10 µm, eluent A: 20 mmol L^{-1} K_2HPO_4; eluent B: 20 mmol L^{-1} K_2HPO_4 and 1 mol L^{-1} NaCl; gradient: 0-10 min: 0-5 % B; 10-115 min: 5-40 % B; 115-120 min: 40-100 % B; flow rate: 1.0 mL min^{-1}; UV absorption wavelength: 254 nm). The fractions of the anion-exchange chromatography were desalted by HPLC reversed-phase C18 chromatography.

B.4.4. Results

In Figure 1, purification of the dinucleotide polyphosphates from adrenal glands is exemplified for Gp_6G. Each chromatography fraction with a significant UV-absorption at 254 nm was fractionated to homogeneity and the underlying substances were identified. The fractions leading to the isolation of Gp_6G are indicated by arrows. These arrows were added to the chromatograms after the fractionation to homogeneity and after identification by analytic methods. Comparable chromatograms are available for the remaining nucleotides.

Figure 1.A shows a characteristic size-exclusion-chromatogram of a deproteinized extract from adrenal glands**.** Retention time comparison with synthetic dinucleoside polyphosphates showed that dinucleoside polyphosphates elute in the retention time interval between 180-280 min. Therefore, each fraction of this retention time range was further fractionated by the chromatographic methods as described in the following.

The subsequent affinity-chromatography of these size-exclusion chromatography fractions by phenyl boronic acid resin allows the separation of mononucleoside and dinucleoside polyphosphates[13]. Next, the desalted and lyophilised eluate of the affinity-chromatography was fractionated by reversed-phase chromatography in the displacement mode. Each resulting fraction of the displacement-chromatography with a significant UV absorption at 254 nm was fractionated by anion-exchange-chromatography. A characteristic anion-exchange chromatogram of one fraction of the displacement-chromatography is given in Figure 1.B. The UV absorption caused by Gp_6G is labelled by an arrow in Figure 1.B.

Next, the eluates of the anion-exchange-chromatographies with a significant UV absorption at 254 nm were fractionated by reversed-phase chromatography. Figure 1.C shows a characteristic reversed-phase chromatogram of the peak labelled by an arrow in Figure 1.B. Each resulting fraction of the reversed-phase chromatography with a significant UV absorption at 254 nm was rechromatographed by reversed-phase chromatography using the same conditions as before. The reversed-phase rechromatogram of the fraction labelled by an arrow in Figure 1.C is given in Figure 1.D.

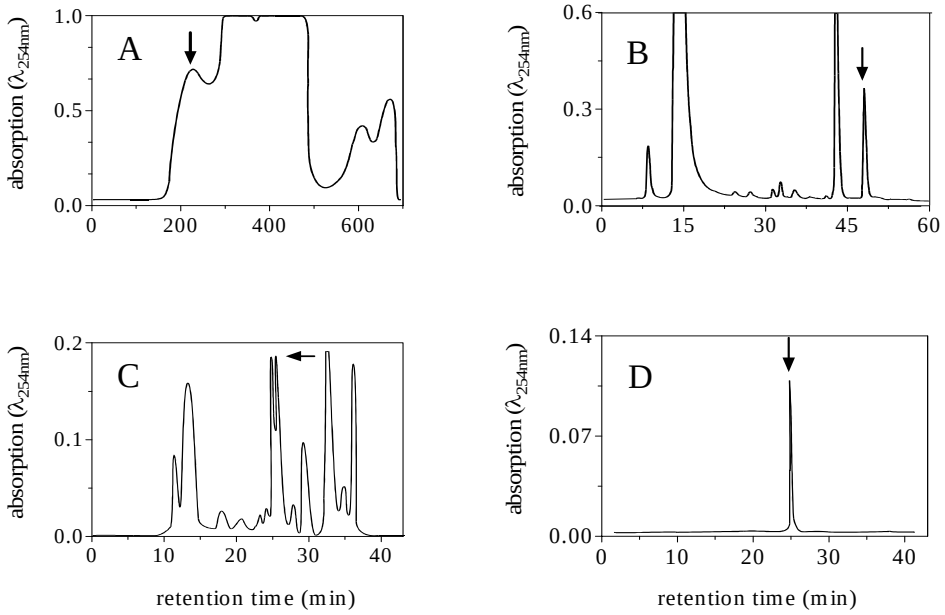

Figure 1: Isolation of dinucleoside polyphosphates from adrenal glands, exemplified for the isolation of diguanosine hexaphosphate. The arrows in the figures indicates the fractions which lead to the identification of Gp_6G.

(A) Size-exclusion chromatography of an extract of adrenal glands (gel: Sephacryl S-100 high-resolution, Pharmacia BioTech, Sweden; column dimension: 1000 x 16 mm; eluent: water; flow rate: 100 µL min^{-1},fraction size: 1.0 mL min^{-1}; abscissa: retention time (min); ordinate: UV-absorption at 254 nm (arbitrary units)).

(B) Anion-exchange chromatography of a fraction of the displacement chromatography (column: TSK DEAE 5 PW, 150 x 20 mm, 10 µm, Tosohaas, Japan; eluent A: 20 mmol L^{-1} K_2HPO_4 in water; pH 8.0; eluent B: 20 mmol L^{-1} K_2HPO_4 and 1 mol L^{-1} NaCl (pH 8) in water; gradient: 0-10 min: 0-5 % B; 10-105 min: 5-35 % B; 105-110 min: 35-100 % B flow rate: 2.0 mL min^{-1}; fraction size: 2 mL; abscissa: retention time (min); ordinate: UV-absorption at 254 nm (arbitrary units)).

(C) Reversed-phase chromatography of the fraction labelled in Figure 1.B by an arrow (column: Supersphere 100 C 18 end., Merck, Germany, 250 x 4 mm, particle size 4 µm; flow rate: 0.5 mL min^{-1}; eluent A: 40 mM triethylammonium acetate in water; eluent B: 100 % acetonitrile; gradient: 0-4 min: 0-2 % B; 4-55 min: 2-7 % B; 55-60 min: 100 % B; abscissa: retention time (min); ordinate: UV-absorption at 254 nm (arbitrary units)).

(D) Rechromatography of the fraction labelled in C. by an arrow (conditions as decribed in legend of C.)

Figure 2.A shows the spectrum of the matrix assisted laser desorption/ionisation mass spectrometry of the fraction labelled in Figure 1.D. The molecular mass of the isolated substance was determined as 1029 Da (1030 Da = $[M+H]^+$). In Figure 2.B the PSD-MALDI-MS spectrum of the substance labelled by an arrow in Figure 1.D is shown. The fragmentation pattern was identical with that of synthetic Gp_6G. In Table 1 the fragmentation pattern of the isolated

substance is deduced from the structure of the dinucleoside polyphosphate Gp_6G. The connection of phosphate groups to the adenosines was investigated by enzymatic analysis using 3´- and 5´nucleotide hydrolase and alkaline phosphatase. Cleavage of the isolated substance (Figure 2.C) with 5´-nucleotide hydrolase (from Crotalus durissus) yielded GMP, as evidenced by retention time comparison with synthetic substances (Figure 2.D). The cleavage pattern was identical with that of synthetic Gp_6G. Incubation of the substance with 3´-nucleotide hydrolase (calf spleen) and alkaline phosphatase yielded no cleavage products (data not shown). The enzymatic cleavage experiments demonstrate that the polyphosphate chain interconnects the adenosines via phosphoester bonds with the 5´-oxygens of the riboses. The retention time of the isolated substance on the reversed-phase chromatography column using TBA as ion-pair reagent (19.6 min) was comparable with the retention time of synthetic Gp_6G (19.0 min). In summary, by the mass-spectrometry, the enzymatic analysis as well as the retention time comparison the isolated substance was identified as diguanosine hexaphosphate (Gp_6G).

In an analogous manner, diadenosine diphosphate (Ap_2A), diadenosine triphosphate (Ap_3A), adenosine guanosine diphosphate (Ap_2G), adenosine guanosine triphosphate (Ap_3G), adenosine guanosine tetraphosphate (Ap_4G), adenosine guanosine pentaphosphate (Ap_5G), adenosine guanosine hexaphosphate (Ap_6G), diguanosine diphosphate (Gp_2G), diguanosine triphosphate (Gp_3G), diguanosine tetraphosphate (Gp_4G), diguanosine pentaphosphate (Gp_5G) and diguanosine hexaphosphate (Gp_6G) were purified from adrenal glands and identified by enzymatic analysis, the signal pattern of the PSD-MALDI-MS fragmentations and retention time comparison.

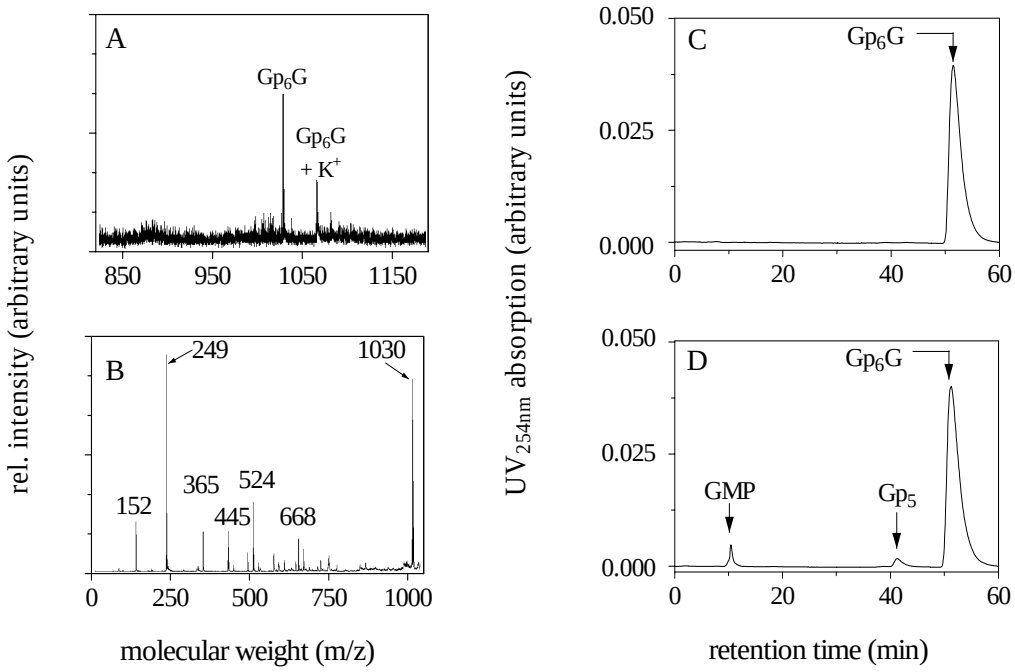

Figure 2:(A) MALDI mass spectrum of the fraction labelled in Figure 1.D by an arrow (abscissa, relative mass/charge, m/z, z = 1; ordinate, relative intensity: arbitrary units).

(B) Positive-ion post source decay (PSD-) MALDI mass spectrum of the fraction labelled by an arrow in Figure 1.D (abscissa: relative mass/charge, m/z, z=1; ordinate: relative intensity: arbitrary units).

(C) Anion exchange chromatography of the fraction labelled by an arrow in Figure 1.D by an arrow before incubation with 5'-nucleotide hydrolase (column: UnoQ-1.7 x 35 mm, BioRad, USA; eluent A: 20 mmol L^{-1} K_2HPO_4, pH 8; eluent B: 20 mmol L^{-1} K_2HPO_4 (pH 8) with 1 mol L^{-1} NaCl; gradient: 0-2 min 0 % B, 2-100 min 0-40 % B, 100-105 min 40-100 % B, 105-116 min 100 % B; flow rate: 0.5 mL min^{-1}).

(D) Anion-exchange chromatography of the fraction labelled in Figure 1.D by an arrow after incubation with 5'-nucleotide hydrolase. 3'-nucleotide hydrolase and alkaline phosphatase had no effect on the molecule (conditions as described in legend of Figure 2.C)

Table 1: Masses of the fragment ions (in Da) obtained by PSD-MALDI mass-spectrometry of each dinucleoside polyphosphate isolated from adrenal glands. (abbreviations: M, protonated parent ion, A´=adenine, G´=guanine, A=adenosine, G=guanosine, p=phosphate group, e. g., Ap_3 =adenosine triphosphate).

Fragment ions	Ap_2A (Da)	Ap_2G (Da)	Gp_2G (Da)	Ap_3A (Da)	Ap_3G (Da)	Gp_3G (Da)	Ap_4G (Da)	Gp_4G (Da)	Ap_5G (Da)	Gp_5G (Da)	Ap_6G (Da)	Gp_6G (Da)
A´	136	136		136	136		136		136		136	
G´		152	152		152	153	153	153	152	152	154	152
A – 2 H_2O	232	233		232	235	232	233		233		233	
G - 2 H_2O		248			252	251	250	249	249	248	249	249
Ap_1	346	349	349	346		349	348		348		350	
Gp_1			364		357			365	364	364	360	365
Ap_2 - H_2O				410	409		409					
Ap_2				429	428		428		428		429	
Gp_2			445		447	445	444	444	444	444	448	445
Ap_3 - H_2O							488		490		490	
Ap_3				509			509		508		509	
Gp_3							523	523	523	523	523	525
M - Gp_2					330		410		490	506		
M - Ap_2									505			
M - Gp_1					410		490	508	572	586	645	668
M - Ap_1				410			509		585		670	
M- A							587		666		747	
M- G + H_2O					609	524	682	605		684	843	764
M - A´- H_2O		524		605			699		778		861	
M - G´- H_2O						617		701		780		859
M - G´					622	638	699			797	861	880
M- A´	634				635		717					
M	678	694	709	757	773	789	853	870	934	949	1016	1030

B.4.5. Discussion

In this study we investigated the question if the dinucleoside polyphosphates Ap_nA (with n=2-3), Ap_nG (with n=2-6) and Gp_nG (with n=2-6) not only appear in granules of human platelets, as previous published[3-5,22], but also in adrenal glands emphasizing the physiological importance of these growth stimulating mediators. Because of the nature of the dinucleoside polyphosphates it is not possible to raise antibodies against these molecules. Therefore, in order to identify dinucleoside polyphosphates in cells or tissues, these molecules have to be purified to homogeneity before they can be identified by physico-chemical methods. The first sample preparation step included the removal of proteins from a bovine adrenal gland extract by per-

chloric acid precipitation. Second, the large numbers of small hydrophobic and hydrophilic substances, such as carbohydrates, were separated from the dinucleotides by reversed-phase chromatography. Next, a highly selective concentration step with a boronate derivative of a cation exchange gel was used, which retains nucleotides containing two or more 1,2-cis-diol groups. Whereas nucleotides like ATP with less than two cis-diol groups do not bind to the boronate gel in the presence of 1 mol L^{-1} ammonium acetate due to charge repulsions between the negative phosphate groups and the carboxyl groups of the cation-exchange gel, the boryl ester formation of the two cis-diol groups of dinucleoside polyphosphates is sufficient to overcome charge repulsion[13]. The eluate of the boronate gel contained salts, which prevent dinucleoside polyphosphates from binding to the anion-exchanger. Therefore, the eluate was desalted by a reversed-phase chromatography gel.

Each fraction with a significant UV-absorption at 254 nm of the reversed-phase chromatography gel was analyzed by matrix assisted laser desorption/ionisation mass-spectrometry (MALDI-MS), post-source decay (PSD-) MALDI-MS, retention time comparison as well as enzymatic analysis. By this procedure we were able to isolate and to identify diadenosine polyphosphates (Ap_nA) (with n=2-3) as well as adenosine guanosine polyphosphates (Ap_nG) and diguanosine polyphosphates (Gp_nG) (with n=2-6) in the tissue of the bovine adrenal glands. Furthermore the results demonstrate that these dinucleoside polyphosphates are stored in the granules of bovine adrenal glands, also. Because of the storage of these dinucleoside polyphosphates in granules, it can be assumed that these molecules are released into the circulation. What will happen to the dinucleoside polyphosphates after they are secreted into the blood?

Dinucleoside polyphosphates are metabolised by enzymes in the extracellular space surrounding vascular endothelial and smooth muscle cells. Several soluble- as well as ecto-enzymes have been described in the past, which are able to hydrolyze dinucleoside polyphosphates symmetrically or asymmetrically. Ecto-hydrolases are present in a broad variety of cell types, like bovine aortic endothelial cells[23] and rat mesangial cells[24]. The substrate specificity of these enzymes mainly depends on the number of phosphates of the dinucleoside polyphosphates. For example, a human diphosphorylated inositol phosphate phosphohydrolase shows a clear preference for Ap_5A and Ap_6A as substrates[25]. Phosphodiesterases, another group of dinucleoside polyphosphate-hydrolysing enzymes, have a broad substrate specifity[26]. In blood dinucleoside polyphosphates are primarily metabolized by plasma enzymes[27,28]. The enzy-

matic breakdown of dinucleoside polyphosphates will lead to the generation of mononucleotides and nucleosides that, in turn, are biologically active in vascular tissues.

While there is compelling evidence for specific membrane receptors for adenine dinucleoside polyphosphates it is undeniable that many of the actions of extracellular dinucleoside polyphosphates can be accommodated simply by activation of known members of the P2 receptor family. The mononucleotide P2 receptor family can be divided into two subfamilies: 1.) the ionotropic P2X receptors (which are ligand-gated ion channels) and 2.) the metabotropic P2Y receptors (which are G-protein coupled receptors)[29]. Various members of each subfamily have been shown to respond potently to diadenosine polyphosphates (e.g.[30-33]).

The P2X ligand-gated ion channels are activated principally by ATP and by very few other naturally occurring substances[34]. Therefore, it is of note that diadenosine polyphosphates are as potent as ATP at many of the P2X receptor subtypes. In addition, there have been five P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) isolated from either human or mammalian cDNA libraries[35]. These mononucleotide receptors show a great disparity in their pharmacological profile, being activated by either purine (adenine, guanine, inosine) or pyrimidine (uridine) nucleotides, or both classes of mononucleotides[34]. Also, some P2Y receptors show a marked preference for nucleoside diphosphates (P2Y1 and P2Y6), and others for nucleoside triphosphates (P2Y2, P2Y4, and P2Y11)[36]. Given the variability in agonist selectivity, it is remarkable that adenine dinucleoside polyphosphates are pharmacologically active at these metabotropic receptors.

A number of inconsistencies and cellular responses invoked by dinucleoside polyphosphates from mononucleotides have led to the hypothesis that classes of dinucleoside polyphosphates-stimulated receptors, which may (P2D) or may not (P4) be stimulated by mononucleoside polyphosphates, do exist[9,37,38]. The prototypical P2D receptor classification arose from radioligand binding studies where dinucleotides and P2 agonists exhibited a pharmacological profile different from any known P2 subtype[10,39]. As of yet there are no cloned receptors fitting the P2D pharmacological profile and only minimal functional studies have defined their signalling pathways. Based upon studies in chromaffin cells demonstrating mobilisation of $[Ca^{2+}\text{-}]_i$ internal stores[9] and activation of protein kinase C[37], P2D receptors are believed to belong to the family of metabotropic Gq-linked G-proteins[38]. Both chromaffin cells and brain

synaptosomes express a very high affinity receptor with equipotent or greater affinity for Ap_4A and adenosine 5´-O-thiodiphosphate[10,39].

The findings reveal that dinucleoside polyphosphates Ap_nG and Gp_nG n=2-6 and Ap_nA n=2-3 described recently[3,5], are also stored in adrenal glands. In earlier studies, besides their structure only their effects on contraction and growth of vascular smooth muscle cells had been elucidated[3,5,6]. The latter compounds had been isolated from human platelets, but due to the restricted capacity of platelets to synthesise enzymes, it is open whether Ap_nG and Gp_nG are produced in human platelets. The adrenal gland apparently is not only capable of generating diadenosine polyphosphates, but also their guanine-containing analogues. The presence of these compounds in secretory granules of the adrenal medulla suggests that the dinucleoside polyphosphates have not only local, but also systemic actions.

In conclusion, the identification of these dinucleoside polyphosphates in granules of adrenal glands emphasizes that these dinucleoside polyphosphates can not only be released from human platelets but also from the adrenal glands.

B.4.6. References

1. Lüthje J, Ogilvie A: The presence of diadenosine 5',5'''-P_1,P_3-triphosphate (Ap_3A) in human platelets. *Biochem Biophys Res Commun* 115:253-260, 1983
2. Schlüter H, Offers E, Brüggemann G, van der Giet M, Tepel M, Nordhoff E, Karas M, Spieker C, Witzel H, Zidek W: Diadenosine phosphates and the physiological control of blood pressure. *Nature* 367:186-188, 1994
3. Jankowski J, Hagemann J, Tepel M, van der Giet M, Stephan N, Henning L, Gouni-Berthold I, Sachinidis A, Zidek W, Schlüter H: Dinucleotides as growth promoting extracellulary mediators: Presence of dinucleoside diphosphates Ap_2A, Ap_2G and Gp_2G in releasable granlues of platelets. *J Biol Chem* 276:8904-8909, 2001
4. Jankowski J, Tepel M, van der Giet M, Tente IM, Henning L, Junker R, Zidek W, Schlüter H: Identification and characterization of P_1, P_7-diadenosine-5'-heptaphosphate from human platelets. *J Biol Chem* 274:23926-23931, 1999
5. Schlüter H, Gross I, Bachmann J, Kaufmann R, van der Giet M, Tepel M, Nofer JR, Assmann G, Karas M, Jankowski J, Zidek W: Adenosine(5') oligophospho-(5') guanosines and guanosine(5') oligophospho-(5') guanosines in human platelets. *J Clin Invest* 101:682-688, 1998
6. van der Giet M, Khattab M, Borgel J, Schlüter H, Zidek W: Differential effects of diadenosine phosphates on purinoceptors in the rat isolated perfused kidney. *Br J Pharmacol* 120:1453-1460, 1997
7. van der Giet M, Schmidt S, Tölle M, Jankowski J, Schlüter H, Zidek W, Tepel M: Effects of dinucleoside polyphosphates on regulation of coronary vascular tone. *Eur J Pharmacol* 448:207-213, 2002

8. Castillo CJ, Moro MA, Del Valle M, Sillero A, Garcia AG, Sillero MA: Diadenosine tetraphosphate is co-released with ATP and catecholamines from bovine adrenal medulla. *J Neurochem* 59:723-732, 1992
9. Castro E, Pintor J, Miras-Portugal MT: Ca^{2+}-stores mobilization by diadenosine tetraphosphate, Ap_4A, through a putative P2Y purinoceptor in adrenal chromaffin cells. *Br J Pharmacol* 106:833-837, 1992
10. Pintor J, Torres M, Miras-Portugal MT: Carbachol induced release of diadenosine polyphosphates-Ap_4A and Ap_5A- from perfused bovine adrenal medulla and isolated chromaffin cells. *Life Sci* 48:2317-2324, 1991
11. Rodriguez del Castillo A, Torres M, Delicado EG, Miras-Portugal MT: Subcellular distribution studies of diadenosine polyphosphates-Ap_4A and Ap_5A-in bovine adrenal medulla: presence in chromaffin granules. *J Neurochem* 51:1696-1703, 1988
12. Schlüter H, Zidek W: Application of non-size related separation effects to the purification of biologically active substances with the size-exclusion gel sephacryl 100 high resolution. *J Chromatogr* 639:17-22, 1993
13. Barnes LD, Robinson AK, Mumford CH, Garrison PN: Assay of diadenosine tetraphosphate hydrolytic enzymes by boronate chromatography. *Anal Biochem* 144:296-304, 1985
14. Schlüter H, Jankowski J: Displacement chromatography, in *Protein Liquid Chromatogaphy*, Eds: Kastner M, Elsevier, Amsterdam, Netherlands, 2000, pp 505-522
15. Hillenkamp F, Karas M: Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. *Methods Enzymol* 193:280-295, 1990
16. Kaufmann R, Kirsch D, Spengler B: Sequencing of peptides in a time-of-flight mass spectrometer: evaluation of postsource decay following matrix-assisted laser desorption ionisation (MALDI). *Int J Mass Spectrom Ion Proc* 131:355-385, 1994
17. Sulkowski E, Laskowski S: Venom exonuclease (phosphodiesterase) immobilized on concanavalin-A-sepharose. *Biochem Biophys Res Commun* 57:463-468, 1974
18. Aunis D, Miras-Portugal MT, Mandel P: Bovine adrenal medullary dopamine-beta-hydroxylase: studies on the structure. *Biochim Biophys Acta* 365:259-273, 1974
19. Pintor J, Rotllan P, Torres M, Miras-Portugal MT: Characterization and quantification of diadenosine hexaphosphate in chromaffin cells: granular storage and secretagogue-induced release. *Anal Biochem* 200:296-300, 1992
20. Ng KE, Orgel LE: The action of water-soluble carbodiimide on adenosine-5'-polyphosphates. *Nucleic Acids Res* 15:3573-3580, 1987
21. Jankowski J, Potthoff W, Zidek W, Schlüter H: Purification of chemically synthesised dinucleoside(5',5') polyphosphates by displacement chromatography. *J Chromatogr B Biomed Sci Appl* 719:63-70, 1998
22. Jankowski J, Schlüter H, Zidek W, Spieker C: Isolation and identification of Ap_3 A, Ap_4 A and Ap_5 A as growth stimulating factor in human platelets. *Biological Chemistry* 377:193, 1996
23. Mateo J, Miras-Portugal MT, Rotllan P: Ecto-enzymatic hydrolysis of diadenosine polyphosphates by cultured adrenomedullary vascular endothelial cells. *Am J Physiol* 273:C918-927, 1997
24. von Drygalski A, Ogilvie A: Ecto-diadenosine 5',5'''-P_1,P_4-tetraphosphate (Ap_4A)-hydrolase is expressed as an ectoenzyme in a variety of mammalian and human cells and adds new aspects to the turnover of Ap_4A. *Biofactors* 11, 2000, pp 179-187
25. Safrany ST, Ingram SW, Cartwright JL, Falck JR, McLennan AG, Barnes LD, Shears SB: The diadenosine hexaphosphate hydrolases from Schizosaccharomyces pombe and Saccharomyces cerevisiae are homologues of the human diphosphoinositol polyphosphate phosphohydrolase. Overlapping substrate specificities in a MutT-type protein. *J Biol Chem* 274:21735-21740, 1999

26. Guranowski A, Sillero A: Enzymes cleaving dinucleoside polyphosphates. in McLennan, AG: *Ap_4A and other dinucleoside polyphosphates*. McLennan AG, CRC Press Inc., Boca Raton, 1992, pp 81-133
27. Lüthje J, Ogilvie A: Diadenosine triphosphate (Ap_3A) mediates human platelet aggregation by liberation of ADP. *Biochem Biophys Res Commun* 118:704-709, 1984
28. Lüthje J, Ogilvie A: Catabolism of Ap_4A and Ap_3A in whole blood. The dinucleotides are long- lived signal molecules in the blood ending up as intracellular ATP in the erythrocytes. *Eur J Biochem* 173:241-245, 1988
29. Ralevic V, Burnstock G: Receptors for purines and pyrimidines. *Pharmacol Rev* 50:413-492, 1998
30. Wildman SS, Brown SG, King BF, Burnstock G: Selectivity of diadenosine polyphosphates for rat P2X receptor subunits. *Eur J Pharmacol* 367:119-123, 1999
31. King BF, Townsend-Nicholson A, Wildman SS, Thomas T, Spyer KM, Burnstock G: Coexpression of rat P2X2 and P2X6 subunits in Xenopus oocytes. *J Neurosci* 20:4871-4877, 2000
32. Liu M, King BF, Dunn PM, Rong W, Townsend-Nicholson A, Burnstock G: Coexpression of $P2X_3$ and $P2X_2$ receptor subunits in varying amounts generates heterogeneous populations of P2X receptors that evoke a spectrum of agonist responses comparable to that seen in sensory neurons. *J Pharmacol Exp Ther* 296:1043-1050, 2001
33. Surprenant A, Schneider DA, Wilson HL, Galligan JJ, North RA: Functional properties of heteromeric P2X(1/5) receptors expressed in HEK cells and excitatory junction potentials in guinea-pig submucosal arterioles. *J Auton Nerv Syst* 81:249-263, 2000
34. Jacobson KA, F. KB, Burnstock G: Pharmacological characterization of P2 (nucleotide) receptors. *Celltransmissions* 16:3-16, 2000
35. King BF, Burnstock G, Boyer JL, Boeynaems J, Weisman GA, Kennedy C, Jacobson KA, Humphries RG, Abbracchio MP, Gachet C, Miras-Portugal MT: Nucleotide receptors: P2Y receptors. in: *The IUPHAR compendium of receptor characterization and classification*, 2000
36. King BF, Townsend-Nicholson A: Recombinant P2Y receptors: the UCL experience. *J Auton Nerv Syst* 81:164-170,
37. Sen RP, Delicado EG, Castro E, Miras-Portugal MT: Effect of P2Y agonists on adenosine transport in cultured chromaffin cells. *J Neurochem* 60:613-619, 1993
38. Miras-Portugal MT, Castro E, Mateo J, Pintor J: The diadenosine polyphosphate receptors: P2D purinoceptors in: *P2 purinoceptors; localization, function and transduction mechanisms.* Edited by Chadwick DJ, Goode JA, Chichester, UK, 1996, pp 35-47
39. Pintor J, Diaz-Rey MA, Miras-Portugal MT: Ap_4A and ADP-beta-S binding to P2 purinoceptors present on rat brain synaptic terminals. *Br J Pharmacol* 108:1094-1099, 1993

B.5. Endogenous diadenosine tetraphosphate, diadenosine pentaphosphate and diadenosine hexaphosphate in human myocardial tissue

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B.5.1. Abstract

Diadenosine polyphosphates have been characterized as extracellular mediators controlling numerous physiological effects. In this study diadenosine tetraphosphate, diadenosine pentaphosphate and diadenosine hexaphosphate were isolated and identified in human myocardial tissue. Human myocardial tissue was homogenized and fractionated by affinity-, displacement-, anion-exchange-, and reversed-phase-chromatographies. In fractions purified to homogeneity, diadenosine tetraphosphate, diadenosine pentaphosphate and diadenosine hexaphosphate were revealed by matrix-assisted laser desorption/ionization mass-spectrometry and UV-spectroscopy. These diadenosine polyphosphates were further identified by enzymatic analysis, which demonstrated an interconnection of the phosphate groups with the adenosines in the 5´-positions of the riboses. Furthermore, diadenosine tetraphosphate, diadenosine pentaphosphate and diadenosine hexaphosphate were found in human cardiac specific granules and the amount of diadenosine tetraphosphate, diadenosine pentaphosphate and diadenosine hexaphosphate was estimated in the range of about 500 µmol L^{-1}.

In conclusion, the experiments show that not only the diadenosine polyphosphates with 2 and 3 phosphate groups occur in human myocardial tissue, but also diadenosine polyphosphates with 4-6 phosphate groups. After being released by cholinergic stimulation, which is known to induce diadenosine polyphosphate release from secretory granules, diadenosine tetraphosphate, diadenosine pentaphosphate and diadenosine hexaphosphate activate P2X purinoceptors in vascular smooth muscle cells and hence can act as vasoconstrictors. From this, it may be inferred that the differential action of both predominantly vasodilator and vasoconstrictor diadenosine polyphosphates allows a fine tuning of myocardial blood flow by locally released diadenosine polyphosphates.

B.5.2. Introduction

Diadenosine polyphosphates (Ap_nA) have attracted growing interest in the past decade with respect to their roles in cardiovascular physiology and pathology (e.g.: [1-5]). The actions of the Ap_nA within the cardiovascular system are mediated by the various purinoceptor subtypes. So

far 14 mammalian purinoceptor subtypes have been cloned[6,7], and six Ap_nA containing 2-7 phosphate groups have been identified in humans[8-11]. The affinities of a given Ap_nA to the various purinoceptor subtypes depends on the number of phosphate groups linking both adenosine moieties[9,12,13]. Moreover, the purinoceptor subtypes are very differently distributed within the cardiovascular system. Depending on the purinoceptor subtypes activated in a given tissue, the Ap_nA are both vasoconstrictors and vasodilators[14,15], both inhibitors and stimulators of platelet aggregation[8,9,11], and modulators of cell proliferation[9,11,16]. Given this diversity of Ap_nA actions it is not surprising that the Ap_nA actions reported in literature widely differ among various species. Currently, it is difficult to decide to what extent species-dependent differences in Ap_nA actions are due to different purinoceptor distribution and to species-specificity of some of the known purinoceptor subtypes.

Obviously, local concentrations of Ap_nA are further important determinants of Ap_nA-mediated effects[8,9]. Hence, the local production of Ap_nA in the cardiovascular system has been studied recently. In earlier experiments both Ap_2A and Ap_3A have been identified in human myocardial tissue[17]. Both Ap_2A and Ap_3A have been characterized as vasodilators[14], whereas Ap_4A, Ap_5A and Ap_6A have vasoconstricting properties[10,14], which increase with increasing number of phosphate groups[15]. Therefore it is of interest whether also Ap_nA with more than three phosphate groups occur in human myocardium. Given powerful local effects of these Ap_nA due to high local concentrations, the presence of vasoconstrictive Ap_nA in human myocardium might have important consequences with respect to pathologic conditions such as myocardial infarction or excess sympathetic nerve stimulation, which is known to stimulate Ap_nA release from chromaffin granules. Therefore, we examined in the present study whether in human myocardium also those Ap_nA are present, which activate P2X purinoceptors in low concentrations and hence are powerful vasoconstrictors, i.e. Ap_nA containing 4-6 phosphate groups. From earlier findings in literature this hypothesis appeared likely, since in chromaffin granules isolated from various tissues mostly several or all known types of Ap_nA occur[18-21]. Our experiments revealed indeed that Ap_nA acting predominantly as vasoconstrictors are also detectable in human myocardium.

The group of Ap_nA polyphosphates have obviously a key position in the regulation cardiovascular system. They are involved in the regulation of most of the organs, cells and body fluids of the cardiovascular system and some Ap_nA acts as agonist or antagonist[22]. Moreover, the ratios of the concentrations of the Ap_nA are different in the organs and body fluids, e.g. the

most abundant dinucleoside polyphosphate in platelets is Ap_3A[21], the most abundant Ap_nA in plasma is Ap_4A[20], and the Ap_nA concentrations dramatically increase in cardiovascular disease, e. g the Ap_nA amount in platelets in chronic renal failure patients[16].

B.5.3. Material and methods

CHEMICALS

HPLC water (gradient grade) and acetonitrile were purchased from Merck (Germany), all other substances from Sigma-Aldrich (Germany).

Diadenosine polyphosphates were extracted from human myocardial tissue as described previously[17]. The extract was concentrated by preparative reversed-phase chromatography (Lichroprep RP-18, 310×25 mm, Merck, Germany). The eluate of the preparative reversed-phase chromatography was fractionated by size-exclusion gel-, preparative anion-exchange-, preparative affinity- and reversed-phase chromatography. The chromatographic conditions were identical to the methods described previously[17]; these conditions are also available online (www.hyper.ahajournals.org).

Next, the desalted and lyophilized eluate of the affinity-chromatography was dissolved in 40 mM TEAA solution and was chromatographed by two connected reversed-phase columns (Superspher 100 RP-18 endcapped, 300×8 mm, Merck, Germany) in the displacement mode[23]. These reversed-phase columns were equilibrated with 40 mM TEAA. The carrier (40 mM TEAA) was pumped through the system at a flow rate of 100 µl min^{-1} during injection of the sample. After the injection, n-butanol (100 mM in 40 mM TEAA) was used as displacer (flow rate 100 µl min^{-1}). Each fraction (1 ml) was lyophilized and was further fractionated by anion-exchange-chromatography. The eluate of the displacement-chromatography was fractionated by analytical anion-exchange- (TSK DEAE-5PW, 75×7.5 mm; TosoHaas, Germany) and desalted by analytical reversed-phase chromatography (Supersphere 100 RP-18 endcapped, 100 × 2.1 mm, 4 µm, Merck, Germany)(conditions as described in:[17]).

PURIFICATION PROCEDURES

The lyophilized eluate of analytical reversed-phase chromatography was analysed by Matrix assisted laser desorption/ionization (MALDI) mass-spectrometry and post-source decay MALDI mass-spectrometry using the conditions as described in Jankowski et. al.[24], UV-spectroscopy (conditions:[17]) as well as enzymatic cleavage experiments (conditions:[20]).

IDENTIFICATION OF Ap_nA FROM HUMAN CARDIAC SPECIFIC GRANULES

Diadenosine polyphosphates were isolated from specific granules of human left ventricular tissue according to the method of Luo et al.[17].

B.5.4. Results

The extracts of human myocardial tissues were fractionated by size exclusion-, anion-exchange-, affinity-, displacement-chromatography, and analytic anion-exchange-chromatography. Each fraction showing a significant UV-absorption at 254 nm in the analytic anion-exchange chromatography was further fractionated by reversed-phase chromatography. Each fraction with a significant absorbance in the 254 nm range of the reversed-phase HPLC was further analyzed by mass spectrometric methods, UV-spectroscopy as well as enzymatic analysis. Figure 1 shows a typical chromatogram of reversed-phase HPLC. The purified substances underlying the fractions labeled by arrows in the figure were analyzed. By MALDI mass-spectrometry, the molecular masses of these fractions (Figure 1) were revealed as 837.2 Da, 917.8 Da and 997.4 Da $[M+H]^+$, which correspond to Ap_4A, Ap_5A and Ap_6A. The UV spectra of these substances were characteristic of adenosine with a maximum at 259 nm and minimum at 230 nm (data not shown). Moreover the fragmentation patterns of the isolated substances were analyzed by PSD-MALDI mass-spectrometry[24,25].

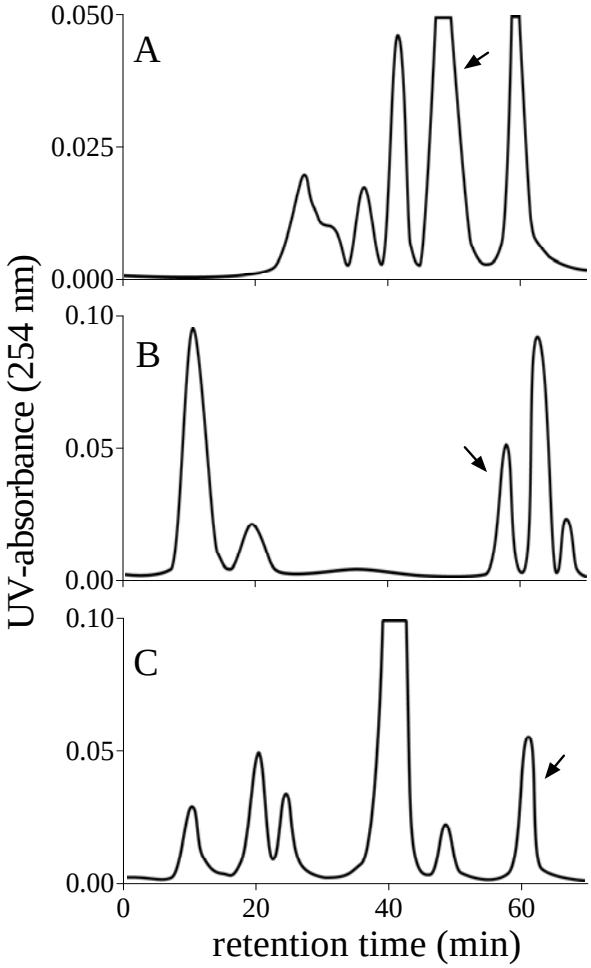

Figure 1: Characteristic chromatograms of reversed-phase HPLC from an extract of human heart tissue after several chromatographies. The reversed-phase HPLC (column: Superspher 100 RP-18 endcapped, 250×4 mm, Merck, Germany) was performed with 40 mM TEAA (eluent A) and 100% ACN (eluent B) and a gradient with eluent B (0-4 min 0-2% B, 4-50 min 2-7% B, 50-56 min 7-60% B, 56-60 min 60-80%) at a flow rate of 0.5 ml min^{-1}. The peaks labeled by arrows lead to the identification of Ap_4A (A), Ap_5A (B) and Ap_6A (C).

The results show that the analyzed substances contained phosphate groups, adenosine, AMP, ADP, ATP, Ap_4, or Ap_5 (Table 1). The fragment patterns of the analyzed fractions were identical to those of synthetic Ap_4A, Ap_5A and Ap_6A (Table 1).

Table 1: Mass signals of the fragment ions (in m/z) obtained by PSD MALDI mass-spectrometry of each diadenosine polyphosphate isolated from human myocardial tissue.

Assignment of the mass	isolated sub-stance (Figure 1.A)	synthetic Ap_4A	isolated sub-stance (Figure 1.B)	synthetic Ap_5A	isolated sub-stance (Figure 1.C)	synthetic Ap_6A
phosphate	97.2	97.3	97.2	97.4	97.0	97.4
adenosine	136.1	135.9	136.1	136.5	136.2	136.3
adenosine-2 H_2O	232.3	232.2	232.4	232.1	232.6	234.0
adenosine-H_2O	250.2	250.6	248.0	--	250.8	249.3
AMP	348.2	349.1	348.0	348.2	348.5	349.2
ADP-H_2O	410.3	410.2	410.1	--	--	410.2
ADP	428.2	428.6	428.1	428.3	428.8	429.4
ATP-H_2O	491.2	492.1	489.9	490.1	490.4	490.3
ATP	509.2	509.0	508.6	508.2	508.3	508.1
AP_4	588.1	589.1	586.4	587.8	588.9	588.4
AP_5			666.5	667.1	668.0	668.3
AP_6					746.9	748.2
parent ion	837.1	837.2	917.0	916.7	997.8	997.4

Furthermore, the interconnection of phosphate groups to the adenosines was analysed by enzymatic analysis using alkaline phosphatase, 3´- and 5´-nucleotide hydrolase. Alkaline phosphatase and 3'-nucleotidase had no effect on these molecules (data not shown). 5'-nucleotidase yielded AMP and ATP (Figure 2.A) after incubation of the fraction labeled in Figure 1.A, AMP and Ap_4 (Figure 2.B) after incubation of the fraction labeled in Figure 1.B, and AMP and Ap_5 (Figure 2.C) after incubation of the fraction labeled in Figure 1.C.

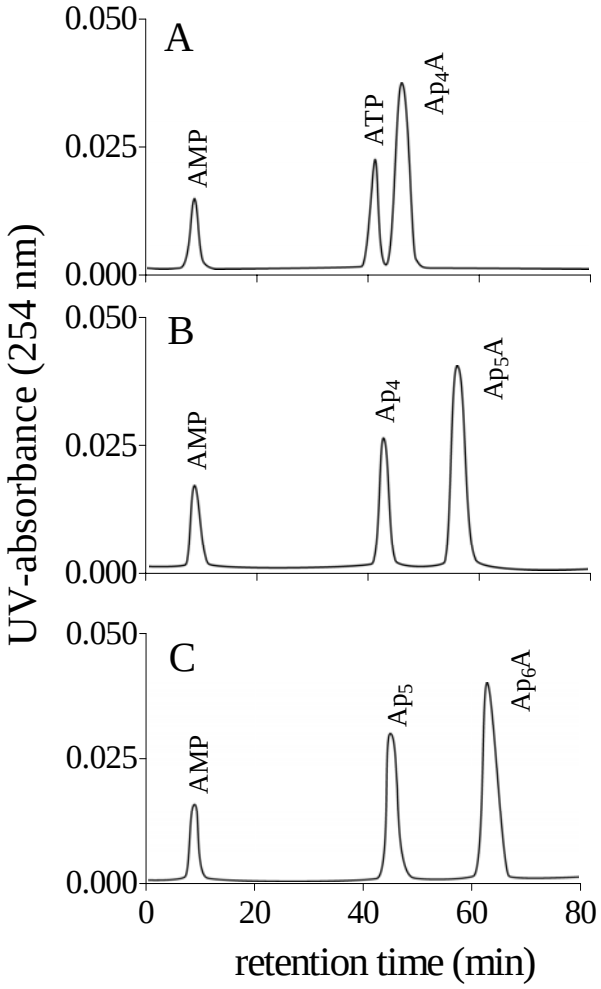

Figure 2: Analytic anion-exchange chromatograms of the isolated diadenosine polyphosphates (labeled by arrows in Figure 1.A-C) after incubation with a 5'-nucleotidase. The analytic anion-exchange chromatography (column: UnoQ-1, 7×3.5 mm, BioRad, USA) was performed at a flow rate of 0.5 ml min^{-1} with 20 mM K_2HPO_4 (eluent A, pH 8.0) and 20 mM K_2HPO_4 in1 M NaCl (eluent B, pH 8.0) by a gradient of 0-2 min 0% B, 2-100 min 0-40% B, and 100-105 min 40-100% B, 105-116 min 100%.

The retention times and the cleavage patterns are in agreement with those of synthetic Ap_4A, Ap_5A and Ap_6A. These results demonstrate that all adenosines in the purified molecules are interconnected via 5'-phosphodiester bonds of the riboses with the phosphates. In summary, by MALDI mass-spectrometry, UV-absorption analysis, PSD MALDI mass-spectrometry as well as by enzymatic analysis, the substances underlying the UV absorption peaks in the reversed-phase HPLC shown in Figure 1 were identified as Ap_4A, Ap_5A and Ap_6A.

To examine if Ap_4A, Ap_5A and Ap_6A are stored in granules, human ventricular specific granules were isolated from myocardial tissue and the nucleotides purified to homogeneity. Fractions from the reversed-phase HPLC were analyzed by MALDI mass-spectrometry. Fractions

with molecular masses of 837.8 Da, 917.3 Da and 996.9 Da were analyzed by PSD MALDI mass-spectrometry. The fragmentation patterns were identical to synthetic Ap_4A, Ap_5A and Ap_6A. Based on the UV absorption at 254 nm and using Ap_8A as internal standard as well as calibration curves of Ap_4A, Ap_5A and Ap_6A, the amount of Ap_4A, Ap_5A and Ap_6A in human myocardial specific granules were estimated at about 500 µmol L^{-1}.

B.5.5. Discussion

Whereas in earlier studies only Ap_2A and Ap_3A had been isolated from human myocardium[17], the present study shows that also Ap_nA containing 4–6 phosphate moieties are found in human myocardial tissue.

We exclusively examined tissue from hearts suffering from severe ischemia, although we used only macroscopically intact tissue. Considering the role of diadenosine polyphosphates as "alarmones" in other tissues or cells, an increased synthesis of these substances under pathologic conditions such as hypoxia cannot be excluded[26]. Despite these limitations due to the material we studied, the presence of dinucleoside polyphosphates in intact tissue may be less representative for the overall population.

Moreover, Ap_4A, Ap_5A and Ap_6A were identified in myocardial specific granules, which are known to release their contents into the extracellular space after stimulation e.g. by cholinergic agonists (e.g. [18,27]). The concentrations of Ap_4A, Ap_5A and Ap_6A (see Result paragraph) in specific granules are not very different from those of Ap_2A and Ap_3A found earlier[17]. Why have Ap_4A, Ap_5A and Ap_6A not been identified in our previous study[17]? Most likely, methodological reasons may be responsible. The purification procedure in this study has been considerably improved with respect to the recovery of Ap_nA compared to the previous study. In this study, but not in the previous one, we used displacement-chromatography before the analytic anion-exchange-chromatography and reversed-phase HPLC. This procedure may be more effective to separate Ap_nA from myocardial tissue, since displacement-chromatography has been shown to be a powerful method for separation of dinucleoside polyphosphates[28].

From the presence of at least 5 different Ap_nA in human myocardial tissue it can be inferred that Ap_nA may have specific functions in human heart. What is the significance of these findings for cardiac physiology and pathology? To answer this question, the cardiovascular effects of Ap_nA have to be considered. Although presently our knowledge on the role of these

molecules is still quite incomplete, animal experiments have given some hints as to potential physiological functions of these molecules. In the coronary vasculature of isolated hearts, Ap_4A, Ap_5A and Ap_6A induce species- and dose-dependent vasodilation, which is mediated by release of either nitric oxide (NO) or prostacyclin (PGI_2). This vasodilation is partially prevented or converted to a vasoconstriction after inhibition of NO or PGI_2 production[22,30]. As to electrophysiological effects on isolated hearts, Ap_4A, Ap_5A and Ap_6A increase action potential duration and refractory period, both effects being mediated by release of NO and PGI_2[30]. In guinea-pig left atria Ap_4A, Ap_5A and Ap_6A inhibited the positive inotropic response elicited by electrical stimulation[31,32]. After beta-adrenergic stimulation Ap_4A and Ap_6A exert negative chronotropic and inotropic effects in animals and human ventricular preparations. In contrast, Ap_4A alone can exert positive inotropic effects[32,33]. In physiologically relevant concentrations Ap_4A, Ap_5A and Ap_6A may serve as endogenous modulators of ryanodine receptor-gated-Ca^{2+}-release channels, as has been demonstrated in membranes prepared from rat cardiac muscle[34]. Furthermore Ap_4A, Ap_5A and Ap_6A are potent inhibitors of myocardial K_{ATP} channels[35-38].

The concentration of Ap_4A in coronary venous blood is increased during ischemia and reperfusion of the heart, whereas it cannot be detected with normal oxygen supply[36]. This increase of Ap_4A during ischemia is probably due to the release of Ap_4A stored in myocardial specific granules and activated platelets in blood[39]. Ap_4A reduces cardiac infarct size and reperfusion injury in the ischemic canine heart. Furthermore, Ap_4A mimics the cardioprotective effect of ischemic preconditioning in the rat heart and significantly improves the postischemic recovery of cardiac function, reducing the leakage of serum creatine kinase[40]. Ap_4A has cardioprotective effects on hypothermic heart storage and cardioplegia[41,42].

Some of these effects of Ap_4A appear to be mediated by activating protein kinase C and mitochondrial K_{ATP} channels via P2Y mimicking in part the effects of ischemic preconditioning[43]. Moreover, Ap_4A is an intracellular regulator ligand of the sarcolemmal K_{ATP} channel[43,44]. Therefore, the intracellular Ap_4A may directly interact with the mitochondrial K_{ATP} channel or is released into the extracellular space to interact with adjacent cells.

Ap_5A was shown to bind to the nucleotide-binding domain of the myocardial K_{ATP} channel[44] thus decreasing channel opening probability. The ischemia-induced decreased Ap_5A levels may thus contribute to open the K_{ATP} channel under ischemic conditions. The K_{ATP} channel is

known to be a sensor of metabolic stress, especially of cellular hypoxia: with decreasing ATP concentrations, the opening probability of this channel increases. This may be an important mechanism for cells undergoing hypoxia to maintain membrane potential despite decreased function of transmembrane ion pumps such as the energy-dependent Na^+-K^+-ATPase.

The regulation of K_{ATP} channels appears to be only a small segment of the potential regulatory functions of myocardial Ap_nA. It may be assumed that not only Ap_5A may have further effects on myocardial ion channels and purinoceptors, but also the other Ap_nA show regulatory effects differing according to the number of phosphate moieties. Taken together with the present findings it may be inferred that the Ap_nA represent a new class of messengers in human myocardial cells, exhibiting cellular protective functions in metabolic or ischemic stress and beyond that, other still unknown effects dependent on their binding to purinoceptors and ion channels.

B.5.6. References

1. Gabriels G, Rahn KH, Schlatter E, Steinmetz M. Mesenteric and renal vascular effects of diadenosine polyphosphates (Ap_nA). *Cardiovasc Res* 56:22-32, 2002
2. Hoyle CHV, Hilderman RH, Pintor JJ, Schlüter H, King BF. Diadenosine polyphosphates as extracellular signal molecules. *Drug Develop Res* 52:260-273, 2001
3. Guranowski A. Specific and nonspecific enzymes involved in the catabolism of mononucleoside and dinucleoside polyphosphates. *Pharmacol Ther* 87:117-139, 2000
4. Ogilvie A, Blasius R, Schulze-Lohoff E, Sterzel RB. Adenine dinucleotides: a novel class of signalling molecules. *J Auton Pharmacol*;16: 325-328, 1996
5. Kisselev LL, Justesen J, Wolfson AD, Frolova LY. Diadenosine oligophosphates (Ap_nA), a novel class of signalling molecules? *FEBS Lett* ;427:157-163, 1998
6. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev*;50:413-492, 1998
7. Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA, Burnstock G. Characterization of the UDP-glucose receptor (re-named here the $P2Y_{14}$ receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci* 24:52-55, 2003
8. Jankowski J, Tepel M, van der Giet M, Tente IM, Henning L, Junker R, Zidek W, Schlüter H. Identification and characterization of P_1,P_7-di(adenosine-5')-heptaphosphate from human platelets. *J Biol Chem* 274:23926-23931, 1999
9. Jankowski J, Hagemann J, Tepel M, van der Giet M, Stephan N, Henning L, Gouni-Berthold I, Sachinidis A, Zidek W, Schlüter H. Dinucleotides as growth promoting extracellulary mediators: Presence of dinucleoside diphosphates Ap_2A, Ap_2G and Gp_2G in releasable granlues of platelets. *J Biol Chem* 276: 8904-8909, 2001
10. Schlüter H, Offers E, Brüggemann G, van der Giet M, Tepel M, Nordhoff E, Karas M, Spieker C, Witzel H, Zidek W. Diadenosine phosphates and the physiological control of blood pressure. *Nature* 367:186-188, 1994
11. Schlüter H, Gross I, Bachmann J, Kaufmann R, van der Giet M, Tepel M, Nofer JR, Assmann G, Karas M, Jankowski J, Zidek W. Adenosine(5') oligophospho-(5')

guanosines and guanosine(5') oligophospho-(5') guanosines in human platelets. *J Clin Invest* 101:682-688, 1998

12. Schlüter H, Tepel M, Zidek W. Vascular actions of diadenosine phosphates. *J Auton Pharmacol* 16:357-362, 1996
13. Flores NA, Stavrou BM, Sheridan DJ. The effects of diadenosine polyphosphates on the cardiovascular system. *Cardiovasc Res* 42:15-26, 1999
14. Busse R, Ogilvie A, Pohl U. Vasomotor activity of diadenosine triphosphate and diadenosine tetraphosphate in isolated arteries. *Am J Physiol* 254:H828-H832, 1988
15. Ralevic V, Hoyle CH, Burnstock G. Pivotal role of phosphate chain length in vasoconstrictor versus vasodilator actions of adenine dinucleotides in rat mesenteric arteries. *J Physiol* 483:703-713, 1995
16. Jankowski J, Hagemann J, Yoon MS, van der Giet M, Stephan N, Zidek W, Schlüter H, Tepel M. Increased vascular growth in hemodialysis patients induced by platelet-derived diadenosine polyphosphates. *Kidney Int* 59:1134-1141, 2001
17. Luo J, Jankowski J, Knobloch M, van der Giet M, Gardanis K, Russ T, Vahlensieck U, Neumann J, Schmitz W, Tepel M, Deng MC, Zidek W, Schlüter H. Identification and characterization of diadenosine 5',5'''-P_1,P_2 - diphosphate and diadenosine 5',5'''-P1,P3-triphosphate in human myocardial tissue. *Faseb J* 13:695-705, 1999
18. Pintor J, Torres M, Miras-Portugal MT. Carbachol induced release of diadenosine polyphosphates -Ap_4A and Ap_5A- from perfused bovine adrenal medulla and isolated chromaffin cells. *Life Sci* 48:2317-2324, 1991
19. Castillo CJ, Moro MA, del Valle M, Sillero A, Garcia AG, Sillero MA. Diadenosine tetraphosphate is co-released with ATP and catecholamines from bovine adrenal medulla. *J Neurochem* 59:723-732, 1992
20. Jankowski J, Jankowski V, Laufer U, van der Giet M, Henning L, Tepel M, Zidek W, Schlüter H. Identification and quantification of diadenosine polyphosphate concentrations in human plasma. *Arterioscler Thromb Vasc Biol* 23:1231-1238, 2003
21. Jankowski J, Jankowski V, Seibt B, Henning L, Zidek W, Schlüter H. Identification of dinucleoside polyphosphates in adrenal glands. *Biochem Biophys Res Commun* 304:365-370, 2003
22. van der Giet M, Schmidt S, Tölle M, Jankowski J, Schlüter H, Zidek W, Tepel M. Effects of dinucleoside polyphosphates on regulation of coronary vascular tone. *Eur J Pharmacol* 448:207-213, 2002
23. Schlüter H, Jankowski J. *Displacement chromatography*, in Protein liquid chromatography. Eds: Kastner M. Elsevier, Amsterdam, Netherlands, 2000, pp 505-524
24. Jankowski J, Grosse-Hüttmann P, Zidek W, Schlüter H. Identification of dinucleoside polyphosphates by matrix-assisted laser desorption/ionisation post-source decay mass spectrometry. *Rapid Commun Mass Spectrom* 17:1189-1194, 2003
25. Hillenkamp F, Karas M. Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. *Methods Enzymol* 193:280-295, 1990

26 Varshavsky A. Diadenosine 5', 5'''-P_1, P_4-tetraphosphate: a pleiotropically acting alarmone? Cell 34:711-712, 1983

27. Pintor J, Rotllan P, Torres M, Miras-Portugal MT. Characterization and quantification of diadenosine hexaphosphate in chromaffin cells: granular storage and secretagogue-induced release. *Anal Biochem* 200:296-300, 1992
28. Jankowski J, Potthoff W, Zidek W, Schlüter H. Purification of chemically synthesised dinucleoside 5',5'-polyphosphates by displacement chromatography. *J Chromatogr B Biomed Sci Appl* 719:63-70, 1998

29. Pohl U, Ogilvie A, Lamontagne D, Busse R. Potent effects of Ap_3A and Ap_4A on coronary resistance and autacoid release of intact rabbit hearts. *Am J Physiol* 260:H1692-H1697, 1991
30. Stavrou BM, Sheridan DJ, Flores NA. Contribution of nitric oxide and prostanoids to the cardiac electrophysiological and coronary vasomotor effects of diadenosine polyphosphates. *J Pharmacol Exp Ther* 298:531-538, 2001
31. Hoyle CH, Ziganshin AU, Pintor J, Burnstock G. The activation of P1- and P2-purinoceptors in the guinea-pig left atrium by diadenosine polyphosphates. *Br J Pharmacol* 118:1294-1300, 1996
32. Vahlensieck U, Boknik P, Knapp J, Linck B, Müller FU, Neumann J, Herzig S, Schlüter H, Zidek W, Deng MC, Scheld HH, Schmitz W. Negative chronotropic and inotropic effects exerted by diadenosine hexaphosphate (Ap_6A) via A1-adenosine receptors. *Br J Pharmacol* 119:835-844, 1996
33. Vahlensieck U, Boknik P, Gombosova IH, Knapp J, Linck B, Luss H, Muller HL, Neumann J, Deng MC, Scheld HH, Jankowski J, Schlüter H, Zidek W, Zimmermann N, Schmitz W. Inotropic effects of diadenosine tetraphosphate (Ap_4A) in human and animal cardiac preparations. *J Pharmacol Exp Ther* 288:805-813, 1999
34. Holden CP, Padua RA, Geiger JD. Regulation of ryanodine receptor calcium release channels by diadenosine polyphosphates. *J Neurochem* 67:574-580, 1996
35. Jovanovic A, Terzic A. Diadenosine-hexaphosphate is an inhibitory ligand of myocardial ATP-sensitive K^+ channels. *Eur J Pharmacol* 286:R1-R2, 1995
36. Jovanovic A, Zhang S, Alekseev AE, Terzic A. Diadenosine polyphosphate-induced inhibition of cardiac KATP channels: operative state-dependent regulation by a nucleoside diphosphate. *Pflugers Arch* 431:800-802, 1996
37. Jovanovic A, Alekseev AE, Terzic A. Intracellular diadenosine polyphosphates: a novel family of inhibitory ligands of the ATP-sensitive K^+ channel. *Biochem Pharmacol* 54:219-225, 1997
38. Jovanovic S, Jovanovic A. Diadenosine tetraphosphate-gating of cardiac K(ATP) channels requires intact actin cytoskeleton. *Naunyn Schmiedebergs Arch Pharmacol* 364:276-280, 2001
39. Flodgaard H, Klenow H. Abundant amounts of diadenosine 5',5'''-P_1,P_4-tetraphosphate are present and releasable, but metabolically inactive, in human platelets. *Biochem J* 208:737-742, 1982
40. Ahmet I, Sawa Y, Nishimura M, Yamaguchi T, Kitakaze M, Matsuda H. Myocardial protection using diadenosine tetraphosphate with pharmacological preconditioning. *Ann Thorac Surg* 70:901-905, 2000
41. Ahmet I, Sawa Y, Nishimura M, Kitakaze M, Matsuda H. Cardioprotective effect of diadenosine tetraphosphate (Ap_4A) preservation in hypothermic storage and its relation with mitochondrial ATP-sensitive potassium channels. *Transplantation* 69:16-20, 2000
42. Ahmet I, Sawa Y, Nishimura M, Matsuda H. Cardioprotective effect of diadenosine tetraphosphate (Ap_4A) cardioplegia in isolated rat hearts. *Heart Vessels* 15:30-34, 2000
43. Ahmet I, Sawa Y, Nishimura M, Ichikawa H, Matsuda H. Diadenosine tetraphosphate (Ap_4A) mimics cardioprotective effect of ischemic preconditioning in the rat heart: contribution of KATP channel and PKC. *Basic Res Cardiol* 95:235-242, 2000
44. Jovanovic A, Jovanovic S, Mays DC, Lipsky JJ, Terzic A. Diadenosine 5',5''-P_1,P_5-pentaphosphate harbors the properties of a signaling molecule in the heart. *FEBS Lett* 423:314-318, 1998

B.5.7. Supplementary Methods

Since not all methods were described in the publication, in this section a more extensive description of the methods is given.

GENERAL INFORMATION

After successful heart transplantation, the recipient's own human myocardial tissue was used for isolation of Ap_nA. Only macroscopically intact tissue from the left ventricle was used for isolation of Ap_nA. The study was approved by the local ethical committee.

PURIFICATION PROCEDURES

Human myocardial tissue was immediately placed in ice-cold physiological saline and cut in small pieces. Tissue was frozen by liquid nitrogen, lyophilized and powdered. The tissue (10 g dry weight) was homogenized with 100 ml perchloric acid (final concentration 0.6 M). The extract was centrifuged at 30,000 g for 30 min at 4°C and the supernatant was neutralized with KOH to pH 8.5. Then it was centrifuged at 6,000 g for 10 min at 4°C, pH of the supernatant was titrated to 6.5 with HCl, and triethylammonium acetate (TEAA, 40 mM final concentration in water) was added. The nucleotides were concentrated from the supernatant by a preparative reversed-phase column (Lichroprep RP-18, 310×25 mm, Merck, Germany). The binding substances were eluted with 40% acetonitrile (ACN) in water at a flow rate of 1.0 ml/min. The 40% acetonitrile-eluate was collected, frozen at -80°C and lyophilized.

Next, size-exclusion chromatography was performed according to Schlüter and Zidek[1]. The high resolution size exclusion gel Sephacryl S-100 (S-100 HR, 1000 x16 mm; Pharmacia BioTech, Sweden) was equilibrated with water. The dried sample from the preparative reversed-phase column resolved in 5 ml water was loaded onto the column. The eluent was pumped with a flow rate of 1 ml/min. The UV absorption of the eluate was detected at 254 nm. All of the following chromatographic steps were also monitored at 254 nm.

The nucleotide-containing eluate of the size-exclusion chromatography was lyophilized, dissolved in 200 ml of 10 mM ammonium acetate (NH_4Ac) and pumped through an anion-exchange column (Fractogel EMD DEAE-650, 300×25 mm, Merck, Germany), equilibrated with 10 mM NH_4Ac (pH 7.4). The sample was eluted with 1.0 M NH_4Ac (pH 7.4) at a flow rate of 3.0 ml/min.

NH_4Ac (final concentration 1.0 M; pH 9.5) was added to the eluate of the anion-exchange col-

umn, and was passed through an affinity-column (150×20 mm). The affinity gel was prepared according to Barnes et al.[2] by derivatizing a cation exchange resin (BioRex 70, Bio-Rad, Germany) with phenylboronic acid. The gel was equilibrated with 1 M NH_4Ac (pH 9.5). Binding substances were eluted with 1 mM HCl at a flow rate of 1 ml/min. The fractions were frozen (-80°C) and lyophilized.

The nucleotide-containing fraction from the affinity chromatography was desalted by reversed-phase high-performance-liquid-chromatography (Supersphere 100 RP-18 endcapped, 250×4 mm, Merck, Germany). The lyophilisate, dissolved in 5 ml 40 mM TEAA, was injected to the reversed-phase column. After a washing period of 10 min with 40 mM TEAA, the nucleotide-containing fraction was eluted with 30% ACN in water at a flow rate of 0.5 ml min^{-1}.

The desalted and lyophilized eluate of the affinity chromatography was dissolved in 40 mM TEAA solution and was chromatographed by two connected reversed-phase columns (Supersphere 100 RP-18 endcapped, 300×8 mm, Merck, Germany) in the displacement mode[3]. These reversed-phase columns were equilibrated with 40 mM TEAA. The carrier (40 mM TEAA) was pumped through the system at a flow rate of 100 µl min^{-1} during injection of the sample. After the injection, n-butanol (100 mM in 40 mM TEAA) was used as displacer (flow rate 100 µl min^{-1}). Each fraction (1 ml) was lyophilized and was further fractionated by anion-exchange chromatography.

The samples from the displacement chromatography dissolved in 10 mM K_2HPO_4, pH 8.0 (eluent A) were injected on an analytical anion-exchange column (TSK DEAE-5PW, 75×7.5 mm; Toso-Haas, Germany), equilibrated with eluent A. Binding substances were eluted by 20 mM K_2HPO_4 and 1.0 M NaCl in water (pH 8.0; eluent B), using the following gradient: 1-10 min 0-5% B, 10-100 min 5-35% B, 100-105 min 35-40% B, and 105-110 min 40-100% B; flow rate: 0.5 ml/min; fraction size: 1 ml.

Fractions of the anion-exchange chromatography were desalted by reversed-phase chromatography (see above). The fractions, dissolved in 40 mM TEAA in water (eluent A), were injected on the reversed-phase column (Supersphere 100 RP-18 endcapped, 100 × 2,1 mm, 4 µm, Merck, Germany). After washing for 10 min by eluent A, the nucleotide-containing fraction was eluted by ACN (eluent B) with the following gradient: 100% ACN, 0-4 min 0-2% B, 4-50 min 2-7% B, 50-56 min 7-60% B, 56-60 min 60-80% B, at a flow rate of 0.5 ml min^{-1}. The eluate was frozen and

lyophilized.

MATRIX ASSISTED LASER DESORPTION/IONIZATION (MALDI) MASS-SPECTROMETRY AND POST-SOURCE DECAY MALDI MASS-SPECTROMETRY

A reflectron type time-of-flight (RETOF) mass spectrometer (Reflex™ III, Bruker, Germany) equipped with nitrogen laser (337 nm, pulse length 1 ns) was used for ion generation and mass analysis[4]. Using a RETOF set-up, structure information from post-source-decay (PSD) fragment ions of precursors produced by MALDI was obtained[5]. For MALDI mass-spectrometry and PSD MALDI mass-spectrometry, centrifuge-vacuum-dried samples from the reversed-phase HPLC were dissolved in 10 µl water. One µl of the 3-hydroxy-picolinic acid matrix solution (50 g L^{-1}) in water was mixed with 0.5 µl of the sample on a flat metallic support. Desorption of ions to be analyzed was performed by laser shots of irradiances in the range of 10^6-10^7 W/cm^2. The ions generated were accelerated with an energy of 20 keV for detection.

UV-SPECTROSCOPY

The substances underlying the fractions of reversed-phase HPLC were analyzed by UV-spectroscopy with a micro-cuvette (UV/Vis-Spectrometer, Jasco V-530, Jasco, Germany). The UV-absorption was scanned from 190 nm to 400 nm with a scan speed of 400 nm min^{-1}.

ENZYMATIC CLEAVAGE EXPERIMENTS

Centrifuge-vacuum dried substances underlying the fractions of the reversed-phase HPLC were dissolved in 10 µl water. One µl of this sample was mixed with 3 mU 5'-nucleotidase (EC 3.1.15.1, from Crotalus durissus, Roche, Germany) and incubated for 10 min at 37°C; one µl of the sample was mixed with 1 mU 3'-nucleotidase (EC 3.1.16.1, from calf spleen, Roche, Germany) and incubated for 60 min at 37°C; another 1 µl of the sample was mixed with 1 mU alkaline phosphatase (EC 3.1.3.1 from calf intestinal mucosa, Roche, Germany) and incubated for 60 min at 37°C. After removing the enzyme with a centrifuge filter (5 kDa cut-off), the samples were separated with anion-exchange chromatography and analyzed by MALDI mass-spectrometry. For anion-exchange chromatography, the enzymatic cleavage products were dissolved in 20 mM K_2HPO_4, pH 8.0 (eluent A) and were injected to an anion-exchange column (UnoQ-1, 7×3.5 mm, BioRad, USA) at a flow rate of 0.5 ml min^{-1}. Binding substances were eluted by eluent B (20 mM K_2HPO_4 in 1 M NaCl, pH 8.0) with a gradient of 0-2 min 0% B, 2-100 min 0-40% B, and 100-105 min 40-100% B, 105-116 min 100%.

IDENTIFICATION OF AP_NA FROM HUMAN CARDIAC SPECIFIC GRANULES

Specific granules were isolated from human left ventricular tissue according to the method of Luo et al.[6]. The specific granule pellet was suspended in 20 ml 50% ethanol with 10 mM K_2HPO_4, and 29 µg Ap_8A as internal standard was added. This mixture was sonicated three times for 20 seconds. TEAA (final concentration: 40 mM) was added to the mixture, and the mixture was concentrated on a reversed-phase column (Supersphere100 RP-18 endcapped, 250 × 8 mm, Merck, Germany). The retended substances were eluented from the reversed phase gel with 30% ACN.

The eluent of reversed-phase chromatography was lyophilized and dissolved in 50 ml 1 M NH_4Ac (pH 9.5). Then affinity chromatography was performed using the conditions as described above. The substances binding to an affinity column were eluted with 1 mM HCl and the resulting fractions were frozen (-80°C) and desalted by reversed-phase chromatography (conditions as described above). The desalted and lyophilized eluate of the affinity chromatography was further purified by an analytic anion-exchange chromatography (column: TSK DEAE-5PW, 75×7.5 mm; TosoHaas, Germany) at a flow rate of 0.5 ml min^{-1} with 10 mM K_2HPO_4 (eluent A, pH 8.0) and 20 mM K_2HPO_4 and 1.0 M NaCl in water (eluent B, pH 8.0) using the following gradient: 1-10 min 0-5% B, 10-100 min 5-35% B, 100-105 min 35-40% B, and 105-110 min 40-100% B. 40 mM TEAA (final concentration) was added to the fractions of the anion-exchanger.

The eluents of the anion-exchange chromatography were purified by reversed-phase chromatography (Supersphere 100 RP-18 endcapped, 100 × 2,1 mm, 4 µm, Merck, Germany).

The nucleotide-containing fractions were eluted by ACN (eluent B) at a flow rate of 0.5 ml min^{-1} using the following gradient B: 0-4 min 0-2% B, 4-50 min 2-7% B, 50-56 min 7-60% B, 56-60 min 60-80% (see above). The fractions were identified by MALDI mass-spectrometry and PSD-MALDI mass-spectrometry. The amount of Ap_4A, Ap_5A and Ap_6A was estimated by using the UV absorption at 254 nm and corresponding calibration curves of Ap_4A, Ap_5A and Ap_6A.

REFERENCES

1. Schlüter H, Zidek W. Application of non-size related separation effects to the purification of biologically active substances with the size-exclusion gel sephacryl 100 high resolution. *J Chromatogr*. 1993;639:17-22
2. Barnes LD, Robinson AK, Mumford CH, Garrison PN. Assay of diadenosine tetraphosphate hydrolytic enzymes by boronate chromatography. *Anal Biochem* 1985;144:296-304
3. Schlüter H, Jankowski J. *Displacement chromatography*, in Protein liquid chromatography.

Eds: Kastner M. Elsevier, Amsterdam, Netherlands, 2000, pp 505-524

4. Hillenkamp F, Karas M. Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. *Methods Enzymol* 1990;193:280-295
5. Jankowski J, Grosse-Hüttmann P, Zidek W, Schlüter H. Identification of dinucleoside polyphosphates by matrix-assisted laser desorption/ionisation post-source decay mass spectrometry. *Rapid Commun Mass Spectrom* 2003;17:1189-1194
6. Luo J, Jankowski J, Knobloch M, van der Giet M, Gardanis K, Russ T, Vahlensieck U, Neumann J, Schmitz W, Tepel M, Deng MC, Zidek W, Schlüter H. Identification and characterization of diadenosine 5',5"'-P_1, P_2 - diphosphate and diadenosine 5',5"'-P_1,P_3-triphosphate in human myocardial tissue. *Faseb J* 1999;13:695-705

B.6. Detection of angiotensin II in supernatants of stimulated mononuclear leukocytes by MALDI-TOF-TOF-mass spectrometric analysis

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B.6.1. Abstract

Angiotensin II (Ang II), acting on the AT1 and AT2 receptors, is the major vasoactive component of the renin-angiotensin system (RAS). Several components of the RAS have been demonstrated in different tissues. Whereas the roles of tissue and renal RAS have been studied in detail, much less is known on whether the corpuscular elements of circulating blood contribute to Ang II production. In the present study, we examined whether besides vasculature also blood cells contribute to the circulating Ang II levels.

Mononuclear leukocytes were obtained from healthy subjects and were incubated for 60 min at room temperature. The resulting supernatant was chromatographed using cation-exchange and reversed-phase chromatographic methods. The vasoconstrictive effects of aliquots of the resulting fractions were tested by using an isolated perfused rat kidney. Each fraction with a vasoconstrictive effect was analysed by MALDI-TOF/TOF mass-spectrometry using the LIFT-technique.

In one fraction with a strong vasoconstrictive effect, Ang II was identified. Mononuclear lymphocytes produced Ang II in amounts sufficient to stimulate AT1 receptors. Moreover, in mononuclear CD8+ (T-lymphocytes) and CD19+ (B-lymphocytes) leukocytes, renin as well as angiotensin converting enzyme (ACE) mRNA expression was detectable by RT-PCR.

These findings demonstrate that human circulating CD8+ cells (T-lymphocytes) and CD19+ cells (B-lymphocytes) are a source of Ang II. Ang II secretion by these cells may play a physiologically relevant role in humoral vascular regulation.

In conclusion, the isolation of Ang II in supernatants of mononuclear leukocytes adds a further physiologic source of Ang II to the current view of angiotensin metabolism. The quantitative role of lymphocyte-derived Ang II secretion compared to the other sources of Ang II should be defined further, but the release found under the present conditions is at least suffi-

cient to elicit vasoconstrictive effects.

B.6.2. Introduction

Whereas Ang II was first regarded merely as a potent vasoconstrictor, at present its functions as a growth factor and as a cytokine are more and more recognized. The classical or renal RAS is known since long to mediate systemic Ang II production[1,2], but Ang II is also produced locally in many tissues[3-5]. This local Ang II production depending on tissue RAS has recently attracted growing interest[6-8].

In the classical RAS, circulating renal-derived renin produces angiotensin I (Ang I) by cleaving angiotensinogen. In the lungs, Ang I is converted to Ang II by the angiotensin converting enzyme (ACE)[9-11]. ACE is found in plasma as well as in most organs, including heart, brain, blood vessels, adrenals, kidney and liver. Tissue ACE may play a role in the regulation of tissue perfusion[12]. Angiotensinogen and ACE, but not renin, have been found to be produced in all layers of the vessel wall[13-15].

Therefore, local generation of Ang II is probably dependent on circulating renin. Of potential relevance to the pathophysiological role of RAS is the recent observation indicating that adipose tissue is a significant source of circulating angiotensinogen and hence possibly contributes to the regulation of blood pressure and sodium homeostasis[16].

Whereas the roles of tissue and renal RAS have been studied in detail, much less is known on whether the corpuscular elements of circulating blood contribute to Ang II production. Therefore, in the present study we examined whether besides vasculature also cellular components of the circulating blood contribute to the circulating Ang II levels. The experiments showed that indeed a fraction of circulating mononuclear leukocytes is a source of Ang II in humans, especially if they are activated. These findings are the more relevant since leukocyte activation and adhesion has been linked to vascular damage[17].

B.6.3. Materials and methods

A: CHEMICALS

HPLC water (gradient grade) and acetonitrile were purchased from Merck (Darmstadt, Germany); all other substances were from Aldrich-Sigma (Taufkirchen, Germany).

B: MONONUCLEAR LEUKOCYTE ISOLATION

PREPARATION OF MONONUCLEAR LEUKOCYTES

Mononuclear leukocytes were obtained from nine healthy subjects who took no medication. The study was approved by the local ethical committee and informed consent was obtained from the blood donors. Their mean age was 25 ± 2 years and mean blood pressure was 119 ± 4 mm Hg systolic and 81 ± 2 mm Hg diastolic. Mononuclear leukocytes were isolated from blood according to established techniques[18]. Briefly, 400 mL heparinized blood was drawn by venipuncture from the antecubital vein and centrifuged at 240 g for 15 min. After removing the supernatant, mononuclear leukocytes were isolated by layering 5 mL diluted blood (1:1 % vol/vol with isotonic NaCl) on 3 mL Histopaque (Sigma-Aldrich, Germany; 5 / 6 % wt / vol Ficoll; density 1.077 g mL^{-1}) and centrifuged at 240 g for 20 min. The interphase of mononuclear leukocytes was carefully aspirated and washed three times in isotonic NaCl by centrifugation at 400 g for 5 min. The isotonic NaCl solution obtained after the third washing of mononuclear leukocytes was kept for analysing the efficiency of the washing procedure. The pellet was resuspended in 1 mL Hanks' balanced salt solution containing (in mmol L^{-1}): NaCl, 136; KCl, 5.4; KH_2PO_4, 0.44; Na_2HPO_4, 0.34; $CaCl_2$, 1; D-glucose, 5.6; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 10; pH of the solution was 7.4.

MEASUREMENT OF CELL VIABILITY OF THE ISOLATED MONONUCLEAR LEUKOCYTES

The cell viability of the isolated mononuclear leukocytes was measured by trypan blue exclusion test[19], by mixing 200 μL of cell suspension with an equal amount of 0.3% Trypan blue solution (Aldrich-Sigma; Germany) in phosphate buffered saline buffer (PBS). After 5 min incubation at room temperature, the number of cells excluding Trypan blue (unstained) was counted using a Burker Turk hemocytometer (Emergo, The Netherlands).

INCUBATION OF MONONUCLEAR LEUKOCYTES

Mononuclear leukocytes were then incubated without or with 10 mg L^{-1} lipopolysaccharide (LPS Serotype 0111:B4 from E. coli; Sigma-Aldrich, Germany) or 200 nmol L^{-1} formyl-Met-Leu-phenylalanine (fMLP)[20]. After incubation for 60 minutes, mononuclear leukocytes were centrifuged and the supernatant analysed.

ISOLATION OF CD8+ (T-LYMPHOCYTES) AND CD19+ (B-LYMPHOCYTES)

For identification of renin, ACE and angiotensinogen and for monitoring of Ang II production in CD8+ and CD19+ lymphocytes, these cells were isolated using a cell isolation kit provided by Miltenyi Biotec (Bergisch-Gladbach, Germany) according to the manufacturers' protocol.

The isolated lymphocytes were divided into two parts: one part was magnetically labelled with CD8 MicroBeads^{TM} (Miltenyi Biotec, Bergisch-Gladbach, Germany), the other part was magnetically labelled with CD19 MicroBeads^{TM} (Miltenyi Biotec, Bergisch-Gladbach, Germany). For this, 10^7 total cells were added to 20 μL MircoBeads suspension according to the manufacturers protocol.

Next, the magnetically labelled CD8 or CD19 cell suspensions were concentrated using a large-scale MACS^{TM} separation column (LS^+ column; Miltenyi Biotec, Bergisch-Gladbach, Germany), which was placed in the magnetic field of a magnetic cell separator (Midi-MACS^{TM}, Miltenyi Biotec, Bergisch-Gladbach, Germany).

After removing cells not-binding to the column with an aqueous buffer (PBS with 2 mmol L^{-1} EDTA and 0.5 % bovine serum albumin), the column was removed from the separator. After applying 5 mL of buffer (PBS with 2 mmol L^{-1} EDTA and 0.5 % bovine serum albumin) to the reservoir of the column, firmly positive cells flush out using the plunger supplied with the column.

Specificity of the positive cell separations was tested by direct immunofluorescence staining of the isolated cells using fluorochrome-conjugated monoclonal antibodies against CD8 and CD19 (Miltenyi Biotec, Bergisch-Gladbach, Germany) on a FACScan flow cytometer (Becton Dickinson, NJ, USA).

The results are expressed as fluorescence histograms plotted on a log scale. Compensation and photomultiplier tube (PMT) voltages were standardized before each run using CalibriteTM beads (Becton Dickinson, NJ, USA). In the final solutions 91 % of the cells were CD8 or CD19 positive.

C: ANALYTICAL TECHNIQUES

The supernatants of mononuclear leukocytes were fractionated by chromatographic methods and the vasoconstrictive substances were analysed by mass-spectrometry. The description of the chromatographic and the mass-spectrometric procedures is available online on

www.hyper.ahajournals.org.

D: MEASUREMENT OF PERFUSION PRESSURE IN THE ISOLATED PERFUSED RAT KIDNEY

The effects of aliquots (1/10) of supernatant of mononuclear leukocytes, of aliquots (1/10) of lyophilised fractions of the reversed-phase chromatography and of synthetic Ang II on vascular tone were evaluated in an isolated rat kidney perfused with a constant flow of 8 mL min^{-1} while perfusion pressure was continuously monitored. Details of the preparation are given elsewhere[21] as well as online on *www.hyper.ahajournals.org*.

Vasoconstrictor responses of the isolated perfused rat kidney were assessed at basal tone after an equilibration period of 30 min. The samples were dissolved in 200 µL of the perfusion solution described above. To characterize the receptor mediating the vasoconstrictive effect, aliquots were also tested after the Ang II receptor antagonist saralasin (50 µmol L^{-1}) was added to the perfusate, 30 min before challenge with the fractions to be tested.

E: MOLECULAR METHODOLOGY

PREPARATION OF RNA AND RT-PCR

The preparation of RNA and the RT-PCR conditions are available online on *www.hyper.ahajournals.org*.

DETECTION OF RENIN AND ACE ACTIVITY OF MONONUCLEAR LEUKOCYTES BY MATRIX ASSISTED LASER DESORPTION/ IONISATION MASS SPECTROMETRY

To detect the renin and ACE activity of mononuclear leukocytes, a previously described method was used[22]. The description of the method is available online on *www.hyper.ahajournals.org*. Briefly, the proteins potentially present in the lysate of mononuclear leukocytes were immobilised to bromine-cyan-(CNBr) activated Sepharose 6 MB beads (Amersham-Pharmacia Biotech, Freiburg, Germany). 25 µL beads containing the immobilized proteins of the mononuclear leukocyte cell suspension filtrate were transferred into a 400 µL reaction vial. 10 µL of a suspension containing either Ang I 8 x 10^{-5} mol L^{-1} or renin-substrate *(*Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser*)* 5 x 10^{-4} mol L^{-1} was added. Moreover, to detect whether angiotensinogen was present in the cytosol of mononuclear leukocytes, 25 µL beads containing the immobilized proteins of the mononuclear leukocyte cell suspension filtrate were incubated with the lysates of mononuclear leuko-

cytes, prepared as described in the online supplement.

Each incubation experiment was performed in the absence and in the presence of an ACE-inhibitor (Bachem Angiotensin-I Converting Enzyme, Cyanoac-Phe-Phe-OH; 10^{-3} mol L^{-1}) and a specific renin inhibitor (D-His-Pro-Phe-His-Leu-Psi-[CH_2NH]-Leu-Val-Tyr), which was used at a concentration achieving maximum renin inhibition (10^{-5} mol L^{-1})[23]. This inhibitor was chosen because it shows a high specificity for renin[23]. From the reaction mixture 0.5 µL aliquots were removed after 5 min, 2, 4, 12 and 48 hours for analysis of the reaction products. The products of the enzymatic activity were analysed by matrix-assisted laser desorption/ionisation mass-analysis using the conditions described above.

VERIFICATION OF RENIN, ACE, ANGIOTENSINOGEN, ANG I AND ANG II CONTENT OF THE ISOTONIC NaCl SOLUTION USED AS WASHING STEP FOR ISOLATION OF MONONUCLEAR LEUKOCYTES

To verify the efficiency of the washing procedure of the mononuclear leukocytes in removing plasma renin, ACE and angiotensin, the proteins potentially present in the isotonic NaCl solution of the third washing step were immobilised to activated CNBr-Sepharose 6 MB beads (Amersham-Pharmacia Biotech, Freiburg, Germany) using the method as described in the supplementary methods (available online on *www.hyper.ahajournals.org*). Next, these CNBr-Sepharose 6 MB beads were incubated with renin substrate *(*Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) or Ang I in the absence or presence of ACE-inhibitor and a renin inhibitor (D-His-Pro-Phe-His-Leu-Psi-[CH_2NH]-Leu-Val-Tyr) as described above. Moreover the amount of Ang I and Ang II in the remaining isotonic NaCl solution of the third washing step was quantified by the chromatographic assays described above.

STATISTICS

All data are presented as mean ± S.E.M.. Where error bars do not appear on figures, errors are within the symbol size. Statistical analyses were performed with the Mann-Whitney test or with the Kruskal-Wallis test for multiple comparisons.

B.6.4. Results

After isolation of human mononuclear leukocytes from human blood by centrifugation, the

cell viability measured by trypan blue exclusion test was 98.1 ± 0.4 % (N=4), after separation by the MACS^{TM} separation column the cell viability was 96.2 ± 0.2 % for CD8 cells and 97.4 ± 0.3 % for CD19 cells (each N=3). After incubation at 25°C for 60 min with or without stimuli, the supernatant was concentrated and fractionated by cation-exchange chromatography. A characteristic cation-exchange chromatogram is illustrated in Figure 1.A. Each fraction of each gradient step of the cation-exchange chromatography was then further chromatographed by reversed-phase chromatography. The reversed-phase chromatography on the one hand allows to desalt the eluate of the cation exchanger and on the other hand to further fractionate the eluate. Each fraction obtained from each reversed-phase chromatography were tested for vasoactivity in the isolated perfused rat kidney. The arrow in Figure 1.A indicates the cation-exchange fraction showing a strong vasoconstrictive effect after desalting and fractionation by reversed-phase chromatography. The corresponding reversed-phase chromatogram of this fraction is shown in Figure 1.B. The vasoconstrictive fraction as detected using the isolated perfused kidney is indicated by the arrows.

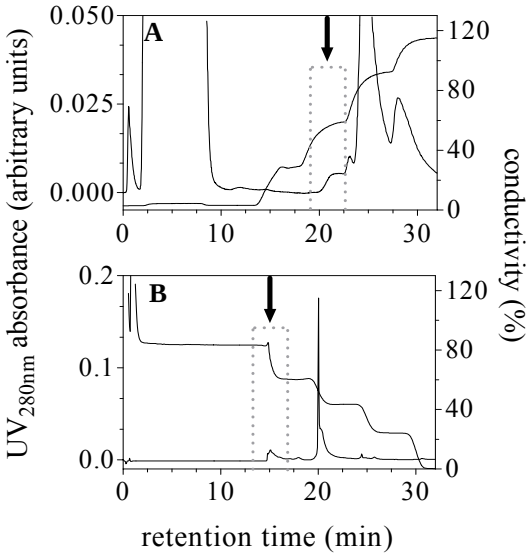

Figure 1: (A) Cation-exchange chromatography of the supernatant of mononuclear leukocytes after 60 min incubation. The vasoactive fraction is indicated by the arrow, the dotted line showing the extent of the fraction.
(B) Reversed-phase chromatography of the selected fraction of the cation-exchange chromatography. The fraction with a vasoconstrictive effect is indicated by the arrow, the dotted line showing the extent of the fraction.

A vasoconstrictive substance contained in the vasoactive fraction labelled in Figure 1.B was identified by MALDI-TOF-TOF mass spectrometry. In this fraction, several peptides with respective molecular weights of m/z of 668.4, 1001.3,1046.5 and 1329.4 were detected (Figure 2). The mass-signal of the peptide with a molecular weight of m/z 1046.5 was the most

prominent one and therefore this peptide was further analysed. This peptide was fragmented to obtain sequence information that could be used for its identification by searching in the Swissprot database. Human Angiotensin II was the highest-ranking match for the peptide at m/z 1046.5, with complete amino acid sequence coverage. This result was confirmed by comparison to a fragment ion spectrum of purified human Ang II (Bachem, Weil am Rhein, Germany).

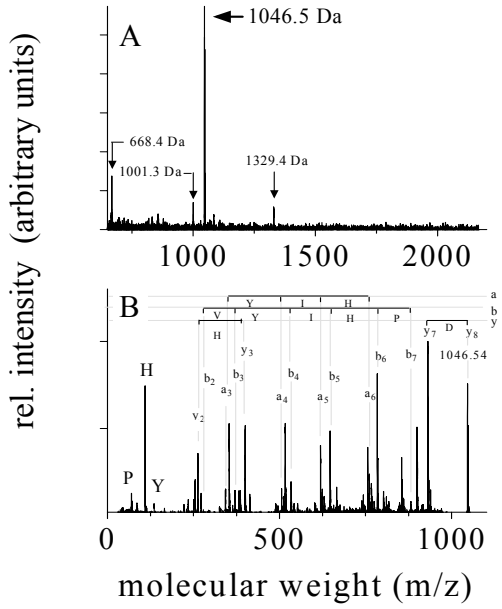

Figure 2: (A) Identification of the substance with the vasoconstrictive effect labelled in Figure 1.B by the arrow. MALDI-TOF mass spectra were obtained from the vasoconstrictive fraction of the RP-HPLC separation.

(B) Fragment ion mass spectrum of the peptide at m/z 1046.5. In the upper part of the panel, the amino acid sequences for the matched a, b, and y-ion series are indicated. In the low mass region of the spectrum, the immonium ions for proline (P), histidine (H), isoleucine (I) and tyrosine (Y) are indicated. Human Ang II was the highest-ranking match for this spectrum.

Figure 3.A shows that indeed both Ang II and the vasoactive fraction obtained from lymphocytes show very similar profiles of vascular effects. In five experiments, the effect of the vasoconstrictive fraction amounted to 74.1 ± 8.4 mmHg (Figure 3.B). In the presence of saralasin the vasoconstrictive effect decreased by 59.8 ± 1.4 %. A comparison to the concentration-response curve of Ang II (Figure 3.C) shows that if the observed hemodynamic changes are attributable of Ang II, indeed physiologically relevant amounts of Ang II can be assumed to be present in this fraction.

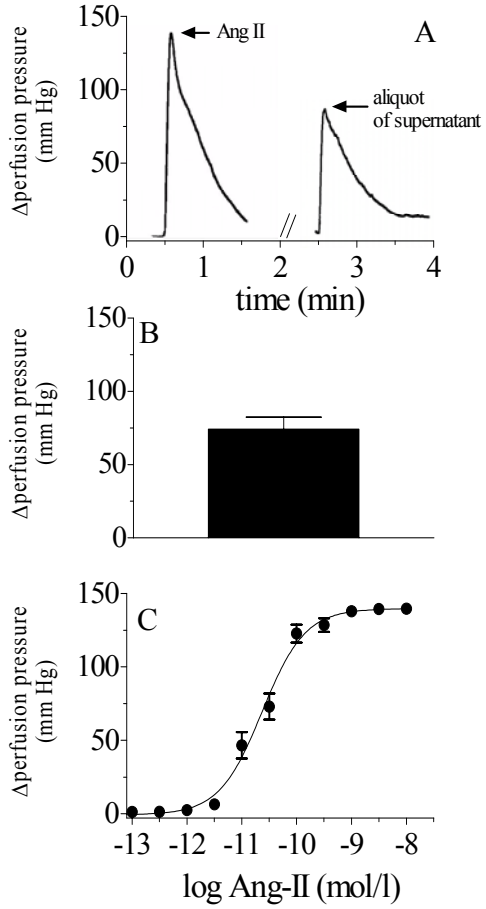

Figure 3: Effect of the supernatant of mononuclear leukocytes stimulated by 10 mg L^{-1} LPS on the perfusion pressure of the isolated perfused rat kidney.

(A) Original curve of the increase of the perfusion pressure of the isolated perfused rat kidney caused by synthetic Ang II (10^{-6} mol L^{-1}) and an aliquot (100 µL) of the supernatant of mononuclear leukocytes.

(B) Effect on the perfusion pressure (N=5).

(C) Dose-response curve of synthetic Ang II on the perfusion pressure of the isolated perfused rat kidney.

From these findings the question arose, which fraction of the mononuclear cells would be responsible for the production of Ang II. Therefore, the different fractions of mononuclear leukocytes were separated and were tested for expression of angiotensinogen, renin and ACE. As shown in Figure 4, CD8+ (Figure 4.A) and CD19+ (Figure 4.B) expressed angiotensinogen-cDNA but also renin- and ACE-cDNA, thus being capable of producing Ang II without the need of being supplied with any of its precursors. Furthermore, in CD8+ and CD19+ lymphocytes Ang II production was monitored separately. In both lymphocyte subsets Ang II production was detectable by mass spectrometry (Figure 4.C, D). The identity of Ang II was confirmed by MS/MS TOF-TOF analysis.

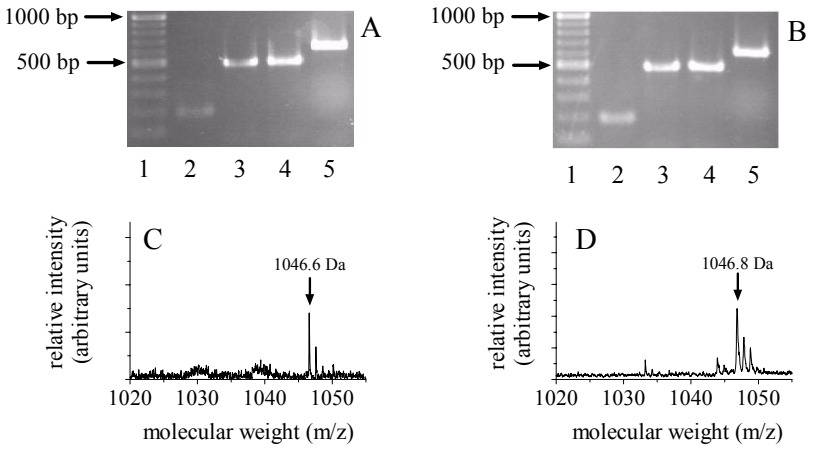

Figure 4: Representative gel electrophoresis out of four experiments of products from RT-PCR using primer against ACE, renin, angiotensinogen and ß-actin cDNA. Lane 1: 1000 and 500 bp-marker. Lane 2: renin; Lane 3: ACE, Lane 4: Angiotensinogen; Lane 5: ß-actin from CD8+ cells (A) and CD19+ cells (B). TOF-TOF mass spectra of the supernatants of CD8+ cells (C) and CD19+ cells (D). The molecular mass labelled in the spectra is conform to that of Ang II. The identity of Ang II was confirmed by MS/MS TOF-TOF analysis.

In order to confirm the detection of lymphocytic angiotensinogen, renin and ACE expression also on a functional level, mass spectrometric Ang I and Ang II measurements were done under conditions chosen to prove the existence of these proteins. Immobilized lymphocytic proteins were incubated with lymphocytic cell lysate for 8 h. Under these conditions Ang II production was observed, but was completely abolished by adding the renin inhibitor (data not shown). Ang I and Ang II production was also demonstrable when instead of lymphocytic cell lysate the synthetic renin substrate, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser was added (Figure 5.A). Ang II production was abolished in the presence of a renin inhibitor (Figure 5.A). Moreover, Ang II production was detectable after incubation with Ang I (Figure 5.B). From these findings (1) the presence of renin in mononuclear leukocytes and (2) the presence of the substrate of renin, angiotensinogen, can be concluded. After adding an ACE inhibitor to the mixture (Figure 5.B), Ang II production was blocked completely, additionally indicating the presence of ACE in the immobilized lymphocyte extract. Moreover, to exclude that Ang I might have been produced from the renin substrate by some other enzyme than renin, since the synthetic renin substrate may be cleaved unspecifically by several peptidases, purified angiotensinogen was added instead of the renin substrate. The experiments revealed that also after addition of angiotensinogen both Ang I and Ang II were produced (Figure 5.C). Again, the Ang II production was abolished by the renin inhibitor (Figure 5.C). After addition of renin substrate, Ang II concentrations in the supernatants exceeded those of

Ang I, whereas after addition of angiotensinogen Ang I was generated in higher concentrations than Ang II. These different patterns may be explained assuming that angiotensinogen has a higher affinity to renin than to renin substrate, so that Ang I is generated faster from renin in the presence of angiotensinogen than in the presence of renin substrate.

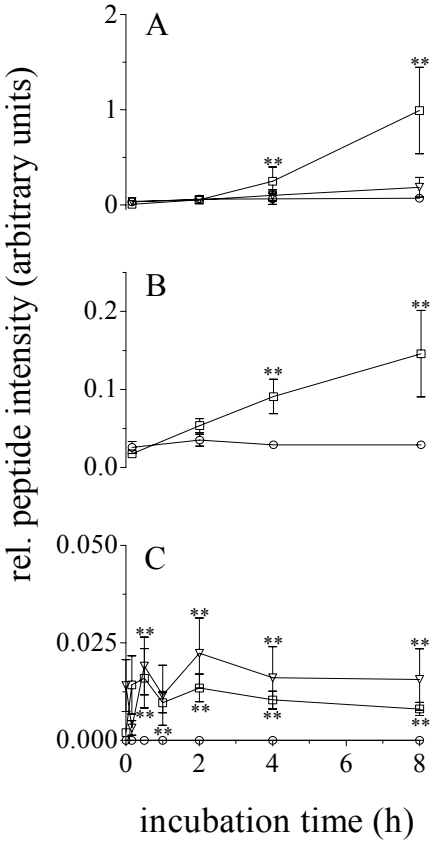

Figure 5: Detection of renin and ACE activity in a protein extract of mononuclear leukocytes by MALDI-MS.

(A) Incubation-time-dependent generation by the protein extract of mononuclear leukocytes in the presence of renin substrate of the reaction products Ang I (∇) in the absence of a renin inhibitor, and of Ang II, either in the absence (□) or in the presence (○) of a renin inhibitor.

(B) Incubation-time-dependent generation of the reaction product Ang II after the incubation of the protein extract of mononuclear leukocytes with Ang I in the absence (□) and in the presence (○) of an ACE inhibitor.

(C) Incubation-time-dependent generation of the reaction products Ang I (∇)in the absence of a renin inhibitor, and Ang II after the incubation of the protein extract of mononuclear leukocytes in the absence (□) and in the presence (○) of a renin inhibitor with purified angiotensinogen. Abscissa: Incubation time. Ordinate: ratios of the relative MALDI spectra signal intensities of the reaction product Ang II (N=5; ∗ indicates significance (calculated by Mann-Whitney-test) of relative peptide intensity in the absence or presence of the inhibitor used: $p < 0.05$).

Next, it had to be excluded that the detected renin and ACE activity was caused by contaminations from plasma renin and ACE. Therefore, the proteins of the isotonic NaCl solution of

the third washing step of the isolation of mononuclear leukocytes were immobilised and incubated with renin substrate and/or Ang I. After an incubation time of 8 h with renin substrate no Ang II production was detected by the MALDI mass spectrometry assay (data not shown). Next, the question arose whether mononuclear leukocytes do not only produce Ang II, but also secrete it into the circulation. The amount of Ang II in the supernatant was quantified (relative to internal standard). After a 120 min-incubation of mononuclear cells without stimulation, Ang II concentration in the supernatant was 0.17 ± 0.051 (rel. Ang II intensity (arbitrary units)). The addition of both fMLP and LPS lead to a significant increase of Ang II concentration in the supernatant (fMLP vs. LPS: 0.37 ± 0.06 vs. 0.36 ± 0.07 (rel. Ang II intensity (arbitrary units)).

This Ang-II amount was produced by a cell suspension which contained 5.08 ± 0.98 x 10^9 mononuclear lymphocytes L^{-1}. From the quantitative relationship between Ang II concentrations and the corresponding MS signals as well as the number of cells releasing this Ang II amounts, it was estimated that after stimulation 9.9 ± 6.6 fmol Ang II/10^6 lymphocytes was secreted per minute. For unstimulated cells the same algorithm leads to an Ang II production rate of 4.7 ± 1.7 fmol Ang II/10^6 lymphocytes/min. Therefore, also unstimulated lymphocytes should contribute amounts of Ang II in the same order of magnitude as stimulated cells. In order to obtain a valid comparison between lymphocytic Ang II secretion rates and human Ang II plasma levels, the latter were also determined by mass spectrometry. In six healthy subjects plasma Ang II concentrations of 180,000 ± 80,000 fmol L^{-1} were found. Therefore, mononuclear lymphocytes (about 7 x 10^9 L^{-1}) contribute to the generation of a physiologically relevant amount of Ang-II in human plasma.

B.6.5. Discussion

The present findings demonstrate that human circulating CD8+ (T lymphocytes) and CD19+ (B lymphocytes) mononuclear leukocytes are a source of Ang II, and that this secretion is sufficient to stimulate AT receptors. The mechanism, whereby LPS stimulates Ang II release has been studied in tissues like liver, fat, adrenal glands and aorta[24,25]. These studies demonstrated that LPS stimulates angiotensinogen mRNA expression and enhances angiotensinogen plasma concentrations. According to the present estimates, the amount of lymphocytes contained in 1 mL blood (about 5 x 10^6 / ml) can produce about 50 fmol Ang II per min after stimulation and 20 fmol Ang II per min without stimulation, thus increasing the concentration by 50 pmol L^{-1} per min after stimulation or 20 pmol L^{-1} per min without stimulation. In con-

sideration of this secretion rate and given an EC_{50} in the nanomolar range[26,27], lymphocytic Ang II production during approximately 30 min would be sufficient to reach EC_{50}.

Obviously, from the rate of synthesis estimated in this study a steady state plasma concentration, which is the net effect of synthesis and degradation, cannot be calculated without reliable data on degradation kinetics. Moreover, the degree of stimulation may be different between *in vivo* and *in vitro* conditions. Therefore the conclusions which can be drawn from lymphocytic Ang II synthesis *in vitro* should be restricted to the point that the order of magnitude of lymphocytic Ang II production is sufficient to contribute to plasma Ang II.

Moreover, by a functional mass spectrometric assay it was demonstrated that angiotensinogen, renin and ACE are not only expressed in human lymphocytes, but are also functionally relevant for lymphocytic Ang II production. Since after blocking each of these components lymphocytic Ang II production was totally neutralized, other pathways of Ang II synthesis are not necessarily involved in these mononuclear leukocytes. From the present findings it appears unlikely that mononuclear leukocytes merely take up Ang II by receptor internalization. The latter mechanism would lead to a continuous decrease of extracellular Ang II concentration with time, whereas in our experiments extracellular Ang II continuously increased. From these findings it is apparent that mononuclear lymphocytes produce and secrete Ang II, but it is not clear whether these cells may also store Ang II. In this study, a specific site of Ang II storage in these cells was not detected. The mass-spectra of this study indicate that the chromatographic fractions from the supernatants of the mononuclear leukocytes not only contain Ang II but also further, yet unknown peptides.

There are several reports pointing out the possibility that in experimental animals and humans Ang II might be produced by immune cells, since the expression of several components of the renin-angiotensin system, such as ACE[28] and angiotensinogen[29] has been demonstrated. A direct proof of secretion of the end-product, Ang II, had up till now not been given, however[30].

Currently, several tissues have been shown to produce Ang II, including vascular endothelial cells[31], macrophages[32] and adipocytes[33]. Moreover, angiotensin peptides are released by rat alveolar macrophages[34]. Local Ang II secretion by these cells and tissues was recognized as an important part of humoral vascular regulation. There have been also reports localizing Ang

II in the cytoplasm of several types of leukocytes by immunocytochemistry. Furthermore, it has been shown that after adding angiotensinogen or Ang I to immune cells Ang II production is increased[35], indicating that all enzymes required for Ang II production are present in these cells. However, until now it was not clear whether Ang II production in these cells was mainly mediated by renin and ACE, like in the classical pathway, or by other renin- or ACE-independent enzymes. Our findings underscore for the first time effective secretion of Ang II by lymphocytes.

What may be the specific role of lymphocyte-derived Ang II? It is tempting to speculate that lymphocyte-derived Ang II has not only a direct vasoconstrictive effect but that it might play also a role in modulating immunologic processes in several ways. Using Agtr1a (-/-) mice, which lack AT1A receptors for Ang II, it was shown that Ang II triggers the proliferation of splenic lymphocytes[36]. These actions of the RAS to promote lymphocyte activation may contribute to inflammatory processes. Moreover, experimental mesangial proliferative glomerulonephritis developing in FcR-deficient mice surviving from lethal initial damage was prevented by an Ang II AT1 blocker[37]. It was shown that glomerular injury in FcR-deficient mice was associated with AT1 receptor-dependent CD4+ T cell infiltration mediated by Ang II-activated renal mesangial cells showing chemotactic activity for T cells[38]. Furthermore, a role of the RAS has been demonstrated in IL-12 secretion by macrophages[39], in hematopoietic processes in multiple cellular lineages including hematopoietic progenitor cells[40,41] and in the regulation of TGFb1 secretion by CD4+ cells[42]. It is well known that Ang II is active as a cytokine stimulating interleukin secretion. In this context, the Ang II production by lymphocytes may be regarded as part of an autocrine loop regulating lymphocytic immune response.

A yet unsolved issue regards the appropriate stimulus of lymphocytic Ang II secretion. Whereas the stimuli of renin secretion, which is the rate-limiting step of Ang II production from circulating angiotensinogen, are well known, the regulation of lymphocytic Ang II production has not been examined yet. Lymphocytes are known to express β_2 adrenergic receptors, which in the iuxtaglomerular apparatus are stimulators of renin secretion. It is difficult to find further analogies between lymphocytic and renal renin secretion, however. Further studies are clearly needed to elucidate the physiologic regulation of lymphocytic Ang II secretion.
In summary, circulating human CD8+ (T) and CD19+ (B) lymphocytes were shown to produce and to secrete Ang II. The findings thus add a further physiologic source of Ang II to the current view of angiotensin metabolism. The physiologic role of lymphocyte-derived Ang II

secretion remains open. Nevertheless, the amounts of Ang II secreted by lymphocytes appear to be sufficient to stimulate AT receptors and to affect vascular tone.

B.6.6. References

1. Peach MJ: Renin-angiotensin system: biochemistry and mechanisms of action. *Physiol Rev* 57:313-370, 1977
2. Reid IA, Morris BJ, Ganong WF: The renin-angiotensin system. *Annu Rev Physiol* 40:377-410, 1978
3. Müller DN, Bohlender J, Hilgers KF, Dragun D, Costerousse O, Menard J, Luft FC: Vascular angiotensin-converting enzyme expression regulates local angiotensin II. *Hypertension* 29:98-104, 1997
4. Dell'Italia LJ, Meng QC, Balcells E, Wei CC, Palmer R, Hageman GR, Durand J, Hankes GH, Oparil S: Compartmentalization of angiotensin II generation in the dog heart. Evidence for independent mechanisms in intravascular and interstitial spaces. *J Clin Invest* 100:253-258, 1997
5. Campbell DJ: Circulating and tissue angiotensin systems. *J Clin Invest* 79:1-6, 1987
6. Engeli S, Negrel R, Sharma AM: Physiology and pathophysiology of the adipose tissue renin-angiotensin system. *Hypertension* 35:1270-1277, 2000
7. Navar GL, Harrison-Bernard LM, Nishiyama A, Kobori H: Regulation of intrarenal angiotensin II in hypertension. *Hypertension* 39:316-322, 2002
8. Dostal DE: The cardiac renin-angiotensin system: novel signaling mechanisms related to cardiac growth and function. *Regul Pept* 91:1-11, 2000
9. Dorer FE, Kahn JR, Lentz KE, Levine M, Skeggs LT: Purification and properties of angiotensin-converting enzyme from hog lung. *Circ Res* 31:356-366., 1972
10. Phillips MI, Speakman EA, Kimura B: Levels of angiotensin and molecular biology of the tissue renin angiotensin systems. *Regul Pept* 43:1-20, 1993
11. Inagami T: A memorial to Robert Tiegerstedt: the centennial of renin discovery. *Hypertension* 32:953-957, 1998
12. Saris JJ, van Dijk MA, Kroon I, Schalekamp MA, Danser AH: Functional importance of angiotensin-converting enzyme-dependent in situ angiotensin II generation in the human forearm. *Hypertension* 35:764-768, 2000
13. Dzau VJ: Multiple pathways of angiotensin production in the blood vessel wall: evidence, possibilities and hypotheses. *J Hypertens* 7:933-936, 1989
14. Naftilan AJ: Role of the tissue renin-angiotensin system in vascular remodeling and smooth muscle cell growth. *Curr Opin Nephrol Hypertens* 3:218-227, 1994
15. Morgan L, Pipkin FB, Kalsheker N: Angiotensinogen: molecular biology, biochemistry and physiology. *Int J Biochem Cell Biol* 28:1211-1222, 1996
16. Gorzelniak K, Engeli S, Janke J, Luft FC, Sharma AM: Hormonal regulation of the human adipose-tissue renin-angiotensin system: relationship to obesity and hypertension. *J Hypertens* 20:965-973, 2002
17. Ross R: Atherosclerosis-an inflammatory disease. *N Engl J Med* 340:115-126, 1999
18. Tepel M, Schlotmann R, Barenbrock M, Kisters K, Klaus T, Spieker C, Walter M, Meyer C, Bretzel RG, Zidek W: Lymphocytic Na^+-H^+ exchange increases after an oral glucose challenge. *Circ Res* 77:1024-1029, 1995
19. Feril LB, Jr., Tsuda Y, Kondo T, Zhao QL, Ogawa R, Cui ZG, Tsukada K, Riesz P: Ultrasound-induced killing of monocytic U937 cells enhanced by 2,2'-azobis(2-amidinopropane) dihydrochloride. *Cancer Sci* 95:181-185, 2004

20. Orie NN, Zidek W, Tepel M: Tyrosine and calcium/calmodulin kinases are common signaling components in the generation of reactive oxygen species in human lymphocytes. *Life Sci* 65:2135-2142, 1999
21. van der Giet M, Khattab M, Borgel J, Schlüter H, Zidek W: Differential effects of diadenosine phosphates on purinoceptors in the rat isolated perfused kidney. *Br J Pharmacol* 120:1453-1460, 1997
22. Jankowski J, Stephan N, Knobloch M, Fischer S, Schmaltz D, Zidek W, Schlüter H: Mass-spectrometry-linked screening of protein fractions for enzymatic activities-a tool for functional genomics. *Anal Biochem* 290:324-329, 2001
23. Wood JM, Gulati N, Forgiarini P, Fuhrer W, Hofbauer KG: Effects of a specific and long-acting renin inhibitor in the marmoset. *Hypertension* 7:797-803, 1985
24. Klett C, Hellmann W, Ganten D, Hackenthal E: Tissue distribution of angiotensinogen mRNA during experimental inflammation. *Inflammation* 17:183-197, 1993
25. Nyui N, Tamura K, Yamaguchi S, Nakamaru M, Ishigami T, Yabana M, Kihara M, Ochiai H, Miyazaki N, Umemura S, Ishii M: Tissue angiotensinogen gene expression induced by lipopolysaccharide in hypertensive rats. *Hypertension* 30:859-867, 1997
26. Le MT, Vanderheyden PM, Szaszak M, Hunyady L, Vauquelin G: Angiotensin IV is a potent agonist for constitutive active human AT1 receptors. Distinct roles of the N-and C-terminal residues of angiotensin II during AT1 receptor activation. *J Biol Chem* 277:23107-23110, 2002
27. Fierens FL, Vanderheyden PM, Roggeman C, van de Gucht P, de Backer JP, Vauquelin G: Distinct binding properties of the AT_1 receptor antagonist ^{3}H-candesartan to intact cells and membrane preparations. *Biochem Pharmacol* 63:1273-1279, 2002
28. Costerousse O, Allegrini J, Lopez M, Alhenc-Gelas F: Angiotensin I-converting enzyme in human circulating mononuclear cells: genetic polymorphism of expression in T-lymphocytes. *Biochem J* 290 :33-40, 1993
29. Gomez RA, Norling LL, Wilfong N, Isakson P, Lynch KR, Hock R, Quesenberry P: Leukocytes synthesize angiotensinogen. *Hypertension* 21:470-475, 1993
30. Kitazono T, Padgett RC, Armstrong ML, Tompkins PK, Heistad DD: Evidence that angiotensin II is present in human monocytes. *Circulation* 91:1129-1134, 1995
31. Leung PS, Chan HC, Wong PY: Immunohistochemical localization of angiotensin II in the mouse pancreas. *Histochem J* 30:21-25, 1998
32. Potter DD, Sobey CG, Tompkins PK, Rossen JD, Heistad DD: Evidence that macrophages in atherosclerotic lesions contain angiotensin II. *Circulation* 98:800-807., 1998
33. Goossens GH, Blaak EE, van Baak MA: Possible involvement of the adipose tissue renin-angiotensin system in the pathophysiology of obesity and obesity-related disorders. *Obes Rev* 4:43-55, 2003
34. Dezso B, Jacobsen J, Poulsen K: Evidence for the presence of angiotensins in normal, unstimulated alveolar macrophages and monocytes. *J Hypertens* 7:5-11, 1989
35. Weinstock JV, Blum AM: Granuloma macrophages in murine schistosomiasis mansoni generate components of the angiotensin system. *Cell Immunol* 89:39-45, 1984
36. Nataraj C, Oliverio MI, Mannon RB, Mannon PJ, Audoly LP, Amuchastegui CS, Ruiz P, Smithies O, Coffman TM: Angiotensin II regulates cellular immune responses through a calcineurin-dependent pathway. *J Clin Invest* 104:1693-1701, 1999
37. Suzuki Y, Shirato I, Okumura K, Ravetch JV, Takai T, Tomino Y, Ra C: Distinct contribution of Fc receptors and angiotensin II-dependent pathways in anti-GBM glomerulonephritis. *Kidney Int* 54:1166-1174, 1998
38. Suzuki Y, Gomez-Guerrero C, Shirato I, Lopez-Franco O, Hernandez-Vargas P, Sanjuan G, Ruiz-Ortega M, Sugaya T, Okumura K, Tomino Y, Ra C, Egido J: Susceptibility to T cell-mediated injury in immune complex disease is linked to local activa-

tion of renin-angiotensin system: the role of NF- AT pathway. *J Immunol* 169:4136-4146, 2002

39. Constantinescu CS, Goodman DB, Ventura ES: Captopril and lisinopril suppress production of interleukin-12 by human peripheral blood mononuclear cells. *Immunol Lett* 62:25-31, 1998
40. Rodgers K, Xiong S, DiZerega GS: Effect of angiotensin II and angiotensin(1-7) on hematopoietic recovery after intravenous chemotherapy. *Cancer Chemother Pharmacol* 51:97-106, 2003
41. Rodgers KE, Xiong S, Steer R, DiZerega GS: Effect of angiotensin II on hematopoietic progenitor cell proliferation. *Stem Cells* 18:287-294, 2000
42. Chen K, Wei Y, Sharp GC, Braley-Mullen H: Inhibition of TGF-β-1 by anti-TGFbeta1 antibody or lisinopril reduces thyroid fibrosis in granulomatous experimental autoimmune thyroiditis. *J Immunol* 169:6530-6538, 2002

B.6.7. Supplementary Methods

Since not all methods were described in the publication, in this section a more extensive description of the methods is given.

ANALYTICAL TECHNIQUES

CATION EXCHANGE CHROMATOGRAPHY OF THE SUPERNATANT

Immediately after incubation the supernatant was chromatographed using a cation exchanger (HiTrap™ SP 1mL; Amersham, Sweden); eluent A: 20 mmol L^{-1} KH_2PO_4, pH 3.5; eluent B: 20 mmol L^{-1} KH_2PO_4 and 2 mol L^{-1} NaCl; pH 3.5; stepwise gradient: 0-5 min: 0 % B; 5-10 min: 5 % B; 10-15 min: 12 % B; 15-20 min: 25 % B; 20-25 min: 37 % B; 25-30 min: 50 % B; 30-35 min: 75 % B. Fractions were collected according to UV absorbance at 280 nm (peak fractionation).

ANALYTICAL REVERSED PHASE CHROMATOGRAPHY

To the vasoactive fraction of the cation exchange chromatography 1 mol L^{-1} triethylammonium acetate (TEAA) was added to a final concentration of 40 mmol L^{-1}. The mixture was loaded on an analytical reversed phase chromatography column (Poros R2/H; 100 x 2.1 mm I.D., Applied Biosystems, USA; equilibration buffer: 40 mmol L^{-1} TEAA in water; flow rate: 500 µL min^{-1}). The retained substances were eluted using 80 Vol-% acetonitrile in water and the following stepwise gradient: 0-10 min: 0 % B; 10-15 min: 25 % B; 15-20 min: 50 % B; 20-25 min: 75 % B; 25-35 min: 100 % B; flow: 500 µL min^{-1}. Fractions were collected according to UV absorbance at 280 nm (peak fractionation), lyophilised and stored at –80°C.

MATRIX-ASSISTED LASER DESORPTION/IONISATION MASS-ANALYSIS

The lyophilised fractions of the reversed-phase chromatography were analyzed by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) and MALDI post-source decay (PSD) fragment ion analysis. The lyophilised fractions were resuspended in 10 µL 0.1% TFA. 1 µL of each fraction was prepared on a prestructured MALDI sample support (MTP AnchorChip™ 400/384, Bruker Daltonics, Germany) using the α-4-hydroxycinnamic acid affinity sample preparation method[1]. All mass spectrometric measurements were performed on a Bruker Ultraflex TOF/TOF instrument (Bruker-Daltonics, Germany). The instrument was equipped with a nitrogen laser (Laser Science, USA), emitting at 337 nm. On

average, the presented spectra are the sums of 200 single-shot spectra for MS mode, and 600 for MS/MS mode. Mass spectra of positively charged ions were analysed in the reflector mode using delayed ion extraction. Fragment ion spectra were recorded using the LIFT option of the instrument. The calibration constants were determined using standard peptides prepared on positions adjacent to the sample, resulting in an error of <100 ppm for the recorded mass spectra. Protein identification using the obtained fragment ion mass data was performed using the software package Mascot (Matrix Science, UK). The adrenocorticotropic hormone (ACTH) fragment 18-39 (Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe) or saralasin (Sar-Arg-Val-Tyr-Val-His-Pro-Ala) (10 µg/sample) was added to the sample as internal standard in the case of kinetic measurements by using MALDI mass spectrometry. Hereby local differences in the peptide concentration on the MALDI spot were neutralized.

MEASUREMENT OF PERFUSION PRESSURE IN THE ISOLATED PERFUSED RAT KIDNEY

The kidney was excised, immediately mounted into the perfusion system and perfused by a peristaltic pump in a single-pass system with a solution containing 115 mmol L^{-1} NaCl, 4.6 mmol L^{-1} KCl, 1 mmol L^{-1} $CaCl_2$, 1.2 mmol L^{-1} $MgSO_4$, 1.2 mmol L^{-1} NaH_2PO_4, 22 mmol L^{-1} $NaHCO_3$, 49 mmol L^{-1} glucose and 35 g of gelatine L^{-1} (Haemaccel; Behring-Werke, Germany), and equilibrated with 95 % O_2 / 5 % CO_2 at 37 °C. The perfusion medium and the kidney were kept constant at 37° C. Perfusion pressure was continuously monitored by a transducer (Gould P23) connected to a bridge amplifier (Hugo Sachs, March-Hugstetten, Germany).

MOLECULAR METHODOLOGY

PREPARATION OF RNA AND RT-PCR

Primers and probes for human Angiotensin-Converting-Enzyme (ACE), renin and angiotensinogen and for the housekeeping gene β-actin were designed using the computer program Primer Express 2.0 (Applied Biosystems, USA) (Table 1). Except for β-actin, primers were located in two different exons.

Total RNA from CD8+ or CD19+ cells was extracted using Qiagen RNeasy-Mini-Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Target RNA (1 - 2 µg) was reversely transcribed using 100 U SuperscriptII Reverse Transcriptase (RT) (Invitrogen,

Karlsruhe, Germany) at 42°C for 80 min in the presence of 50 mmol L^{-1} Tris-HCl (pH 8.3), 5.7 mmol L^{-1} KCl, 3 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} DTT, 0.5 mmol dNTPs, 8 U RNasin (Promega Corp., Mannheim, Germany) and 5 µmol L^{-1} Olgio$(dT)_{16}$ (Applied Biosystems, Forster City, Ca, USA). For every reaction set, one RNA sample was performed without SuperscriptII RT (RT^- reaction) to provide a negative control in subsequent PCR reaction.

Control PCR assays were performed to check cDNA amount, as well as RT efficiency and cDNA quality, using specific primers to human ß-actin [2]. Reactions were carried out in a total volume of 50 µL, containing 5 µL of each RT-sample, 5 µL RT buffer (Life Technologies, Germany), 2 mmol L^{-1} $MgCl_2$, 0.4 mmol L^{-1} dNTPs, 0.1µmol L^{-1} of each primer, DNase and RNAse free water and 2.5 U Taq polymerase (Life Technologies, Germany). The PCR temperature profile consisted of 26 cycles at 94°C for 30 s, 57°C for 1 min and 72°C for 30 s followed by an additional 6 min extension period at 72°C. PCR was carried out in a total volume of 50 µL containing 5 µL of RT in 50 µL PCR buffer (Life Technologies, Germany), 1.5 mmol L^{-1} $MgCl_2$, 0.4 mmol L^{-1} dNTPs, 0.1µmol L^{-1} of each specific primer, DNase and RNAse free water and 2.5 U Taq polymerase (Life Technologies, Germany). The PCR temperature profile for ACE-, renin- and angiotensinogen- cDNA consisted of 30 cycles at 94°C for 30 s, 57°C for 1 min and 72°C for 30 s followed by an additional 6 min extension period at 72°C, using the primers described in table 1. Control reactions for RT and PCR were performed by using water instead of mRNA or cDNA in reaction mixtures. The presence and size of the obtained PCR products were analyzed on ethidium bromide-stained agarose gels (2 %). Furthermore, the specificity of the obtained cDNA product was checked by digesting the amplificate with specific restriction enzymes.

DETECTION OF RENIN AND ACE ACTIVITY OF MONONUCLEAR LEUKOCYTES BY MATRIX ASSISTED LASER DESORPTION/ IONISATION MASS SPECTROMETRY

Mononuclear leukocytes were isolated from 200 mL blood as described in the regular paper. The mononuclear leukocytes were stored at –20°C for 10 h. After thawing, 4 mL distilled water was added to the mononuclear leukocytes. The cell suspension was filtered with a centrifuge filter (100 kDa cut-off). The proteins of the filtrate (750 µL) were immobilised by mixing with 750 µl $NaHCO_3$ and activated CNBr-Sepharose 6 MB beads (Amersham-Pharmacia Biotech, Germany) as described elsewhere[3]. The mixture was incubated for 2

hours at room-temperature. After incubation the beads were washed three times with double distilled water.

Table 1: Primers and human ACE, renin, angiotensinogen and for ß-actin; all cDNA sequences were obtained from the genbank database

	Name	Sequence (5'-3')	Amplicon length (bp)
ACE	ACEhumfor ACEhumrev	ATG GCA CTG GAA AAA ATT GC CAG CCC AGG ACC TCG CCG TT	500
renin	Renhumfor Renhumrev	GTG GGT GGA ATC ACG GTG TGT TGT AGT AGA AAG AGA AGA CGT CC	195
angiotensinogen	Aoghumfor Aoghumrev	CTG GTG CTA GTC GCT GCA AA AAC CTG TCA ATC TTC TCA GC	500
human β-actin	ßachumfor ßachumrev	CCT CGC CTT TGC CGA TCC GGA TCT TCA TGA GGT AGT CAG TC	630

REFERENCES

1. Gobom J, Schuerenberg M, Mueller M, Theiss D, Lehrach H, Nordhoff E: Alpha-cyano-4-hydroxycinnamic acid affinity sample preparation. A protocol for MALDI-MS peptide analysis in proteomics. *Anal Chem* 73:434-438, 2001
2. Raff T, van der Giet M, Endemann D, Wiederholt T, Paul M: Design and testing of beta-actin primers for RT-PCR that do not co- amplify processed pseudogenes. *Biotechniques* 23:456-460, 1997
3. Jankowski J, Stephan N, Knobloch M, Fischer S, Schmaltz D, Zidek W, Schlüter H: Mass-spectrometry-linked screening of protein fractions for enzymatic activities-a tool for functional genomics. *Anal Biochem* 290:324-329, 2001

C. Original publications relating to uraemia

C.1. A novel lymphocyte-derived vasoactive angiotensin peptide

Vera Jankowski, Raymond Vanholder, Markus van der Giet, Lars Henning, Sevil Karadogan, Günter Giebing, Markus Tölle, Jens Furkert, Alexander Oksche, Hartmut Schlüter,Michael Bienert, Ute Bahr, Walter Zidek, Joachim Jankowski

C.1.1. Abstract

The angiotensin peptides play a central role in cardiovascular physiology and pathology. Among these peptides, angiotensin II (Ang II) has been investigated most intensively. The other angiotensin peptides nevertheless may importantly contribute to vascular regulation as well, showing different affinities to the angiotensin receptors and hence different vascular effects.

The present experiments revealed that after incubation of human mononuclear leukocytes a vasocontrictive fraction appeared time dependently. After chromatographic purification structural analysis by matrix-assisted laser desorption/ionisation and electrospray ionization mass spectrometry revealed an angiotensin-octapeptide with the sequence Ala-Arg-Val-Tyr-Ile-His-Pro-Phe, which differs from Ang II in Ala^1 in stead of Asp^1.

In the presence of lymphocytes Ang II is converted to des[Asp^1]-[Ala^1]-Ang II by decarboxylation of the Asp^1 group of Ang II. Des[Asp^1]-[Ala^1]-Ang II had a same affinity to the AT_1 receptor as Ang II, but was a weaker vasoconstrictor, suggesting only partial AT_1 receptor agonism. In addition, this compound showed a higher AT_2 receptor affinity than genuine Ang II. These two characteristics point to a specific action profile compared to Ang II. Therefore the generation of des[Asp^1]-[Ala^1]-Ang II from Ang II may modulate the Ang II effects on vasculature. In chronic renal failure, the relative ratio des[Asp^1]-[Ala^1] / Ang II is significantly increased compared to healthy subjects (1.42 ± 0.29 vs 0.38 ± 0.08; N=5; $p < 0.01$).

In summary des[Asp^1]-[Ala^1]-Ang II is a newly identified human active angiotensin peptide which is transformed from angiotensin II by lymphocyte-derived aspartate-decarboxylase. For the first time, the generation by a non-proteolytic enzyme of a peptide hormone exhibiting specific actions is demonstrated.

in preparation

C.1.2. Introduction

The octapeptide, angiotensin II (Ang II) is since long accepted to play a central role in the physiology and pathology of vascular regulation. The peculiar therapeutic success of ACE inhibitors and AT_1 blockers in cardiovascular protection recently stressed that the role of Ang II in the physiopathology of atherosclerosis and hypertension might be even more important than previously accepted.

In general, less attention has been paid to further members of the angiotensin family, including angiotensin III and IV and angiotensin (1-7). These alternative angiotensin peptides nevertheless deserve interest, since at least some of them have vascular effects different from those of Ang II. Angiotensin (1-7) has been shown to be a vasodilator[1]. Similarly, angiotensin IV is an agonist to the AT_4 receptor mediating vasodilation[2]. These actions of the various angiotensin peptides may gain importance especially under the conditions of ACE inhibition, when Ang II levels decrease while in most cases increased or at least stable amounts of the other angiotensin peptides are formed via ACE-independent pathways[3].

The differential effects of the various angiotensin peptides are closely linked to their different affinities to the angiotensin II receptor subtypes. When the first two subtypes, the AT_1 receptor and the AT_2 receptor had been distinguished on a pharmacological basis and then had been identified by expression cloning, it soon appeared that both displayed striking differences with respect to their actions.

Most importantly, the AT_2 receptor appeared to mediate a number of beneficial vascular Ang II effects like vasodilation and inhibition of smooth muscle cell growth, whereas the AT_1 receptor appeared to be the mediator of unfavourable effects such as vasoconstriction and vascular smooth muscle proliferation[4].

Among the members of the currently known angiotensin peptide family none appears to show a higher affinity to the AT_2 receptor than Ang II. On the background of the delicate interplay between the AT_1 and AT_2 receptor in the pathogenesis of cardiovascular disease, the novel member of the angiotensin family described below may be relevant to human cardiovascular physiopathology.

A recent study documents that also human mononuclear leukocytes are a further source of

Ang II. Ang II secretion by these cells may play a physiologically relevant role in humoral vascular regulation. The isolation of Ang II in supernatants of mononuclear leukocytes adds a further physiologic source of Ang II to the view of angiotensin metabolism[5].

In this recent study[5], supernatant of stimulated human mononuclear leukocytes were screened for vasoconstrictive compounds. Ang II was identified in one fraction with a strong vasoconstrictive effect by mass-spectrometric methods. Moreover, mass-signals of yet unknown peptide were detected in the corresponding mass-spectra (see Chapter B.6; Figure 2.A). One of these peptides, a new vasoactive angiotensin peptide, was isolated, identified and characterized in the present study. Since the ratio of the new peptide and Ang II is significantly increased in plasma of chronic renal failure patients compared to healthy control subjects, a pathophysiologic relevance of this peptide is likely in this setting.

C.1.3. Methods

CHEMICALS

HPLC water (gradient grade) and acetonitrile were purchased from Merck (Germany), all other substances from Sigma (Germany).

PREPARATION OF MONONUCLEAR LEUKOCYTES

Mononuclear leukocytes were obtained from healthy subjects according to established techniques[6]. Briefly, 400 ml heparinized blood was drawn by venipuncture from the antecubital vein and centrifuged at 240 g for 15 min. After removing the supernatant, mononuclear leukocytes were isolated by layering 5 mL diluted blood (1:1 vol with isotonic NaCl) on 3 mL Histopaque (Sigma-Aldrich, Germany; 5 / 6% wt / vol Ficoll; density 1.077 g mL^{-1}) and centrifugation at 240 g for 20 min. The mononuclear leukocyte interphase was carefully aspirated, washed three times in isotonic NaCl by centrifugation at 400 g for 5 min, and resuspended in Hanks' balanced salt solution containing (in 10^{-3} mol L^{-1}): NaCl, 136; KCl, 5.4; KH_2PO_4, 0.44; Na_2HPO_4, 0.34; $CaCl_2$, 1; D-glucose, 5.6; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 10; pH 7.4. Mononuclear leukocytes were resuspended in 2 ml physiologic salt solution (0.9 % NaCl). After incubation for 2 h at room temperature, mononuclear leukocytes were centrifuged (1,500 g; 5 min).

CATION-EXCHANGE CHROMATOGRAPHY OF THE SUPERNATANT

Immediately after incubation, the supernatant was chromatographed using a cation-exchanger

(HiTrap™ SP 1 mL; Amersham Pharmacia, Sweden; eluent A: KH_2PO_4; pH 3.5; eluent B: KH_2PO_4 and 2 mol L^{-1} NaCl; pH 3.5; stepwise gradient: 0-5 min: 100% A; 5-10 min: 5% B; 10-15 min: 12% B; 15-20 min: 25% B; 20-25 min: 37% B; 25-30 min: 50% B; 30-35 min: 75% B). Fractions were collected according to their UV absorbance at 280 nm (peak fractionation) as well as their conductivity.

ANALYTICAL REVERSED-PHASE CHROMATOGRAPHY

To the fractions of the cation exchange chromatography 1 mol L^{-1} TEAA was added to a final concentration of 40 mmol L^{-1}. The mixture was loaded to an analytical reversed-phase chromatography column (Poros R2/H; 100 x 2.1 mm I.D., Perseptive Biosystems, Germany; equilibration buffer: 40 mmol L^{-1} TEAA in water; flow rate: 500 µL min^{-1}). The retained substances were eluted using 80 vol-% acetonitrile in water and the following stepwise gradient: 0-10 min: 100% A; 10-15 min: 25% B; 15-20 min: 50% B; 20-25 min: 75% B; 25-35 min: 100% B; flow: 500 µL min^{-1}.

MEASUREMENTS OF PERFUSION PRESSURE IN THE ISOLATED PERFUSED RAT KIDNEY

The effects of aliquots of the desalted and lyophilised fractions of the cation-exchange chromatography on vascular tone were evaluated in an isolated rat kidney perfused with a constant flow of 8 mL min^{-1} while perfusion pressure was continuously monitored. Details of the preparation are given elsewhere[7]. Briefly, the kidney was excised and immediately mounted into the perfusion system. The isolated rat kidney was perfused by a peristaltic pump in a single-pass system with a solution containing 115 mmol L^{-1} NaCl, 4.6 mmol L^{-1} KCl, 1 mmol L^{-1} $CaCl_2$, 1.2 mmol L^{-1} $MgSO_4$, 1.2 mmol L^{-1} NaH_2PO_4, 22 mmol L^{-1} $NaHCO_3$, 49 mmol L^{-1} glucose and 35 g of gelatine / L^{-1} (Haemaccel; Behringwerke, Germany), and equilibrated with 95% O_2 / 5% CO_2. The perfusion medium and the kidney were kept constantly at 37° C. Perfusion pressure was continuously monitored by a transducer (Gould P23, USA) connected to a bridge amplifier (Hugo Sachs, Germany).

Vasoconstrictor responses of the isolated perfused rat kidney to fractions to be tested were assessed at basal tone after an equilibration period of 30 min. Fractions with vasoconstrictive effects were next tested in the presence of the angiotensin-receptor antagonist (AT_1) EXP 3174. The AT_1 antagonist EXP 3174 (1 µmol L^{-1}) was added to the perfusate 30 min before challenge with fractions to be tested.

MATRIX-ASSISTED LASER DESORPTION/IONISATION MASS-ANALYSIS

The lyophilised fractions of the reversed-phase chromatography were examined by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) and post-source decay (PSD)-MALDI-MS. A reflectron type time-of-flight (RETOF) mass spectrometer (Reflex III, Bruker, Germany) was used according to Hillenkamp and Karas[8]. The sample was mounted on an x, y, z movable stage allowing irradiation of selected sample areas. In this study, a nitrogen laser (VSL-337 ND, Laser Science, USA) with an emission wavelength of 337 nm and 3 ns pulse duration was used. The laser beam was focused to a diameter of 50 µm at an angle of 45° to the surface of the target. The laser focussing was assisted by microscopic sample observation using a video device. 10-20 single spectra were accumulated to obtain a better signal-to-noise ratio.

Sample preparation for MALDI- and PSD-MALDI experiments was identical. 5 µL bidest. water was added to the lyophilised sample. 1.5 µL of the analyte solution was mixed with 1.5 µL of matrix solution (α-cyano-4-hydroxy-cinnamic acid in water/acetonitrile 50/50 vol-%). Before introduction into the mass spectrometer the mixture was gently dried on a target using the AnchorChip^{TM} Technology (Bruker-Daltronic, Germany). AnchorChip^{TM} targets are equipped with hydrophilic patches in hydrophobic surroundings. The mass accuracy was in the range of approximately 0.01%.

ELECTROSPRAY IONIZATION MASS-ANALYSIS

Liquid chromatography / mass spectrometric measurements were performed on an orthogonal-acceleration time-of-flight instrument (qTOF MS Mariner, PE/ PerSeptive Biosystems, Framingham, USA). For chromatography a column (0.3 mm x 100 m) packed with nonporous silica-based C-18 stationary phase was used. 0.1% formic acid in water was used as eluent A and 0.1% formic acid in acetonitrile as eluent B. The capillary of the electrospray source was heated at 190°C. Nitrogen was used both as drying gas and nebulizing gas at flow rates of 35 and 10 l/h, respectively. The total-ion current chromatogram was obtained in the positive-ion mode.

INCUBATION OF MONONUCLEAR LEUKOCYTES WITH ANGIOTENSIN II

To test whether des[Asp^1]-[Ala^1]-Ang II is synthetized in the presence of mononuclear leuko-

cytes 10^{-6} mol L^{-1} AT-II were added to the supernatant. After 0, 0.2, 0.5, 1 and 3 hours aliquots of the supernatant were analysed by MALDI mass spectrometry. Adrenocorticotropic hormone (ACTH, 10^{-5} mol L^{-1}) was added to the MALDI matrix (α-cyano-4-hydroxycinnamic acid saturated in acetonitrile and water 50/50 (vol-%) with 0.01% trifluoroacetic acid) for internal calibration of the MALDI mass signal intensity. The MALDI experimental conditions were the same as described above. This approach was repeated with mononuclear leukocytes thermically denaturated by heating at 100 °C for 10 min in a reaction vial.

SYNTHESIS OF THE IDENTIFIED PEPTIDE

The identified peptide was synthesized automatically by the solid-phase method using standard Fmoc chemistry in continuous flow mode (TentaGel S Random-Access Memory (RAM) resin 0.21 mmol/g for peptide amides, TentaGel S p-hydroxybenzoic acid (PHB) resin (Rapp Polymere Tuebingen, Germany) for the free acid of urocortin, o-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), 2 equiv of n,n-diisopropylethylamine (DIEA), coupling 20 min, deblocking with 20% piperidine in N,N-dimethyl formamide (DMF) for 15 min, final cleavage with 95% TFA/5% water for 3 h). Purification of crude peptide was carried out by preparative HPLC on PolyEncap A300 (10 µm particle size, 250 mm x 20 mm i.d., Bischoff Analysentechnik GmbH, Leonberg, Germany) in water with increasing concentrations of ACN as mobile phase. An eluent gradient of 5-70% (v / v) ACN/water (0.1% TFA) over 70 min with a flow rate of 10 mL/min was used. The purified peptide was lyophilized. The peptide was characterized by MALDI mass spectroscopy on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA) using R-cyano-4-hydroxycinnamic acid and sinapinic acid as matrix and gave the expected $[M + H]^+$ mass peaks[9].

BINDING STUDIES

All binding studies were performed as displacement studies. For binding studies of des[Asp^1]-[Ala^1]-Ang II with angiotensin type 1 (AT_1) and angiotensin type 2 receptors (AT_2), Human Embryonic Kidney (HEK) 293 cells were transiently transfected with human AT_1- and AT_2-receptors. cDNA encoding the human AT_1 (pcDNA3.AT_1)- and AT_2 (pcDNA3.AT_2)-receptor was kindly provided by Tadashi Inagami (Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennesse, USA).

CELL CULTURE AND TRANSFECTION METHODS

HEK 293 cells were cultured at 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, penicillin (100 U mL^{-1}), and streptomycin (100 µg mL^{-1}). Cells were grown on poly-L-lysine-coated plastic material to improve adherence. Cells were transfected with Lipofectamin according to the supplier's recommendations. For a 15-mm-diameter well of a 24-well plate, 5 X 10^4 HEK 293 cells were transfected with 250 ng of plasmid DNA and 2 mL of Lipofectamin. After removal of the transfection reagent, cells were incubated for 48 h.

MEMBRANE PREPARATION FOR RECEPTOR BINDING

HEK 293 cell expressing either the AT_1- or AT_2-receptor protein were grown on 100-mm Petri dishes, washed twice with 5 ml of PBS (137 mmol L^{-1}, NaCl, 2.7 mmol L^{-1} KCl, 1.5 mmol L^{-1} KH_2PO_4, 8.0 mmol L^{-1} Na_2HPO_4, pH 7.4), harvested with a rubber policeman and centrifuged at 400 g for 10 min. The pellet was resuspended in Tris-BAME buffer (50 mmol L^{-1} Tris, 0.15 mmol L^{-1} bacitracin, 0.0015% aprotinin, 10 mmol L^{-1} $MgCl_2$, 2 mmol L^{-1} EGTA, pH 7.3), and the suspension was homogenized with a glass/Teflon homogenizer (10 strokes), and centifuged at 26,000 g for 30 min. The pellet was rehomogenized in Tris-BAME and aliquots of the resulting suspension were stored at -70°C until use.

^{125}I-SAR1,ILE^8-ANG II DISPLACEMENT BINDING ANALYSIS

HEK 293 cell membranes (5 µg) were incubated in a final volume of 200 µl of Tris/BAME buffer containing 1 µM ^{125}I-Sar^1,Ile^8-Ang II alone or in the presence of increasing concentrations of des[Asp^1]-[Ala^1]-Ang II and Ang II (1 x 10^{-12} to 1 x 10^{-6} mol L^{-1}) for 2 h at 25°C at 300 rpm in a shaking water bath. The samples were then transferred onto GF/C filters (Whatman International Ltd., UK), pretreated with 0.1% (w/v) polyethylenimine and washed rapidly twice with PBS using a Brandel cell harvester. Filters were finally transferred into 5-ml vials and radioactivity was determined in a liquid scintillation counter. Data were analyzed with RadLig software 4.0 (Cambridge, UK), and graphs were generated with Prism Software 2.02 (GraphPad, USA). Saturation analysis yielded K_D values of 1 µmol L^{-1} and 100 nmol L^{-1} for the AT_1- and AT_2-receptor. The values were used for calculations of the K_i values of unlabeled ligands.

CALCULATION OF DISPLACEMENT CURVES

For AT-receptor displacement studies, non-specific binding was accounted for the determination of the number of counts measured in the presence of 1 µmol L^{-1} Sar^1,Ile^8 Ang II. The values obtained were deduced from the total counts measured to obtain specific binding.

Basal values (dpm, mean ± S.E.M., n=12 – 15) of 100% binding were 2670 ± 157 (AT_1-receptor) and 6015 ± 213 (AT_2-receptor). Specific binding in the sole presence of radioligands was normalized to 100%, and the remaining data were normalized to this figure. IC_{50}-values were calculated using a non-linear curve fitting function (Prism™, Graph Pad, USA).

ISOLATION, IDENTIFICATION AND QUANTIFICATION OF des[ASP^1]-[ALA^1]-ANG II FROM HUMAN BLOOD

Peripheral blood (30 ml) was obtained by catheterization of the cubital vein of 5 end-stage renal failure patients (stage 5) and 5 healthy control subjects. The blood of the donors was collected in tubes containing K_2-EDTA (7.2 mg). The clinical and biochemical characteristics of the patients and the controls are given in Table 1. The blood samples were centrifuged at 800 g for 10 min at 25°C for isolation of plasma.

Healthy control subjects did not use any drugs. The cause of end-stage renal failure was hypertensive nephropathy in 1, diabetic nephropathy in 2 and unknown in 2 patients. One patient was treated with a beta-blocker and one patient with a calcium channel blocker. Smokers were not included into the study. The c-reactive protein concentration of the patient group was slightly increased (90.1 ± 33.4 mg/mL). One patient suffered from coronary heart disease and one from peripheral arterial occlusive disease. All received hemodialysis three times a week using a F 17 (Fresenius, Germany) hemodialysis membrane.

The plasma (15 ml) was fractionated by size-exclusion chromatography. The size-exclusion gel “Sephacryl S-100 High Resolution” (1,000 x 16 mm, S100 HR, Pharmacia BioTech, Sweden) was equilibrated with 0.9 w-% NaCl in water. The sample was loaded onto the equilibrated column. The eluent (0.9 w-% NaCl water) was pumped with a flow rate of 1 ml/min. The elution was monitored with a UV-detector at 280 nm.

Each fraction from size exclusion chromatography was further separated by reversed-phase HPLC (“Chromolith® SpeedROD” (50 x 4.6 mm I.D., Merck, Darmstadt, Germany)). The fractions dissolved in eluent A (40 mM TEAA) were injected to the HPLC and were pumped onto the column using a flow rate of 1.0 mL/min. The substances was eluted from the column using 80 vol-% acetonitrile in water as eluent B and the following gradient: 0-10 min 0 % B, 10-60 min 0-40 % B, 60-65 min 40-100 % B, 65-70 min 100 % B (flow rate: 1.0 mL/min). The wavelength of the UV-detector was 280 nm. 1.5 ml fractions were collected and lyophi-

lised. Each fraction was analysed by MALDI-mass spectrometry using the condition as described.

Table 1: Clinical and biochemical characteristics of the patients (values are mean ± SEM) and control subjects

	end-stage renal patients (N=5)	control subjects (N = 5)	p value
age (years)	65 ± 2	57 ± 7	n.s.
sex (m / f)	3/2	3/2	n.s.
blood pressure (mm Hg)	129 ± 8 / 71 ± 6	119 ± 6 / 66 ± 2	n.s.
red blood cells ($10^6 \mu l^{-1}$)	3.6 ± 0.3	3.9 ± 0.4	n.s.
white blood cells ($10^3 \mu l^{-1}$)	10.5 ± 2.0	6.5 ± 0.1	n.s.
platelets ($10^3 \mu l^{-1}$)	231 ± 48	271 ± 67	n.s.
serum creatinin (µmol L^{-1})	475.1 ± 86.9	82.0 ± 5.6	<0.01
blood urea nitrogen (BUN; mmol L^{-1})	19.6 ± 1.7	4.8 ± 0.8	<0.01

STATISTICS

All data are given as means ± S.E.M. of the individual values. For the binding experiments, Friedman's test was used to compare 100% corrected means of controls with the binding observed in the presence of displacing substances.

C.1.4. Results

HUMAN LYMPHOCYTES RELEASE ONE OR MORE VASOCONSTRICTIVE SUBSTANCES

After isolation of human mononuclear leukocytes by centrifugation, washing with an isotonic

salt solution and incubation at 25°C for 2 h, the supernatant was concentrated and fractionated by cation-exchange chromatography. A characteristic cation-exchange chromatogram is given in Figure 1.A.

Because of the unphysiologic salt concentration of the cation-exchange chromatography fractions, a direct screening of the vasoconstrictive properties of these fractions was not possible. Therefore, each fraction of the cation-exchange chromatography was desalted by reversed phase chromatography before the screening experiments. The reversed-phase chromatography allows to desalt the eluate of the cation exchanger and to further fractionate the eluate.

One of the fractions from the reversed phase chromatography with a strong vasoconstrictive effect is labelled in Figure 1.B by an arrow. The corresponding cation-exchange-fraction is labelled in Figure 1.A also by an arrow. The underlying substances were identified by the procedure as described. The different UV absorption intensities of this fraction in the cation-exchange and the reversed-phase chromatography may be caused by different flow rates and the resulting dilution of the fraction.

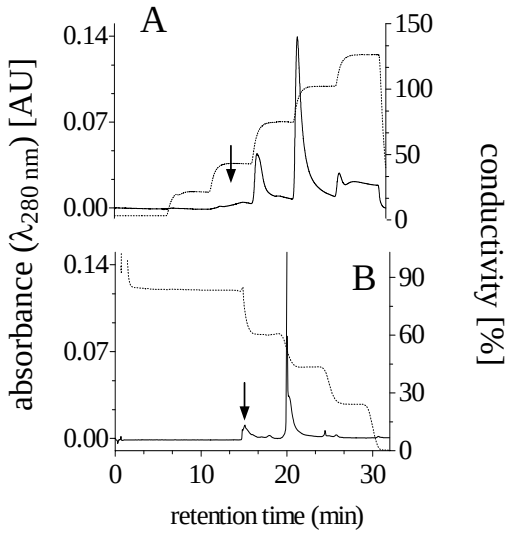

Figure 1: (A) Cation-exchange chromatography of the supernatant of mononuclear leukocytes after 2 h incubation (conditions: cation exchange column: HiTrap™ SP 1 mL; Amersham Pharmacia (Sweden), eluent A: KH_2PO_4; pH 3.5; eluent B: KH_2PO_4 and 2 mol L^{-1} NaCl; pH 3.5; stepwise gradient: 0-5min: 100% A; 5-10 min: 5% B; 10-15 min: 12% B; 15-20 min: 25% B; 20-25 min: 37% B; 25-30 min: 50% B; 30-35 min: 75% B; flow: 1 mL min^{-1}). The arrow indicates the fraction further submitted to reversed-phase chromatography shown in Figure 1.B.

(B) Reversed-phase chromatography of the eluate of the cation-exchange chromatography labelled by an arrow (conditions: reversed-phase: Poros R2/H, 100 x 2.1 mm I.D., Perseptive Biosystems, Germany; eluent A*:* 40 mmol L^{-1} TEAA in water; eluent B: 80 % acetonitrile in water; stepwise gradient: 0-10 min: 100% A; 10-15 min: 25% B; 15-20 min: 50% ACN; 20-25 min: 75% B; 25-35 min: 100% B; flow: 500 µL min^{-1}). The vasoconstrictive fraction is indicated by the arrow.

The reversed-phase chromatography obtained with this fraction is shown in Figure 1.B. The fractions obtained from each reversed-phase chromatography elution step were again tested for vasoactivity in the isolated perfused rat kidney. The vasoconstrictive fraction is again indicated by the arrow in the figure.

IDENTIFICATION OF AN ANGIOTENSIN PEPTIDE CONTAINING ALA^1 INSTEAD OF ASP^1

The reversed-phase fraction with a strong vasoconstrictive effect (Figure 1.B) was analysed by MALDI mass spectrometry as well as electrospray ionisation (ESI) ion-trap mass spectrometry. In these spectra, a mass-signal at 1001.3 Da was detected (Figure 2.A); the ESI MS/MS mass spectrum of this peptide is given in Figure 2.B.

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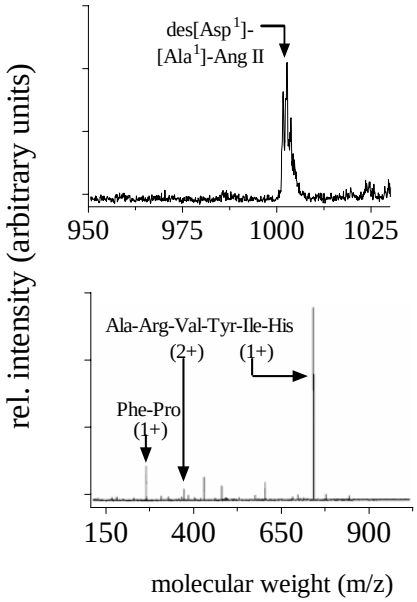

Figure 2: MALDI- (A) and ESI-MS/MS- (B) mass spectra of the fraction from the reversed-phase chromatography (Figure 1.B) labelled by the arrow.

Interpretation of the ESI MS/MS data by de-novo sequencing techniques, identified the underlying substance as a peptide with the sequence Ala- Arg - Val - Tyr - Ile - His - Pro - Phe. Obviously, the vasoactive peptide isolated differs from Ang II by the replacement of Asp^1 with Ala^1. The amino acid sequences of Ang II as well as of the newly detected vasoactive peptide identified are given in Figure 3.

Ang II:	**Asp** - Arg - Val - Tyr - Ile - His - Pro – Phe
des[Asp^1]-[Ala^1]-Ang II:	**Ala** - Arg - Val - Tyr - Ile - His - Pro - Phe

Figure 3: Amino acid sequence of angiotensin II (Ang II) and des[Asp^1]-[Ala^1]-Ang II.

Des[Asp^1]-[Ala^1]-Ang II could be synthesised from Ang II by decarboxylation of Asp^1. After identification of the peptide, we tested the hypothesis that des[Asp^1]-[Ala^1]-Ang II is enzymatically synthesised from Ang II in lymphocytes. In Figure 4 MALDI mass spectra of the supernatant of mononuclear leukocytes before (Figure 4.A) and after an incubation period of 2 h (Figure 4.B) with Ang II (added to the supernatant) are given. During the incubation of lymphocytes in the presence of Ang II in the supernatant, the amount of des[Asp^1]-[Ala^1]-Ang

II in the supernatant increased significantly compared to the amount before the incubation period (Figure 4.A and Figure 4.B). The generation of des[Asp^1]-[Ala^1]-Ang II over time in the supernatant is quantified in Figure 4.C. There was no increase in the des[Asp^1]-[Ala^1]-Ang II signal detectable using denatured mononuclear leukocytes (data not shown).

Using the experimental conditions as described, the available endogenous Ang II contributes only to a lesser extent to the synthesis of the des[Asp^1]-[Ala^1]-Ang II based due to high exogenously added concentrations of Ang-II.

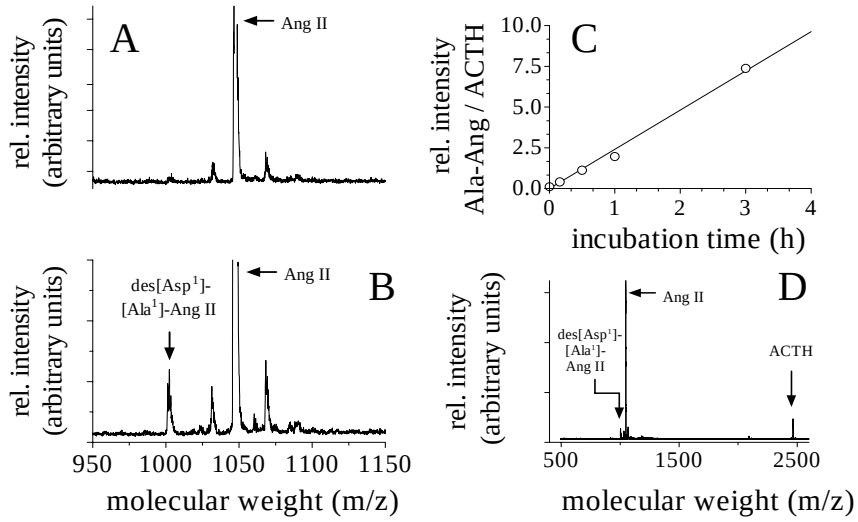

Figure 4: MALDI mass spectra of the supernatant of mononuclear leukocytes before (A) and after an incubation period of 2 h (B) with Ang II (added to the supernatant). In (C) the generation of des[Asp^1]-[Ala^1]-Ang II in the supernatant over time is quantified (relative to internal standard ACTH). (D): Mass spectrum showing the whole mass range including the internal standard, ACTH.

Des[Asp^1]-[Ala^1]-Ang II is a partial AT1 receptor agonist showing the same affinity as Ang II and has a higher AT2 receptor affinity than Ang II. At basal tone, des[Asp^1]-[Ala^1]-Ang II caused a dose-dependent vasoconstriction (Figure 5). In the presence of the angiotensin-receptor antagonist AT(1) EXP 3174 the vasoconstrictive effect of des[Asp^1]-[Ala^1]-Ang II was abolished. The vasoconstrictor EC_{50} value (mol L^{-1}) of des[Asp^1]-[Ala^1]-Ang II ($(4.43 \pm 1.95) \times 10^{-7}$) exceeded that of Ang II ($(5.20 \pm 2.52) \times 10^{-8}$, Figure 5). The minimal effective concentration of des[Asp^1]-[Ala^1]-Ang II was in the range of 10^{-8} mol L^{-1}. Thus, des[Asp^1]-[Ala^1]-Ang II appeared to be a less active vasoconstrictor than Ang II.

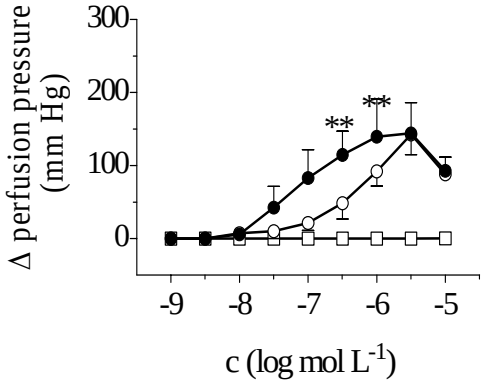

Figure 5: Change of perfusion pressure in the isolated perfused rat kidney induced by des[Asp^1]-[Ala^1]-Ang II (○) and Ang II (●) and des[Asp^1]-[Ala^1]-Ang II in the absence or presence of angiotensin-receptor antagonist AT(1) EXP 3174 (□) (abscissa: concentration of agonist (log mol L^{-1}); ordinate: change in perfusion pressure (mm Hg)). Each point is the mean of at least 6 determinations (n = 6) and vertical lines show the ± S.E.M.; **, $p < 0.01$ des[Asp^1]-[Ala^1]-Ang II vs Ang II.

The binding of des[Asp^1]-[Ala^1]-Ang II and Ang II to the AT_1- and AT_2-receptor was characterized by displacement of the radioligand [^{125}I]-Sar^1, Ile^8 angiotensin II by unmarked Sar^1, Ile^8 angiotensin II (Figure 6). IC_{50}-values in HEK 293 cells transiently transfected with the human AT_1-or AT_2-receptor for des[Asp^1]-[Ala^1]-Ang II were [-log IC_{50}] 9.54 ± 0.04 or 9.92 ± 0.04, respectively, and for Ang II [-log IC_{50}] 9.49 ± 0.06 or 9.59 ± 0.04, respectively. The IC_{50} for des[Asp^1]-[Ala^1]-Ang II was significantly lower in AT_2-transfected cells compared to Ang II ($p<0.05$), whereas no significant difference for des[Asp^1]-[Ala^1]-Ang II and Ang II IC_{50} was observed in AT_1-transfected cells.

Finally we quantified the des[Asp^1]-[Ala^1]-Ang II and Ang II ratio in human plasma by MALDI mass spectrometry. The des[Asp^1]-[Ala^1]-Ang II / Ang II ratio in chronic renal failure patients is significantly increased compared to healthy control subjects (1.42 ± 0.29 vs 0.38 ± 0.08; each N=5; $p < 0.01$).

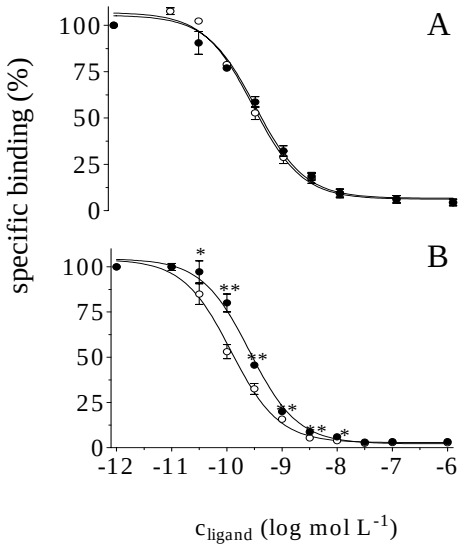

Figure 6: Displacement of [I^{125}] Sar^1, Ile^8 angiotensin II by des[Asp^1]-[Ala^1]-Ang II (○) and Ang II (●) in HEK 293 transiently transfected with AT_1- (A) and AT_2-receptors (B). Means ± S.E.M., n = 5 in duplicate. *, $p < 0.05$; **, $p < 0.01$ des[Asp^1]-[Ala^1]-Ang II vs Ang II.

C.1.5. Discussion

The present findings show that des[Asp^1]-[Ala^1]-Ang II is a novel angiotensin peptide occurring in increasing amounts with increasing incubation times in the supernatant of human lymphocytes, suggesting that Ang II or des[Asp^1]-[Ala^1]-Ang II is secreted into plasma. After the addition of Ang II to the suspension of leukocytes, Ang II is converted to des[Asp^1]-[Ala^1]-Ang II. Therefore, it can be speculated, that *in vivo* Ang II is released from leukocytes and thereafter converted to des[Asp^1]-[Ala^1]-Ang II in the supernatant. The hypothesis, that des[Asp^1]-[Ala^1]-Ang II is a product of Ang II, is also supported by the fact, that no DNA sequence coding des[Asp^1]-[Ala^1]-Ang II was found in a data-base covering the human genome[9].

The findings furthermore indicate that des[Asp^1]-[Ala^1]-Ang II is presumably synthesized by an enzyme decarboxylating Asp to Ala. A non-enzymatic decarboxylation was excluded by the finding that after heat-denaturation of intact mononuclear leukocytes des[Asp^1]-[Ala^1]-Ang II synthesis is abolished. At present, the nature of this decarboxylase is not known, including its substrate specificity and tissue distribution. Potentially, also in other tissues besides lymphocytes des[Asp^1]-[Ala^1]-Ang II can be produced. Des[Asp^1]-[Ala^1]-Ang II plasma concentration in healthy subjects is about 30-40 % of the Ang II plasma concentration.

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The affinity of des[Asp^1]-[Ala^1]-Ang II to the AT_1 receptor is nearly equal to that of Ang II, but the vasoconstrictive effect is significantly lower, indicating partial agonism. Des[Asp^1]-[Ala^1]-Ang II shows a higher affinity to the AT_2 receptor than Ang II.

The results suggest that des[Asp^1]-[Ala^1]-Ang II may play a role in human vascular physiology and pathology. Both the binding of des[Asp^1]-[Ala^1]-Ang II to the AT_1 receptor and the AT_2 receptor are modulating, most likely mitigating the Ang II effects. Des[Asp^1]-[Ala^1]-Ang II plasma concentrations in healthy controls are lower than those of Ang II, but Ang II is continuously metabolised to des[Asp^1]-[Ala^1]-Ang II in the presence of human lymphocytes. Therefore, des[Asp^1]-[Ala^1]-Ang II concentrations may locally reach levels similar to those of Ang II. Hence des[Asp^1]-[Ala^1]-Ang II may modulate the Ang II effects on human vasculature by at least two mechanisms.

A factor is very likely present in chronic renal failure patients, which stimulates the activity of the decarboxylase generating des[Asp^1]-[Ala^1]-Ang II. This stimulation leads to an increased des[Asp^1]-[Ala^1]-Ang II / Ang II ratio in plasma of these patients. This increase des[Asp^1]-[Ala^1]-Ang II / Ang II ratio leads to enhanced activation of the AT_2 receptor of these patients. Hereby, some beneficial vascular effects like vasodilation and inhibition of smooth muscle cell growth may be entailed.

In general, the interactions of des[Asp^1]-[Ala^1]-Ang II with the AT_2 receptor have not been assessed in depth in the present study. The mechanisms by which the AT_2 receptor is a mediator of vasodilation are still under debate. Several authors favor an indirect mechanism, such as stimulation of the B_2 bradykinin receptor, possibly via intracellular acidification and resulting in an increased NO and cGMP production[11,12]. Furthermore, there are reports suggesting that phosphotyrosine activity is increased following AT_2 receptor stimulation[13-15]. Further elucidation of AT_2 receptor signalling potentially will help to study the downstream effects of des[Asp^1]-[Ala^1]-Ang II binding to AT_2 receptor.

The above findings show that human lymphocytes produce and secrete a novel Ang II peptide, des[Asp^1]-[Ala^1]-Ang II. Des[Asp^1]-[Ala^1]-Ang II shows the same affinity to AT_1 receptor as Ang II, being a partial agonist, and a higher affinity to the AT_2 receptor, potentially antagonising AT_1 receptor stimulation. Des[Asp^1]-[Ala^1]-Ang II is produced by decarboxyla-

tion of Ang II, thereby possibly modulating Ang II effects in a negative feedback loop. Des[Asp^1]-[Ala^1]-Ang II may therefore play a role in the regulation of vascular tone and growth in normal and atherosclerotic or hypertrophied vessels.

C.1.6. References

1. Ren Y, Garvin JL, Carretero OA. Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension* 39(3):799-802, 2002
2. de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev* 52(3):415-72, 2000
3. Ferrario CM, Chappell MC, Dean RH, Iyer SN. Novel angiotensin peptides regulate blood pressure, endothelial function, and natriuresis. *J Am Soc Nephrol* 9(9):1716-22, 1998
4. Henrion D, Kubis N, Levy BI. Physiological and pathophysiological functions of the AT_2 subtype receptor of angiotensin II: from large arteries to the microcirculation. *Hypertension* 38(5):1150-7, 2001
5. Jankowski V, Vanholder R, van der Giet M, Henning L, Tölle M, Giebing G, Schönfelder G, Krakow A, Karadogan S, Gustavsson N, Gobom J, Webb J, Lehrach H, Schlüter H, Zidek W, Jankowski J. Detection of angiotensin II in supernatants of stimulated mononuclear leukocytes. *Hypertension* (in press, 2005)
6. Tepel M, Schlotmann R, Barenbrock M, Kisters K, Klaus T, Spieker C, Walter M, Meyer C, Bretzel RG, Zidek W. Lymphocytic Na^+-H^+ exchange increases after an oral glucose challenge. *Circ Res* 77(5):1024-9, 1995
7. van der Giet M, Khattab M, Borgel J, Schlüter H, Zidek W. Differential effects of diadenosine phosphates on purinoceptors in the rat isolated perfused kidney. *Br J Pharmacol* 120(8):1453-60, 1997
8. Hillenkamp F, Karas M. Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. *Methods Enzymol* 193:280-95, 1990
9. Beyermann M, Fechner K, Furkert J, Krause E, Bienert M: A single-point slight alteration set as a tool for structure-activity relationship studies of ovine corticotropin releasing factor. *J Med Chem* 39:3324-3330, 1996
10. Japan Biological Information Research Center. H-invitanational database (human genome database). http://www.h-invitational.jp/
11. Tsutsumi Y, Matsubara H, Masaki H, Kurihara H, Murasawa S, Takai S, Miyazaki M, Nozawa Y, Ozono R, Nakagawa K and others. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. *J Clin Invest* 104(7):925-35, 1999
12. Gohlke P, Pees C, Unger T. AT_2 receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism. *Hypertension* 31:349-55, 1998
13. Tsuzuki S, Matoba T, Eguchi S, Inagami T. Angiotensin II type 2 receptor inhibits cell proliferation and activates tyrosine phosphatase. *Hypertension* 28(5):916-8, 1996
14. Bedecs K, Elbaz N, Sutren M, Masson M, Susini C, Strosberg AD, Nahmias C. Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase. *Biochem J* 325(Pt 2):449-54, 1997
15. Horiuchi M, Hayashida W, Akishita M, Tamura K, Daviet L, Lehtonen JY, Dzau VJ. Stimulation of different subtypes of angiotensin II receptors, AT_1 and AT_2 receptors, regulates STAT activation by negative crosstalk. *Circ Res* 84(8):876-82, 1999

in preparation

C.2. Characterization of p-hydroxy-hippuric acid as an inhibitor of Ca^{2+}-ATPase in end-stage renal failure

Joachim Jankowski, Martin Tepel, Nina Stephan, Markus van der Giet, **Vera Breden**, Walter Zidek and Hartmut Schlüter

C.2.1. Abstract

In patients with end-stage renal failure, disturbances of Ca^{2+} metabolism are common. Besides hormonal changes, inhibition of cellular Ca^{2+}-ATPase was postulated to contribute to uremic toxicity. We purified a potent inhibitor of Ca^{2+}-ATPase from the hemofiltrate of patients with end-stage renal failure by multiple steps of high-performance liquid chromatography to homogeneity and identified the isolated inhibitor by mass spectrometric methods as p-hydroxy-hippuric acid. The enzyme used for the Ca^{2+}-ATPase assay system was isolated from red blood cells by cross-flow filtration. The activity of the Ca^{2+}-ATPase was measured spectrophotometrically as the difference in hydrolysis of ATP in the presence and absence of Ca^{2+} with different concentrations of ATP and p-hydroxy-hippuric acid. Ca^{2+}-ATPase was found to be inhibited by p-hydroxy-hippuric acid at a concentration above 11.7 µmol L^{-1}. P-hydroxy-hippuric acid inhibited erythrocyte Ca^{2+}-ATPase by reducing v_{max} and increasing the K_M-value. The EC_{50} (log mol L^{-1}; mean ± SEM) for p-hydroxy-hippuric acid was calculated as 4.82 ± 0.14. In conclusion, p-hydroxy-hippuric acid may play an important role in disturbed Ca^{2+} metabolism in end-stage renal failure.

C.2.2. Introduction

Ca^{2+} metabolism is disturbed in most patients with end-stage renal failure. Hormonal mechanisms are long known to contribute to these disturbances: secondary hyperparathyroidism[1], impaired biosynthesis of calcitriol[2], acidosis and hyperphosphatemia are generally accepted to play an important role in the pathogenesis of uremic bone disease and of altered Ca^{2+} homeostasis. However, there may be further mechanisms contributing to disturbed Ca^{2+} metabolism in uremia. One important finding in uraemia is a decreased transmembrane Ca^{2+} transport. This finding prompted the hypothesis that one or several circulating inhibitors of the plasmalemmal Ca^{2+}-ATPase accumulated in uremia. Consequently, several authors have shown that plasma from patients with end-stage renal failure can inhibit the activity of the Ca^{2+}-ATPase[3,4]. Nevertheless, to our knowledge the identification of these circulating inhibitors has proven difficult. Recently, Lindner et al[5] presented evidence of a lipophilic inhibitor but did not identify the structure of this substance. Here we describe the identification and charac-

terization of a low molecular weight hydrophilic Ca^{2+}-ATPase inhibitor, p-hydroxy-hippuric acid.

C.2.3. Material and methods

CHEMICALS

HPLC water (gradient grade) and acetonitrile were purchased from Merck (Germany), all other substances from Sigma-Aldrich (Germany).

CLINICAL AND BIOCHEMICAL CHARACTERISTICS OF PATIENTS WITH END-STAGE RENAL FAILURE AND CONTROL SUBJECTS

After giving informed consent, 13 healthy control subjects (m/f 7/6) with normal renal function (serum creatinine (mean ± SEM): 1.0 ± 0.2 mg/dl) and 9 patients (m/f 5/4) with end-stage renal failure (serum creatinine (mean ± SEM): 9.3 ± 1.1 mg/dl), who had been undergoing maintenance hemodialysis for 35.7 ± 13.4 months (mean ± SEM), were enrolled in this study. The cause of end-stage renal failure was chronic interstitial nephritis in 3 patients, chronic glomerulonephritis in 3, diabetic nephropathy in 2, polycystic kidney disease in 1. The blood urea nitrogen concentration (mean ± SEM) was significantly higher compared to healthy control subjects (21.31 ± 2.99 vs 3.16 ± 1.17 mmol/l), the hemoglobin concentration (mean ± SEM) was significantly lower compared to healthy control subjects (9.5 ± 0.6 vs 14.5 ± 1.1 g/dl). All patients received hemodialysis three times a week. As confirmed by the clinical examination and routine laboratory examination, all patients were stable and free from intercurrent illness.

FRACTIONATION OF THE HEMOFILTRATE

The hemofiltrate was collected during a hemofiltration using a high-flux membrane. Hemofiltrate (500 ml) from the above mentioned patients was concentrated to dryness in a vacuum concentrator (Freeze Dryer, Snijders, The Netherlands) (step 1). The dried hemofiltrate was dissolved in 5 ml of 40 mmol L^{-1} triethylammonium acetate (TEAA) in water (eluent A) and chromatographed (flow 0.1 ml min^{-1}) on a C18 reversed-phase column (Nucleosil RP C18 250 x 4.6 mm, Merck, Germany) in the displacement mode (displacer: 160 mmol L^{-1} n-butanol in eluent A) (step 2). After each chromatographic step, the influence of the fractions on the activity of the Ca^{2+}-ATPase was assessed. In step 3, the fractions inhibiting Ca^{2+}-ATPase were fractionated (flow, 0.5 ml min^{-1}) on a C18 reversed-phase HPLC column (Lichrosorb RP C18, 250 x 4.6 mm, Merck, Germany) with 0.1 % TFA in H_2O as eluent A

and 0.1 % TFA in CH_3CN as eluent B under following gradient conditions: 0 to 5 min: 0 to 20 % B; 5 to 10 min: 20 % B; 10 to 30 min: 20 to 40 % B; 30 to 40 min: 40 to 60 % B; 40 to 45 min: 60 to 100 % B; 45 to 50 min: 100 % B; 50 to 55 min: 100 to 0 % B. Thereafter, the fractions showing Ca^{2+}-ATPase inhibition were chromatographed (flow, 0.5 ml min^{-1}) on an other reversed-phase column (Supersphere RP C18 endcapped, 250 x 4.6 mm, Merck, Germany) with the following gradient: 0 to 10 min: 100 % A (20 mmol L^{-1} TEAA in water); 10 to 20 min: 0 to 20 % B (20 mmol L^{-1} TEAA in CH_3CN); 20 to 40 min: 20 to 40 % B; 40 to 50 min: 40 to 60 % B; 50 to 55 min: 60 to 100 % B; 55 to 60 min: 100 % B; 60 to 62 min: 100 to 0 % B) (step 4).

ANALYTICAL PROCEDURE

The hemofiltrate fractions purified to homogenity were analysed by gas-chromatography / mass spectrometry. For that propose, speed-vac-dried samples were dissolved in N-methyl-N-trimethylsilylfluoroacetic acid amide and incubated for one hour at 70ºC. The fractions were separated by gas-chromatography (0 to 35 min: 80 to 280 ºC; column: 150 RCN; carrier gas: helium; flow: 2 ml min^{-1}) and identified by electron impact (EI) mass spectrometry (Finnigan MAT 8200, USA).

PREPARATION OF THE ERYTHROCYTE MEMBRANE

The membrane was purified from human red blood cells from the normotensive subjects. The red blood cells were isolated from 500 mL blood and the cells were separated from plasma by centrifugation at 4,000 g and 4 °C for five minutes. After centrifugation the buffy coat was removed[6]. The red blood cells were washed twice with an isotonic NaCl solution and once with isotonic sodium hydrogen carbonate buffer. By then using a hypotonic sodium hydrogen carbonate buffer the washed red blood cells were hemolyzed after which they were washed in a cross-flow filtration device (constant-volume mode) with a 300 kDa cut-off membrane with 24 litres of a solution containing tris(hydroxymethyl)amino methane hydrochloride (25 mmol L^{-1}), sodium chloride (75 mmol L^{-1}), potassium chloride (25 mmol L^{-1}), magnesium chloride (1 mmol L^{-1}), ethylene glycol-bis(-aminoethylether)-N,N,N',N'-tetraacetic acid (1 mmol L^{-1}), phenylmethyl-sulfonylfluoride (0.1 mmol L^{-1}), benzamide (1 mmol L^{-1}), sodium azide (100 µmol L^{-1}). After washing the membranes, ethylene-glycol-bis(-aminoethylether)-N,N,N',N'-tetraacetic acid was removed by a buffer containing phenylmethylsulfonylfluoride (1 mmol L^{-1}), benzamide (1 mmol L^{-1}) and tris-HCl (28 mmol L^{-1}). The membranes were

concentrated by ultra-centrifugation at 100,000 g at 4 °C for 30 min and frozen in liquid nitrogen. Protein concentration was determined according to the method of Bradford[7].

Ca^{2+}-ATPASE ASSAY

Ca^{2+}-ATPase activity was spectrophotometrically measured as the difference in hydrolysis of ATP in absence and presence of Ca^{2+} (0.25 mmol L^{-1}). The assay system included magnesium chloride hexahydrate (2 mmol L^{-1}), tris(hydroxymethyl) amino methane (12.5 mmol L^{-1}), ouabain octahydrate (100 µmol L^{-1}), tris-HCl (12.5 mmol L^{-1}), EGTA (100 µmol L^{-1}), NaCl (75 mmol L^{-1}), KCl (25 mmol L^{-1}), and 570 µg membrane protein. Water free calcium was prepared by affinity chromatography (chelex 100, BioRad, USA). To determine the maximum activity of the Ca^{2+} / Mg^{2+} ATPase, this activity was measured in the presence of calmodulin (10 µmol L^{-1})[8]. To obtain the dose-response relationship, the p-hydroxy-hippuric acid concentration was varied in small intervals (1–316 µmol L^{-1}), whereas the concentration of adenosine-5'-triphosphate was kept constant at 80 µmol L^{-1}. Then, the activity of Ca^{2+}-ATPase was measured at different concentrations of adenosine-5'-triphosphate (0.004-1.0 mmol L^{-1}), while the concentrations of p-hydroxy-hippuric acid were kept constant at 0, 10 and 100 µmol L^{-1}. After initiating the reaction with adenosine-5'-triphosphate, the assay medium was incubated for 90 minutes at 37 °C. Reactions were terminated by icing the samples and adding 2 ml of a mixture consisting of malachite green (2.1 mmol L^{-1}), hydrochloric acid (6 mol L^{-1}), polyvinyl alcohol (300 µmol L^{-1}) and ammonium molybdate (46.2 mmol L^{-1}). Five minutes after addition of the malachite green-solution phosphate was measured by UV extinction at 578 nm. Ca^{2+}-ATPase activity was expressed as PO_4^{3-} production in µmol L^{-1} $g_{protein}^{-1}$ h^{-1}.

NMR SPECTROSCOPY

The proton NMR (^{1}H-NMR) measurements were carried out using a Bruker AMX 500 FT spectrometer (Bruker Analytische Messtechnik, Germany) operating at a field strength of 11.7 T. A signal turn surface coil with 5 mm inner diameter was used. All spectra were recorded at a controlled probe temperature of 25 °C. The signal from deuterium oxide was used to optimise the homogeneity of the magnetic field, and the fine adjustment was done by inspection of the free induction decay obtained without water suppression. A field-frequency was provided by detecting the deuterium signal of deuterium oxide. The ^{1}H-NMR spectra were obtained by using a flip angle of 90 degrees (6.2 µs). Pulse conditions were 128 accumulations collected into 32 K computer points using 90 degree pulses and a relaxation delay of 6 s. The spectral width comprised 5 kHz. Using these conditions, spectra were fully T_1-

relaxed and, for sharp resonances, T_2-relaxation during the period 3t was not observed[9]. The water resonance (4.8 ppm) was suppressed by presaturation during relaxation- and mixing-time with a 90° sequence (d_1-90°-d_0-90°-d_9-90°-free induction decay), following a modified procedure of Bodenhausen et al.[10]. The spinning rate was 18 rotations per second. The accumulation time was 10.4 min. Chemical shifts were referenced to 3-trimethylsilyl-[2,2,3,3,-$^2H_4$]-propionate at 0 ppm. Calibration curves with identified substances revealed a linear relationship between concentration and peak height. Therefore, the peak height of the respective substance was used for quantitation[11].

C.2.4. Results

In Figure 1.A a reversed-phase displacement chromatogram of lyophilized hemofiltrate is shown. The fraction labelled in Figure 1.A had an inhibitory effect on Ca^{2+}-ATPase activity. This fraction was purified to homogeneity by further chromatographic methods. Figure 1.B shows a typical chromatogram of the reversed-phase chromatography of the peak labelled in Figure 1.A by an arrow. The fraction labelled in the figure by an arrow again inhibited Ca^{2+}-ATPase activity. Therefore this fraction was chromatographed by further reversed-phase chromatography using different reversed-phase gels and different eluents. In the last chromatographic step, a single UV peak was obtained (Figure 1.C). The isolated substance was again shown to inhibit Ca^{2+}-ATPase

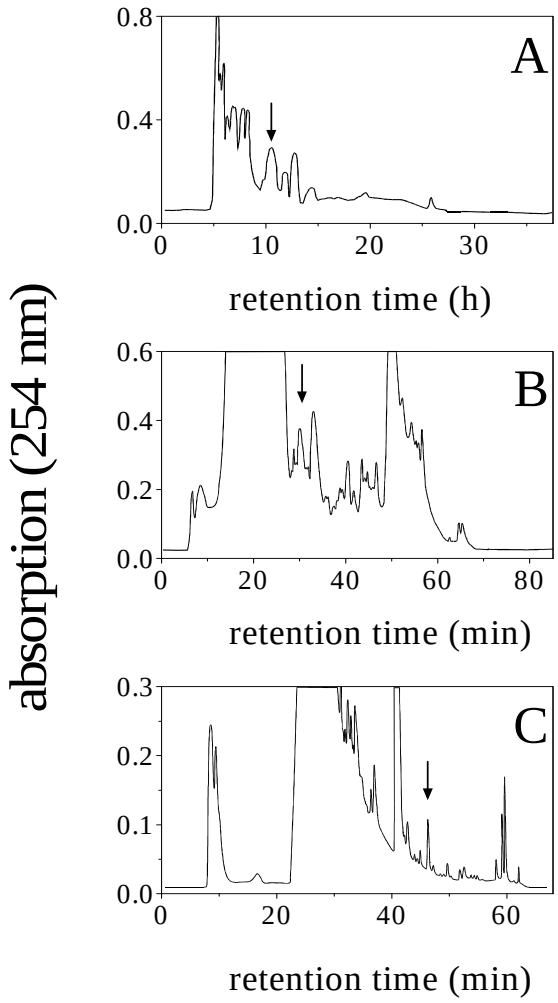

Figure 1: Isolation of p-hydroxy-hippuric acid from hemofiltrate of patients with end-stage renal failure.

(A) Chromatography of lyophilised hemofiltrate of plasma of patients with end-stage renal failure with a C18 reversed-phase column.

(B) Reversed-phase chromatography of the fraction labelled in Figure 1.A on an analytical reversed-phase high performance liquid chromatographic column.

(C) Reversed-phase chromatography of the fraction labelled in Figure 1.B on an analytical reversed-phase high performance liquid chromatographic column. The arrows indicate the fractions with the strongest inhibitory effect on the Ca^{2+}-ATPase activity, which leads to the identification of p-hydroxy-hippuric acid.

The molecular mass as well as the fragmentation pattern of this Ca^{2+}-ATPase inhibitor were determined by gas-chromatography / mass-spectrometry. The mass spectrum is shown in Figure 2. The analysis of this mass spectrum and comparison of this spectrum with mass spectra of a database led to the identification of p-hydroxy-hippuric acid.

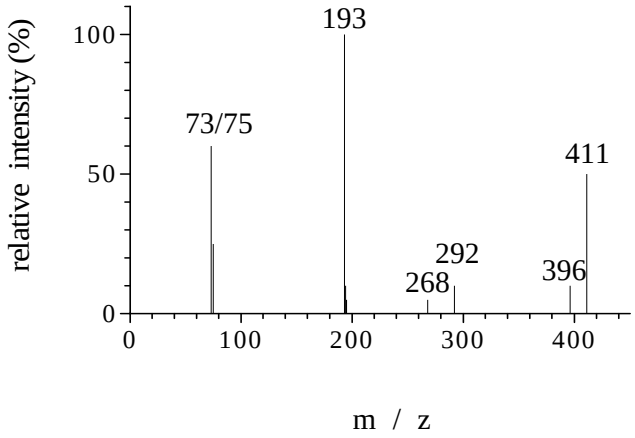

Figure 2: Mass spectrum of the fraction labelled in Figure 1.C by an arrow (abscissa: relative mass/charge, m/z, z=1; ordinate: relative intensity, arbitrary units). By data bank analysis the underlying substance was identified as p-hydroxy-hippuric acid.

Commercially available p-hydroxy-hippuric acid (Sigma-Aldrich, Germany) caused a dose-dependent inhibition of the Ca^{2+}-ATPase (Figure 3). Maximum inhibition of Ca^{2+}-ATPase was achieved with p-hydroxy-hippuric acid concentrations above 100 µmol L^{-1}. Using that concentration p-hydroxy-hippuric acid decreased the Ca^{2+}-ATPase by 64.6 % compared to the basal Ca^{2+}-ATPase. The calculated EC_{50} (log mol L^{-1}; mean ± SEM) for p-hydroxy-hippuric acid was –4.82 ± 0.14. Therefore, the EC_{50} value of p-hydroxy-hippuric acid is comparable to the EC_{50} values of dimethylguanosine, phenylethylamine and phenylacetic acid[12].

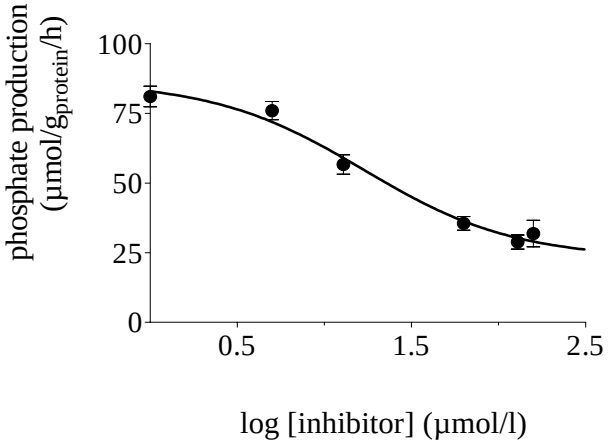

Figure 3: Effect of different concentrations of p-hydroxy-hippuric acid on the activity of Ca^{2+}-ATPase at constant concentration of ATP (log $c_{p\text{-hydroxy hippuric acid}}$ = 0 - 2,5 log µmol L^{-1}; c_{ATP} = 80 µmol L^{-1}). The data are the means ± SEM of three experiments.

The minimal inhibitory concentration of p-hydroxy-hippuric acid on the activity of Ca^{2+}-ATPase was 11.7 µmol L^{-1}. The effect of various concentrations of ATP in the presence of p-hydroxy-hippuric acid on the activity of the Ca^{2+}-ATPase is shown in Figure 4.

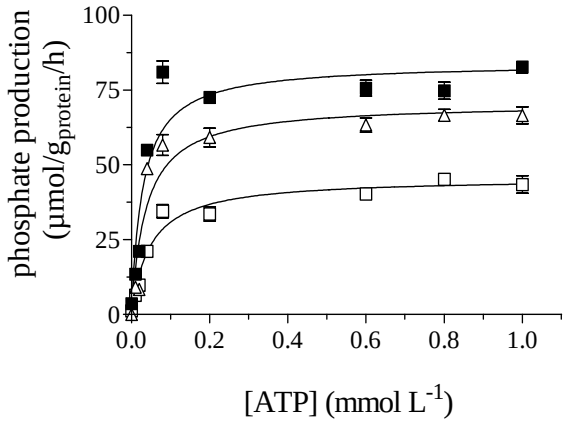

Figure 4: Effect of p-hydroxy-hippuric acid on the activity of the Ca^{2+}-ATPase at varying concentrations of ATP ($c_{p\text{-hydroxy-hippuric acid}}$ (■)= 0 µg L^{-1}, (Δ)= 16 µg L^{-1}, (□) = 160 µg L^{-1}; c_{ATP} = 0.004–1.0 mmol L^{-1}). The data are means ± SEM for four similar experiments.

The inhibitory effect of p-hydroxy-hippuric acid was not abolished by increased concentrations of ATP. Therefore a non-competitive inhibition of the activity of Ca^{2+}-ATPase by p-hydroxy-hippuric acid is supposed.

Are the concentrations used in the present *in vitro* studies physiologically relevant for end-stage renal failure patients? The concentration of p-hydroxy-hippuric acid in sera of patients with end-stage renal failure before hemodialysis as estimated by ^{1}H-NMR spectra was 94 ± 12 µmol L^{-1} although some resonances of p-hydroxy-hippuric acid and of hippuric acid overlap in ^{1}H-NMR spectra. This concentration is in the range of the calculated EC_{50} for p-hydroxy-hippuric acid. At the end of dialysis, the serum concentration of p-hydroxy-hippuric acid of patients with end-stage renal failure was decreased by 53 %. In sera of healthy controls p-hydroxy-hippuric acid was not detectable by NMR spectroscopy.

C.2.5. Discussion

Circulating Ca^{2+}-ATPase inhibitors are important factors for disturbed cellular Ca^{2+} metabolism in patients with end-stage renal failure. In the present study, p-hydroxy-hippuric acid was

identified as an inhibitor of Ca^{2+}-ATPase in hemofiltrate of patients with end-stage renal failure.

The results of our study document that p-hydroxy-hippuric acid accumulates in sera of patients with end-stage renal failure and that this substance is a further potent inhibitor of the erythrocytic Ca^{2+}-ATPase. In the present study, a Ca^{2+}-ATPase isolated from human erythrocytes was used to test the effects of the chromatographic fractions from hemofiltrates as well as authentic p-hydroxy-hippuric acid. Therefore, the observed effects of p-hydroxy-hippuric acid appear to be direct effects the Ca^{2+}-ATPase, and an indirect action by mediators is unlikely. The inhibition of Ca^{2+}-ATPase by p-hydroxy-hippuric acid is apparently elicited by an allosteric inhibition of the enzyme, i.e. the interaction with ATP is non-competitive.

It is well-known that the concentration of p-hydroxy-hippuric acid correlates negatively with motor nerve conduction velocity[13]. P-hydroxy-hippuric acid was associated with neurophysiological variables while urea, creatinine, urate and phosphate were not.

The p-hydroxy-hippuric acid concentration decrease by only 53 % during hemodialysis is explainable on the one hand by the protein-binding of this compound and on the other hand by the rapid refilling of the vascular compartment from extravascular sources. In healthy control subjects p-hydroxy-hippuric acid is not detectable by NMR spectroscopy. Therefore, the concentration of p-hydroxy-hippuric acid was apparently lower than 5 µmol L^{-1}, the limit of detection of a 500 MHz- NMR spectrometer. Hence, it can be assumed that decreased renal function causes an accumulation of p-hydroxy-hippuric acid comparable to that of other low molecular weight compounds like creatinine or urea.

Only a part of p-hydroxy-hippuric acid may penetrate the plasma membrane into the intracellular space to inhibit the intracellular Ca^{2+}-ATPase because of the protein-binding of p-hydroxy-hippuric acid[14]. This may explain why patients with end-stage renal failure show functioning transmembrane Ca^{2+} transport activities although the plasma concentration of p-hydroxy-hippuric acid may be in the range of maximal Ca^{2+}-ATPase inhibition.

In summary p-hydroxy-hippuric acid was identified as an inhibitor of the plasma membrane Ca^{2+}-ATPase. This effect may contribute to the inhibition of plasma membrane Ca^{2+}-ATPase activity in end-stage renal disease.

C.2.6. References

1. Tsuchihashi K, Takizawa H, Torii T, Ikeda R, Nakahara N, Yuda S, Kobayashi N, Nakata T, Ura N, Shimamoto K: Hypoparathyroidism potentiates cardiovascular complications through disturbed calcium metabolism: possible risk of vitamin D(3) analog administration in dialysis patients with end-stage renal disease. *Nephron* 84:13-20, 2000
2. Daisley-Kydd RE, Mason NA: Calcitriol in the management of secondary hyperparathyroidism of renal failure. *Pharmacotherapy* 16:619-630, 1996
3. Gafter U, Malachi T, Barak H, Djaldetti M, Levi J: Red blood cell calcium homeostasis in patients with end-stage renal disease. *J Lab Clin Med* 114:222-231, 1989
4. Nieman LK, Davis FB, Davis PJ, Cunningham EE, Gutman S, Blas SD, Schoenl M: Effect of end-stage renal disease on responsiveness to calmodulin and thyroid hormone of calcium-ATPase in human red blood cells. *Kidney Int Suppl* 16:S167-170, 1983
5. Lindner A, Hinds TR, Joly A, Schreiner GF: Neutral lipid from proteinuric rat urine is a novel inhibitor of the red blood cell calcium pump. *J Am Soc Nephrol* 10:1170-1178, 1999
6. Penniston JT, Filoteo AG, McDonough CS, Carafoli E: Purification, reconstitution, and regulation of plasma membrane Ca^{2+}- pumps. *Methods Enzymol* 157:340-351, 1988
7. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254, 1976
8. Gafter U, Malachi T, Barak H, Levi J: Red blood cell calcium level in chronic renal failure: effect of continuous ambulatory peritoneal dialysis. *J Lab Clin Med* 116:386-392, 1990
9. Bell JD, Brown JCC, Sadler PJ: High resolution proton magnetic resonance studies of human cerebrospinal fluid. *Clin Sci* 72:563-570, 1987
10. Bodenhausen G, Kogler H, Ernst RR: Selection of coherence-transfer pathway in NMR pulse experiments. *J Magn Res* 58:370-378, 1984
11. Bell JD, Lee JA, Lee HJ, Sadler PJ, Wilkie DR, Woodham RH: Nuclear magnetic resonance studies of blood plasma and urine from subjects with chronic renal failure. *Biochim Biophys Acta* 1096:101-107, 1991
12. Jankowski J, Luftmann H, Tepel M, Leibfritz D, Zidek W, Schlüter H: Characterization of dimethylguanosine, phenylethylamine, and phenylacetic acid as inhibitors of Ca^{2+} ATPase in end-stage renal failure. *J Am Soc Nephrol* 9:1249-1257, 1998
13. Schoots AC, De Vries PM, Thiemann R, Hazejager WA, Visser SL, Oe PL: Biochemical and neurophysiological parameters in hemodialyzed patients with chronic renal failure. *Clin Chim Acta* 185:91-107, 1989
14. De Smet R, Vogeleere P, Van Kaer J, Lameire N, Vanholder R: Study by means of high-performance liquid chromatography of solutes that decrease theophylline/protein binding in the serum of uremic patients. *J Chromatogr A* 847:141-153, 1999

C.3. The AN69 hemodialysis membrane has a decreasing effect on the intraplatelet diadenosine pentaphosphate concentration

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C.3.1. Abstract

The type of hemodialysis membrane may have an impact on the outcome of end-stage renal failure patients. In the present study, the effects of hemodialysis on the intracellular amount of diadenosine pentaphosphate (Ap_5A), a hydrophilic, anionic substance with a low molecular weight, was investigated. The intracellular Ap_5A concentrations were measured before and after hemodialysis, using either polyacrylonitrile (AN69; n = 10) or polysulfone (n = 23) membranes. Ap_5A was isolated from platelets using affinity chromatography and reversed-phase chromatography methods. The Ap_5A concentrations were quantified by ultraviolet absorption at 254 nm. The Ap_5A concentrations were significantly higher in platelets from the patients with end-stage renal failure as compared with 21 healthy control subjects (136 ± 50 vs. 9 ± 6 fg/platelet; mean ± SEM, $p < 0.01$). Before hemodialysis, the intracellular Ap_5A concentrations in platelets from 10 patients with end-stage renal failure using an AN69 membrane were not significantly different from those in platelets from 23 patients using a polysulfone membrane (93 ± 39 vs. 155 ± 70 fg/platelet). However, after the hemodialysis session, the intracellular Ap_5A concentrations in platelets from patients with end-stage renal failure using an AN69 membrane were significantly lower as compared with those in platelets before hemodialysis (51 ± 18 vs. 93 ± 39 fg/platelet, $p < 0.05$) as well as compared with those in platelets from patients using a polysulfone membrane (51 ± 18 vs. 250 ± 59 fg/platelet, $p < 0.05$). It was found that hemodialysis by using an AN69 membrane has a direct effect on the intraplatelet amount of Ap_5A and that changes of intraplatelet hydrophilic substances are dependent on the hemodialysis membrane used.

C.3.2. Introduction

Ap_5A is an intra- and extracellular mediator controlling numerous physiological functions[1]. Reviews about the role of Ap_5A in the cardiovascular system have been published recently[2,3,4]. After release, local Ap_5A concentrations in the range of 10^{-5} mol/l have been demonstrated[5]. An increased Ap_5A concentration has been shown in platelets from hemodialysis patients[6]. This may enhance growth of vascular smooth muscle cells and may be a further important atherogenic factor[6]. In order to evaluate the effects of hemodialysis on intracellular Ap_5A concentrations, diadenosine polyphosphates were determined in platelets from patients

with end-stage renal failure before and after hemodialysis using reversed phase chromatography. We used Ap_5A as the target substance. We observed that polyacrylonitrile (AN69) and polysulfone membranes have different effects on the intracellular amount of diadenosine pentaphosphates.

C.3.3. Material and Methods

CHEMICALS

All substances were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Deisenhofen, Germany) if not indicated otherwise. Diadenosine octaphosphate (Ap_8A) was synthesised according to Ng and Orgel[7] and purified by a method described by Jankowski et al.[8].

CLINICAL AND BIOCHEMICAL CHARACTERISTICS OF HEMODIALYSIS PATIENTS AND HEALTHY CONTROLS

After giving informed consent, 33 patients with end-stage renal failure who had been undergoing maintenance hemodialysis for 45 ± (SEM) 12 months were enrolled in this study. Ten patients used an AN69 membrane, and 23 patients used a polysulfone membrane (F16, Fresenius, Germany). The dialysates used were bicarbonate based. The clinical and biochemical characteristics of the patients with end-stage renal failure before and after the dialysis sessions are given in Table 1. The causes of end-stage renal failure were diabetic nephropathy in 10 patients, nephrosclerosis in 11 patients, chronic glomerulonephritis in 7 patients, interstitial nephritis in 1patient, and unknown in 4 patients. All patients underwent hemodialysis sessions three times a week. As confirmed by clinical and routine laboratory examinations, the patients were in good clinical condition. All patients were stable and free from intercurrent illness. Blood samples were collected immediately before and after the hemodialysis sessions from the arterial line of the hemodialysis equipment. Twenty-one healthy age-matched subjects without major medical illness and intake of medications served as controls.

Table 1: Clinical and biochemical characteristics of patients with end-stage renal failure before and after a regular hemodialysis session using AN69 or polysulfone membranes and of healthy controls

	patients with end-stage renal failure (ESRD) (N=33)			healthy control subjects (N=21)	
	before hemodialysis	after hemodialysis	p value before vs. after hemo-dialysis		p value healthy control subjects vs. ESRD before hemodialysis
age (years)	60.3 ± 2.8		n.s.	41.5 ± 12.9	n.s.
male/female ratio	22 / 11		--	10 / 11	n.s.
heart rate (beats/min)	74.5 ± 1.5	78.8 ± 1.5	<0.05	74.1 ± 5.8	n.s.
blood pressure (mm Hg)					
systolic	146.7 ± 4.0	131.5 ± 3.1	<0.05	125.6 ± 6.8	<0.05
diastolic	76.5 ± 1.5	70.9 ± 1.8	<0.05	79.0 ± 3.1	n.s.
serum creatinine (mg/dl)	9.1 ± 0.5	3.8 ± 0.3	<0.05	0.9 ± 0.1	<0.05
platelet count (x 10^3/mm^3)	255.0 ± 15.6	243.0 ± 25.1	n.s.	192.6 ± 43.3	n.s.

ISOLATION, IDENTIFICATION AND QUANTIFICATION OF DIADENOSINE PENTAPHOSPHATE AP_5A FROM HUMAN PLATELETS

The methods for isolation, identification and quantification of Ap_5A has been extensively described[6]. Briefly, for the quantification of the amount of Ap_5A in intact human platelets, a 4 ml blood sample per donor was collected. The biochemical characteristics of the subjects are given in Table 1. Platelets were isolated from the plasma by centrifugation, washed with physiological NaCl solution, and were frozen at –30 ° C for 1 day. After thawing, 1 µg Ap_8A as internal standard was added to the samples. The samples were deproteinized by using perchloric acid neutralized with potassium hydroxide. The platelets were counted in a solution of resuspended platelets directly after the sample was drawn. Ammonium acetate was added to the sample (final concentration 1 mol/l) and pH was adjusted to 9.5. Thereafter, the sample was passed through a boronate gel (derivative of a cation exchange gel, BioRex 70, BioRad, Germany; column: 10 mm x 100 mm), which was prepared according to Barnes et al.[9]. The

column was washed with 1 mol/l NH_4Ac (pH 9.5) with a flow rate of 2 ml/min. Ap_5A was eluted with 10 mmol/l HCl in water (flow rate: 2 ml/min). The eluate of the affinity chromatography was concentrated by reversed-phase C18 HPLC. A reversed-phase column (Supersphere, 250 x 4 mm, Merck, Germany) was equilibrated with 40 mmol/l triethylammonium acetate. The sample was pumped with a flow rate of 0.5 ml/min into the column. After washing the column with 15 ml 40 mmol/l triethylammonium acetate in water, Ap_5A was eluted with 35 % acetonitrile in water.

To quantify the amount of Ap_5A, the eluate of the reversed phase chromatography was chromatographed by a reversed-phase column (Poros R 2/H, 100 x 2.1 mm, Perseptive Biosystems, USA). Eluent A = 2 mmol/l tetrabutylammonium hydrogensulfate in a phosphate buffer (10 mmol/l K_2HPO_4, pH 6.8); eluent B = water:acetonitrile (20 : 80, v/v); gradient 0-1 min: 100 % eluent A, gradient 1-30.5 min: 0-30 % eluent B, gradient 30.5-31.5 min = 30-50 % eluent B; flow rate = 300 µl/min). Ultraviolet absorption was measured at 254 nm.

For identification of the individual Ap_5A peak fractionated lyophilized eluates of the reversed phase chromatography were dissolved in 100 µl 20 mmol/l K_2HPO_4 (pH 8) and subjected to an anion exchange column (MiniQ PC 1.6/5, Pharmacia, Uppsala Sweden; equilibration and sample buffer = 20 mmol/l K_2HPO_4 (pH 8); flow rate: 100 µl/min). Retained substances were eluted by 1 mol/l NaCl in 20 mmol/l K_2HPO_4 in water; 1 mol/l triethylammonium acetate in water was added to the eluate of the anion-exchange chromatography to a final concentration of 40 mmol/l. The mixture was loaded to a reversed phase column (Supersphere RP-C18, 2.1 x 100 mm, Merck, Germany; equilibration and sample buffer = 40 mmol/l triethylammonium acetate in water; flow rate: 100 µl/min). Ap_5A was eluted by 20 % acetonitrile in water. The lyophilised fractions from the reversed phase chromatography were examined by matrix assisted laser desorption/ ionisation mass spectrometry (MALDI-MS) as well as post-source decay (PSD)-MALDI-MS. A reflectron type time-of-flight mass spectrometer (Reflex III, Bruker, Germany) was used employing the method of Hillenkamp and Karas[10]. Ten to 20 single spectra were accumulated for a better signal-to noise ratio. The sample preparation for MALDI-MS and PSD-MALDI-MS experiments[11] was identical. The concentrations of the analysed substances were 1-10 µmol/l in double distilled water. One microliter of the analyte solution was mixed with 1 µl of matrix solution (50 mg/ml 3-hydroxy-picolinic acid in water). To this mixture cation exchange beads (AG 50 W-X12, 200-400 mesh, Bio-Rad, USA) equilibrated with NH_4^+ as counterion were added to remove Na^+ and K^+ ions. The mixture

was gently dried on an inert metal surface before introduction into the mass spectrometer. The mass accuracy was in the range of approximately 0.01 %.

C.3.4. Results

Figure 1 shows a representative reversed-phase chromatogram of a platelet extract before hemodialysis. The peak of diadenosine pentaphosphate is indicated by an arrow. The identity of the underlying Ap_5A was confirmed by MALDI-MS, ultraviolet spectroscopy, enzymatic cleavage experiments as well as retention time comparisons as described elsewhere[12].

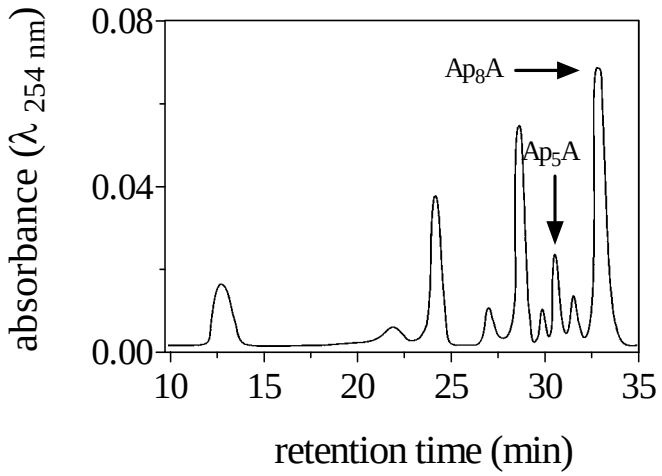

Figure 1: Reversed phase chromatography of a platelet extract from a patient with end-stage renal failure obtained before hemodialysis

The intracellular Ap_5A concentrations were significantly higher in platelets from the 33 patients with end-stage renal failure as compared with the 21 healthy control subjects (136 ± 50 vs 9 ± 6 fg/platelet, mean ± SEM, $p<0.01$).

We analysed the effects of a conventional hemodialysis in 10 patients using AN69 membranes and in 23 patients using low flux polysulfone membranes. The clinical and biochemical characteristics are given in Table 2.

Figure 2 shows the effect of hemodialysis using AN69 (Figure 2.A) and polysulfone membranes (Figure 2.B) on the amounts of Ap_5A in platelets. After a hemodialysis session the Ap_5A concentrations in platelets from patients with end-stage renal failure using an AN69 membranes were significantly decreased (51 ± 18 vs 93 ± 39 fg/platelet, $p<0.05$) (Figure 2.A). There were no significant differences in the Ap_5A concentrations after hemodialysis using low flux polysulfone membranes (Figure 2.B).

Table 2: Clinical and biochemical characteristics of patients with end-stage renal failure hemodialyzed using AN69 and low flux polysulfone membranes

	AN69 (n = 10)	Polysulfone (n = 23)	p value
age, years	56.9 ± 6.5	61.7 ± 2.8.	n.s.
male/female ratio	7 / 3	15 / 8	n.s.
body mass index (kg / m^2)	23.2 ± 1.0	24.8 ± 0.7	n.s.
heart rate (beats/min)	77.5 ± 2.9	73.2 ± 1.7	n.s.
blood pressure (mm Hg)			
systolic	130.6 ± 8.6	153.7 ± 3.6	<0.05
diastolic	79.4 ± 2.4	75.2 ± 1.8	n.s.
serum creatinine (mg/dl)	10.2 ± 0.7	8.7 ± 0.6	n.s.
platelet count (x $10^3/mm^3$)	250.8 ± 19.0	256.9 ± 23.1	n.s.

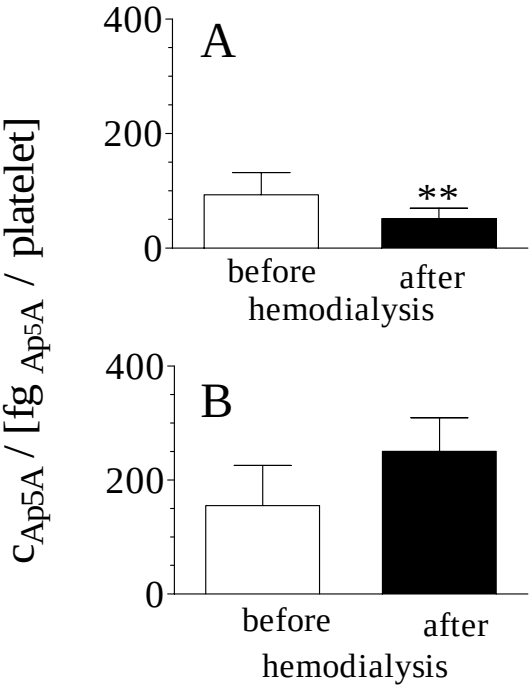

Figure 2: Concentration of Ap_5A in platelets before and after regular hemodialysis using an AN69 membrane (A) and a low flux polysulfone membrane (B). (Data are means ± SEM; **: $p<0.05$ (before vs. after hemodialysis)).

C.3.5. Discussion

The present study confirms that Ap_5A concentrations are significantly higher in platelets from patients with end-stage renal failure as compared with healthy control subjects[12]. How can the increased amount of Ap_nA in platelets from hemodialysis patients be explained? Principally, either an increased production or a decreased breakdown has to be considered. Which mechanism definitely leads to the increased Ap_nA concentration in platelets of chronic renal failure patients cannot be clarified yet. An increase of the synthesis rate is more likely because there is only a difference in the Ap_nA concentration in platelets and not in plasma. Ap_nA are not synthesised but degraded by plasma, but they are synthesised and degraded by platelets. Because the Ap_nA concentration is increased in CRF platelets, but not in CRF plasma, a modification of the synthesis rate is more likely because an effect on the degradation rate would result in a similar effect on the Ap_nA concentration in plasma and platelets.

There is a significant effect of hemodialysis on the difference of the Ap_5A concentration before and after a single hemodialysis session. The elevated Ap_5A amounts in platelets of patients with end-stage renal failure are affected by different membranes in a different way.

Since Ap_5A is related to vasoconstriction[13] and to growth of vascular smooth muscle cells[12] the increased intracellular Ap_5A concentration in platelets from patients with end-stage renal failure may contribute to the increased arteriosclerotic risk in these patients. Several approaches have been proposed to reduce the increased arteriosclerotic risk in patients with end-stage renal failure. One approach has been the use of biocompatible membranes with large pores, such as AN69. The present study indicates that the AN69 membrane has obviously a significant effect on the amount of Ap_5A. In contrast, hemodialysis by using a low flux polysulfone membrane did not significantly affect the intraplatelet amount of Ap_5A.

On the one hand, these different effects may be explained by different charges of the membrane surfaces. In contrast to the AN69 membrane, the low flux polysulfone membrane is not electrostatically charged. These different charge conditions of the hemodialysis membranes may result in different Ap_5A removal rates. First, the hemodialysis membranes may stimulate platelets in a different way, resulting in subsequent differences in generation of Ap_5A. Next, the released Ap_5A may be hemodialysed by the membranes, resulting in a different Ap_5A plasma concentration and different Ap_5A amounts in platelets. Therefore, in future studies not only the Ap_5A amount of platelets of CRF patients but also the Ap_5A plasma concentration should be determined.

On the other hand, the different effects may be explained by different removal rates of yet unknown uremic toxins by the AN69 membrane. These unknown uremic toxins may decrease the activity of Ap_5A degrading enzymes, or may increase the activity of Ap_5A synthesizing enzymes. Both effects would result in an increasing Ap_5A plasma concentration or an increasing Ap_5A amount of platelets, and a decrease upon removal of these toxins by AN69.

In conclusion, the increased atherogenic potential of Ap_5A in chronic renal failure patients can be affected by hemodialysis using different hemodialysis membranes.

C.3.6. References

1. Preston N. Determination of Dinucleoside Polyphosphates, in: McLennan AG: *Ap_4A and other dinucleoside polyphosphates*. Eds: McLennan AG. CRC Press Inc, London, UK, 1992, pp 30-54
2. Flores NA, Stavrou BM, Sheridan DJ: The effects of diadenosine polyphosphates on the cardiovascular system. *Cardiovasc Res* 42:15-26, 1999
3. Schlüter H, Tepel M, Zidek W: Vascular actions of diadenosine phosphates. *J Auton Pharmacol* 16:357-362, 1996

4. Hoyle CHV, Hilderman RH, Pintor JJ, Schlüter H, King BF: Diadenosine polyphosphates as extracellular signal molecules. *Drug Develop Res* 52:260-273, 2001
5. Ogilvie A: Extracellular functions of Ap_n A, in *Ap_4A and other dinucleoside polyphosphates* , edited by McLennan AG, Boca Raton, Florida, CRC Press, Inc., 1992, pp 229-273
6. Jankowski J, Hagemann J, Yoon MS, van der Giet M, Stephan N, Zidek W, Schlüter H, Tepel M: Increased vascular growth in hemodialysis patients induced by platelet-derived diadenosine polyphosphates. *Kidney Int* 59:1134-1141, 2001
7. Ng KE, Orgel LE: The action of water-soluble carbodiimide on adenosine-5'-polyphosphates. *Nucleic Acids Res* 15:3573-3580, 1987
8. Jankowski J, Potthoff W, Zidek W, Schlüter H: Purification of chemically synthesised dinucleoside(5',5') polyphosphates by displacement chromatography. *J Chromatogr B Biomed Sci Appl* 719:63-70, 1998
9. Barnes LD, Robinson AK, Mumford CH, Garrison PN: Assay of diadenosine tetraphosphate hydrolytic enzymes by boronate chromatography. *Anal Biochem* 144:296-304, 1985
10. Hillenkamp F, Karas M: Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. *Methods Enzymol* 193:280-295, 1990
11. Kaufmann R, Kirsch D, Spengler B: Sequencing of peptides in a time-of-flight mass spectrometer: evaluation of postsource decay following matrix-assisted laser desorption ionisation (MALDI). *Int J Mass Spectrom Ion Proc* 131:355-385, 1994
12. Jankowski J, van der Giet M, Jankowski V, Schmidt S, Hemeier M, Mahn B, Giebing G, Tolle M, Luftmann H, Schlüter H, Zidek W, Tepel M: Increased plasma phenylacetic acid in patients with end-stage renal failure inhibits iNOS expression. *J Clin Invest* 112:256-264, 2003
13. Schlüter H, Offers E, Brüggemann G, van der Giet M, Tepel M, Nordhoff E, Karas M, Spieker C, Witzel H, Zidek W: Diadenosine phosphates and the physiological control of blood pressure. *Nature* 367:186-188, 1994

C.4. Increased plasma phenylacetic acid in patients with end-stage renal failure inhibits iNOS expression

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C.4.1. Abstract

Nitric oxide (NO) prevents atherogenesis and inflammation in vessel walls by inhibition of cell proliferation and cytokine-induced endothelial expression of adhesion molecules and pro-inflammatory cytokines. Reduced NO production due to inhibition of either endothelial nitric oxide synthase (eNOS) or inducible nitric oxide synthase (iNOS) may therefore reinforce atherosclerosis. Patients with end-stage renal failure show markedly increased mortality due to atherosclerosis. In the present study we tested the hypothesis that uremic toxins are responsible for reduced iNOS expression. Lipopolysaccharide-induced iNOS expression in mononuclear leukocytes was studied using real-time-PCR. iNOS expression in mononuclear leukocytes was blocked by addition of plasma from patients with end-stage renal failure, whereas plasma from healthy controls had no effect. Hemofiltrate obtained from patients with end-stage renal failure was concentrated, fractionated by preparative reversed-phase chromatography, analytical reversed-phase chromatography in the displacement- and gradient-mode with trifluoroacetic acid as well as triethylammonium acetate as ion-pair reagent. The chromatographic procedures revealed a homogenous fraction with a strong inhibitory effect on the iNOS expression in mononuclear leukocytes. Using gas-chromatography/mass-spectrometry and comparison of the data of the mass-spectrometry with pertinent databases the inhibitor of lipopolysaccharide-induced iNOS expression was identified to be phenylacetic acid. Authentic phenylacetic acid inhibited iNOS expression in a dose-dependent manner. Plasma concentrations of phenylacetic acid were determined by magnetic nuclear resonance. In healthy control subjects plasma concentrations were below the detection level, whereas patients with end-stage renal failure had a phenylacetic acid concentration of 3.49 ± 0.33 mmol L^{-1} (n=41). It is concluded that accumulation of phenylacetic acid, a metabolite of phenylalanine, in patients with end-stage renal failure inhibits iNOS expression. That mechanism may contribute to increased atherosclerosis and cardiovascular morbidity in patients with end-stage renal failure.

J Clin Invest 112 (2): 256-264, 2003

C.4.2. Introduction

Nitric oxide (NO) is an important signalling molecule that mediates a variety of essential physiological processes including neurotransmission, vasodilation, and host cell defence[1]. NO is synthesized from L-arginine by nitric oxide synthase (NOS). Three distinct isoenzymes of NOS are known. Two calcium/calmodulin-dependent constitutive NOS isoenzymes dominantly expressed in the brain and endothelium and a calcium-independent, cytokine-inducible NOS isoenzyme (iNOS) have been identified so far. NO inhibits cell proliferation, cytokine-induced endothelial expression of adhesion molecules and proinflammatory cytokines[2-4].

NO generally is protective against atherogenic stimuli in the vessel wall[5]. Vascular NO can principally be produced by two cell types, namely endothelial cells and vascular smooth muscle cells (VSMC)[6,7]. In normal vessels endothelial NO production is sufficient to meet the requirements of vascular regulation. NO production by VSMC is not activated[6]. On the other hand, under the conditions of endothelial dysfunction, NO production by endothelial cells may not be sufficient to defend the vascular wall against oxidative damage. In this setting, VSMC increase their NO synthesis as a compensatory mechanism[8,9]. Therefore, VSMC-derived NO can be assumed to play a critical role under pathological conditions characterized by endothelial dysfunction. VSMC-derived NO is solely produced by inducible NO synthase (iNOS), which is also expressed in leukocytes, but not in endothelial cells. Excess NO production by iNOS is implicated in the pathogenesis of vascular remodelling and atherosclerosis[10], as it causes inhibition of cell proliferation and apoptosis of VSMC[3,11], mesangial cell proliferation and extracellular matrix synthesis[12]. Obviously, studies on iNOS expression in human VSMC are limited by the difficulty to obtain samples of vascular tissue. Since iNOS is expressed in both VSMC and leukocytes, the latter may be a suitable model to study iNOS expression in human pathology. Therefore in the present study mononuclear leukocytes are used as a model for examining the effects of uremic toxins on iNOS expression.

Atherosclerosis is a major cause of morbidity and mortality in chronic renal failure (CRF)[13,14]. Increased cardiovascular mortality in patients with CRF is in part due to the prevalence of established risk factors for atherosclerosis such as hypertension or an increased serum level of low-density lipoproteins, or lipoprotein (a)[14,15]. Abnormalities of NO synthetic pathway have a key role in the pathogenesis of atherosclerosis in patients with CRF[14,16]. In CRF patients NO bioavailability is reduced[17]. A marked reduction of NO, in the face of continuous local generation of vasoconstrictor and mitogenic substances, contributes to intraglomerular hyperten-

sion[18], cell proliferation and atherosclerosis. In glomeruli soon after surgical ablation of renal mass inflammatory mediators, such as platelet-derived growth factor (PDGF)[19] and transforming growth factor β (TGF-β)[20], are formed in excessive amounts. PDGF and TGF-β are both potent inhibitors of NO synthesis and dose-dependently block IL-1β-induced iNOS mRNA in rat mesangial cells[21]. Reduced NO production may be ameliorated by pharmacological tools like L-arginine[22].

To identify factors decreasing the iNOS expression in CRF we investigated the effects of plasma and hemofiltrate obtained from patients with end-stage renal failure on iNOS expression. Hemofiltrate was fractionated by chromatographic methods, and the effect of the resulting fractions on the iNOS expression was examined. The chromatographic procedures revealed a homogenous fraction, that reduces iNOS expression in mononuclear leukocytes. Using gas-chromatography/mass-spectrometry and comparison of the data of the mass-spectrometry with pertinent databases the inhibitor of lipopolysaccharide-induced iNOS expression was identified to be phenylacetic acid (PAA). Using nuclear magnetic resonance increased plasma concentrations of PAA could be measured in patients with end-stage renal failure.

C.4.3. Methods

PATIENTS

The study was approved by the local ethical committee and informed consent was obtained from the patients. 41 patients with end-stage renal failure who had been undergoing maintenance hemodialysis for 40 ± 5 months (mean ± SEM) were enrolled in this study. Moreover, 39 subjects with normal renal function were used as a control group. The cause of end-stage renal failure was diabetic nephropathy in 16 cases, nephrosclerosis in 9 cases, chronic glomerulonephritis in 4 cases, and unknown in 12 cases. Patients were stable, and free from intercurrent illness. All of the patients were routinely dialyzed for four to five hours thrice weekly using biocompatible polysulfone hemodialysis membranes (F60, Fresenius Medical Care, Bad Homburg, Germany) with no dialyzer reuse. Water and dialysate used in hemodialysis were in accordance with the recommendations of the American Association of Medical Instrumentation. Bacterial growth was less than 50 c.f.u. ml^{-1} in water and less than 200 c.f.u. ml^{-1} in dialysate as described in[23]. We obtained 500 ml hemofiltrate from 5 patients with end-stage renal failure undergoing regular treatment with hemofiltration. Dialysis adequacy was estimated using Kt/V values (the amount of plasma cleared of urea divided by the urea distribu-

tion volume), which were determined using the formula Kt/V= -ln (R-0.03) + (4-3.5 x R) x UF / W; with R=post/pre plasma urea nitrogen ratio; UF=ultrafiltrate volume (liters) removed; W= postdialysis weight (kg)[24,25]. Kt/V-values were 1.2 ± 0.1 (mean ± SEM). The clinical and biochemical characteristics of patients and control subjects are given in Table 1.A. From a subset of patients and controls (Table 1.B) plasmas were used for measurements of iNOS mRNA expression by real-time PCR.

Plasma from patients with end-stage CRF was obtained before the regular hemodialysis session. Blood was taken from the arterial side of the hemodialysis fistula immediately before starting the dialysis session by use of an EDTA vacutainer (K_2-EDTA, 1.8 mg ml^{-1}) and immediately centrifuged at 4,000 g and 20 °C for 5 min. Thereafter plasma was stored at –20 °C.

Table 1.A: Clinical and biochemical characteristics of patients with end stage renal failure and healthy control subjects (Data are mean ± SEM).

	patients with end stage renal failure (n = 41)	healthy control subjects (n = 39)	p value
age (years)	45 ± 2	61 ± 3	n.s.
sex (male/female)	25 / 16	17 / 22	n.s.
body weight (kg)	74 ± 2	66 ± 3	<0.01
duration of dialysis (months)	40 ± 5	0	<0.01
systolic blood pressure (mmHg)	149 ± 4	125 ± 2	<0.01
diastolic blood pressure (mmHg)	81 ± 2	70 ± 2	<0.01
hemoglobin (g dl^{-1})	11.3 ± 0.3	12.9 ± 0.3	<0.01
serum creatinine (mg dl^{-1})	7.2 ± 0.4	1.0 ± 0.1	<0.01
blood urea nitrogen (mg dl^{-1})	27 ± 2	17 ± 1	<0.01
total protein (g L^{-1})	73 ± 2	71 ± 1	n.s.

CHROMATOGRAPHY OF THE HEMOFILTRATE

The sequence of chromatographic separation steps described below was chosen for the following reasons: in the first step, displacement chromatography allows to get rid of a bulk of interfering substances without overloading the column. As further steps different reversed-

phase procedures were chosen, dealing with considerably less material than the first rough separation steps. To obtain optimum effects of a sequence of reversed-phase chromatographies, different ion-pair reagents were applied.

Hemofiltrate (500 ml) was concentrated to dryness in a vacuum concentrator (Freeze Dryer, Snijders, Tilburg, The Netherlands). The dried hemofiltrate was dissolved in 5 ml of 40 mmol L^{-1} triethylammonium acetate (TEAA) in water (eluent A) and chromatographed (flow: 1.0 ml min^{-1}) on a C18 reversed-phase column (Lichroprep, Typ B, Merck, Darmstadt, Germany) in the displacement mode (displacer: 160 mmol L^{-1} n-butanol in eluent A). After each chromatographic step, the influence of the fractions on the iNOS-expression was assayed.

Next, the fractions decreasing the iNOS-expression were fractionated (flow: 0.5 ml min^{-1}) on a C18 reversed-phase HPLC column (Lichrosorb RP C18, 250 x 4.6 mm, Merck, Darmstadt, Germany) with 0.1% trifluoroacetic acid (TFA) in H_2O as eluent A and 0.1% TFA in CH_3CN as eluent B under following gradient condition: 0 to 5 min: 0 to 20% B; 5 to 10 min: 20% B; 10 to 30 min: 20 to 40% B; 30 to 40 min: 40 to 60% B; 40 to 45 min: 60 to 100% B; 45 to 50 min: 100% B.

Subsequently, the fractions decreasing the iNOS-expression were chromatographed (flow: 0.5 ml min^{-1}) on a further reversed-phase column (Supersphere RP C18 endcapped, 250 x 4.6 mm, Merck, Darmstadt, Germany) with the following gradient: 0 to 10 min: 100% A (20 mmol L^{-1} TEAA in water); 10 to 20 min: 0 to 20% B (20 mmol L^{-1} TEAA in CH_3CN); 20 to 40 min: 20 to 40% B; 40 to 50 min: 40 to 60% B; 50 to 55 min: 60 to 100% B; 55 to 60 min: 100% B).

The final purification step was a chromatography (flow: 0.5 ml min^{-1}) on a reversed-phase HPLC column (Supersphere RP C18 endcapped, 250 x 4.6 mm, Merck, Darmstadt, Germany). The column was run in the gradient mode with 0.1% TFA in 40% CH_3CN / H_2O (eluent A) and 0.1% TFA in 99.9% CH_3CN (eluent B) (gradient: 0 to 10 min: 0 to 20% B; 10 to 20 min: 20 to 30% B; 20 to 40 min: 30 to 100% B; 40 to 45 min: 100% B).

MASS-ANALYSIS

The purified fraction was examined with gas-chromatography / mass spectrometry. Therefore the speed-vac-dried sample was dissolved in N-methyl-N-trimethylsilylfluoroacetic acid am-

ide and incubated for one hour at 70ºC. The fractions were separated by gas-chromatography (0 to 35 min: 80 to 280ºC; column: 150 RCN; carrier gas: helium; flow: 2 ml min^{-1}) and identified by mass spectrometry (Finnigan MAT 8200, San Jose, USA).

PREPARATION AND STIMULATION OF MONONUCLEAR LEUKOCYTES

Mononuclear leukocytes were obtained from healthy subjects according to established techniques[26]. Briefly, 20 ml heparinized blood was drawn by venipuncture from the antecubital vein and centrifuged at 240 g for 15 min. After removing the supernatant, mononuclear leukocytes were isolated by layering 5 ml diluted blood (1:1 vol with isotonic NaCl) on 3 ml Histopaque (Sigma-Aldrich, Germany; 5 / 6% wt-%/vol-% Ficoll; density 1.077 g ml^{-1}) and centrifugation at 240 g for 20 min. The mononuclear leukocyte interphase was carefully aspirated, washed three times in isotonic NaCl by centrifugation at 400 g for 5 min, and resuspended in Hanks' balanced salt solution containing (in mmol L^{-1}): NaCl, 136; KCl, 5.4; KH_2PO_4, 0.44; Na_2HPO_4, 0.34; $CaCl_2$, 1; D-glucose, 5.6; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 10; pH 7.4. Cells were counted using a cell counter (CASY 1 Model TT, Schaerfe System, Reutlingen, Germany). Cells were centrifuged at 400 g for 5 min, and resuspended in RPMI 1640 (Sigma-Aldrich, Deisenhofen, Germany) with 10% FCS (Invitrogen, Karlsruhe, Germany) and 25 mmol L^{-1} HEPES. Cells were deposited in 60 mm^2 tissue flasks with a total of 1.8 x 10^7 cells. Cells were incubated for 2 h in a humidified incubator at 37 °C and 5% CO_2. Cells were then stimulated for 6 h in a humidified incubator at 37 °C and 5% CO_2 with 100 U ml^{-1} γ-interferon (γ-IFN) (Sigma-Aldrich, Deisenhofen, Germany) and 1 μg ml^{-1} lipopolysaccharide (LPS Serotype 0111:B4 from *E. coli*, Sigma-Aldrich, Deisenhofen, Germany). Incubation of cells was done in the absence or presence of PAA in various concentrations (0.1 - 2 mmol L^{-1}), in the presence of plasma from patients with end-stage renal failure (n=6) on regular hemodialysis and healthy control subjects (n=6), or in the presence of phenylalanine (1 mmol L^{-1}), homogentisic acid (1 mmol L^{-1}), and phenylethylamine (1 mmol L^{-1}). After incubation cells were harvested and centrifuged (400 g for 5 min). The pellet was immediately frozen in liquid nitrogen and stored at -80 °C for further analysis.

PREPARATION AND STIMULATION OF RAW 264.7 CELLS

The murine macrophage cell line of RAW 264.7 was obtained from European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in Dulbecco's modified eagle medium/F12 (DMEM/F-12) supplemented with 2.44 g L^{-1} $NaHCO_3$, 2 mmol L^{-1} L-glutamine, 1 mmol L^{-1} sodium pyruvate, 10% FCS, and penicillin (100 U ml^{-1}) / streptomycin (100 mg ml^{-1}). Cultures were maintained in a humidified incubator in 5% CO_2 at 37 °C. Cells were plated at a concentration of 1 x 10^5 ml^{-1} and used for the experiment when they reached 80% confluency. Cells were then stimulated for 6 h or 12 h in an humidified incubator at 37 °C and 5% CO_2 with 1 µg ml^{-1} lipopolysaccharide (LPS Serotype 0111:B4 from *E. coli*, Sigma-Aldrich, Deisenhofen, Germany). Incubation and further processing of the cells were done as described above for mononuclear leukocytes. In the experiments, cells were serum-starved by incubation in a serum-reduced medium Dulbecco's modified eagle medium/F12 (DMEM/F-12) supplemented with 2.44 g L^{-1} $NaHCO_3$, 2 mmol L^{-1} L-glutamine, 1 mmol L^{-1} sodium pyruvate, 1.0% FCS, penicillin (100 U ml^{-1}), streptomycin (100 mg ml^{-1}) for 24 h before use.

PREPARATION AND STIMULATION OF ENDOTHELIAL CELLS

To test the influence of PAA on eNOS, human umbilical vein endothelial cells (ECV304) were used. ECV304 is a spontaneously transformed, immortal endothelial cell line established from the vein of an apparently normal human umbilical cord. Cells were provided by the European Collection of Animal Cell Cultures (Salisbury, UK). Cells were grown in medium M199 supplemented with 10% FCS, 100 U ml^{-1} penicillin, and 100 µg ml^{-1} streptomycin. Cells were maintained in a standard culture incubator with humidified air containing 5% CO_2 at 37 °C. In the experiments, cells were serum-starved by incubation in a serum-free medium M199 containing antibiotics for 48 h before use.

PREPARATION OF RNA AND REALTIME PCR

Primers and probes for murine and human iNOS and for the housekeeping gene β-actin were designed using the computer program Primer Express 2.0 (Perkin Elmer / Applied Biosystems, Foster City, CA, USA) (Table 2). Except for β-actin, primers were located in two different exons.

Table 2: Primer and probe sequences for murine and human iNOS and for ß-actin (146 bp) (TP: fluorogenic probe, FAM-labelled at the 5'end and TAMRA-labelled at the 3'end; RP: reverse primer; FP: forward primer; all cDNA sequences were obtained from the genbank database; iNOS, inducible nitric oxide synthase).

	Name	Sequence (5'-3')	Amplicon length (bp)
murine iNOS	miNOS-TP miNOS-RP miNOS-FP	CGG GCA GCC TGT GAG ACC TTT GA CAT TGG AAG TGA AGC GTT TCG CAG CTG GGC TGT ACA AAC CTT	95
murine β-actin	Mβact-TP Mβact-RP Mβact-FP	CAC TGC CGC ATC CTC TTC CTC CC CAA TAG TGA TGA CCT GGC CGT AGA GGG AAA TCG TGC GTG AC	148
human iNOS	hiNOS-TP hiNOS-RP hiNOS-FP	TCC GAC ATC CAG CCG TGC CA CAG GAG AGT TCC ACC AGG ATG TCA AAT CTC GGC AGA ATC TAC AAA	66
human β-actin	Hβact-TP Hβact-RP Hβact-FP	TCA AGT ATC ATT GCT CCT CCT GAG CGC GCC GAT CCA CAC GGA GTA CT CTG GCA CCC AGC ACA ATG	65

Total RNA from murine macrophage cell line (RAW 264.7) or mononuclear leukocytes was extracted using Qiagen RNeasy-Mini-Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Target RNA (1 - 2 µg) was reverse transcribed using 100 U SuperscriptII RT (Invitrogen, Karlsruhe, Germany) at 42 °C for 80 min in the presence of 50 mmol L^{-1} Tris-HCl (pH 8.3), 5.7 mmol L^{-1} KCl, 3 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} DTT, 0.5 mmol dNTPs, 8 U RNasin (Promega Corp., Madison, WI, USA) and 5 µmol L^{-1} Olgio$(dT)_{16}$ (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA). For every reaction set, one RNA sample was performed without SuperscriptII RT (RT^- reaction) to provide a negative control in subsequent PCR reaction.

Real-time PCR was done by using the ABI prism 7700 sequence detector (TaqMan; Perkin Elmer/ Applied Biosystems, Foster City, CA, USA). The method and the quantification procedure is extensively described elsewhere[27]. Commercial reagents (TaqMan PCR Reagent Kit, Perkin Elmer, Foster City, CA, USA) and conditions according to the manufacturer's protocol were applied (2.5 µl of cDNA and oligonucleotides at a final concentration of 200 nmol L^{-1}). Each reaction also contained 100 nmol L^{-1} of the corresponding detection probe (Table 2).

Each PCR amplification was performed in triplicate wells, using the following conditions:

2 min at 50 °C and 10 min at 94 °C, followed by a total of 40 or 45 two-temperature cycles (15 s at 94 °C and 1 min at 60 °C).

PREPARATION OF PROTEINS FROM RAW 264.7 CELLS, POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING

Harvested RAW 264.7 cells after stimulation were lysed in lysis buffer containing 50 mmol L^{-1} Tris-Cl (pH 7.4), 150 mmol L^{-1} NaCl, 100 μg ml^{-1} phenylmethylsulfonyl fluoride (PMSF), 1% Nonident P-40 and 4% protease inhibitor cocktails. Protein concentrations in the cell lysates were stored at -70 °C until further measurements.

Aliquots were subjected to SDS-PAGE on 7.5% polyacrylamide slabe gels and blotted onto polyvinylidene difluoride membranes. Polyvinylidene difluoride blots were blocked for 1 h in Tris-buffered saline (TBS, 150 mmol L^{-1}, NaCl, 20 mmol L^{-1}, Tris, pH 7.5) containing 5% non-fat milk, and incubated overnight at 4 °C with antibody against iNOS (1:10 000 dilution, Santa Cruz biotechnology, Heidelberg, Germany) in TBS containing 5% non-fat milk. Membranes were washed in TBS and incubated with goat anti-rabbit alkaline phosphatase-conjugated antibody (1:3 000 dilution) for 1.5 h. After further washing with TBS, blots were detected by the enhanced chemiluminescene method using an immunoblot assay kit (BioRad, München, Germany).

MEASUREMENT OF NO PRODUCTION

NO production was assayed by measuring the accumulation of nitrite in the culture medium by the Griess reaction using sodium nitrite as a standard. Aliquots of culture medium were mixed with an equal volume of Griess reagent (1% sulphanilamide / 0.1% N-(1-napthyl) ethylenediamine dihydrochloride in 5% phosphoric acid), the mixture incubated at room temperature for 10 min and the absorbance at 540 nm was measured using a photometer (iEMS Reader, Labsystems, Helsinki, Finland). Standard curves were constructed using known concentrations of sodium nitrite. Net NO production after stimulation with LPS was calculated by subtracting basal NO production from total production, and expressed as the percentage release of nitric oxide relative to control. NO production in ECV304 cells was measured after stimulation with acetylcholine (1 μmol L^{-1}).

CELL VIABILITY

The cell viability was assessed using Trypan blue dye. Trypan blue dye solution (0.4%) was

added to the cell suspension at a ratio of 1:2. After mixing, the cells were observed under the microscope. Cells not stained with the dye were regarded as living, and cell viability was expressed as the percentage of living to total cells.

NMR SPECTROSCOPY

In order to quantitate PAA concentrations, proton-NMR (^{1}H-NMR) measurements were carried out using a Bruker AMX 400 FT spectrometer (Bruker Analytische Messtechnik, Bremen, Germany) operating at a field strength of 400 MHz. To samples (500 µl) 50 µl D_2O and 50 µl of a solution of 0.75% 3-trimethylsilyl-[2,2,3,3,-$^2H_4$]-propionate in D_2O was added and mixed thoroughly by vortex for 3 min at room temperature. The sample was poured into a glass NMR test tube with an inner diameter of 5 mm. All spectra were recorded at a controlled probe temperature of 25 °C. The signal from deuterium oxide was used to optimise the homogeneity of the magnetic field, and the fine adjustment was done by inspection of the free induction decay obtained without water suppression. A field-frequency was provided by detecting the deuterium signal of deuterium oxide. The proton spectra were obtained by using a flip angle of 90 degrees (6.2 µs). Pulse conditions were 128 accumulations collected into 32 K computer points using 90 degree pulses and a relaxation delay of 6 s. The spectral width comprised 5 kHz. Using these conditions spectra were fully T_1-relaxed and, for sharp resonances, T_2 relaxation during the period 3t was not observed[28]. The water resonance (4.8 ppm) was suppressed by presaturation during relaxation- and mixing-time with a 90° sequence (d_1-90°-d_0-90°-d_9-90°-free induction decay)[29]. The spinning rate was 20 rotations per second. The accumulation time was 10.4 min. Shifts were referenced to 3-trimethylsilyl-[2,2,3,3,-$^2H_4$]-propionate (TSP) at 0 ppm. The relative intensities of the resonances of phenylacetic acid were determined by comparison with the intensity of the resonance of TSP. Resonance intensity can be used to determine concentration provided that the linewidths are comparable[30]. Using the internal reference, concentrations were calculated from the resonance intensity of identified substance. Calibration curves with identified substances revealed a linear relationship between concentration and resonance intensity. Therefore, the resonance intensity of the respective substances was used for quantification[30]. To quantify protein binding of PAA, native plasma samples were divided into two portions, one of which underwent NMR spectoscopy immediately, and the other that was deproteinized with 0.6 mol L^{-1} perchloric acid and centrifuged at 4,000 rpm and 4 °C for 5 min. The supernatant was neutralized by KOH. After deproteination this sample was analysed by NMR spectroscopy, too.

STATISTICS

All data are presented as mean ± SEM. Statistical analyses were done with GraphPad Prism version 3.0 (San Diego, USA). Comparisons between the groups were made using the non-parametric Wilcoxon rank sum test and non parametric Wilcoxon matched pairs test, as appropriate. A two-tailed $p < 0.05$ was considered significant.

C.4.4. Results

As shown in Figure 1.A, the stimulation of mononuclear leukocytes from healthy subjects with 1 μg ml^{-1} LPS and 100 U ml^{-1} γ-interferon for 6 h caused a significant increase in iNOS expression. In the presence of plasma from patients with end-stage renal failure on regular hemodialysis the LPS- and γ-IFN-induced iNOS mRNA expression was dose-dependently blocked, whereas plasma from healthy persons did not reduce LPS- and γ-IFN-induced iNOS-expression.

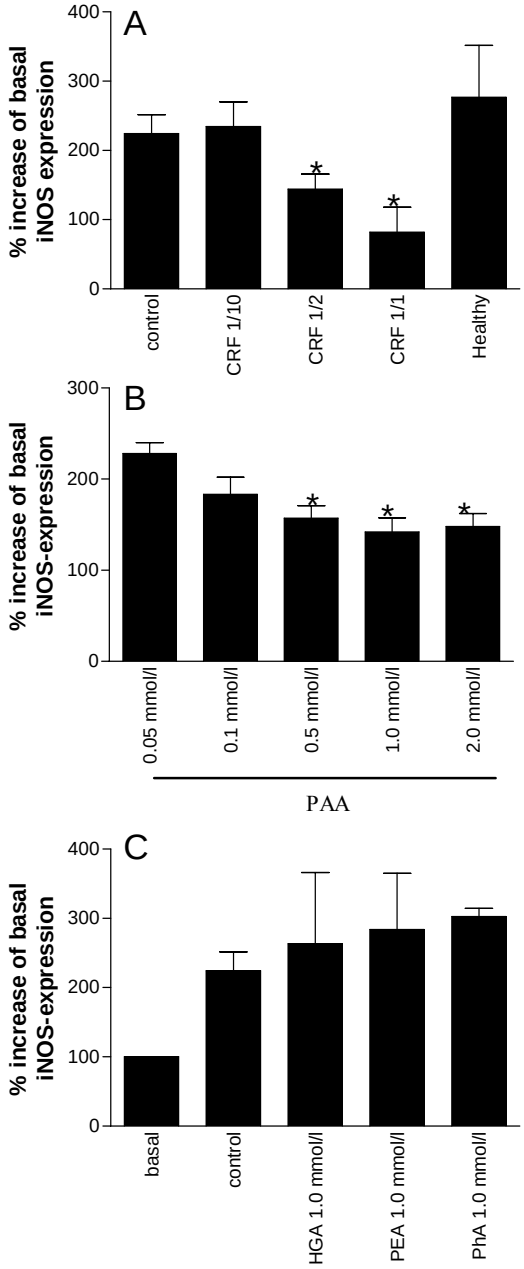

Figure 1: Effects of plasma from patients with end-stage renal failure and healthy control subjects, of phenylacetic acid and some of its derivatives on iNOS mRNA expression measured by real-time PCR without (basal, set to 100%) and after stimulation by LPS (1 μg ml^{-1}) and γ-IFN (100 U ml^{-1}) (control) in mononuclear leukocytes.

(A) iNOS-expression in the presence of various amounts of plasma diluted in PBS (1/10, 1/2, or 1/1) of patients with CRF on regular hemodialysis (end-stage renal failure) or in the presence of plasma from healthy persons (healthy). Basal, unstimulated iNOS expression was set to 100%; each n=6.

(B) iNOS expression in the presence of various concentrations of phenylacetic acid (PAA; 0.05 mmol L^{-1}, 0.1 mmol L^{-1}, 0.5 mmol L^{-1}, 1.0 mmol L^{-1}, 2.0 mmol L^{-1}). Control value as in A. Basal, unstimulated iNOS expression was set to 100%.

(C) iNOS expression in the presence of homogentisic acid (HGA 1.0 mmol L^{-1}), phenylethyl amine (PEA 1.0 mmol L^{-1}), or phenylalanine (PhA 1.0 mmol L^{-1}); n=6; *$p<0.05$ significant difference from control.

For the fractionation of the hemofiltrate from patients with end-stage renal failure a preparative reversed-phase column was used. Because of the great amount of substances in the hemofiltrate, the chromatography was performed in the displacement modus. Moreover for this separation step an anionic ion pair reagent was used. By this procedure the large amount of hemofiltrate was separable by one chromatographic step. One fraction of the displacement-chromatography eluting at 630.3 min had a strong decreasing effect on the LPS-induced expression of iNOS mRNA in mononuclear leukocytes.

This fraction was next separated by reversed-phase chromatography with 0.1% TFA in water as an anionic ion pair reagent. For this separation step an analytical reversed-phase column was used in the gradient modus. The analytical reversed-phase column in the gradient modus allows to effectively separate the hemofiltrate substances while being applicable at this stage of separation without overloading the column, since the bulk of interfering substances has been removed in the preceding steps.

One fraction with a strong decreasing effect on the iNOS-expression was determined at a retention time of 55.9 min. Next, reversed-phase chromatography of this fraction was performed with 40 mmol L^{-1} triethylammonium acetate (TEAA) in water as a cationic ion pair reagent. For this fraction step another analytic reversed-phase column with alternative reversed-phase gel was used in the gradient modus. For further variation of the separation conditions instead of TFA as ion pair reagent again TEAA was used as ion-pair reagent.

One fraction with a strong decreasing effect on the iNOS expression was next separated by the identical reversed-phase gel but in presence of TFA as anionic ion-pair reagent. The separation of the fraction eluting at 33.1 min by reversed-phase chromatography with 0.1% TFA in water and ACN as eluent revealed one obviously homogeneous fraction decreasing iNOS expression and eluting at 44.6 min.

The underlying substance of this chromatographic fraction had a blocking effect on the LPS plus γ-IFN-induced expression of iNOS mRNA in mononuclear leukocytes. This substance was analyzed by mass-spectrometry. The MS data are given in Table 3. By interpretation of the mass-spectrum and also by comparison with a pertinent database (NIST Mass Spectral Library/Standard Reference Database, National Institute of Standards and Technology, Gaithersburg, MD, USA) the underlying substance was identified as PAA. The mass spec-

trum of PAA was clearly distinct from that of other derivatives of phenylalanine, such as homogentisic acid, phenylethylamine, and phenylalanine. After PAA had been isolated and identified as an endogenous iNOS inhibitor, the effects of authentic PAA were assessed in detail. First, the effects of PAA on iNOS mRNA expression were examined. Authentic PAA inhibited LPS-induced plus γ-IFN-iNOS expression in a dose-dependent manner (Figure 1.B).

Table 3: Data of the mass spectrum of the GC-MS-analysis of the substance showing an inhibitory effect on the iNOS expression, identified as phenylacetic acid by interpretation of the mass-spectrum. The fragment masses are identical with those found in a pertinent database (NIST Mass Spectral Library/Standard Reference Database, National Institute of Standards and Technology, Gaithersburg, MD, USA).

fragment mass (Da)	relative intensity (arbitrary units)
45	8.2
47	6.0
65	10.0
73	100.0
74	12.0
75	46.8
76	3.4
77	4.6
89	5.0
90	6.2
91	15.0
117	2.6
118	1.2
121	1.0
137	6.0
164	18.0
165	8.0
193	16.0
194	3.0
195	2.0

To test whether this PAA effect was specific for PAA and did not reflect unspecific toxicity, several phenylalanine derivatives were tested for their effects on iNOS expression. In the presence of phenylalanine (1 mmol L^{-1}), homogentisic acid (1 mmol L^{-1}), and phenylethylamine (1 mmol L^{-1}) the LPS- and γ-IFN-induced iNOS-expression was not significantly affected (Figure 1.C). Moreover, viability of RAW 264.7 cells was not affected by PAA (Figure 2).

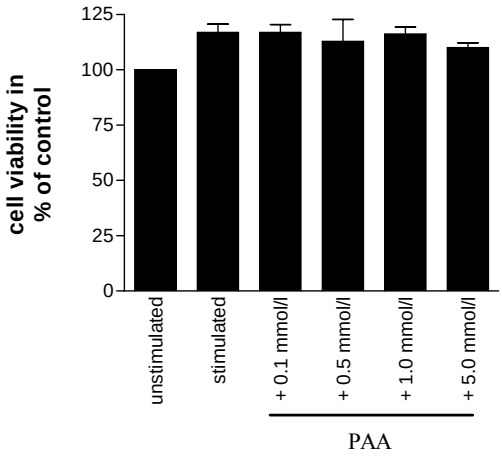

Figure 2: Cell viability of RAW 264.7 cells in the presence of phenylacetic acid (PAA). Cell viability was assessed using Trypan blue dye. RAW 264.7 cells were stimulated (stimulated) or not (unstimulated) for 12 h with LPS (1 μg ml^{-1}). Viability of cells was measured after stimulation with LPS and in the presence of various concentrations of PAA (0.1 mmol L^{-1}, 0.5 mmol L^{-1}, 1.0 mmol L^{-1}, and 5.0 mmol L^{-1}). Data are mean ± SEM, n=6, *$p<0.05$ compared with stimulted.

From the above findings the question arose, whether PAA itself was the effective agent or metabolites of PAA affected iNOS mRNA expression. The NMR spectra obtained from PAA after incubation for 0 and 60 min with mononuclear leukocytes were essentially unchanged. The PAA signal at 60 min was 98 ± 6% of the 0 min signal, indicating that PAA was not metabolized during this time period in significant amounts. Furthermore, the PAA effects on iNOS mRNA expression were also tested in RAW 264.7 cells. Real-time PCR revealed a significant expression of iNOS mRNA in these cells after 6 h stimulation with 1 μg ml^{-1} LPS. Furthermore, in these cells PAA showed a similar concentration-dependent effect on iNOS mRNA expression as in mononuclear leukocytes (Figure 3.A).

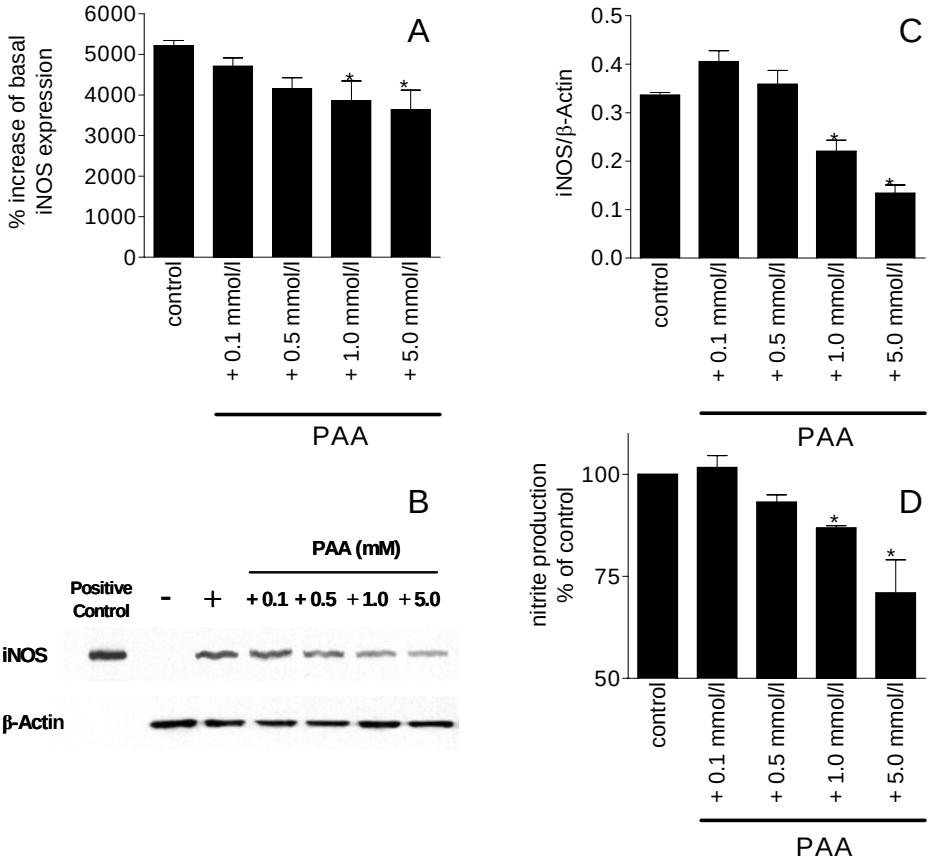

Figure 3: iNOS-expression measured by real-time PCR, protein-blotting of iNOS or nitrite formation in RAW 264.7 cells.

(A) iNOS-expression measured by real-time PCR after stimulation by LPS (1 µg ml^{-1}) (control) and in the presence of various concentrations of phenylacetic acid (PAA; 0.1 mmol L^{-1}, 0.5 mmol L^{-1}, 1.0 mmol L^{-1}, and 5.0 mmol L^{-1}). Basal, unstimulated iNOS expression was set to 100%; n=6; * $p<0.05$ significant difference from control.

(B) Representative protein-blotting of iNOS and ß-actin after 12 h stimulation (+) or without stimulation (-) of RAW 264.7 cells with LPS (1 µg/ml) and in the presence of PAA (0.1 mmol L^{-1}, 0.5 mmol L^{-1}, 1.0 mmol L^{-1}, and 5.0 mmol L^{-1}). iNOS protein was detected as a band with a molecular mass of ~125 kDa.

(C) Signals of iNOS were quantified and normalized to those of ß-actin using a bioimaging analyzer. Data represent means of triplicate determinations from each of three protein preparations. n=3; *$p<0.05$ significant difference from control.

(D) Effect of various concentrations of PAA (0.1 mmol L^{-1}, 0.5 mmol L^{-1}, 1.0 mmol L^{-1}, and 5.0 mmol L^{-1}) on LPS-induced nitrite production in RAW 264.7 cells. Data are means ± S.E.M (n=6). *$p<0.05$ compared with control (+).

To confirm the functional relevance of PAA effects on iNOS mRNA expression, iNOS protein as well as LPS-induced nitrite production were determined in RAW 264.7 cells. In the presence of PAA in concentrations > 1 mmol L^{-1} the amount of iNOS protein was significantly ($p<0.05$) reduced (Figure 3.B, C). Likewise, nitrite production by iNOS induced by 1 µg ml^{-1} LPS was significantly reduced by PAA (Figure 3.D). On the other hand, the acetyl-

choline-induced nitrite production by eNOS of endothelial ECV304 cells was not significantly affected by the administration of PAA (Figure 4).

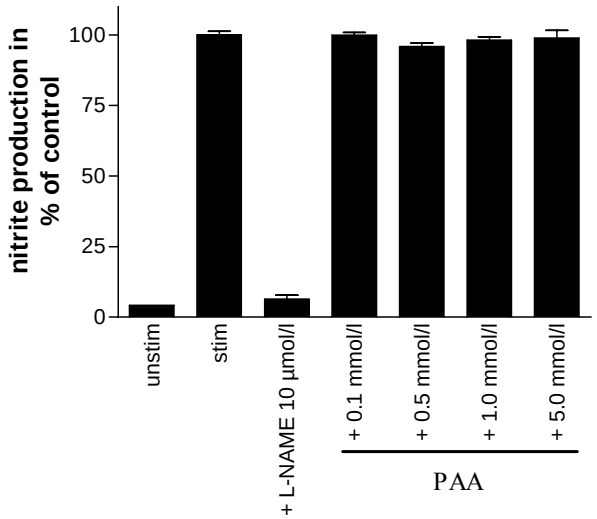

Figure 4: Nitrite formation in ECV304 cells. Nitrite accumulation was measured 30 min with (stim) or without (unstim) stimulation of ECV304 with acetylcholine (1 µmol L^{-1}). ECV304 were stimulated with acetylcholine in the absence (stim) or presence of phenylacetic acid (PAA, 0.1 mmol L^{-1}, 0.5 mmol L^{-1}, 1.0 mmol L^{-1}, and 5.0 mmol L^{-1}) and L-NAME (10 µmol L^{-1}). Data are mean ± SEM, n=6, *p<0.05 compared with stimulation.

In plasma of healthy subjects (n=39) PAA was not detectable. Experiments with authentic PAA revealed a detection limit by NMR analysis in the range of 5-10 µmol L^{-1}. On the other hand, in patients with end-stage renal failure, plasma concentrations of PAA were 3.49 ± 0.33 mmol L^{-1} (n=41, p<0.01 vs. control). These concentrations were obtained in plasma from patients with end-stage renal failure before a regular hemodialysis session. The plasma PAA concentrations of the patients with end-stage renal failure used for the experiments of Figure 1.A were 2.5 ± 0.5 mmol L^{-1} (n=6). Next we quantified the portion of protein-bound PAA in blood plasma from end-stage renal failure patients. After deproteinization, 69.8 ± 22.5% of PAA detected in untreated samples was found (n=8). Accordingly, in unprocessed ultrafiltrate from end-stage renal failure patients 52.7 ± 12.6% of the plasma PAA concentration was measured by NMR (n=10).

C.4.5. Discussion

Several reports indicate that in CRF NO production is reduced [31,32]. Given the important function of NO in maintaining vascular function and integrity, the deficient NO production in renal failure may be one key step leading to the functional and structural vascular changes in renal failure. The present study focused on the PAA effects on iNOS expression, since it is known that increased iNOS expression is a compensatory mechanism when endothelial NO production is inadequate[33].

The present study may offer one explanation of the decreased NO bioavailability in renal failure patients. Plasma from patients with CRF inhibited the expression of iNOS whereas plasma from healthy subjects had no effect. eNOS activity was unaffected by PAA. Using hemofiltrate we isolated and characterized the iNOS inhibitor in patients with end-stage renal failure to be phenylacetic acid. PAA is a degradation product of the amino acid, phenylalanine. Phenylalanine is primarily metabolized by phenylalanine hydroxylase to tyrosine. In addition, phenylalanine is decarboxylated to phenylethylamine, 90% of which is oxidized to phenylacetic acid, and the remainder to mandelic acid[34]. Using nuclear magnetic resonance we measured plasma concentrations of phenylacetic acid in patients with end-stage renal failure and healthy control subjects. As suggested by the findings obtained with hemofiltrate, plasma phenylacetic acid concentrations in renal failure patients far exceeded those in healthy in controls.

The principal finding of the present study, that PAA accumulates in patients with end-stage renal failure and inhibits iNOS expression and, consequently, NO production, may help to understand the vascular and hemodynamic changes in end-stage renal failure for several reasons:

Firstly, reduced iNOS expression may contribute to accelerated atherosclerosis[13,35] and hence to increased cardiovascular morbidity in these patients. Nitric oxide (NO) metabolism has been implicated in the pathogenesis of arteriosclerosis[36]. NO helps to maintain the integrity of the vascular endothelium by inhibiting the expression of adhesion molecules. NO inhibits ADP-induced human platelet aggregation, mediated by a cGMP-dependent mechanism[37]. Moreover, NO attenuates leukocyte adhesion and chemotaxis, important steps in atherogenesis. An enhanced leukocyte adhesion to endothelium has been observed during infusion of NOS inhibitors in cats. This effect was reversed by infusion of L-arginine and an antibody to

the adhesion complex CD11b/CD18 on the leukocyte[38]. Monocyte chemotaxis is inhibited by NO via a cGMP-dependent pathway[39]. These observations, along with others, strongly suggest that NO blocks inflammatory cell adhesion and migration into the subintimal space, thereby limiting the deleterious effects of the inflammatory cascade and the subsequent development of arteriosclerosis.

Secondly, there is increasing evidence that decreased renal NO production plays a key role in causing and/or mediating the complex changes of renal hemodynamics associated with the progression of CRF[16,40]. It has been proposed that NO production by iNOS in medullary thick ascending limbs serves to maintain and regulate medullary blood flow and oxygenation[41]. There is also evidence that NO induces natriuresis by antagonizing the effect of angiotensin II on sodium reabsorption in proximal tubuli[41] and by directly inhibiting renal tubular Na^+/K^+ ATPase[42].

On the other hand, the role of iNOS in modulating the development of atherosclerosis is less clear than should be expected from those beneficial effects of NO on vasculature. NO has both antioxidant and oxidant effects. The latter mainly depend on further reactions of NO with reactive oxygen species, producing a variety of reactive nitrogen species, which are powerful oxidants: NO and O_2^- form peroxynitrite ($ONOO^-$), and myeloperoxidase can produce NO_x from NO^{2-}, which is a reaction product of NO, and H_2O_2 [43]. Both NO_x and $ONOO^-$ are known to induce protein nitration, an important mechanism of oxidative injury[44,45]. Possibly as a consequence of reduced iNOS-induced oxidative stress, in apoE/iNOS double-knockout mice, a combined genetic model of iNOS deficiency and atherosclerosis, iNOS deficiency was protective against atherosclerosis[46].

Increased NO production may thus divergently affect the progression of atherosclerosis. Moreover, the present findings may have implications on iNOS expression in at least two cell types: (1) iNOS is expressed in both VSMC and mononuclear leukocytes. The effects of PAA on iNOS expression in mononuclear leukocytes may therefore be extrapolated to iNOS expression in VSMC. Indeed, in several experimental models iNOS expresion in mononuclear cells and VSMC was found to be inducible by the same experimental procedures[47-52]. (2) Macrophages and mononuclear leukocytes are known to play an important role in the development of atherosclerosis[53]. Decreased iNOS expression in these cells has consistently been observed and, as detailed above, may be of pathogenetic relevance[54-56]. Therefore, beyond the

extrapolation of the leukocyte findings to VSMC, the present data may also be relevant to iNOS expression in mononuclear cells infiltrating the vessel wall.

The experiments demonstrating an inhibitory effect of PAA on iNOS expression may be relevant to the clinical setting of end-stage renal failure: plasma PAA levels in end-stage renal failure patients as determined by NMR are similar to those completely inhibiting iNOS expression in isolated mononuclear leukocytes. This remains also true, when the percentage of protein-bound PAA is considered. Both in ultrafiltrate and in supernatant obtained after deproteinization, about 60% of total PAA were recovered, with the percentage of free PAA found in perchloric acid-treated samples being slightly higher, probably resulting from additonal PAA release from precipitated proteins. The mechanism whereby PAA inhibits iNOS expression is still open. Apparently, PAA is taken up by mononuclear leukocytes in sufficient amounts to be effective intracellularly.

In conclusion, we demonstrated that PAA is a potent inhibitor of iNOS-expression accumulating in patients with end-stage renal failure. This mechanism may contribute to increased atherosclerosis and cardiovascular morbidity in CRF patients.

C.4.6. References

1. Moncada S, Higgs EA. Molecular mechanisms and therapeutic strategies related to nitric oxide. *Faseb J* 9(13):1319-1330, 1995
2. Katsuyama K, Shichiri M, Marumo F, Hirata Y. NO inhibits cytokine-induced iNOS expression and NF-κB activation by interfering with phosphorylation and degradation of IkappaB-alpha. *Arterioscler Thromb Vasc Biol* 18(11):1796-1802, 1998
3. Cornwell TL, Arnold E, Boerth NJ, Lincoln TM. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol* 267:C1405-1413, 1994
4. De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA, Jr., Shin WS, Liao JK. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* 96(1): 60-68, 1995
5. Gewaltig MT, Kojda G. Vasoprotection by nitric oxide: mechanisms and therapeutic potential. *Cardiovasc Res* 55(2): 250-260, 2002
6. Andrew PJ, Mayer B. Enzymatic function of nitric oxide synthases. *Cardiovasc Res* 43(3): 521-531, 1999
7. Beasley D, Schwartz JH, Brenner BM. Interleukin-1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J Clin Invest* 87(2):602-608, 1991
8. Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG, Marsden PA. Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol* 17(11): 2479-2488, 1997

9. Yan ZQ, Yokota T, Zhang W, Hansson GK. Expression of inducible nitric oxide synthase inhibits platelet adhesion and restores blood flow in the injured artery. *Circ Res* 79(1):38-44, 1996
10. Collins T. Endothelial nuclear factor-kappa B and the initiation of the atherosclerotic lesion. *Lab Invest* 68(5):499-508, 1993
11. Fukuo K, Hata S, Suhara T, Nakahashi T, Shinto Y, Tsujimoto Y, Morimoto S, Ogihara T. Nitric oxide induces upregulation of Fas and apoptosis in vascular smooth muscle. *Hypertension* 27:823-826, 1996
12. Garg UC, Hassid A. Inhibition of rat mesangial cell mitogenesis by nitric oxide-generating vasodilators. *Am J Physiol* 257:F60-66, 1989
13. Raine AE, Margreiter R, Brunner FP, Ehrich JH, Geerlings W, Landais P, Loirat C, Mallick NP, Selwood NH, Tufveson G and others. Report on management of renal failure in Europe, XXII, 1991. *Nephrol Dial Transplant* 7 (Suppl 2):7-35, 1992
14. Moeslinger T, Spieckermann PG. Urea-induced inducible nitric oxide synthase inhibition and macrophage proliferation. *Kidney Int* 59 Suppl 78:S2-8, 2001
15. Gupta A, Robinson K. Hyperhomocysteinaemia and end stage renal disease. *J Nephrol* 10(2):77-84, 1997
16. Aiello S, Noris M, Remuzzi G. Nitric oxide/L-arginine in uremia. *Miner Electrolyte Metab* 25(4-6):384-390, 1999
17. Passauer J, Bussemaker E, Range U, Plug M, Gross P. Evidence in vivo showing increase of baseline nitric oxide generation and impairment of endothelium-dependent vasodilation in normotensive patients on chronic hemodialysis. *J Am Soc Nephrol* 11(9):1726-1734, 2000
18. Aiello S, Remuzzi G, Noris M. Nitric oxide/endothelin balance after nephron reduction. *Kidney Int Suppl* 65:S63-67, 1998
19. Floege J, Burns MW, Alpers CE, Yoshimura A, Pritzl P, Gordon K, Seifert RA, Bowen-Pope DF, Couser WG, Johnson RJ. Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. *Kidney Int* 41(2):297-309, 1992
20. Lee LK, Meyer TW, Pollock AS, Lovett DH. Endothelial cell injury initiates glomerular sclerosis in the rat remnant kidney. *J Clin Invest* 96(2): 953-964, 1995
21. Ketteler M, Border WA, Noble NA. Cytokines and L-arginine in renal injury and repair. *Am J Physiol* 267:F197-207, 1994
22. Wheeler MA, Smith SD, Saito N, Foster HE, Jr., Weiss RM. Effect of long-term oral L-arginine on the nitric oxide synthase pathway in the urine from patients with interstitial cystitis. *J Urol* 158(6):2045-2050, 1997
23. Lonnemann G. Assessment of the quality of dialysate. *Nephrol Dial Transplant* 13(Suppl 5):17-20, 1998
24. Daugirdas JT. Simplified equations for monitoring Kt/V, PCRn, eKt/V, and ePCRn. *Adv Ren Replace Ther* 2(4):295-304, 1995
25. Daugirdas JT. Rapid methods of estimating Kt/V: three formulas compared. *ASAIO Trans* 36(3):M362-364, 1990
26. Tepel M, Schlotmann R, Barenbrock M, Kisters K, Klaus T, Spieker C, Walter M, Meyer C, Bretzel RG, Zidek W. Lymphocytic Na^+-H^+ exchange increases after an oral glucose challenge. *Circ Res* 77(5):1024-1029, 1995
27. Overbergh L, Valckx D, Waer M, Mathieu C. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine* 11(4):305-312, 1999
28. Bell JD, Brown JCC, Sadler PJ. High resolution proton magnetic resonance studies of human cerebrospinal fluid. *Clin Sci* 72:563-570, 1987

29. Bodenhausen G, Kogler H, Ernst RR. Selection of coherence-transfer pathway in NMR pulse experiments. *J Magn Res* 58:370-378, 1984
30. Bell JD, Lee JA, Lee HJ, Sadler PJ, Wilkie DR, Woodham RH. Nuclear magnetic resonance studies of blood plasma and urine from subjects with chronic renal failure. *Biochim Biophys Acta* 1096:101-107, 1991
31. MacAllister RJ, Whitley GS, Vallance P. Effects of guanidino and uremic compounds on nitric oxide pathways. *Kidney Int* 45(3):737-742, 1994
32. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* 339(8793):572-575, 1992
33. Hecker M, Cattaruzza M, Wagner AH. Regulation of inducible nitric oxide synthase gene expression in vascular smooth muscle cells. *Gen Pharmacol* 32(1):9-16, 1999
34. Oberdoerster J, Guizzetti M, Costa LG. Effect of phenylalanine and its metabolites on the proliferation and viability of neuronal and astroglial cells: possible relevance in maternal phenylketonuria. *J Pharmacol Exp Ther* 295(1):295-301, 2000
35. London GM, Drueke TB. Atherosclerosis and arteriosclerosis in chronic renal failure. *Kidney Int* 51(6):1678-1695, 1997
36. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. *Cell* 78(6):915-918 1994
37. Mellion BT, Ignarro LJ, Ohlstein EH, Pontecorvo EG, Hyman AL, Kadowitz PJ. Evidence for the inhibitory role of guanosine 3', 5'-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood* 57(5):946-955, 1981
38. Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* 88(11):4651-4655, 1991
39. Bath PM. The effect of nitric oxide-donating vasodilators on monocyte chemotaxis and intracellular cGMP concentrations in vitro. *Eur J Clin Pharmacol* 45(1):53-58, 1993
40. Dumont Y, D'Amours M, Lebel M, Lariviere R. Supplementation with a low dose of L-arginine reduces blood pressure and endothelin-1 production in hypertensive uraemic rats. *Nephrol Dial Transplant* 16(4):746-754, 2001
41. Ito S. Nitric oxide in the kidney. Curr Opin Nephrol *Hypertens*;4(1):23-30 1995
42. Morrissey JJ, McCracken R, Kaneto H, Vehaskari M, Montani D, Klahr S. Location of an inducible nitric oxide synthase mRNA in the normal kidney. *Kidney Int* 45(4):998-1005, 1994
43. Hazen SL, Zhang R, Shen Z, Wu W, Podrez EA, MacPherson JC, Schmitt D, Mitra SN, Mukhopadhyay C, Chen Y and others. Formation of nitric oxide-derived oxidants by myeloperoxidase in monocytes: pathways for monocyte-mediated protein nitration and lipid peroxidation In vivo. *Circ Res* 85(10):950-958, 1999
44. Leeuwenburgh C, Hardy MM, Hazen SL, Wagner P, Oh-ishi S, Steinbrecher UP, Heinecke JW. Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J Biol Chem* 272(3):1433-1436, 1997
45. Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, van der Vliet A. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391(6665):393-397, 1998
46. Kuhlencordt PJ, Chen J, Han F, Astern J, Huang PL. Genetic deficiency of inducible nitric oxide synthase reduces atherosclerosis and lowers plasma lipid peroxides in apolipoprotein E- knockout mice. *Circulation* 103(25):3099-3104, 2001
47. Akyurek LM, Fellstrom BC, Yan ZQ, Hansson GK, Funa K, Larsson E. Inducible and endothelial nitric oxide synthase expression during development of transplant arteriosclerosis in rat aortic grafts. *Am J Pathol* 149(6):1981-1990, 1996

48. Hattori Y, Matsumura M, Kasai K. Vascular smooth muscle cell activation by C-reactive protein. *Cardiovasc Res* 58(1):186-195, 2003
49. Behr-Roussel D, Rupin A, Sansilvestri-Morel P, Fabiani JN, Verbeuren TJ. Histochemical evidence for inducible nitric oxide synthase in advanced but non-ruptured human atherosclerotic carotid arteries. *Histochem J* 32(1):41-51, 2000
50. Russell ME, Wallace AF, Wyner LR, Newell JB, Karnovsky MJ. Upregulation and modulation of inducible nitric oxide synthase in rat cardiac allografts with chronic rejection and transplant arteriosclerosis. *Circulation* 92(3):457-464, 1995
51. Hunter GC, Henderson AM, Westerband A, Kobayashi H, Suzuki F, Yan ZQ, Sirsjo A, Putnam CW, Hansson GK. The contribution of inducible nitric oxide and cytomegalovirus to the stability of complex carotid plaque. *J Vasc Surg* 30(1):36-49, 1999
52. Clark RS, Kochanek PM, Schwarz MA, Schiding JK, Turner DS, Chen M, Carlos TM, Watkins SC. Inducible nitric oxide synthase expression in cerebrovascular smooth muscle and neutrophils after traumatic brain injury in immature rats. *Pediatr Res 3*9(5):784-790, 1996
53. Binder CJ, Chang MK, Shaw PX, Miller YI, Hartvigsen K, Dewan A, Witztum JL. Innate and acquired immunity in atherogenesis. *Nat Med* 8(11):1218-1226, 2002
54. Li AC, Glass CK. The macrophage foam cell as a target for therapeutic intervention. *Nat Med* 8(11):1235-1242, 2002
55. Niu XL, Yang X, Hoshiai K, Tanaka K, Sawamura S, Koga Y, Nakazawa H. Inducible nitric oxide synthase deficiency does not affect the susceptibility of mice to atherosclerosis but increases collagen content in lesions. *Circulation* 103(8):1115-1120, 2001
56. Ihrig M, Dangler CA, Fox JG. Mice lacking inducible nitric oxide synthase develop spontaneous hypercholesterolaemia and aortic atheromas. *Atherosclerosis* 156(1):103-107, 2001

D. General conclusion and future perspectives

Hypertension affects approximately one billion individuals worldwide. As the population ages, the prevalence of hypertension will increase even further unless broad and effective preventive measures are implemented. An increased blood pressure increases the risk for heart attack, heart failure, stroke and kidney disease[1].

The cause of essential hypertension is still unknown and the pathogenesis of essential hypertension is far from being understood completely, although some vasoconstrictive factors have been identified. However, hemodynamic disturbances are obviously primordial. These lead to the modification of humoral and mechanical signalling pathways and result in vascular wall thickening and increased vascular tone, whereby a vicious circle is created[2]. Several hypotheses of the pathophysiology of essential hypertension have been postulated[3-5], but none of these hypotheses leads to a clear explanation of the pathophysiology of this disease (**Chapter A.1.1.** and **A.1.2.**).

For clarification of essential hypertension, the knowledge of vasoconstrictive factors and vasoregulatory systems is essential. Thus, the factors causing an acute increase of blood pressure are of great interest, as well as the mechanisms leading to a chronic increase of blood pressure in essential hypertension, e.g. premature arteriosclerosis and disturbances of Ca^{2+} metabolism. This thesis proposes a number of arguments in favour of the hypothesis that in addition to the known vasoregulatory systems and vasoconstrictive factors, still several other previously unknown elements, such as vasoconstrictive dinucleoside polyphosphates or inhibitors of the Ca^{2+}-APTase, may contribute to essential hypertension as well (**Chapter A.1.3.** and **A.1.4.**).

Hypertension is part of the clinical picture of chronic renal disease (**Chapter A.1.5.**). Its frequency depends on the type of nephropathy and the stage of chronic renal disease. Whatever the primary renal disease, more than eighty percent of patients beginning dialysis suffer from hypertension, and hypertension also contributes substantially to the co-morbidities of patients with renal failure not yet on dialysis. Nevertheless, few of the pathophysiologic aspects of hypertension in chronic renal failure patients are known, such as the hyperactivity of the renin-angiotensin system and a decrease of the capacity to excrete sodium accompanying the decrease in glomerular filtration rate[6]. Therefore, it is of great interest to identify yet unknown

uremic compounds with potential pathophysiologic impact on hypertension and new strategies to remove these compounds from plasma of chronic renal failure patients. Such factors might be active in the non-uremic population as well. But because of the continuous removal of the factors by the kidneys in these patients, the plasma concentration is significantly lower than in uremic patients, and therefore the factors cause less effects than in the uremic population.

In recent years, chromatographic and mass-spectrometric methods have improved substantially (**Chapter A.2.** and **A.3.**). These changes now offer the possibility to isolate and to identify unknown biomolecules and to clarify unknown pathogenetic mechanisms. Applying these new possibilities to study mechanisms of hypertension has provided us new insights not only relating to hypertension but also to vascular regulation in general.

Consequently, the first part of this thesis focuses on the identification of unknown vasoconstrictive factors and their physiologic pathways with a potential role in vascular regulation (**Chapter B.1.-B.6. and C.1.**). The second part of the thesis deals with both the isolation and identification of unknown uremic solutes, the description of their relevance for hypertension in uremia, and the possibilities to influence their concentration (**Chapter C.1.-C.4.**).

The thesis initially focuses on the development of a chromatographic assay system for the baseline separation of diadenosine polyphosphates using new chromatographic methods (**Chapter B.1.**)[7].

Diadenosine polyphosphates are known to affect vascular tone via purinoceptors and may be involved in the regulation of blood pressure. Modulating actions of diadenosine phosphates are demonstrated in numerous vascular models influencing most of the physiologically important elements of blood pressure regulation[8]. Mostly, the vasoactive action depends on the number of phosphates in the diadenosine phosphates[9]. Vasodilation can be observed in intact vessels after application of Ap_2A, Ap_3A and Ap_4A whereas Ap_5A and Ap_6A elicit contraction. Vasoconstriction induced by the diadenosine phosphates in vascular smooth muscle cells is mediated by an increase in intracellular free Ca^{2+} [10]. In vivo, intravenous injection of Ap_4A lowered blood pressure whereas injections of Ap_5A and Ap_6A caused a prolonged increase in blood pressure. In blood, in contrast to ATP, diadenosine phosphates are relatively long-lived molecules, suggesting that the action is characterized by a longer time span[11]. In a similar

manner to the vasoconstrictor angiotensin II, diadenosine phosphates also act as mitogens[12]. It can be assumed that diadenosine phosphates may be involved in circulatory pathophysiological events including hypertension and atherosclerosis[8]. It is therefore conceivable that a reliable determination method is needed which allows a clear separation of Ap_nA in an acceptable time span. We sought to develop new determination methods showing such characteristics.

By screening a combination of different chromatographic media and chromatographic conditions, we were able to develop a determination method for analysis of dinucleoside polyphosphate based on a monolithic reversed-phase chromatography column (**Chapter B.1.**). This assay is characterized by (a) high sensitivity and high resolution of the chromatography and (b) compatibility with mass-spectrometric methods. Therefore, further dinucleoside polyphosphate analytic assays should be based on monolithic silica C18 columns. Altogether, this method offers the possibility of isolation and quantification of yet unknown dinucleoside polyphosphates.

We used this innovative assay system to show that diadenosine polyphosphates are present in human plasma in µmolar concentrations (**Chapter B.2.**)[13]. These findings are of relevance in view of the fact that these biomolecules are involved in manifold ways in the regulation of cardiovascular functions, and possibly other processes are modulated by purinergic signal transduction in humans.

On the one hand, the role of extracellular diadenosine polyphosphates in physiology has been evaluated in a number of recent studies[14-16], and our study (**Chapter B.2.)**[13] demonstrates that diadenosine polyphosphates occur in human plasma. But on the other hand, an endocrine organ in humans releasing diadenosine polyphosphates had not yet been identified. Findings in animal experiments suggested that adrenal glands might be a source of diadenosine polyphosphates[17-20]. Therefore, we quantified the diadenosine polyphosphate concentration in adrenal veins, vena cava inferior, vena cava superior and vena suprarenalis. Since in blood from adrenal veins significantly higher diadenosine polyphosphate concentrations were measured than in blood from the vena cava, it can be assumed that in humans, beside platelets, the adrenal medulla is a source of diadenosine polyphosphates. Plasma concentrations of diadenosine polyphosphates quantified in this thesis are obviously sufficient to cause systemic as well as local vasoactive effects (**Chapter B.2.**)[13].

These data are in good accordance with earlier data describing the release of Ap_4A and Ap_5A from bovine adrenal glands[18]. However, after the identification of the guanosine containing dinucleoside polyphosphates Ap_nG (n=3-6) and Gp_nG (n=3-6) in human platelets[21], the question arose, whether these substances were also released by adrenal glands. The results of a further study show that the adrenal glands not only release the diadenosine polyphosphates Ap_4A and Ap_5A[18], but also Ap_2A, Ap_3A and Ap_6A, as well as Ap_nG (n=3-6) and Gp_nG (n=3-6) (**Chapter B.4.**)[23]. The identification of these dinucleoside polyphosphates emphasizes the importance of dinucleoside polyphosphates because the presence of these compounds in secretory granules of the adrenal medulla suggests that dinucleoside polyphosphates have not only local but also systemic actions.

Because of the identification of the guanosine containing dinucleoside polyphosphates Ap_nG (n=3-6) and Gp_nG (n=3-6) in adrenal glands (**Chapter B.4.)**[23] and the physiologic effects of these substances, the question arises whether these substances are also components of human plasma. This question cannot be completely answered yet. We assume that the adrenal glands release Ap_nG (n=3-6) and Gp_nG (n=3-6) into the plasma to influence physiologic and pathophysiologic processes such as the proliferation of vascular smooth muscle cells. To clarify this assumption we will search in the future for these substances in plasma of healthy subjects. In yet unpublished pilot experiments we were already able to isolate diguanosine pentaphosphate (Gp_5G) from human plasma. Plasma levels of Gp_5G from 11 healthy young male normotensive subjects were in the range of 9.5 nmol L^{-1}. Future experiments have to clarify whether besides Gp_5G also Ap_nG (with n=3-6) and Gp_nG (with n=3-4; 6) are components of the plasma.

Altogether, dinucleoside polyphosphates fulfil the requirements of endocrine hormones: they are released by an organ (adrenal glands[18,23]) and the blood moves them from these organs to their target organs (e. g. heart or kidney[24]), or their target cells (e. g. vascular smooth muscle cells[25]). The physiologic and pathophysiologic functions of the target organs or target cells are affected by the dinucleoside polyphosphates, e.g. increasing the proliferation rate of mesangial cells[24] or vascular smooth muscle cells[25]. Next, the hypothesis that diadenosine polyphosphates act not only as endocrine hormones but also as paracrine hormones was considered. We evaluated whether diadenosine polyphosphates are components of the human myocardial tissue (**Chapter B.5.)**[26]. The physiologic and pathophysiologic effects of diadenosine

polyphosphates in the cardiac tissue have been long known, e. g. the vasoconstrictive effect on the coronary resistance[8] and on positive heart inotropy[27], and the activation of P1- and P2-purinoceptors of the left atrium[28]. However, only the less potent diadenosine polyphosphates Ap_2A and Ap_3A had so far been isolated from human myocardial tissue[29]. In the framework of this thesis, we were able to isolate the potent diadenosine polyphosphates Ap_4A, Ap_5A and Ap_6A from human myocardial tissue. These physiologic data and the isolation and identification of these diadenosine polyphosphates in heart tissue substantiate now the function of diadenosine polyphosphates as paracrine hormones influencing myocardial function and coronary perfusion.

During the last two decades evidence has been raised that disturbances of the endothelium may be one of the causes of hypertension[30,31]. At the site of endothelial lesions, leukocyte adhesion and subsequently platelet aggregation occur, which consequently results in the release of vasoconstrictive substances. Also, the endothelium *per se* is a source of vasoregulating factors[32-34]. Therefore, the next concern of the thesis was to check whether endothelial cells release not only NO and endothelin but also further vasoregulatory messengers. We cultivated endothelial cells, stimulated these cells with different stimuli, and tested the vasoconstrictive properties of the resulting supernatants. During this procedure, the yet unknown dinucleoside polyphosphate Up_4A was identified as a potent vasoconstrictive factor released by endothelial cells (**Chapter B.3.**)[35]. For the first time, a dinucleoside polyphosphate containing both purine and pyrimidine moieties was isolated from human cells. Up_4A is a novel potent non-peptidic endothelium-derived vasoconstricting factor (EDCF). In contrast to the known peptidic EDCF, endothelin, only some of the physiologic effects of Up_4A have yet been investigated. The vasoregulatory effects, plasma concentrations and the release upon endothelial stimulation however strongly suggest that Up_4A is a potent vasoregulator.

Future studies should clarify the pathophysiologic implications of Up_4A and structurally related compounds concerning the genesis of essential hypertension. Subsequent studies also have to show whether Up_4A not only has a direct effect on the vascular tone but also affects the growth of vascular smooth muscle cells. An effect on vascular smooth muscle cells is likely because the proliferation of vascular smooth muscle cells is mediated by P2Y receptors[14] and Up_4A stimulated P2Y receptors[35]. Moreover, further studies also have to clarify whether Up_4A affects the proliferation rate of endothelial cells. In this case, Up_4A has to be considered as an autocrine hormone. The most interesting point will be the quantification of

Up_4A in essential hypertensive patients, in chronic renal failure patients and in healthy control subjects. Because of the very time-consuming quantification assay, some efforts are necessary to automate this assay before starting extensive clinical studies.

If these studies indicate significant differences, two different ways of intervention will be possible. On the one hand, a purinoceptor antagonist for Up_4A has to be developed. Presently some purinoceptor antagonists such as PPADS are available, but the yet available substances are toxic and therefore not suitable for use in humans. On the other hand, we were able to show in subsequent, yet unpublished pilot experiments, that an enzymatic synthesis of Up_4A is likely. After the identification of this yet unknown enzyme, a potent, non-toxic inhibitor for this enzyme should be generated. This inhibitor will lead to a decrease of the Up_4A plasma concentration. A comparable approach has already been successful: the inhibition of the angiotensin-II synthesis by angiotensin-converting-enzyme (ACE) inhibitors leads to a significant blood pressure decrease in hypertensive patients.

From the foregoing, it is obvious that apart from the peptides and nucleotides, which are known vasoregulatory messengers, there are several other as yet unknown moieties, which are also intimately involved in the generation of hypertension. These messengers may be secreted by organs or organ systems such as the adrenal glands, the myocardial tissue, or endothelial cells. However, these messengers may also be released by circulating blood cells such as the mononuclear leukocytes. Therefore, to gain more insight into the pathogenesis of hypertension, deciphering the mononuclear leukocyte secretome may be especially helpful. A link has been demonstrated between inflammation and hypertension[36] and vascular damage[4]. To contribute to the identification of the mononuclear leukocyte secretome, we isolated mononuclear leukocytes from human blood and stimulated them with different stimuli. We were able to identify Ang-II in the mononuclear leukocyte secretome (**Chapter B.6.**). Ang II is obviously not solely produced by the classical renin-angiotensin-pathway[37,38] and locally in tissues[39-41], but is obviously also produced by corpuscular elements of streaming blood. The Ang-II release determined in this study is sufficient to stimulate angiotensin receptors, to contribute to plasma Ang II concentration and to elicit vasoconstrictive effects, and may therefore contribute to the pathogenesis of hypertension.

While the first part of this thesis focuses on isolation, identification and characterisation of vasoregulatory hormonal messengers (**Chapter B.1.-B.6.**), the second part of the thesis deals

with potentially hypertensive agents in chronic renal failure patients (**Chapter C.1.–C.4.)**. This effort is relevant in view of the frequent presence of hypertension in renal failure, which is the origin of accelerated cardio-vascular damage and further deterioration of kidney function. Further identification of agents provoking hypertension might be useful in developing specific preventive therapeutic means.

There were some hints in the mass-spectra of the reversed-phase chromatography fractions from the supernatants of stimulated mononuclear leukocytes (Figure 2. A of **Chapter B.6**) indicating that these fractions not only contain Ang-II but also further vasoactive peptides. We extended our attempts to identify these unknown peptides, and subsequently we were able to identify a novel lymphocyte-derived vasoactive angiotensin peptide des[Asp^1]-[Ala^1]-Ang II, which differs from Ang II in Ala^1 instead of Asp^1 (**Chapter C.1.**).

Des[Asp^1]-[Ala^1]-Ang II had a same affinity to the AT_1 receptor as Ang II, but was a weaker vasoconstrictor, suggesting only partial AT_1 receptor agonism. In addition, this compound showed a higher AT_2 receptor affinity than genuine Ang II. In chronic renal failure patients the des[Asp^1]-[Ala^1]-Ang II / Ang II ratio dramatically increased in comparison to healthy control subjects. Further investigations now have to clarify the importance of this peptide in chronic renal failure patients. Moreover, it has to be investigated whether des[Asp^1]-[Ala^1]-Ang II is also produced by cell systems involved in the classical renin-angiotensin-pathway or locally in tissues.

A disturbance of Ca^{2+}-signaling is one of the important causes for a blood pressure increase. In patients with chronic renal failure the intracellular calcium amount is significantly increased[42]. In principle, the intracellular calcium concentration increase may be caused by two different mechanisms: (1) an increased calcium influx into the intracellular space via calcium channels or (2) a decrease of calcium efflux from the intracellular space into the extracellular space via Ca^{2+}-ATPase. Therefore, the identification of substances causing a decrease of Ca^{2+}-ATPase activity is of great importance and interest for chronic renal failure patients. We were able to identify and quantify one of these Ca^{2+}-ATPase inhibitors as p-hydroxy-hippuric acid (**Chapter C.2.**)[43]. Conventional hemodialysis decreases the p-hydroxy-hippuric acid plasma concentration of chronic renal failure patients by only 53 %. Because p-hydroxy-hippuric acid plasma concentration in healthy control subjects is below the detection limit, the plasma of chronic renal failure patients obviously contains significantly increased p-hydroxy-hippuric

acid plasma concentrations, even after hemodialysis. Elevated p-hydroxy-hippuric acid plasma concentration very likely leads to a chronic Ca^{2+}-ATPase inhibition in these patients. Therefore, the permanently increased p-hydroxy-hippuric acid plasma concentration may contribute to hypertension of chronic renal failure patients. Further studies should focus on modifications of renal replacement strategies in such a way that p-hydroxy-hippuric acid is removed more adequately. Possibly, removal is hampered by the protein binding of this molecule. This might be a clue in developing removal techniques based on e.g. adsorption effects.

A further reason for hypertension and arteriosclerosis in chronic renal failure may be decreased nitric oxide (NO) production in cells and plasma. In general, NO prevents atherogenesis and inflammation in vessel walls by inhibition of cell proliferation and cytokine-induced endothelial expression of adhesion molecules and proinflammatory cytokines. A large NO amount is produced by the iNOS enzyme. We were able to show that phenylacetic acid is a potent inhibitor of the iNOS activity and that this substance accumulates in plasma of chronic renal failure patients (**Chapter C.4.**)[44]. By conventional hemodialysis only 47.3 % of the plasma phenylacetic acid is removed. The phenylacetic acid concentration in renal failure also after hemodialysis is sufficient to noticeably decrease the iNOS activity and to decrease the NO amount in these patients, hence also inhibiting the protective effect of NO on the endothelium.

Because of the insufficient removal by dialysis of the uremic toxins p-hydroxy-hippuric acid and phenylacetic acid, future investigations have to focus on the modification of hemodialysis procedures to increase the removal rate of these uremic toxins and on the development of alternative procedures. The use of specific adsorption materials for the uremic toxin removal may be an alternative to the present dialysis approaches. Pilot experiments with adsorption materials are already performed in the network of the European Uremic Toxin Working Group (EUTox), of which our research group is also a member. Thereby, the protein binding of these molecules might offer a clue for the nanotechnologic development of adsorptive systems.

Alternatively, these protein bound uremic toxins should be removed by modification of the current standard dialysis timeframes. This can be achieved by prolonged hemodialysis. There is a constant ratio of protein-bound and protein-unbound uremic toxins according to the mass-action expression. If uremic toxins are continuously removed from the blood into the dialysate

and the hemodialysis period is increased, the uremic toxin plasma concentration is subsequently decreased.

In the future, studies have to be extended to evaluate the benefit of these approaches in detail. In one study, we have already documented that the modification of hemodialysis conditions may have a distinctive effect on the concentration of the solutes, which are the subject of this thesis (**Chapter C.3.**)[45]. The use of different hemodialysis membranes obviously has a significant effect on the intrathrombocytic Ap_nA concentration.

In the framework of this thesis, some unknown substances with strong effects on the vasoregulatory system were isolated, identified and characterised. These identifications document that the human vasoregulatory system is not yet completely known. It is obvious that beside the well-known mechanisms, further vasoregulatory systems are essential. The isolation of the substances involved in these systems provides the basis for further investigations. Some of the identified substances, such as the des[Asp^1]-[Ala^1]-Ang II, are obviously enzymatically synthesised, but the underlying mechanisms regulating these systems are presently unknown. Furthermore, the synthesis pathway of other identified substances is still unknown. Development of innovative therapies cannot be realised without this knowledge. Therefore, future investigations should be focused on detailing the underlying vasoregulatory systems of the identified substances. Subsequently, the investigations should focus on the development of innovative drugs and therapies to influence these systems.

In summary, the data of this thesis indicate that (a) many yet unknown factors such as endothelium derived Up_4A and mononuclear leukocyte derived des[Asp^1]-[Ala^1]-Ang II are involved in vasoregulation, (b) organs and cells such as heart cells, endothelium, adrenal glands and mononuclear leukocytes release as yet unknown vasoregulatory factors, and (c) that uremic toxins such as phenylacetic acid and p-hydroxy-hippuric influence the vasoregulation. Some of these factors, tissues, cells, and metabolites were isolated and identified and their physiologic and pathophysiologic effects were characterized in the framework of this thesis.

This thesis substantiates the hypothesis that hypertension is a disease caused by many different factors and a fraction of these factors have been identified in the framework of this thesis. It is very likely that there are still many other unknown factors which have to be identified

before the genesis of hypertension will be sufficiently known. Each of these identifications offers the possibility to develop new and more appropriate therapeutic approaches.

References

1. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., Jones DW, Materson BJ, Oparil S, Wright JT, Jr., Roccella EJ: The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. 289:2560-2572, 2003
2. Touyz RM: Molecular and cellular mechanisms regulating vascular function and structure-implications in the pathogenesis of hypertension. *Can J Cardiol* 16:1137-1146, 2000
3. Kaplan NM: Treatment of hypertension: insights from the JNC-VI report. *Am Fam Physician* 58:1323-1330, 1998
4. Schoen: Blood Vessel, in: *Robbins Pathologic basis of disease*, 6th edition. Eds: W.B. Saunders Company, Philadelphia, USA, 1999, pp 493-541
5. Hingorani AD, Brown MJ: Identifying the genes for human hypertension. *Nephrol Dial Transplant* 11:575-586, 1996
6. Laville M: Hypertension in patients with chronic renal disease. *Rev Prat* 54:641-645, 2004
7. Jankowski V, Vanholder R, Henning L, Karadogan S, Zidek W, Schlüter H, Jankowski J: Isolation and quantification of dinucleoside polyphosphates by using monolithic reversed phase chromatography columns. *J Chromatogr B Analyt Technol Biomed Life Sci* 819:131-139, 2005
8. Schlüter H, Tepel M, Zidek W: Vascular actions of diadenosine phosphates. *J Auton Pharmacol* 16:357-362, 1996
9. Pohl U, Ogilvie A, Lamontagne D, Busse R: Potent effects of Ap_3A and Ap_4A on coronary resistance and autacoid release of intact rabbit hearts. *Am J Physiol* 260:H1692-1697, 1991
10. Schlüter H, Offers E, Brüggemann G, van der Giet M, Tepel M, Nordhoff E, Karas M, Spieker C, Witzel H, Zidek W: Diadenosine polyphosphates and the physiological control of blood pressure. *Nature* 367:186-188, 1994
11. Lüthje J, Ogilvie A: Catabolism of Ap_4A and Ap_3A in whole blood. The dinucleotides are long lived signal molecules in the blood ending up as intracellular ATP in the erythrocytes. *Eur J Biochem* 173:241-245, 1988
12. Erlinge D, You J, Wahlestedt C, Edvinsson L: Characterisation of an ATP receptor mediating mitogenesis in vascular smooth muscle cells. *Eur J Pharmacol* 289:135-149, 1995
13. Jankowski J, Jankowski V, Laufer U, van der Giet M, Henning L, Tepel M, Zidek W, Schlüter H: Identification and quantification of diadenosine polyphosphate concentrations in human plasma. *Arterioscler Thromb Vasc Biol* 23:1231-1238, 2003
14. Ogilvie A, Blasius R, Schulze-Lohoff E, Sterzel RB: Adenine dinucleotides: a novel class of signalling molecules. *J Auton Pharmacol* 16:325-328, 1996
15. Jankowski J, Hagemann J, Tepel M, van der Giet M, Stephan N, Henning L, Gouni-Berthold I, Sachinidis A, Zidek W, Schlüter H: Dinucleotides as growth promoting extracellulary mediators: Presence of dinucleoside diphosphates Ap_2A, Ap_2G and Gp_2G in releasable granules of platelets. *J Biol Chem* 276:8904-8909, 2001
16. Hoyle CHV, Hilderman RH, Pintor JJ, Schlüter H, King BF: Diadenosine polyphosphates as extracellular signal molecules. *Drug Develop Res* 52:260-273, 2001

17. Pintor J, Rotllan P, Torres M, Miras-Portugal MT: Characterization and quantification of diadenosine hexaphosphate in chromaffin cells: granular storage and secretagogue-induced release. *Anal Biochem* 200:296-300, 1992
18. Pintor J, Torres M, Miras-Portugal MT: Carbachol induced release of diadenosine polyphosphates-Ap_4A and Ap_5A- from perfused bovine adrenal medulla and isolated chromaffin cells. *Life Sci* 48:2317-2324, 1991
19. Rodriguez del Castillo A, Torres M, Delicado EG, Miras-Portugal MT: Subcellular distribution studies of diadenosine polyphosphates-Ap_4A and Ap_5A-in bovine adrenal medulla: presence in chromaffin granules. *J Neurochem* 51:1696-1703, 1988
20. Castillo CJ, Moro MA, Del Valle M, Sillero A, Garcia AG, Sillero MA: Diadenosine tetraphosphate is co-released with ATP and catecholamines from bovine adrenal medulla. *J Neurochem* 59:723-732, 1992
21. Schlüter H, Gross I, Bachmann J, Kaufmann R, van der Giet M, Tepel M, Nofer JR, Assmann G, Karas M, Jankowski J, Zidek W: Adenosine(5') oligophospho-(5') guanosines and guanosine(5') oligophospho-(5') guanosines in human platelets. *J Clin Invest* 101:682-688, 1998
22. Pintor J, Torres M, Miras-Portugal MT: Carbachol induced release of diadenosine polyphosphates (Ap_4A and Ap_5A) from perfused bovine adrenal medulla and isolated chromaffin cells. *Life Sci* 48:2317-2324, 1991
23 Jankowski J, Jankowski V, Seibt B, Henning L, Zidek W, Schlüter H: Identification of dinucleoside polyphosphates in adrenal glands. *Biochem Biophys Res Commun* 304:365-370, 2003
24. Heidenreich S, Tepel M, Schlüter H, Harrach B, Zidek W: Regulation of rat mesangial cell growth by diadenosine phosphates. *J Clin Invest* 95:2862-2867, 1995
25. Jankowski J, Hagemann J, Yoon MS, van der Giet M, Stephan N, Zidek W, Schlüter H, Tepel M: Increased vascular growth in hemodialysis patients induced by platelet-derived diadenosine polyphosphates. *Kidney Int* 59:1134-1141, 2001
26. Luo J, Jankowski V, Gungar N, Neumann J, Schmitz W, Zidek W, Schlüter H, Jankowski J: Endogenous diadenosine tetraphosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate in human myocardial tissue. *Hypertension* 43:1055-1059, 2004
27. Vahlensieck U, Boknik P, Gombosova IH, Knapp J, Linck B, Luss H, Müller HL, Neumann J, Deng MC, Scheld HH, Jankowski H, Schlüter H, Zidek W, Zimmermann N, Schmitz W: Inotropic effects of diadenosine tetraphosphate (Ap_4A) in human and animal cardiac preparations. *J Pharmacol Exp Ther* 288:805-813, 1999
28. Hoyle CH, Ziganshin AU, Pintor J, Burnstock G: The activation of P1- and P2-purinoceptors in the guinea-pig left atrium by diadenosine polyphosphates. *Br J Pharmacol* 118:1294-1300, 1996
29. Luo J, Jankowski J, Knobloch M, van der Giet M, Gardanis K, Russ T, Vahlensieck U, Neumann J, Schmitz W, Tepel M, Deng MC, Zidek W, Schlüter H: Identification and characterization of diadenosine 5',5'''-P1,P2 - diphosphate and diadenosine 5',5'''-P1,P3-triphosphate in human myocardial tissue. *Faseb J* 13:695-705, 1999
30. Lüscher TF, Boulanger CM, Dohi Y, Yang ZH: Endothelium-derived contracting factors. *Hypertension* 19:117-130, 1992
31. Higashi Y, Sasaki S, Nakagawa K, Matsuura H, Oshima T, Chayama K: Endothelial function and oxidative stress in renovascular hypertension. *N Engl J Med* 346:1954-1962, 2002
32. Furchgott RF, Zawadzki JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373-376, 1980
33. Palmer RM, Ferrige AG, Moncada S: Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327:524-526, 1987

34. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332:411-415, 1988
35. Jankowski V, Tölle M, Vanholder R, Schönfelder G, van der Giet M, Henning L, Schlüter H, Paul M, Zidek W, Jankowski J: Uridine adenosine tetraphosphate: a novel endothelium- derived vasoconstrictive factor. *Nat Med* 11:223-227, 2005
36. Ross R: Atherosclerosis-an inflammatory disease. *N Engl J Med* 340:115-126, 1999
37. Peach MJ: Renin-angiotensin system: biochemistry and mechanisms of action. *Physiol Rev* 57:313-370, 1977
38. Reid IA, Morris BJ, Ganong WF: The renin-angiotensin system. *Annu Rev Physiol* 40:377-410, 1978
39. Müller DN, Bohlender J, Hilgers KF, Dragun D, Costerousse O, Menard J, Luft FC: Vascular angiotensin-converting enzyme expression regulates local angiotensin II. *Hypertension* 29:98-104, 1997
40. Dell'Italia LJ, Meng QC, Balcells E, Wei CC, Palmer R, Hageman GR, Durand J, Hankes GH, Oparil S: Compartmentalization of angiotensin II generation in the dog heart. Evidence for independent mechanisms in intravascular and interstitial spaces. *J Clin Invest* 100:253-258, 1997
41. Campbell DJ: Circulating and tissue angiotensin systems. *J Clin Invest* 79:1-6, 1987
42. Gafter U, Malachi T, Barak H, Levi J: Red blood cell calcium level in chronic renal failure: effect of continuous ambulatory peritoneal dialysis. *J Lab Clin Med* 116:386-392, 1990
43. Jankowski J, Tepel M, Stephan N, van der Giet M, Breden V, Zidek W, Schlüter H: Characterisation of p-hydroxy-hippuric acid as an inhibitor of Ca^{2+}-ATPase in end-stage renal failure. *Kidney Int* 59 Suppl 78:S84-88, 2001
44. Jankowski J, van der Giet M, Jankowski V, Schmidt S, Hemeier M, Mahn B, Giebing G, Tolle M, Luftmann H, Schlüter H, Zidek W, Tepel M: Increased plasma phenylacetic acid in patients with end-stage renal failure inhibits iNOS expression. *J Clin Invest* 112:256-264, 2003
45. Jankowski J, Schlüter H, Henning L, van der Giet M, Jankowski V, Zidek W, Tepel M: The AN69 Hemofiltration Membrane Has a Decreasing Effect on the Intracellular Diadenosine Pentaphosphate Concentration of Platelets. *Kidney Blood Press Res* 26:50-54, 2003

E. Curriculum vitae

Vera Jankowski
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married to PD Dr. Joachim Jankowski
two children (Melanie, born 14.9.1986; Christian, born 29.6.1988)

I. Education

A. Vocational
1982 – 1985 Tax specialized aid, Witten, Germany

B. Secondary school (1992 – 1994)
Secondary school level I certificate Dortmund, Germany

C. Higher education
Study of human medicine, University of Bochum, Germany (7 university semesters)
Study of biochemistry, University of Bochum, Germany (4 university semesters)
Study of biochemistry, Free university of Berlin, Germany (3 university semesters)

II. Diplomas and certificates

July 6th, 1982	first public examination in secondary school
June 4th, 1985	tax specialized aid
June 19th, 1995	high-school diploma
September 26th, 2002	Bachelor of Science / Biochemistry

III. Working experience

1985 – 1986	Tax specialized aid
1999 – 2000	Regular tutorial assistant of the Medical Clinic I, Marienhospital, University of Bochum, Germany (Director: Prof. Dr. med. W. Zidek)
2001-2002	Regular tutorial assistant of the Medical Clinic IV, Charité, Berlin, Germany (Director: Prof. Dr. med. W. Zidek)
since September 2002	Scientific assistant, Medical Clinic IV, Charité (CBF), Berlin, Germany (Director Prof. Dr. med W. Zidek)

IV. Original publications

A. Articles in journals of the Science Citation Index

1. Jankowski J, Tepel M, Stephan N, van der Giet M, Breden V, Zidek W, Schlüter H. Characterisation of p-hydroxy-hippuric acid as an inhibitor of Ca^{2+}-ATPase in end-stage renal failure. *Kidney Int* 59 (78): 84-88, 2001

2. Jankowski J, Jankowski V, Laufer U, van der Giet M, Tepel M, Zidek W, Schlüter H. Identification and quantification of diadenosine polyphosphate concentrations in human plasma. *Arterioscler Thromb Vasc Biol* 23 (7): 1231-1238, 2003

3. Jankowski J, Schlüter H, Jankowski V, Hemeier M, van der Giet M, Luftmann H, Zidek W, Tepel M. Increased plasma phenylacetic acid in patients with end-stage renal failure inhibits iNOS expression. *J Clin Invest* 112 (2): 256-264, 2003

4. Jankowski J, Schlüter H, Henning L, van der Giet M, Jankowski V, Zidek W, Martin Tepel. The AN69 hemofiltration membrane has a decreasing effect on the intracellular diadenosine pentaphosphate concentration of platelets. *Kidney and Blood Pressure Research* 26: 50-54, 2003

5. Jankowski J, Jankowski V, Seibt B, Henning L, Zidek W, Schlüter H. Identification of dinucleoside polyphosphates in adrenal glands. *Biochem Biophys Res Commun* 304(2): 365-370, 2003

6. Luo J, Jankowski V, Gungar N, Neumann J, Schmitz W, Zidek W, Schlüter H, Jankowski J. Endogenous diadenosine tetraphosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate in human myocardial tissue. *Hypertension* 43(5): 1055-1059, 2004

7. Yoon MS, Jankowski V, Montag S, Zidek W, Henning L, Schlüter H, Tepel M. Characterisation of advanced glycation endproducts in saliva from patients with diabetes mellitus. *Biochem Biophys Res Commun* 323 (2) 377-381, 2004

8. Jankowski V, Vanholder R, Tölle M, Schönfelder G, van der Giet M, Henning L, Schlüter H, Zidek W, Jankowski J. Identification of uridine adenosine tetraphosphate (Up_4A) as an endothelially derived vasoconstrictive factor. *Nature Medicine* 11 (2): 223-227, 2005

9. Jankowski V, Vanholder R. Henning L, Karadogan S, Zidek W, Schlüter H, Jankowski J. Isolation and quantification of dinucleoside polyphosphates by using monolithic reversed-phase chromatography columns. Journal of Chromatography *J Chromatogr B* 819 (1):131-139, 2005

10 Jankowski V, Vanholder R, van der Giet M, Henning L, Tölle M, Giebing G, Schönfelder G, Krakow A, Karadogan S, Gustavsson N, Gobom J, Webb J, Lehrach H, Schlüter H, Zidek W, Jankowski J. Detection of angiotensin II in supernatants of stimulated mononuclear leukocytes. *Hypertension* (in press, 2005)

11. Obermaier C, Jankowski V, Schmutzler C, Bauer J, Wildgruber R, Infanger M, Köhrle J, Weber G, Grimm D. Improved Sample Preparation for Free Flow-Isoelectric Focusing: Identification of Human Thyroid Cancer Cell Proteins. *Electrophoresis* 26:2109-16, 2005

12. Tölle M, Levkau B, Brinkmann V, Schönfelder G, Schäfers M, Lipinski K, Jankowski J, Jankowski V, Chun J, Zidek W, van der Giet M. The immunosuppresant FTY720 induces eNOS-dependent arterial vasodilation via the lysophospholipid receptor $S1P_3$. *Circulation Research* 96 (8):913-920, 2005

13. Tölle M, Giebing G, Tietge U, Jankowski J, Jankowski V, Henning L, Zidek W, van der Giet M. Diguanosinepentaphosphate: an endogenous activator of rho-kinase. (*Hypertension* in revision)

14. Jankowski V, Vanholder R, van der Giet M, Henning L, Karadogan S, Schönfelder G, Giebing G, Tölle M, Furkert J, Oksche A. Bienert M, Bahr U, Zidek W, Schlüter H. Jankowski J. A novel lymphocyte-derived vasoactive angiotensin peptide. (in preparation)

B. Abstracts

Oral presentations

1. Jankowski V, Zidek W, Jankowski J: Nachweis von Dinukleosidpolyphosphaten in den Granula der Nebenniere. 32. Rostocker Gespräche über kardiovaskuläre Funktionen und Hypertonie, Rostock, Germany, 2003

2. Jankowski V, Zidek W, Tepel M, Jankowski J: Eine Ursache der Atherosklerose im Rahmen der chronischen Niereninsuffizienz. 33. Rostocker Gespräche über kardiovaskuläre Funktionen und Hypertonie, Rostock, Germany, 2004

3. Jankowski V, Zidek W, Jankowski J: The adrenal gland: source of diadenosine polyphosphate endocrine hormones. European Society of Hypertension, Paris, France, 2004

4. Jankowski V, van der Giet M, Zidek W, Tepel M, Jankowski J: Platelet-derived diadenosine polyphosphates: Possible reason of the increase of vascular growth in hemodialysis patients? ERA-EDTA, Lisbon, Portugal, 2004

5. Jankowski V, Tepel M, Zidek W, Jankowski J: Inhibition der iNOS Expression bei chronischer Niereninsuffizienz, 34. Rostocker Gespräche über kardiovaskuläre Funktionen und Hypertonie, Rostock, Germany, 2005

6. Jankowski V, Vanholder R, Zidek W, Jankowski J: Identification of uridine adenosine tetraphosphate (Up_4A) in endothelia cells. European Society of Hypertension, Milan, Italy, 2005

Poster presentations

1. Jankowski J, Jankowski V, Zidek W, Tepel M: The AN69 hemofiltration membrane has an decreasing effect on the intracellular diadenosine pentaphosphate concentration of platelets. European Society for Artificial Organs, Aachen, Germany, 2003

2. Jankowski J, Jankowski V, Zidek W, Tepel M: Dialysis membranes have a direct effect on diadenosine pentaphosphate concentrations of platelets of chronic renal failure patients. European Society of Hypertension YII Meeting, Milano, Italy, 2003

3. Jankowski V, Tepel M, Zidek W, Jankowski J: Isolation and identification of dinucleoside polyphosphates in bovine adrenal glands. European Society of Hypertension YII Meeting, Milano, Italy, 2003

4. Jankowski V, Zidek W, Jankowski J: Nachweis von Dinukleosidpolyphosphaten in den Granula der Nebenniere. 32. Rostocker Gespräche über kardiovaskuläre Funktionen und Hypertonie, Rostock, Germany, 2003

5. Jankowski J, Jankowski V, van der Giet M, Tepel M, Zidek W: Novel endocrine function of the human adrenal medulla. Dtsch Med Wochenschr Supplement 3; 128:S149-S184, 2003

6. Jankowski J, van der Giet M, Jankowski V, Zidek W, Tepel M: Identification and characterization of an iNOS-inhibitors. Dtsch Med Wochenschr Supplement 3; 128:S149-S184, 2003

7. Jankowski V, Zidek W, Jankowski J: New messanger molecules of the adrenal glands. Dtsch Med Wochenschr Supplement 3; 128:S149-S184, 2003

8. Jankowski J, van der Giet M, Jankowski V, Zidek W, Tepel M: The intracellular diadenosinpentaphosphat amount in human platelets can be modified by hemofiltration. Dtsch Med Wochenschr Supplement 3, 128:S149-S184, 2003

9. Jankowski J, Jankowski V, van der Giet M, Tepel M: Novel endocrine function of the human adrenal medulla. Dtsch Med Wochenschr Supplement 3, 128:S149-S184, 2003

10. Jankowski J,van der Giet M, Zidek W, Jankowski V, Tepel M: The inhibitor of the inos-expression in chronic renal failure patients: Phenylacetic acid. ERA-EDTA, Lisbon, Portugal, 2004

11. Jankowski V, van der Giet M, Tepel M, SchlÜter H, Zidek W, Jankowski J: The adrenal gland – source of diadenosine polyphosphate endocrine hormones. ERA-EDTA, Lisbon, Portugal, 2004

12. Jankowski J, Van der Giet M, Zidek W, Jankowski V, Tepel M: INOS-Expression decreasing factors in plasma of chronic renal failure patients. European Society of Hypertension, Paris, France, 2004

13. Jankowski V, Van der Giet M, Tepel M, Schlüter H, Zidek M, Jankowski J: Novel endocrine function of the human adrenal medulla. European Society of Hypertension, Paris, France, 2004

14. Jankowski V, van der Giet M, Tepel M, SchlÜter H, Zidek W, Jankowski J: Adrenal Glands: One source of dinucleoside polyphosphates. European Society of Hypertension, Paris, France, 2004

15. Jankowski V, van der Giet M, Zidek W, Tepel M, Jankowski J: Platelet-derived diadenosine polyphosphates: Reason of increased vascular growth in hypertensive chronic renal failure patients. European Society of Hypertension, Paris, France, 2004

16. Jankowski V, Zidek W, Tepel M, Jankowski J. Isolation and quantification of advanced glycation endproducts in saliva from patients with diabetes mellitus. 28. Wissenschaftliche Kongress Hypertonie, Hannover, Germany, 2004

17. Jankowski J, Jankowski V, Zidek W. Vascular actions of diadenosine phosphates. 28. Wissenschaftliche Kongress Hypertonie, Hannover, 2004

18. Jankowski V, Zidek W, Jankowski J. Identification of endogenous diadenosine tetraphosphate, diadenosine pentaphosphate and diadenosine hexaphosphate in human myocardial tissue. 28. Wissenschaftliche Kongress Hypertonie P114, Hannover, 2004

19. Jankowski V, Zidek W, Jankowski J. Diadenosine polyphosphates increase the proliferation rate of vascular smooth muscle cells of CRF patient. American Society of Nephrology 37th Annual Meeting and Scientific Exposition, St. Louis, USA, 2004

20. Jankowski V, Tepel M, Zidek W, Jankowski J: Inhibition der iNOS Expression bei chronischer Niereninsuffizienz, 34. Rostocker Gespräche über kardiovaskuläre Funktionen und Hypertonie, Rostock, Germany, 2005

21. Jankowski V, Luo J, Zidek W, Jankowski J: Human myocardial tissue: one aource of diadenosine tetraphosphate, diadenosine pentaphosphate, and diadenosine hexaphospate. European Society of Hypertension, Milan, Italy, 2005

22. Karadogan S, Jankowski V, Tepel M, Zidek W, Jankowski J: Advanced Glycation Endproducts: Isolation and characterisation in salviva from patients with diabetes mellitus. European Society of Hypertension, Milan, Italy, 2005

23. Giebing G, Toelle M, Jankowski V, Jankowski J, Zidek W, van der Giet M: The recently identified endothelially derived constrictive factor uridine adenosine tetraphosphate acts via P2x and P2Y receptors. European Society of Hypertension, Milan, Italy, 2005

24. Jankowski V, Toelle M, van der Giet M, Zidek W, Jankowski J: A novel endothelial derived vasoconstrictor. European Society of Hypertension, Milan, Italy, 2005

Awards

1. Travel award, European Society of Hypertension, Milan, Italy, 2003

2. Travel award, European Society of Hypertension, Paris, France, 2004

3. Travel award, ERA-EDTA, Lisbon, Portugal, 2004

4. Poster award, 34. Rostocker Gespräche über kardiovaskuläre Funktionen und Hypertonie, Rostock, Germany, 2005

5. Travel award, European Society of Hypertension, Milan, Italy, 2005

Acknowledgement

The thesis was conducted at the Faculty of Medicine and Health Sciences, Department of Internal Medicine, Nephrology Division, University of Gent (Belgium) and the Charitè, Medical Clinic IV, Berlin (Germany), during years 2001-2005.

Foremost, I want to express my greatest gratitude to my promotor Prof. Dr. R. Vanholder. Without him, this thesis would not have been possible. His continuous pursuit of excellence, exceptionally extensive knowledge of scientific work and biochemistry, have made the execution of this thesis a memorable learning-experience, his patience and continual encouragement in the last years. I thank him for his enlightening view and criticism, which enabled me to significantly improve the coherence and quality of the manuscripts and my thesis.
I would like to express also my greatest thanks to Prof. Dr. W. Zidek. I am grateful to him for introducing me into this interesting research area since 1999. I deeply thank him for his patience and encouragement and for his insights and suggestions that helped to shape my research skills, who guided this work and helped whenever I was in need.

I am deeply grateful to Joachim Jankowski, the group manager of my lab at the Charitè and - last but not least- my husband. I have appreciated his permanent encouragements for solving many not only scientific problems. I wish also to thank him for his patience and his deep human understanding. Simply, I would like to thank him to be a very amiable and open-minded boss and husband.

I am grateful to the members of the Faculties of Medicine in Berlin and Gent for their support and their comradeship, especially to Lars Henning, Markus van der Giet, Markus Tölle, Markus Giebing, Min-Sung Yoon, Tim Westhoff, Martin Tepel, Anja Krakow, Bertram Seibt, Sevil Karadogan and Griet Glorieux. Thanks for the friendly working atmosphere.

Moreover, I would like to express my deepest gratitude for the constant support, understanding and love that I received from Melanie and Christian.

Finally, I would like to thank all those people who made this thesis possible and an enjoyable experience for me.