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Identification, characterization and physiological significance of metabolites of the B-type natriuretic peptide

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1 INTRODUCTION

1.1 The natriuretic peptide system

The natriuretic peptide system (NPS) has been well characterized over the last decades. It represents a dynamic hormonal system involved in physiological, but also pathophysiological processes[1]. The NPS regulates salt and body-fluid balance as well as vascular tone and cardiac remodeling, thus playing a pivotal role in the maintenance of blood pressure and cardiovascular function[2-4]. Hence, the system strongly influences the development of cardiovascular diseases like hypertension[5] and heart failure[6] including the formation of cardiac hypertrophy and fibrosis[7]. These effects are in part also resulting from its antagonistic actions towards other blood pressure-regulating peptide systems in the body e.g. the renin-angiotensin system (RAS)[5, 8] and the endothelin system[9]. Additionally, the NPS is essentially involved in fetal development[10, 11] including bone growth[12], and cardiac maturation[11, 13] as well as in other elementary mechanisms like lipid metabolism[14, 15].

The NPS is conserved in all vertebrates ranging from simple species like Drosophila to humans[16]. Recently, there was even evidence for the existence of a similar system in plants[17].

The NPS is composed of the natriuretic peptides and the natriuretic peptide receptors, which mediate the intracellular signaling upon binding of the natriuretic peptides and are in part also involved in the clearance of them[18]. Additionally, several enzymes are associated with the NPS regulating the natriuretic peptide generation, processing[19], and inactivation[20].

1.1.1 Discovery of natriuretic peptides

The three main peptide hormones of the natriuretic peptide system are the atrial natriuretic peptide (ANP), the B-type natriuretic peptide (BNP) and the C-type natriuretic peptide (CNP). The existence of ANP was first described in 1981 by de

Bold *et al.,* who injected extracts of atrial tissues into rats and observed a decrease in arterial pressure as well as an increase in urinary water and sodium excretion in these animals[21]. Two years later, the responsible peptide has been isolated and its 28-amino acid (aa) sequence was deciphered[22]. According to its origin and its natriuretic properties, the newly identified substance was named atrial natriuretic peptide. In 1988, Sudoh *et al.* identified a second structurally related natriuretic peptide consisting of 32 amino acids in porcine brain and named it accordingly brain natriuretic peptide or BNP[23]. In the following years, it was revealed that BNP is mainly expressed in ventricular and also atrial cardiomyocytes rather than in the brain, where it was discovered[24, 25]. However, both, ANP and BNP transcripts were also found in the central nervous system, lung, thyroid, adrenal, kidney, spleen, small intestine, ovary, and uterus in levels approximately 1-2 orders of magnitude lower than in cardiac tissues[26]. In 1990 the third member of the natriuretic peptide system, the 22-amino acid long CNP, was also discovered in porcine brain by Sudoh *et al.*[27]. Now it is known that CNP is abundantly produced in vascular endothelium[28] and also the central nervous system[29].

The existence of two more endogenously expressed natriuretic peptides has been reported in the literature. Urodilatin (URO) was extracted from human urine in 1988[30]. The 32-amino acid residue peptide is generated by alternative processing of the ANP precursor peptide taking place in the kidney tubules[30, 31]. However, URO seems to have less biological activity compared to ANP[31]. Dendroaspis natriuretic peptide (DNP), harboring 38 amino acids, was originally isolated from the venom of the green mamba in 1992[32]. Due to the discovery of DNP-like immunoactivity in human plasma and atrial tissue, the existence of DNP in men is anticipated[33, 34].

1.1.2 Structure and secretion of the mature natriuretic peptides

The mature forms of the three classical natriuretic peptides, ANP, BNP, and CNP, are similar in their amino acid sequence and secondary structure. They are derived from distinct cleavage of their precursors, the pro-natriuretic peptides, leading to the formation of two peptide portions, the N-terminal fragments, so called NT-pro-natriuretic peptides and the C-terminal fragments[35]. These C-terminal fragments represent the mature and bioactive forms of the natriuretic peptides (**Figure 1**). The

bioactive ANP consists of 28 aa (ANP1-28)[22]; BNP of 32 aa (BNP1-32)[36], and CNP of 22 aa (CNP1-22)[37]. These bioactive forms are often just named ANP, BNP, and CNP without any indication of their sequence length. All three mature peptides share a 17-aa ring structure formed by an intramolecular disulfide bridge. This structure is likely formed by cardiac protein disulfide isomerases, whose expression has been reported to be up-regulated during cardiopathological conditions[38]. The ring structure is essential for the biological activity of these natriuretic peptides[39]. High sequence similarity between the peptides is present within this cyclic structure (**Figure 1**, highlighted in grey), while the C- and N-terminal regions are very divergent, especially for CNP, which lacks a C-terminal extension.

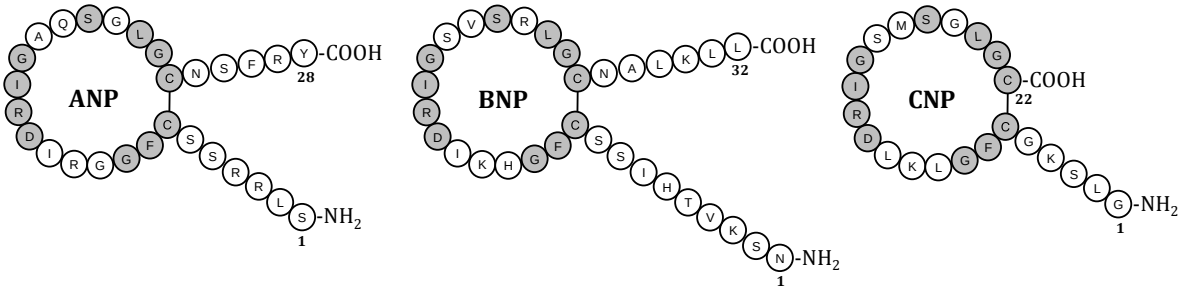


Figure 1: Secondary structures of the bioactive murine atrial natriuretic peptide (ANP) with 28 amino acids, B-type natriuretic peptide (BNP) with 32 amino acids, and C-type natriuretic peptide (CNP) with 22 amino acids. Conserved amino acids are highlighted in grey. The first amino acid at the N-terminus and the last amino acid at the C-terminus are numbered.

Upon synthesis, ANP is transported through the Golgi complex and packaged into membrane bound atrial granules[40]. It has been suggested that BNP is also transported towards such granules and that both, ANP and BNP, are stored together[41]. The main stimulus for ANP and BNP secretion is wall stretch of the myocardium induced by raising blood pressure accompanied with atrial and ventricular volume overload[42-44]. Moreover, a constitutive release through passive diffusion in the absence of stimuli has been described as well as basal secretion depending on the degree of newly synthesized pepetide[45-47]. CNP secretion is not accompanied with increased wall stretch, since CNP is neither predominantly expressed in heart tissue nor stored in secretory granules[48]. Its release from endothelial cells seems to be regulated through paracrine secretion[28]. It was shown that ANP and BNP stimulate the production and secretion of CNP from endothelial cells[49]. Beside, ANP and BNP, also cytokines like

tumor necrosis factor alpha (TNFα) and interleukin 6 (Il6) are able to induce CNP release[28].

1.1.3 Natriuretic peptide receptors and principal signaling

Biological actions of the natriuretic peptides are mediated by membrane-bound cell-surface receptors, the natriuretic peptide receptors A, B, and C (NPRA, NPRB and NPRC). The relative effectiveness of the peptides to bind and activate NPRA has been reported as: ANP ≥ BNP >> CNP, whereby for CNP no significant activation of the NPRA has been detected[50, 51]. On the contrary, the affinity of CNP towards the NPRB receptor is 50- to 500-fold higher than for ANP and BNP (CNP >> ANP = BNP). Thus, ANP and BNP are ligands for NPRA[50], while CNP binds to NPRB[50, 51] (**Figure 2**).

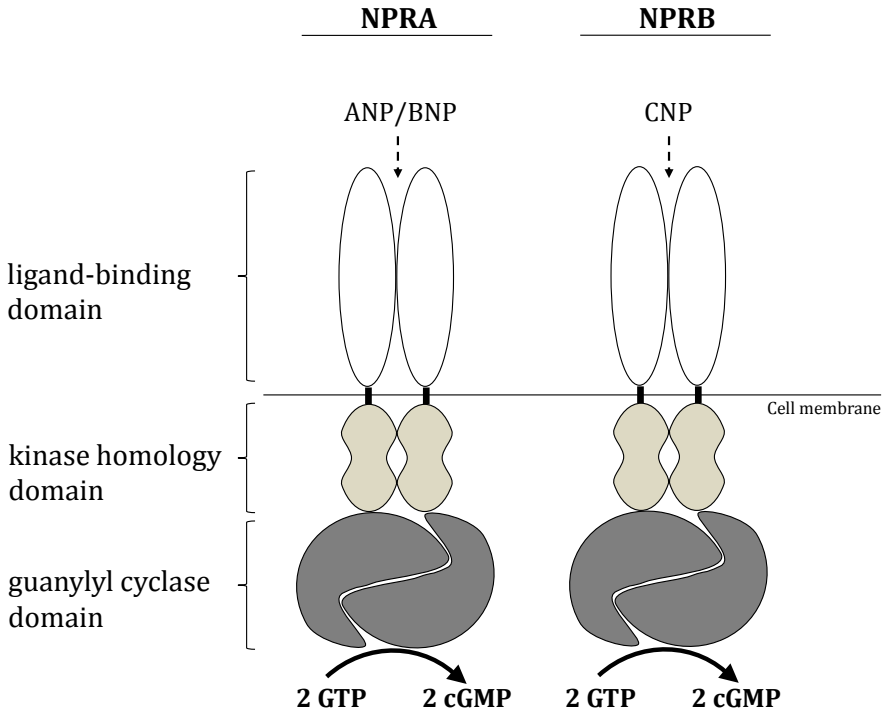


Figure 2: Natriuretic peptide receptors A (NPRA) and B (NPRB) with their ligands atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Upon ligand binding, a conformational change of the kinase honology domain is induced, which relieves its inhibitory effect on the guanylyl cyclase domain. Active guanylyl cyclases generate cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP)[56,57].

Both, NPRA and NPRB, contain two intracellular kinase homology (KH) domains and guanylyl cyclase (GC) domains[52]. Upon extracellular binding of the ligand, the binding domain of the receptor undergoes a twist motion, which is suggested to facilitate the binding of adenosine triphosphate (ATP) on the KH domain. Once ATP is bound, a

conformational change occurs in the KH domain relieving its normal inhibitory effect on the GC domains[53-55]. Subsequently, the two GC domains associate with each other and form two active sites per receptor[55]. Activated GC is catalyzing the formation of the second messenger cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP)[56, 57]. Intracellular cGMP stimulates a broad variety of effector molecules, e.g. cGMP-dependent protein kinases (PKG), cGMP-dependent phospho-diesterases (PDE), and cGMP-dependent ion-gated channels (CNG)[58]. These effectors influence a vast number of signaling pathways mainly by regulating intracellular Ca^{2+} release[59] and protein kinases, like mitogen-activated protein kinase (MAPK)[60, 61] as well as protein kinase C (PKC)[62]. Physiological effects of the natriuretic peptide/NPRA and natriuretic peptide/NPRB axis are described in more detail in section 1.1.5.

The extracellular domain of NPRC shares high structural similarity with that of the NPRA and NPRB[18] (**Figure 3**) and possesses high and similar affinity for all natriuretic peptides, which has been reported as: ANP ≥ CNP ≥ BNP[50]. However, NPRC is lacking the intracellular GC domain[63], but is associated with G_i-proteins and binding of the ligands to the NPRC activates G_i-protein-dependent pathways[64, 65]. In cardiomyocytes the $G_{i\alpha}$-subunit inhibits adenylyl cyclase leading to a reduction of intracellular cAMP and inhibition of Ca^{2+} influx. In cardiac fibroblasts the $G_{i\beta\gamma}$ subunit activates phopsholipase C stimulating Ca^{2+} influx[66]. Despite these actions, NPRC is commonly considered to be a clearance receptor for the natriuretic peptides[67, 68].

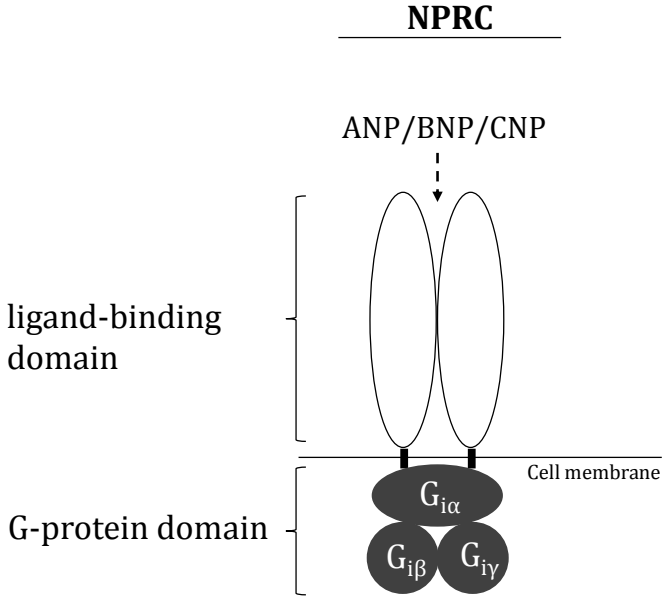


Figure 3: Natriuretic peptide receptors C (NPRC) with its ligands atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Upon binding of the ligand, Gi-protein domains (G_{α}-, G_{β}- and G_{γ}-subunits) are activated[64,65].

1.1.4 Physiological effects of the natriuretic peptide system

1.1.4.1 The ANP & BNP/NPRA axis

The discovery of ANP and BNP, and their secretion from the myocardium revealed that the heart is not only a pump driving the circulation of blood, but is also an endocrine organ regulating the cardiovascular system. The NPRA axis counteracts plasma volume expansion and thus regulates blood pressure homeostasis[35, 69]. On the one hand, this is achieved through its ability to induce natriuresis as well as diuresis, and by its direct vasodilatory effects[35, 57, 70]. At the same time, it regulates thirst and salt appetite[71, 72] as well as the sympathetic nervous system by inhibiting sympathetic activity[73, 74]. The NPRA axis also counteracts the development and progression of cardiac remodeling e.g. cardiac hypertrophy[75] and fibrosis[76]. Notably, it was discovered, that NPRA expression is downregulated in heart failure[77].

Several genetically altered animal models have been developed for the investigation of the physiological functions of ANP and BNP through NPRA. The group around O. Smithies generated mice with a disruption of the gene encoding for the ANP precurser proANP[78]. In the homozygous model no circulating ANP can be found. These animals suffer from substantially increased blood pressure. In contrast to this, BNP knockout mice do not exhibit hypertension, but develop severe cardiac fibrosis[76]. In NPRA knockout mice, the lack of all ANP- and BNP- mediated effects can be observed[79]. Such animals exhibit chronic hypertension and significant reductions in natriuresis and diuresis in response to volume overload. Moreover, these knockout mice exert cardiac hypertrophy with extensive interstitial fibrosis resulting in increased lethal vascular events similar to those observed in untreated hypertensive patients[80]. It was predicted that the ANP- and BNP- mediated stimulation of NPRA leads to the inhibition of the Ca^{2+} channels known to positively regulate 'calcineurin-nuclear factor of activated T cells' (NFAT) - signaling, which is involved in the development of cardiac hypertrophy through increasing the hypertrophy-related gene expression[81, 82] as demonstrated in **Figure 4**. It was also revealed that NPRA-signaling leads to an inactivation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and thus to a reduction of extracellular remodeling including fibrosis[83] (**Figure 4**).

Interestingly, mice absent for NPRA in cardiomyocytes only, also develop cardiac hypertrophy, suggesting that the NPRA axis acts as a local antihypertrophic regulator[84].

Smooth muscle cell-specific deletion of the NPRA does not alter blood pressure, but abolishes ANP-mediated vasodilation of isolated vessels[85]. On the other hand, endothelial-specific deletion of NPRA is accompanied with high blood pressure but the direct vasodilatory effect of ANP is preserved[86]. This indicates that endothelial NPRA is critical for water homoestasis, and smooth muscle cell NPRA plays a critical role for the acute regulation of vasodilation, respectively.

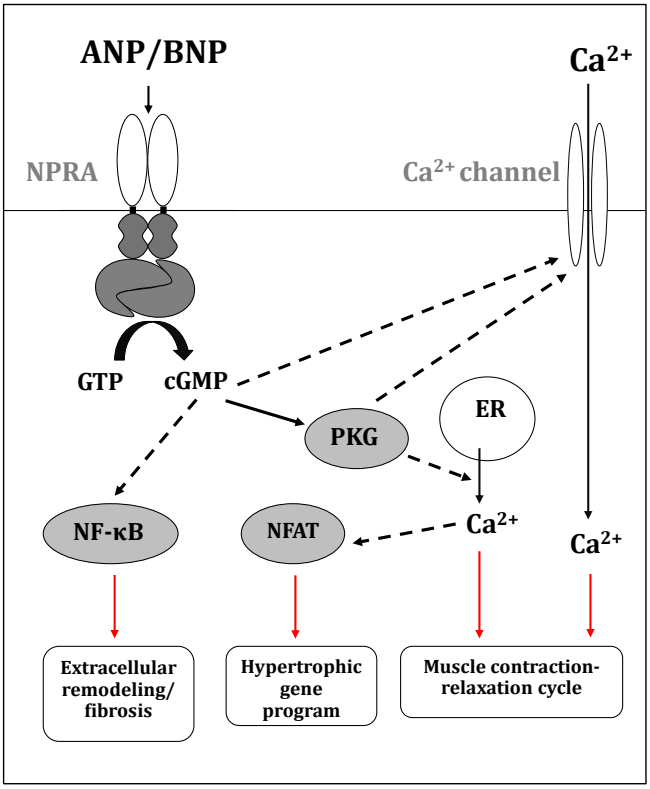


Figure 4: Schematic diagram of how atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP)-signaling via the natriuretic peptide receptor A (NPRA) prevents the extracellular remodeling, induction of the hypertrophic gene program and induces vasorelaxation. Inhibitory pathways are highlighted with dashed arrows. The overview is evolved from references 81-83. Abbreviations: guanosine triphosphate (GTP), cyclic guanosine monophosphate (cGMP), cGMP-dependent protein kinase (PKG), calcineurin-nuclear factor of activated T cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), endoplasmatic reticulum (ER).

1.1.4.2 The CNP/NPRB axis

In contrast to ANP and BNP, it was shown that CNP acts anti-natriuretic, since infusions of the peptide increases renal sodium reabsorption[87]. Nevertheless,

infusions of CNP decrease mean arterial blood pressure and it was shown that CNP acts as local regulator of vascular tone[88] and also remodeling[89, 90].

Wang *et al.* developed an animal model with cardiomyocyte-specific overexpression of CNP[91]. Since CNP is not expressed in the heart under physiological conditions, the peptide levels increased up to 100-fold in the right ventricle. Notably, in healthy animals no increase of plasma CNP and no influence on blood pressure or heart morphology were observed. However, they found that CNP overexpression significantly prevents myocardial infarction-induced cardiac hypertrophy compared to wild-type controls, indicating that also the NPRB axis plays a role in the regulation of hypertrophy. Supporting this hypothesis, NPRB dominant-negative mutants, which exert reduced CNP but not ANP-NPRA-dependent cGMP generation, display progressive, blood pressure-independent cardiac hypertrophy[92]. Other than for the NPRA knockouts, these animals do not show fibrosis.

NPRB knockout mice, generated by the replacement of exons encoding for the extracellular ligand binding domain of the NPRB by a neomycin resistance gene, show a very severe phenotype[93]. These mice have a drastically reduced survival and develop strong dwarfism through impairment of endochronal ossification. Animals that survived beyond 100 days were only 60-70% in nasoanal length and had only 50% of the body weight compared to their wild-type controls. Notably, also mice lacking CNP develop severe dwarfism with reduced survival[94]. Thus, signaling through the NPRB axis seems to be important for the bone development. Moreover, NPRB knockout females are infertile showing abnormal development of the uterus and ovary, whereas no changes were observed in male reproductive organs[94].

1.1.4.3 The natriuretic peptides/NPRC axis

The NPRC is the most widely and abundantly expressed natriuretic peptide receptor and the most represented one in endothelial cells[64]. At time of the discovery of NPRC, it was described as a silent receptor with the only function to remove natriuretic peptides from the circulation[95]. Indeed, up- or downregulation of NPRC influences the clearance rate of natriuretic peptides[64]. NPRC is highly expressed in adipocytes, and weight reduction of obese individuals goes along with increased natriuresis and diuresis probably caused by the reduced clearance of ANP and BNP and thus higher activity on the ANP/BNP/NPRA axis[96]. Other than for NPRA expression, which is

decreased in failing hearts, NPRC expression is increased[97, 98]. Very recently a cross-talk between NPRC and NPRA has been suggested, which might cause this effect[99].
Several evidences for a functional NPRC axis involving the action of all three natriuretic peptides have been accumulated. It was shown that ANP interacts with NPRC in atria and ventricles activating calmodulin through the G_i protein leading in part to its hypotensive, natriuretic and diuretic effects[100]. A functional interaction between NPRC and BNP has also been suggested, possibly mediating antiproliferative actions in cardiac fibroblasts[101].
The NPRC also plays a critical role in bone growth. NPRC deficient mice show long bone over-growth, possibly due to the lack of CNP clearance and thus an increased signaling through the NPRB[68].

1.1.4.4 The natriuretic peptide system in fetal development

It was shown that natriuretic peptides are present in fetal circulation and constitute a functional endocrine system responsive to cardiac filling pressure[11, 102]. Levels of ANP are higher in the fetal circulation than in adults[103], and fetal ventricles express higher levels of ANP than adult ventricles[104]. Peaks of ANP and BNP expression during gestation are associated with significant events in cardiac organogenesis, suggesting the involvement in embryonic heart development[10]. Supportingly, NPRA knockout mice present significant, blood pressure-independent cardiac hypertrophy at birth, suggesting that the natriuretic peptide/NPRA-axis is involved in myocyte growth during fetal development[105]. ANP and BNP may also help to regulate blood supply to the fetus by acting as vasodilators in the placental vasculature[106]. No CNP has been detected in developing hearts[10].
Cardiac ANP and BNP are also increased in fetuses having trisomy in comparison to healthy fetuses, which might be the consequence of the high incidences of congenital heart defects seen in these individuals and thus may lead to the development of nuchal translucency[11, 107].
Natriuretic peptides have also effects on the development of other tissues than heart. As stated before, NPRC knockout mice[68] and also BNP-overexpressing mice[108] develop skeletal overgrowth, whereas CNP knockout fetuses show dwarfism and bone malformation[94].

1.1.5 Clearance and Inactivation of natriuretic peptides

1.1.5.1 Half-life of natriuretic peptides

Natriuretic peptides have short half-life times and are cleared from the circulation within minutes[109]. Two main mechanisms are contributing to this process: binding to natriuretic peptide receptors with subsequent internalization[95, 110] and enzymatic inactivation by peptidases[110, 111]. Pharmacokinetic measurements revealed a half-life of about 2 to 3 min for ANP and CNP in healthy human subjects[112, 113]. The half-life for BNP lasts slightly longer. Mukoyama *et al*. determined half-life times of 3.9 min for BNP and 1.7 min for ANP in normal men[114]. The half-life of porcine BNP, which was injected to human circulation, has been reported to be 3 min[115] and in sheep, endogenous BNP secreted after acute volume overload was determined with a half-life of about 5 min[116].

1.1.5.2 Clearance by natriuretic peptide receptors

Upon binding of the natriuretic peptides to the natriuretic peptide receptors, endocytotic receptor-ligand internalization takes place[110, 117]. In a subsequent process, the natriuretic peptides are hydrolysed in the lysosome while a big portion of the receptor is rapidly recycled leading to a constant receptor level on the cell surface[117, 118]. The clearance receptor NPRC is thought to be the receptor mainly responsible for the clearance of the natriuretic peptides also because of its abundant distribution and high expression[64]. In transgenic mice, in which NPRC was genetically ablated, it was shown that ANP had a 66% longer half-life compared to wilde-type animals[68]. Furthermore, it was shown recently that NPRC, expressed in mouse brain capillaries, mediates brain-to-blood efflux of ANP at the blood-brain barrier as a pathway of cerebral ANP clearance[119].

1.1.5.3 Inactivation by the peptidase neprilysin

The main enzyme catabolizing natriuretic peptides was thought to be neprilysin (neutral endopeptidase, NEP, EC 3.4.24.11), a zinc-dependent membrane-bound metallopeptidase, inactivating natriuretic peptides by a hydrolytic attack on the peptides' ring structure[120-122]. This hydrolysis opens the typical ring structure of the peptides, which is essential for biological activity, and thus leads to the formation of inactive fragments[123]. However, some studies questioned the role of NEP as the

central degrading enzyme for all natriuretic peptides, since degradation times for BNP last much longer compared to ANP[124, 125]. In 2004, Walther *et al.* reported that, in contrast to ANP, no differences in the BNP degradation rates in NEP knockout mice and their wild-type controls can be observed[125]. These *in vivo* studies and additional experiments with recombinant NEP revealed that ANP and CNP are indeed quickly degraded by NEP, but in comparison to them, BNP is inert to the hydrolytic attack of NEP[125]. Own studies investigating the structural requirements of natriuretic peptides to be substrates for NEP, highlighted N- and C-terminal regions of the peptides to be decisive for the degradation by NEP[20] as presented in **Figure 5**.

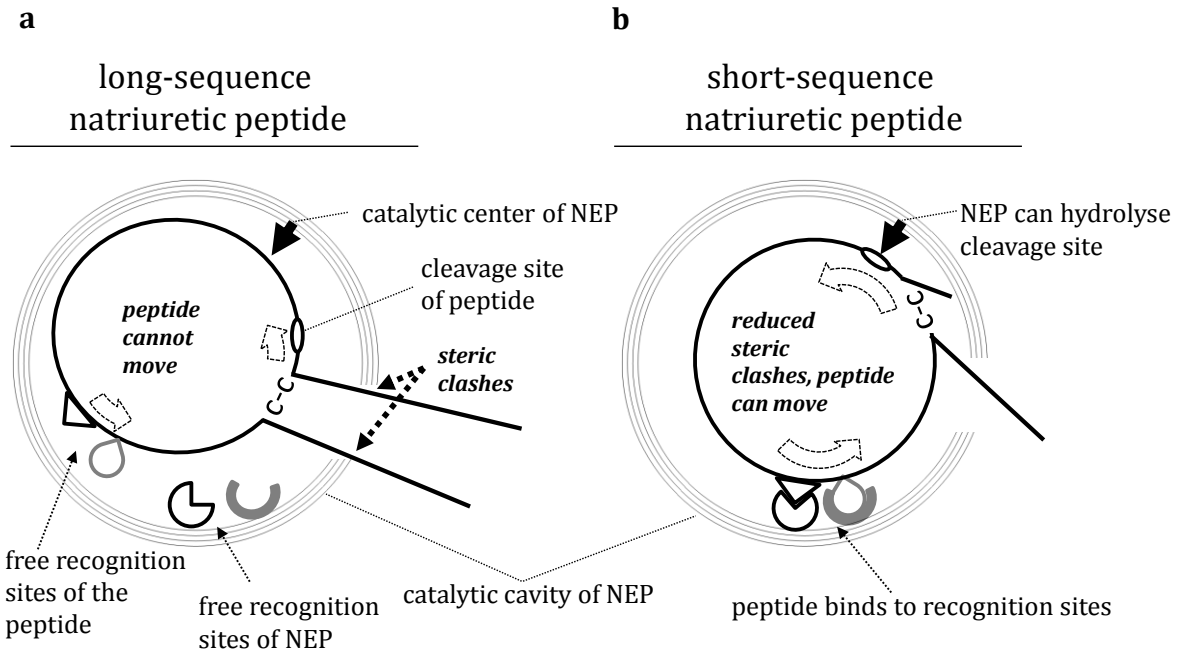


Figure 5: Simplified mechanism of the orientation of short and long natriuretic peptides within the catalytic center of neprilysin (NEP). **a)** Due to steric clashes caused by long N- and C-terminal regions, the peptide cannot be adjusted towards the catalytic center of NEP and the cleavage site is not hydrolyzed. **b)** Shorter N- and C-terminal regions allow the peptide to move and bind to the recognitions sites in the cavity. Thus, the orientation of the cleavage site towards the catalytic center of NEP is favored and hydrolysis can take place. Adapted from Pankow & Schwiebs *et al.*[20]

Although computer modeling revealed that BNP fits into the catalytic cavity of NEP, it was shown that BNP is not hydrolyzed by the enzyme. Reason for this is an impaired adjustment of the peptide towards the catalytic site of NEP. This impairment is caused by its long N- and C-termini, which hinder the peptide to bind to the recognition sites within the catalytic cavity of NEP. As a general conclusion it was highlighted that shorter natriuretic peptides like ANP (28 aa) and CNP (22 aa) are faster degraded by NEP compared to peptides with longer amino acid sequences like BNP (32 aa) and DNP (38 aa).

Fitting the model, it was shown recently for a human ANP variant having a 12-amino acid extension on the C-terminus caused by a frameshift mutation (fsANP), that its degradation through NEP was significantly reduced compared to the mature ANP[126].

1.1.6 Design of natriuretic peptides

Besides the naturally occurring natriuretic peptides, several variants composed of different parts of the endogenous natriuretic peptides, e.g. CU-NP (CNP and URO)[127], CD-NP (CNP and DNP)[128], or ACNP (ANP and CNP)[129, 130] have been designed. These synthetic peptides are designed to increase proteolytic resistance or biological activity, respectively. ACNP is a chimeric peptide composed of the disulfide-linked ring of endogenous CNP and the N- and C-terminal part of human ANP (**Figure 6**).
In own work it was found that ACNP has altered receptor specificity[129]. While ANP binds to NPRA and CNP binds to NPRB, ACNP is able to bind to both of these receptors and stimulate significant cGMP generation. Thus, ACNP combines bioactivity of ANP and CNP.

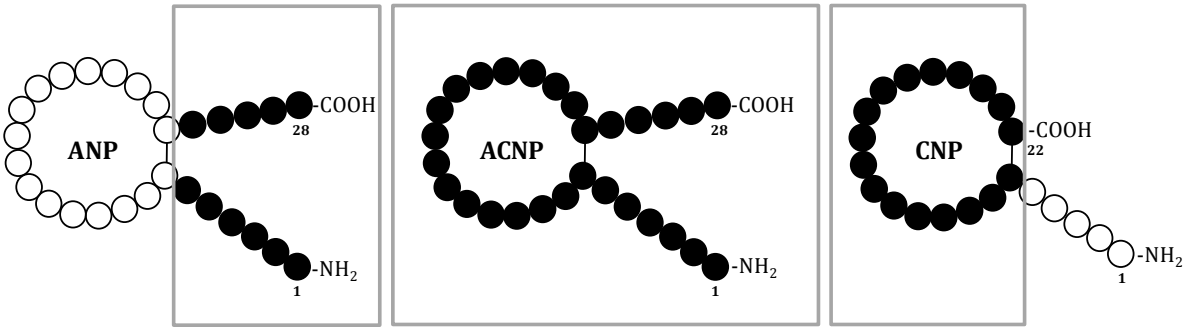


Figure 6: Schematic design of ACNP from the N- and C-terminal regions of the atrial natriuretic peptide (ANP) and the ring structure of the C-type natriuretic peptide (CNP). The first amino acid at the N-terminus and the last amino acid at the C-terminus are numbered.

1.2 The B-type natriuretic peptide

1.2.1 Endogenous BNP: Gene expression and peptide processing

BNP gene expression takes place in atrium and ventricle, while ventricular expression increases drastically upon the development of cardiac diseases mainly as a response to myocardial stretch[131, 132]. The synthesis of BNP starts with the transcription of a gene composed of 1.3 kb including three exons and two introns located upstream from the ANP gene on chromosome 4 in mice[133] and chromosome 1 in humans[134]. The promoter of the BNP contains gene elements, which regulate cardiac-specific gene expression[135, 136]. A study using transgenic animals carrying a luciferase gene coupled to the proximal region of the BNP promoter showed that this segment is sufficient to mediate the ventricle-specific expression and thus expression is higher in ventricles than in atrium[137, 138]. The translation of the mRNA encoding the precursor of the peptide results in the generation for the pre-proBNP with 134 amino acids[139, 140]. Already during synthesis, an N-terminal 26-amino acid signal peptide is removed resulting in the formation of proBNP (108 aa)[141] (**Figure 7**).

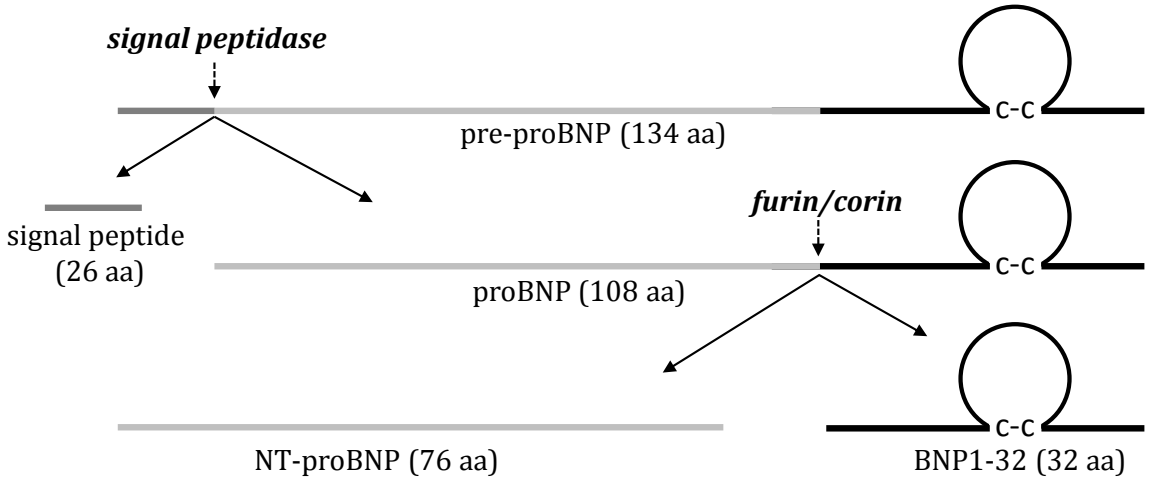


Figure 7: Processing of pre-proB-type natriuretic peptide (pre-proBNP). After removal of the signal peptide, furin and corin are generating the N-terminal fragment NT-proBNP with 76 amino acids (aa) and the C-terminal fragment BNP1-32 with 32 amino acids[19,141,144].

It might be, that the signal peptide is already removed before the synthesis of the precursor is completed and therefore pre-proBNP does probably not exist as an entity[142]. However, in case pre-proBNP is generated, it can in theory be anchored in

cell membranes or be associated with lipid compounds in the circulation since the signal peptide is highly hydrophobic. A truncated fragment of the signal peptide has been identified in the circulation, indicating that the pre-proBNP might have been secreted from the cell or shedded from the cell surface resulting in the generation of this fragment[143].

ProBNP is considered to be cleaved by two convertases, intracellular furin and membrane-bound corin, both generating two portions of BNP: the N-terminal part named NT-proBNP (76 aa) and the bioactive C-terminal part named BNP1-32 (32 aa)[19, 144] (**Figure 7**). The genes of furin and BNP are co-expressed in cardiomyocytes and expression of furin is also elevated under cardiopathological conditions[145]. It has been shown that inhibition of furin *in vitro* decreases pro-BNP processing and cleavage of proBNP in furin-deficient cells is reduced by up to 60%[19]. Furin is mainly located in the trans-Golgi network, while corin, a serine protease, is anchored transmembranal and is also present in circulation[146, 147]. It is thought to cleave proBNP and also proANP upon secretion from the cell generating the bioactive fragments[146, 148, 149]. Corin knockout mice develop sustained hypertension [122, 150] and very recently corin gene-mutations have been suggested to be associated with pre-eclampsia (= pregnancy-induced hypertension)[151].

Both peptides, NT-proBNP and BNP1-32, as well as their precursor proBNP have been shown to circulate in the body[152, 153].

In rodents an additional bioactive BNP peptide was found resulting from a differential processing of proBNP releasing a C-terminal BNP peptide of 45 amino acids instead of 32[154, 155]. This peptide, namely BNP1-45, was not found in humans, respectively.

1.2.2 Metabolism of the bioactive BNP1-32 by peptidases

In circulation, peptides are exposed towards the proteolytic activity of enzymes in blood and on cellular membranes of organs, leading to their degradation. However, it was shown for some vasoactive peptides e.g. for the vasoconstrictory peptides Angiotenisn II and endothelin or for the vasorelaxing peptide bradykinin, that before degradation, they are metabolised into truncated, but bioactive peptide fragments[156-158]. Only very few studies have shown that BNP1-32 is also processed into shorter fragments before being cleared form the circulation[159, 160]. Such fragments still contain the 17-aa ring structure, which is the prerequisite for

biological activity of natriuretic peptides[39] and thus, such BNP fragments are called metabolites rather than degradation products. In 2002, Shimizu *et al.* identified in plasma of heart failure patients that has been spiked with BNP1-32 *in vitro*, that the peptide is metabolized into an N-terminally truncated metabolite[152]. This BNP metabolite is shortened by two amino acids compared to the mature BNP1-32 and was subsequently named BNP3-32. In 2006, Brandt *et al.* reported that the conversion of BNP1-32 to BNP3-32 is mediated by dipeptidyl-peptidase IV (DPPIV)[159]. DPPIV is a prolyloligopeptidase that occurs on cell-surfaces as well as in soluble form in plasma[161]. In 2007, Pankow *et al.* reported the cleavage of murine BNP1-32 by renal meprin A resulting in the generation of the BNP metabolite BNP7-32[160] as presented in **Figure 8**.

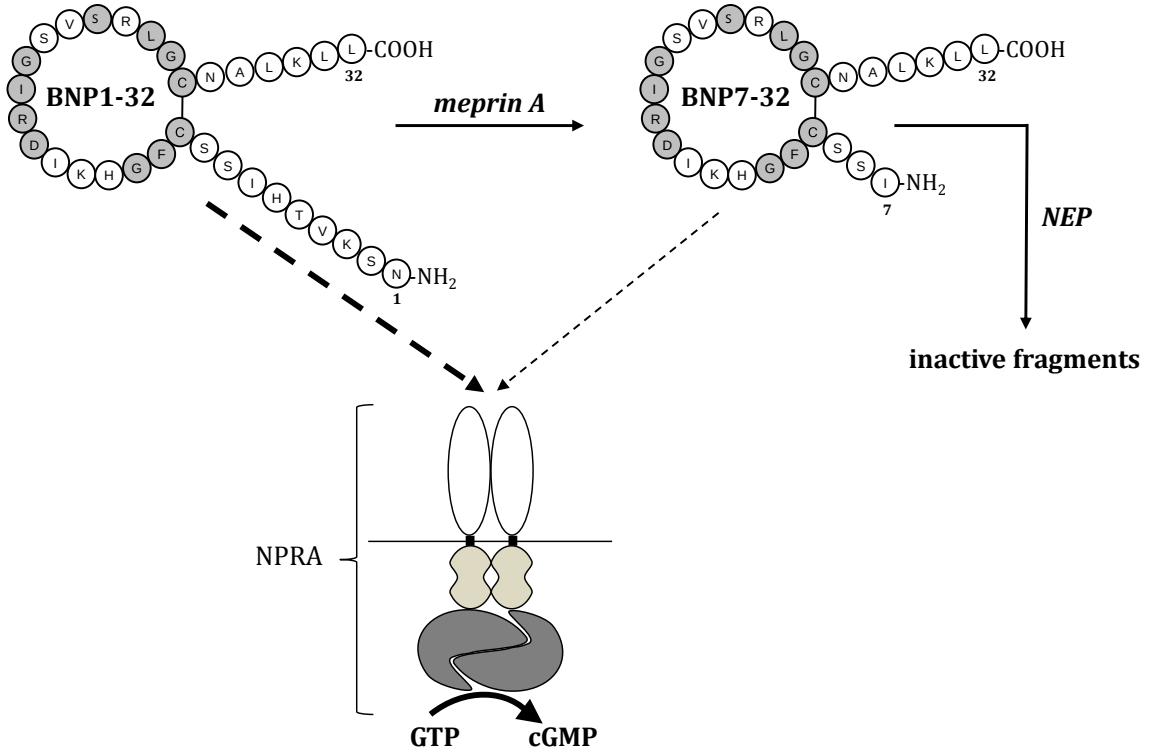


Figure 8: Metabolism of murine B-type natriuretic peptide 1-32 (BNP1-32) to the metabolite BNP7-32 by meprin A. BNP7-32 is less biologically active compared to BNP1-32, since its ability to stimulate cGMP generation through the natriuretic peptide receptor A (NPRA) is reduced. Moreover, BNP7-32 can be inactivated by neprilysin (NEP). Adapted from Pankow *et al.*[159]

Both BNP metabolites have strongly reduced bioactivity compared to the mature BNP1-32. A lack of vasodilatory actions and also natriuresis and diuresis was shown for BNP3-32 infused into dogs compared to BNP1-32[162]. Similarly, it was shown that the ability of BNP7-32 to induce the generation of cGMP via the NPRA *in vitro* was approximately 50% less compared to BNP1-32[160]. Furthermore, it was revealed that BNP7-32 is no longer resistant towards the proteolytic activity of NEP. According to the model introduced in section 1.1.5.3, BNP7-32 can be degraded into inactive fragments, due to its shorter N-terminus leading to a better adjustment of the peptide in the catalytic center of NEP and thus the peptide can be inactivated through a hydrolytic attack within the ring-structure[20].

1.2.3 Physiological effects of BNP and involvement in (cardiovascular) diseases

Besides its systemic actions, like induction of natriuresis[163] and vasorelaxation[164], BNP has direct inhibitory effects on cardiac and vascular remodeling[6, 7]. It mediates cell growth and proliferation, and thus counteracts cardiac hypertrophy via NPRA mediated signaling[141] and acts as a local anti-fibrotic factor in the heart[165] (see also **Figure 4**). Numerous studies have demonstrated an increase of BNP levels in cardiovascular disease states including ischemia, arrhythmias, fibrosis, cardiac hypertrophy, coronary endothelial dysfunction, and hypertension[166-169]. In addition, plasma BNP concentrations are markedly increased in the early phase of acute myocardial infarction, when plasma ANP levels are increased only very slightly[170]. These observations suggest that elevation of BNP levels represent a final common pathway for many cardiovascular diseases in order to decrease blood pressure and volume, and to prevent extensive cardiac remodeling[141, 167-169, 171-173].

Nakao *et al.* generated a BNP knockout mouse by replacing exon 1 and 2 of the mouse BNP gene with a neomycin-resistance gene leading to the generation of a disrupted BNP allele[76]. This disruption results in a complete loss of BNP mRNA and peptide. BNP knockout mice exert extensive fibrosis in the ventricles shown by increased collagen mRNA levels developing from week 15 of age and thereafter. These mice also exert increased expression of mRNA of the Angiotensin-converting enzyme (ACE), the enzyme being responsible for the generation of the vasoconstrictory peptide Angiotensin II[174]. However, these animals do not exert significant differences in blood

pressures compared to wildtype animals, suggesting that BNP might not be involved in the regulation of blood pressure under physiological conditions in normal healthy subjects. On the other hand, ANP mRNA expression and ANP levels were markedly increased in the ventricle of these mice, probably representing a compensatory mechanism.

Transgenic mice, overexpressing BNP in liver driven by a serum amyloid P component promoter, show a 100-fold increase in BNP and cGMP plasma levels and have a significant lower blood pressure and heart weight suggesting that BNP especially plays a regulatory role when it circulates in large quantities like in cardiopathological states[175]. Moreover, BNP overexpressing animals that underwent unilateral nephrectomy, are protected against glomerular fibrosis and hypertrophy, suggesting that BNP can, besides acting intracardially, also act as an antifibrotic and antihypertrophic factor in other organs through systemic release[176]. BNP overexpression also has dramatical effects on skeletal development[108]. Such mice develop skeletal abnormalities like kyphosis and become progressively hump-backed. Moreover, they have significantly increased bone length without exhibiting an increase in body weight. Explanations for these effects are inconclusive so far, since it was shown before, that the NPRB axis is responsible for skeletal development[93] (see section 1.1.4.2), but BNP1-32 is not able to activate this axis (see section 1.1.3).

1.2.4 BNP as a therapeutic drug

In 2001, the Federal Drug Administration (FDA) approved the use of recombinant human BNP1-32, named Nesiritide®, for the treatment of acutely decompensated congestive heart failure[177]. A literature search to review preclinical and clinical information related to Nesiritide® revealed that it has beneficial effects for the treatment of heart failure through stimulation of arterial and venous dilatation, enhancement of natriuresis and diuresis, and suppression of the renin-angiotensin and sympathetic nervous systems[178-180]. The study 'Fusion I' revealed a significant improvement of all-cause mortality and all-cause hospitalization in high-risk patients receiving Nesiritide® in comparison to standard care[179]. However, its clinical usefulness is discussed[181], since studies were made in which Nesiritide® was described to have adverse effects on renal function[182-184] and was associated with an increased risk of death in patients with previous renal dysfunction[185]. However, in

2011, O'Conner *et al*.[183] published data of a cohort of 7141 patients with acute heart failure receiving Nesiritide® or placebo. They could not associate the treatment with worsening of renal function or risk of death. However, they also couldn't find any significant improvements of the patients disease' status caused by the administration of Nesiritide® in comparison to the placebo group. Thus, the usefulness of Nesiritide® is rather unclear.

1.2.5 BNP as a biomarker: clinical applications

A dramatically increase of BNP levels occurs with the progression of cardiac diseases[186-189], which made BNP a useful biomarker[190-193] and measurements of BNP levels extremely clinically relevant[166, 194]. Plasma BNP concentrations were shown to correlate with echocardiographic parameters obtained from patients with heart failure[195], which made the determination of such levels precise enough for the diagnosis of heart failure independently of the examination of cardiologists[196].

The diagnostic use of BNP has been evaluated in many clinical trials. The study 'Breathing Not Properly' showed the usefulness of determining BNP for implicating or excluding congestive heart failure in patients with dyspnoea[171]. Thus, BNP1-32 has emerged as valuable biomarker for patients presenting with chest pain or dyspnoea[196-199]. It was shown by Maisel *et al.* that increasing BNP levels correlate with heart failure stage[172]. It was disclosed that after acute myocardial infarction (MI), BNP levels rise dependent on the severity of the infarct, revealing BNP as a tool to monitor etiopathology[200]. Already in 1999, the first report was published that suggested to utilize measurements of plasma concentrations of natriuretic peptides as a save method of adjusting the therapy of moderate heart failure[201]. Since the change of plasma BNP levels of patients follows improvement or decline of the disease, decreasing BNP values predict improvement of cardiac function and disease outcome[202-204]. It was shown in patients with congestive heart failure and MI, that increasing concentrations of BNP correlate with enlargement, decreased contractility, and increased stiffness of the left ventricle[205]. Ganem *et al.* showed recently, that pre-operative BNP levels predict the adverse outcome for patients undergoing cardiac surgery[206].

At the same time, the utilization of BNP measurements entails economic advantages. Importantly, knowledge of BNP levels reduces medical costs (e.g. reduction of

echocardiography) and reduces examination time in the emergency room[207]. As evaluated in the 'IMPROVE' study[207], the rehospitalisation rate over a 60-day period was significantly reduced when BNP levels where known and interpreted. In 2006, Roche Diagnostics' proposed at the American Heart Association's Scientific Sessions that the introduction of BNP testing could generate yearly savings up to $600 million in the U.S. Healthcare system[208].

However, there are a few studies like the 'Framingham' study[173] and the 'CONSENSUS II' study[209] describing rather poor sensitivity and prognostic values of BNP measurements compared to echocardiography or other established techniques, in some cardiac diseases. However, in general BNP measurements have turned out to be an important non-invasive tool for the diagnosis, risk stratification, and guidance of therapy of patients with heart failure[200, 210, 211]. Thus, utilization of BNP measurements is anchored in the guidelines for the diagnosis and management of heart failure of the American Heart Association[212] and in the guidelines for cardiac risk assessment of the European Heart Failure Society[213].

1.2.6 Techniques to detect BNP in patients

Since BNP level measurements gained increased importance in the routine examination of patients in the emergency departments[212, 213], several FDA approved tests from different companies have been developed. In 2000, the Triage® BNP test (Biosite Diagnostics) was introduced as a rapid point-of-care test for determination of BNP levels in human plasma[214]. This non-competitive immunofluorometric assay is using a monoclonal antibody against an epitope within the disulfide-linked ring structure of the peptide. The method was developed for BNP determination in ambulatory or emergency units, where usually only a few samples have to be measured in a short time[214]. The SHIONOSPOT® BNP test (Shionogi & Co., Ltd) uses two monoclonal antibodies directed against the ring structure and the C-terminus of BNP[215]. These two antibodies are also used in the Bayer ADVIA Centaur® BNP assay, the first fully automated BNP test[216]. The NT-proBNP test from Roche Diagnostics is designed to measure NT-proBNP and is eligible for high throughput[217].

Many of the commercial tests are specific for detection of either NT-pro BNP or the bioactive BNP1-32, but all tests have in common that they do crossreact with their

precursor proBNP harboring both BNP forms. The Triage® test e.g. does have as much as 5 to 20% crossreactivity towards proBNP[218].

Another pitfall of all these tests is that besides the crossreactivity towards proBNP, various other forms of BNP that might circulate in the body and harbor the epitope in their sequence, are detected, e.g. the BNP metabolites BNP3-32 and BNP7-32 (see section 1.2.2). Thus, such tests do not just measure the concentration of BNP1-32, but rather the whole pool of different BNP forms. Considering that the bioactivity of various BNP metabolites is different, e.g. BNP7-32 has just 50% of the bioactivity of BNP1-32[160], the determination of the total BNP activity[i] in plasma does not represent 100% of that of BNP1-32. Thus, predictions about the biological effectiveness of the BNP levels measured in the patient's plasma are not precise. Other techniques to detect BNP in plasma, such as a quantitative mass spectrometry immunoassay, have been developed[219]. Such techniques, based on mass spectrometry, are more specific compared to tests utilizing antibodies only, since they can distinguish distinct forms of BNP. Disadvantageous is the high complexity and time-consuming procedure, which excludes clinical usage.

Another problem with the detection of BNP-levels is that the tests do not provide information about the particular cardiac disease of the patient. Thus, the concentration of the whole BNP pool measured cannot be used to distinguish between e.g. dilated cardiomyopathy, intrinsic cardiomyopathy, coronary artery disease, coronary endothelial dysfunction, hypertension and so forth. Many diagnostic strategies using biomarkers are rather unspecific[220].

[i] BNP activity = activity of the whole BNP pool including all metabolites

1.3 Aim of the study

The B-type natriuretic peptide (BNP1-32) is an endogenously generated, vasoactive and cardioprotective peptide that is utilized as a biomarker for the diagnosis of heart failure and as a pharmaceutical drug for the therapy of such. Upon its generation and secretion, it is transported within the blood stream to and through organs. Thus, the peptide is constantly exposed towards proteolytic activity present in blood and on cellular membranes of organs.
The central hypothesis of this thesis is that BNP1-32 is, similar to other vasoactive peptides, metabolized by peptidases into shorter peptide fragments with analog or altered biological activity, before it is inactivated by degradation.

This work aims to investigate the metabolism of BNP1-32 to evolve a comprehensive model of BNP processing from synthesis towards clearance. The following main points are of particular interest:

1 The identification of potential BNP metabolites
2 The disclosure of enzymes responsible for the formation of BNP metabolites
3 The determination of the biological activity of potential BNP metabolites towards the natriuretic peptide receptors in comparison to the mature BNP1-32 by the use of *in vitro, ex vivo,* and *in vivo* methods.

Based on this work, it is aimed to initially assess the functional role and physiological consequence of BNP1-32 metabolism and to reveal the potential of BNP metabolites to serve as specific biomarkers or pharmaceutical drugs in the therapy of cardiovascular diseases.

2 MATERIALS and METHODS

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals and reagents used were of high purity grade and are listed in **Table 1**.

Table 1: Chemicals and reagents.

Name	**Company**
Acetic acid	Sigma-Aldrich
Acetonitrile (ACN) gradient grade	Th.Geyer
Acrylamide	Sigma-Aldrich
Ammonium persulfate (APS)	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
Bisacrylamide	Sigma-Aldrich
Bovine serum albumin (BSA)	Roth
Braunol	Ratiopharm
Bromophenol blue	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
Chloroform, Biotech grade	Sigma-Aldrich
Coomassie-Brilliant-Blue R 250	Bio-Rad
Dimethyl sulfoxide (DMSO)	Roth
Ethanol	Stockmeier Chemie Dillenburg
Ethylenediaminetetraacetic acid (EDTA)	Roth
Glycerol	Sigma-Aldrich
HEPES	Sigma-Aldrich

Hydrochloric acid	Sigma-Aldrich
Isoflurane	Baxter
Isopropanol	Sigma-Aldrich
Ketamine	Inresa Arzneimittelwerk
Magnesium sulfate	Roth
Methanol	VWR
Monopotassium phosphate	Roth
N-[Tris(hydroxymethyl)methyl]glycine (tricine)	Sigma-Aldrich
Pancuronium	Inresa Arzneimittelwerk
Perchloric acid	Sigma-Aldrich
PolyFect®	QIAGEN
Potassium chloride	Roth
Saline for injections, sterile	Diaco
Sodium chloride	Roth
Sodium dihydrogen phosphate monohydrate	Merck
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
Sodium hydrogen carbonate	Sigma-Aldrich
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Trifluoracetic acid for spectroscopy	Merck
trishydroxymethylaminomethane (Tris)	Sigma-Aldrich
Trizol®	Life Technologies
Tryptone	Sigma-Aldrich
Urea	Roth
Water for injections, sterile	Diaco
Xylocaine	AstraZeneca
Yeast Extract	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich

2.1.2 Recombinant natriuretic peptides

All natriuretic peptides used are listed in **Table 2** and have been synthesized at BIOSYNTAN Berlin, Germany. The cysteines forming the disulfide-linked ring structure are highlighted in bold.

Table 2: Recombinant natriuretic peptides and their sequences.

Name	Sequence
ANP	SLRRSS**C**FGGRMDRIGAQSGLG**C**NSFRY
BNP1-32	NSKVTHISS**C**FGHKIDRIGSVSRLG**C**NALKLL
BNP1-30	NSKVTHISS**C**FGHKIDRIGSVSRLG**C**NALK
BNP1-29	NSKVTHISS**C**FGHKIDRIGSVSRLG**C**NAL
BNP7-32	ISS**C**FGHKIDRIGSVSRLG**C**NALKLL
BNP7-30	ISS**C**FGHKIDRIGSVSRLG**C**NALK
BNP1-45	SQGSTLRVQQRPQNSKVTHISS**C**FGHKIDRIGSVSRLG**C**NALKLL
CNP	GLSKG**C**FGKLKDRIGSMSGLG**C**

2.1.3 Media and solutions for cell culture

The media and solutions used for cell culture are listed in **Table 3**.

Table 3: Media and solutions for cell culture.

Name	Company
Gibco® Dulbecco's Modified Eagle Medium (DMEM)	Life Technologies
Endothelial Cell Basal Medium	PromoCell
Endothelial Cell Supplement Kit	PromoCell
Gibco® Fetal Bovine Serum (FBS)	Life Technologies
Gibco® Glutamine	Life Technologies
Gibco® Non-essential amino acids (NAEE) 100x	Life Technologies
Gibco® Penicillin/streptomycin 100x	Life Technologies
Gibco® RPMI 1640	Life Technologies
Gibco® Sodium Pyruvate 100mM	Life Technologies
Gibco® Trypsin/EDTA	Life Technologies

2.1.4 Buffers, solutions and media

All buffers, solutions and media (**Table 4**) were prepared using water from the Milli-Q Synthesis (Millipore) water purification system.

Table 4: Composition of general buffers, solutions and media.

Name	Composition
Acrylamide-bisacrylamide [SDS-PAGE]	46.5 g acrylamide, 3 g bisacrylamide in 100 ml water
Anode buffer [SDS-PAGE]	100 mM Tris- HCl, pH 8.9
Cathode buffer [SDS-PAGE]	100 mM Tris, 100 mM tricine, 0.1% SDS
Denaturating sample buffer [SDS-PAGE]	100 mM Tris-HCl, pH 6.8; 4% SDS , 20% glycerol , 4% β-mercaptoethanol , 0.005% (w/v) bromophenol blue
Destaining solution [SDS-PAGE]	10% (v/v) Ethanol, 7% (v/v) Acetic acid
DMEM culture medium	10 % FBS, 1% Sodium Pyruvate, 1% NEAA, 1% Streptomycin/Penicillin in DMEM
Gel buffer [SDS-PAGE]	3 M Tris-HCl, 0.3 % SDS, pH 8.45
Krebs-Henseleit-solution (KHS)	11 mM D-Glucose , 1 mM $MgSO_4$, 1 mM KH_2PO_4, 4.6 mM KCL, 12 mM NaCl
Luria-Bertani (LB) medium	1 % tryptone, 1 % NaCl, 0.5 % yeast extract in water
NaH_2PO_4 buffer	100 mM NaH_2PO_4 x H_20, pH 8.0
Perchloric acid solution	350 mM perchloric acid in ultrapure water
Pre-perfusion solution	5 mM HEPES, 4 nM $CaCl_2$ in <u>Krebs-Ringer solution:</u> 120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24.4 mM $NaHCO_3$
Solvent A	0.05% TFA in acetonitrile
Solvent B	0.05% TFA in ultrapure water

Staining solution [SDS-PAGE]	0.1% Coomassie-Brilliant-Blue R 250 , 50% Methanol , 10% Acetic acid
TE buffer	100 mM Tris, 12 mM EDTA, pH 7.4
TFA solution for HPLC and LCMS	0.05 % TFA in deionized water
Tris/BSA buffer	50 mM Tris-HCl, traces of BSA, pH 7.4

2.1.5 Kits and Assays

Kits and Assays used to determine levels of poteins and peptides, to isolate DNA as well as for quantitative real-time polymerase chain reaction are listed in **Table 5**.

Table 5: Kits and Assays.

Name	Company
BCA Protein Assay Kit	Bio-Rad
cGMP complete ELISA Kit for cells	Enzo Life Sciences
cGMP complete ELISA Kit for blood	Enzo Life Sciences
Plasmid Maxi Kit	QIAGEN
QuantiTect® Reverse Transcription Kit	QIAGEN
QuantiTect® SYBR® Green PCR Kit	QIAGEN

2.1.6 Recombinant enzymes

Table 6: Recombinant enzymes.

Name	Company
Angiotensin-converting enzyme (ACE)	R&D
Angiotensin-converting enzyme 2 (ACE2)	R&D
Carboxypeptidase A (CPA)	Life Technologies
Carboxypeptidase B (CPB)	Life Technologies
Carboxypeptidase C/Cathepsin A (Cath A)	Life Technologies
Collagenase Type II	Life Technologies
Endothelin-converting enzyme 1 (ECE-1)	R&D
Meprin A	R&D
Neprilysin (NEP)	R&D

Prolylcarboxypeptidase (PRCP)	R&D
Proteinase K	AppliChem
Trypsin	R&D

2.1.7 Enzyme Inhibitors

Table 7: Enzyme inhibitors.

Name	**Company**
Actinonin	Santa Cruz Biotechnology
Alliskiren	Selleck Chemicals LLC
Amastatin	Sigma-Aldrich
Aprotinin	Santa Cruz Biotechnology
Bestatin	Roche Applied Science
Candoxatrilat	Gift from Dr. W.E. Siems, FMP, Germany
Captopril	Santa Cruz Biotechnology
Carboxypeptidase inhibitor (CP inhib.)	Sigma-Aldrich
Chymostatin	Santa Cruz Biotechnology
Diprotin A	Bachem
EDTA	Sigma-Aldrich
F480	Gift from Prof. V. Dive, iBiTec-S, France
GEMSA	Calbiochem
Kallistop	American Peptide
Leupeptin	Santa Cruz Biotechnology
Pepstatin	Bachem
Roche complete [without EDTA]	Roche Applied Science
SM19712	Sigma-Aldrich
Trp-inhibitor (trp.inhib.)	Merck

2.1.8 Consumables

General laboratory consumables (tubes, tips, cell culture dishes and so forth) were obtained from Sarstedt or from the suppliers listed in **Table 8**.

Table 8: Consumables and their suppliers.

Name	Company
Cryo-tubes	Greiner Bio-One
HPLC vials	Chromacol
Needles	BD Microlance
Osmotic Minipumps [Model 2002]	Alzet
PCR tubes	Thermo Scientific
Scalpels	Feather
Seriological Pipets	Becton Dickinson
Silk Silicam®	Braun
Suture	Ethicon
Syringes	Braun

2.2 Methods

2.2.1 Synthesis of BNP1-32 and BNP metabolites

All BNPs have been synthesized at the BIOSYNTAN GmbH in Berlin, Germany via a simultaneous multiple peptide synthesis method using the Fmoc/But strategy according to SHEPPARD[221]. Briefly, the linkage has been performed using Fmoc-aa-OH/TBTU/N-Methylmorpholin (1:1:2), which was coupled to the polymeric matrix Tentagel S Trityl resin (RAPP Polymere, Germany). The cyclisation was performed using PBS-buffer with 10% DMSO. The following protective groups have been used: Cys(Trt), Arg(Pbf), Ser(But), Thr(But), Tyr(But), Asp(OBut), Glu(OBut), Asn(Trt), Gln(Trt), Lys(Boc), His(Trt), Trp(Boc). The elimination of the protection groups was achieved with trifluoacetic acid/thioanisole/thiocresol (95:2.5:2.5) within 3 h in the presence of 3% triethylsilane and following administration of 5% Me_3SiCl[222].

2.2.2 Incubation studies

2.2.2.1 Preparation of organ membranes

Organs, in particular lungs, livers, kidneys, spleens, and hearts (ventricles and atria) have been explanted from male C57BL/6 mice in the age of 4 to 6 month directly after cervical dislocation of the animals. Membranes of the organs were prepared by homogenizing them in an ice-cold 50 mM Tris buffer (pH 7.4) with a homogenizer. After filtration through a nylon gaze samples were centrifuged at 40.000 *g* to separate membranes from the cytosol. After a washing step with 50 mM Tris buffer, the pellet was resuspended in the same buffer and stored at -80°C until further processing. The total protein contents of the membrane preparations were determined by BCA Protein Assay Kit (Bio-Rad, Germany) and adjusted depending on the experiment (see section 2.2.2.3).

2.2.2.2 Preparation of serum, citrate- and heparin blood

Blood was taken from male C57BL/6 mice in the age of 4 to 6 weeks directly after cervical dislocation by cardiac puncture. To prepare serum, blood was left at room temperature for 20 min to reach sufficient clotting. After this, the blood was centrifuged at 7000 *g* for 10 min and supernatant was snap frozen in liquid nitrogen. Citrate- and heparin blood have been taken with syringes containing 10% citrate or heparin. Immediately after blood withdrawal, samples have been centrifuged at 6000 *g* for 10 min and supernatants have been snap frozen in liquid nitrogen.

2.2.2.3 Protein quantification of membrane and blood preparations

Protein concentration has been determined with the BCA Protein Assay Kit (Bio-Rad, Germany) according to their instructions based on the method of Bradford *et al.*[223]. In brief, the samples have been put in duplicates on a microplate and mixed with a reagent containing Coomassie brilliant blue G-250 dye, phosphoric acid, and methanol. After 30 min of incubation at 37°C and cooling down to room temperature the absorbance has been measured at 562 nm on the $VERSA_{max}$ *tunable* microplate reader (Molecular Devices, California).

2.2.2.4 Incubation of BNP1-32 and BNP metabolites with organ membrane preparations

To investigate the metabolism of BNP1-32 or BNP metabolites by the proteolytic activity of cellular membranes of organs, appropriate peptides were incubated with organ membrane preparations (see section 2.2.2.1) in a 50 mM Tris/BSA buffer. The buffer was supplemented with traces of BSA to minimize adhesion of the peptides on the tube walls. Samples have been incubated at 37°C under constant shaking at 300 U/min using an HLC Cooling-ThermoMixer MKR 13 (DITABIS, Germany). Reactions were stopped after appropriate incubation times by inducing a pH change by supplementation of ½ vol 0.35 M perchloric acid. After centrifugation at 12.000 *g* supernatants were analyzed by High Performance Liquid Chromatography (HPLC) and Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Conditions for the different experimental settings are described in the following passages:

- For the investigation of proteolysis rates of BNP1-32 with organ membrane preparations 10 µM of the peptide have been incubated with 60 µg protein of the organ preparations in a reaction volume of 100 µl for 90 min.
- For the investigation of initial product peaks of BNP1-32 metabolism by organ membrane preparations, 10 µM of BNP1-32 have been incubated with 60 µg protein of the organ membrane incubations in a reaction volume of 100 µl for the following times: 90 min for heart and spleen, 60 min for lung, 50 min for liver, and 15 min for kidney incubations.

2.2.2.5 Incubation of BNP1-32 with organ membrane preparations in the presence of enzyme inhibitors

Ten µM of BNP1-32 were incubated with 60 µg protein of organ membrane preparations in a 50mM Tris/BSA buffer in the presence of the following enzyme inhibitors: actinonin [10 µM], alliskiren [10 µM], aprotinin [10 µM], candoxatrilat [10 µM], captopril [10 µM], CP.inhib [10 µM], Chymostatin [5µM], diprotin A [10 µM], EDTA [5 µM], F480 [1 µM], GEMSA [10 µM], Kallistop [10 µM], Leupeptin [10 µM], pepstatin [10 µM], roche cocktail 10x [1 µl], SM19712 [10 µM], tryp.inhib [10 µM], ZPP [100 µM] in a reaction volume of 100 µl. The buffer was supplemented with traces of BSA to minimize adhesion of the peptides on the tube walls. Samples have been incubated at 37°C under constant shaking at 300 U/min using an HLC

Cooling-ThermoMixer MKR 13 (DITABIS, Germany). Reactions were stopped after the following incubation times: 120 min for heart, atrium, ventricle, and spleen, 60 min for lung, 50 min for liver by inducing a pH change by supplementation of ½ vol 0.35 M perchloric acid. After centrifugation at 12.000 *g* supernatants were analyzed by HPLC and MALDI-TOF-MS.

2.2.2.6 Incubation of BNP1-32 and BNP metabolites with serum, citrate- and heparin plasma with or without enzyme inhibitors

To investigate the metabolism of BNP1-32 or BNP metabolites by the proteolytic activity in blood, appropriate peptides were incubated with blood preparations in a 50 mM Tris/BSA buffer. The buffer was supplemented with traces of BSA to minimize adhesion of the peptides on the tube walls. Samples have been incubated at 37°C under constant shaking at 300 U/min using an HLC Cooling-ThermoMixer MKR 13 (DITABIS, Germany). Reactions were stopped after appropriate incubation times by inducing a pH change by supplementation of ½ vol 0.35 M perchloric acid. After centrifugation at 12.000 *g* supernatants were analyzed by HPLC and MALDI-TOF-MS. Conditions for the different experimental settings are described in the following passages:

- For the investigation of BNP1-32 proteolysis rates and identification of BNP metabolites, 10 µM BNP1-32 have been incubated with 140 µg protein of the blood preparations in a reaction volume of 100 µl for 90 min.
- For the investigation of proteolytic stability of BNP1-32 and BNP metabolites, 10 µM BNP1-32 have been incubated with 14 µg protein of the blood preparations in in a reaction volume of 100 µl for 10, 20, 30, and 40 min. For BNP1-29 and BNP1-32 incubations additional time points have been investigated (0-240 min).
- For the investigation of proteolysis rates of BNP1-30 and BNP7-30 in serum in the presence of GEMSA, 10 µM BNP1-30 or BNP7-30 were incubated with 14 µg protein of serum in the presence of 10 µM GEMSA in a reaction volume of 100 µl for 10 min

2.2.2.7 Incubation of BNP1-32 and BNP metabolites with recombinant enzymes

ANP, CNP, BNP1-32, or BNP metabolites were incubated with one of the following recombinant enzymes: ACE, ACE2, CathA, CPA, CPB, ECE-1, meprin A, or NEP in a

50 mM Tris/BSA buffer. Specific activities of the enzymes are indicated in the figure legends of the graphs demonstrating the results of the particular approaches. The concentrations of ANP, CNP, BNP1-32, or BNP metabolites in the incubation were 5 µM for the incubations with PRCP and CathA or 10 µM for all other incubations. The buffer was supplemented with traces of BSA to minimize adhesion of the peptides on the tube walls. Samples have been incubated at 37°C under constant shaking at 300 U/min using an HLC Cooling-ThermoMixer MKR 13 (DITABIS, Germany). Reactions were stopped after appropriate incubation times that are indicated in the figure legends of the particular approaches by inducing a pH change by supplementation of ½ v 0.35 M perchloric acid. After centrifugation at 12.000 *g* supernatants were analyzed by HPLC and MALDI-TOF-MS.

2.2.3 Analysis and quantification of natriuretic peptides with High Performance Liquid Chromatography (HPLC)

After the incubation studies, samples have been investigated for emerging product peaks resulting from the cleavage of BNP1-32 or BNP metabolites (see section 2.2.2). Furthermore, these product peaks have been quantified. Peptide analysis was performed by reversed phase chromatography using the UFLC-2020 System with UV-detection (Shimadzu, Germany). After a sample was centrifuged for 10 min at 12.000 *g*, 100 µl of the supernatant were loaded by the autosampler onto a RP Nucleosil 100 C12 column (Phenomenex, Germany; column dimensions: 250 x 4.6 mm, 5 µm). The sample and the mobile phase have been pumped through the system with a flow rate of 1 ml/min. The temperature of the column and the samples was regulated by a column oven and a cooling system connected to the autosampler. The temperature has been set to 25°C during all measurements. After getting initial chromatograms, HPLC methods have been optimized using these samples or recombinant peptides to allow good separation of peaks of BNP1-32, BNP metabolites, and other natriuretic peptides like ANP and CNP (Method 1-3).

Furthermore, a cleaning method and methods for the recovery of the column have been developed (Method 4-7):

Method 1 = Separation of BNP metabolites in organ membrane preparations
Method 2 = Separation of BNP metabolites in blood preparations
Method 3 = Separation on ANP and CNP
Method 4 = Cleaning
Method 5 = Recovery of initial solvent mixtures (Method 1)
Method 6 = Recovery of initial solvent mixtures (Method 2)
Method 7 = Recovery of initial solvent mixtures (Method 3)

For all methods different linear gradients using 0.05% trifluoracetic acid in acetonitrile (Solvent A) and 0.05% trifluoracetic acid in ultrapure water (Solvent B) were used (**Table 9**). Every ten injections the column has been cleaned with a cleaning method (Method 4) followed by a short method to recover the initial solvent mixture (Method 5-7). The peaks were detected at 205 nm and peak areas have been determined by peak integration with the *LabSolutions software version 5.11.160* (Shimadzu, Germany). A linear correlation between peak areas and peptide concentrations was confirmed before analysis.

Table 9: Methods used for High Performance Liquid Chromatography to separate natriuretic peptides. Solvent A: 0.05% trifluoroacetic acid in acetonitrile; Solvent B: 0.05% trifluoroacetic acid in ultrapure water.

	Gradient phase			**Recovery phase**		
	Solvent A [%]	**Solvent B [%]**	**Time [min]**	**Solvent A [%]**	**Solvent B [%]**	**Time [min]**
Method 1	18-35	82-65	20	18	82	5
Method 2	17-26	83-74	10	17	83	5
Method 3	20-32	80-68	9	20	80	5
Method 4	1-70	99-30	50	-	-	-
Method 5	-	-	-	18	82	10
Method 6	-	-	-	17	83	10
Method 7	-	-	-	20	80	10

2.2.4 Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS has been performed in collaboration with Prof. Lochnit at the Protein Analytics facility, University of Giessen, Germany. MALDI-TOF-MS was performed on an *Ultraflex I TOF/TOF* mass spectrometer equipped with a nitrogen laser and a LIFT-MS/MS facility (Bruker Daltonics, Germany). The instrument was operated in the positive-ion reflectron mode. Sum spectra consisting of 200–400 single spectra were acquired. One µl of the sample has been mixed with one µl of the matrix consisting of 2.5-dihydroxybenzoic acid and methylendiphosphonic acid dissolved in a solution of trifluoroacetic acid, acetonitrile, and water. The sample has been placed on the MALDI target (*MPT AnchorChip 800-384*, Bruker Daltonics, Germany). After the solvents were vaporized, the analyte molecules were left embedded into the recrystallized matrix, which has been analysed. For external calibration a peptide standard (Bruker Daltonics, Germany) was used. For data processing and instrument control the *Compass 1.1* software package consisting of *FlexControl 2.4*, *FlexAnalysis 3.0* and *BioTools 3.0* (Bruker Daltonics, Germany) was used.

2.2.5 Quantitative real-time polymerase chain reaction

The quantitative real-time polymerase chain reaction (RT-PCR) was carried out in three steps including (I) isolation of RNA, (II) reverse transcription, and (III) SYBR Green-based RT-PCR. All steps are described in the following sections.

2.2.5.1 (I) Isolation and quantification of RNA

RNA has been isolated from cardiac tissue of male spontaneously hypertensive rats (SHR) after appropriate experiments (see section 2.2.11.3). A small piece of the ventricle has been homogenized in 1 ml TRIzol (100 mg organ in 1 ml TRIzol). To one ml of the homogenate 0.2 ml of chloroform has been added and mixed firmely. After 30 min centrifugation at 4°C and 10,000 *g*, ½ v isopropanol has been added to the supernatant. After 10 min of incubation at room temperature, the samples have been centrifuged 10 min at 4°C and 12,000 *g*. 1.5 ml ice-cold 70% ethanol has been transferred to the dried pellet. After mixing and centrifugation for 8 min at 4°C and 7,500 *g* the pellet has been air-dried again. The pellet was resuspended in RNAse free

water. The samples have been used for RNA quantification with a NanoDrop™ Spectrophotometer (ThermoScientific, Delaware). One microliter of a sample was applied to the target and absorption has been measured at 260 nm. The purity was evaluated by the A260/A280 value. A ratio of ~2.0 was generally accepted as pure for RNA.

Subsequently, one microliter of RNA of each sample has been used for reverse quantification.

2.2.5.2 (II) Reverse transcription

The *QuantiTect® Reverse Transcription Kit* (QIAGEN, Germany) was used according to the manufacturers' instructions[224]. In brief, the procedure comprised two main steps: the elimination of genomic DNA using a *wipeout buffer* and the reverse transcription using a reverse transcriptase with RNA-dependent DNA-polymerase activity and RNase H activity. Generated cDNA was directly used for RT-PCR.

2.2.5.3 (III) SYBR Green-based RT- PCR

Quantitative RT-PCR was performed with the *QuantiTect SYBR Green PCR* (QIAGEN, Germany) according to the manufacturers recommendations[225] using the *iQ5 Real Time PCR system* (Bio-Rad, Germany). Primer pairs used for the amplification of specific cDNA regions are listed in **Table 10**.

Table 10: Primer sequences of reverse primer (R) and forward primer (F) used for quantitative real-time polymerase chain reaction.

Name	Sequence
COL1A1 rat **R**	5'-TGC CAG CGG GAC CAA CAG CG-3'
COL1A1 rat **F**	5'-GCC CGT GGA CAG GCT GGT GTC-3'
ANP rat **R**	5'-TCC TCC AGG TGG TCT AGC AGG TTC TTG-3'
ANP rat **F**	5'-GGC CTT TTG GCT CCC AGG CCA-3'
BNP rat **R**	5'-AGG TGG TCC CAG AGC TGG GGA AAG A-3'
BNP rat **F**	5'-CGC TTG GGC TGT GAC GGG CT-3'
HPRT1 rat **R**	5'-CCC TTC AGC ACA CAG AGG GCC AC-3'
HPRT1 rat **F**	5'-CCC TCA GTC CCA GCG TCG TGA TTA GT-3'

Reactions were carried out in total volumes of 20 µl and included 200 nM of each, forward (F) and reverse (R) primer, 1 µl of the primed cDNA, 12.5 µl of *SYBR Green-based RT-PCR Master Mix* (QIAGEN, Germany), and sterile water. The RT-PCR conditions are listed in **Table 11**.

Table 11: Conditions used for quantitative real-time polymerase chain reaction.

Steps	Time	Temperature	Cycles
Initial Heat activation	15 min	95	1
Denaturation	15 sec	94	40
Annealing	30 sec	58	
Elongation	30 sec	72	
Melting point analysis	1 min		1

The cycle threshold (C_T) was determined in each sample for both, the *BNP* gene amplification and the amplification of the house keeping gene *HPRT1*, encoding for the hypoxanthine phosphoribosyltransferase 1, during the beginning of the exponential phase. The relative mRNA expression levels were calculated using the formula $2^{\Delta\Delta C_T}$. Melting curve analyses were performed after RT-PCR reactions to confirm correct amplification.

2.2.6 Development of tricine- sodium dodecyl sulfate polyacrylamide gel electrophoresis for separation of BNP metabolites

For the separation of BNP metabolites a protocol was developed on the basis of the tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (*Tricine-SDS-PAGE)* from H. Schaegger[226]. The gel was made from three single gels, the separating gel, the spacer gel and the stacking gel. **Table 12** presents the composition of each gel. All gels were, upon mixing and loading on a vertical electrophoresis apparatus (Bio-Rad, Germany), left for 20 min to polymerise. Isopropanol was applied on the top of each gel to allow the formation of a smooth surface. Ten µg of the peptide was mixed 1:1 with the denaturating sample buffer and water and were incubated for 15 min at 37°C. Ten µl of each sample has been loaded onto the stacking gel and electrophoresis was performed until the sample buffer left the gel.

Table 12: Composition of gels used in the tricine- sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE).

Reagent	Separating gel	Spacer gel	Stacking gel
Acrylamide-bisacrylamide buffer	10 ml	6 ml	1 ml
Gel buffer	10 ml	10 ml	6 ml
Glycerol	-	3 g	-
Urea	10.8 g	-	-
Water	30 ml	30 ml	12 ml
APS (10%)	100 µl	150 µl	90 µl
TEMED	10 µl	15 µl	9 µl

The staining of proteins on the gels was performed with Coomassie-Brilliant-Blue R 250 solution for 20 min at room temperature. The excess staining was removed with destaining solution.

2.2.7 Microbiological methods

2.2.7.1 Transformation of competent *Escherichia coli DH5α*

50 µl of a competent cell suspension of *Escherichia coli DH5α* was thawed on ice. Plasmid DNA with genes either encoding for the natriuretic peptide receptor A or B was added to the bacterial cells and incubated for 30 min on ice. A thermal shock of 42˚C was applied for 45 sec and the cells were cooled on ice for 1-2 min afterwards. After addition of 1 ml LB-medium, cell suspensions have been incubated for 1 h at 37°C. Subsequently cells were spread on agar plates with the appropriate antibiotic selection and incubated overnight at 37˚C.

2.2.7.2 Over Night culture and amplification of the plasmids

A single colony of transfected bacterial cells was picked with a sterile pipette tip and incubated in 5 ml LB-medium over night at 37°C with the appropriate anitibiotics. This culture was diluted with 200 ml of LB-medium with appropriate antibiotics and cultured for 16 to 18 h at 37°C. Thereafter, cells were harvested by centrifugation.

2.2.7.3 Preparation of glycerol stocks

800 µl of an overnight culture were mixed with 200 µl of sterile glycerol and stored at -80˚C.

2.2.7.4 Isolation of plasmid DNA from *Escherichia coli DH5α*

Cells were centrifuged at 3,000 *g* for 10 min. Plasmid DNA was isolated using the Plasmid Maxi Kit (QIAGEN, Germany) according to the manufacturer's protocol. In brief, cells have been lysed by an alkaline lysis buffer, followed by binding of plasmid DNA to an anion-exchange resin under appropriate low salt and pH conditions. After three washing-steps with a washing buffer provided in the Kit, plasmid DNA has been eluted with 10 mM Tris buffer, pH 8.5.

2.2.8 Cell culture

2.2.8.1 Defrosting of mammalian cell stocks

Mammalian cells were stored in DMEM containing 10% DMSO in liquid nitrogen. The cells were thawed and immediately resuspended in DMEM to dilute the DMSO. Cells were centrifuged at 1000 *g* for 10 min at 4˚C. The medium was removed and the cells were resuspended in fresh DMEM and seeded on a 10 cm cell culture dish. Human embryonic kidney (HEK293) cells were cultures in DMEM culture medium (see **Table 4**). Human dermal microvascular endothelial cells (HDMEC) were cultured in Endothelial Cell Basal Medium supplemented with growth factors present in the Endothelial Cell Supplement Kit. The cultures were maintained at 37°C in a 5% CO_2 humidified incubator.

2.2.8.2 Passaging of mammalian cell lines

All mammalian cell lines were split in the same way. After removing the medium, cells were rinsed with PBS and incubated at 37˚C with 0.05% trypsin/EDTA until they were detached from the culture dish. The reaction was stopped by adding DMEM culture medium. After centrifugation at 1000g for 10 min at 4°C, cells were resuspended in fresh culture medium. HEK293 cells were split every 2-3 days when they reached 70-80% confluency at a ratio of 1:3 to 1:5. HDMEC were split once per week at a ratio of 1:3.

2.2.8.3 Preparation of cryostocks of mammalian cell lines

To prepare the cells for cryoconservation in liquid nitrogen, a confluent monolayer from a 10 cm plate was trypsinised with trypsin/EDTA, resuspended in cold DMEM culture medium and centrifuged at 1000 *g* for 10 min at 4°C. The pellet was resuspended in 900 µl DMEM culture medium and 100 ml DMSO. The cells were put in cryotubes at -20°C over night and were then transferred to liquid nitrogen on the following day.

2.2.8.4 Transfection of HEK293 cells

One day after seeding, HEK293 cells were transferred into 24-well cell culture dishes. Another 24 h later, transfections with the plasmid encoding NPRA or NPRB, or the empty plasmid were performed using PolyFect® reagent in DMEM culture medium with 1% FCS instead of 10% FCS. For that 0.25 µg DNA per well was incubated with 5 µl Polyfect® and 5 µl DMEM for 15 min at room temperature. Transfection solution was carefully applied to the cells without disrupting the monolayer. Cells transfected with the empty plasmid served as controls. After 24 hours cells were used for bioactivity measurements of natriuretic peptides (see section 2.2.9.1)

2.2.8.5 Isolation and culture of vascular smooth muscle cells

Vascular smooth muscle cells have been isolated from the explants of the thoracic aortas[227] of 4 to 6 weeks old male C57BL/6 mice. Vessels were opened longitudinally and incubated for 30 min with 4 mg/ml collagenase type II. Cells were seeded in small 6 cm culture dishes and grown in DMEM culture medium supplemented with 2 mmol/l glutamine[160]. Cells were harvested for passaging every 2 to 3 weeks. Cells have been used for stimulation experiments at passage 2 to 3. For experiments on the bioactivity of BNP metabolites, cells have been seeded into 24-well plates.

2.2.8.6 Isolation and culture of mesangial cells

Mesangial Cells have been isolated from explanted kidneys of 4 to 6 weeks old male C57BL/6 mice[228]. The kidneys were decapsulated and minced in ice-cold RPMI 1640 medium. Homogenates were dispersed with 5 ml chilled RPMI 1640 medium to a 100 µm nylon filter and then the filtrate transferred to a 70 µm nylon filter. After decapsulated, the glomerular 'cores' were digested with collagenase type II for approximately 30 min, then centrifuged at 1000 *g* for 10 min. The pellet was

resuspended in RPMI 1640 medium supplemented with 17% FCS, penicillin/streptomycin, and 0.1 U/ml insulin, and cultured at 37°C in a humidified 5% CO_2 incubator[228]. Cells were harvested for passaging every 2 weeks. Cells have been used for stimulation experiments at passage 2 to 3. For experiments on the bioactivity of BNP metabolites, cells have been seeded into 24-well plates.

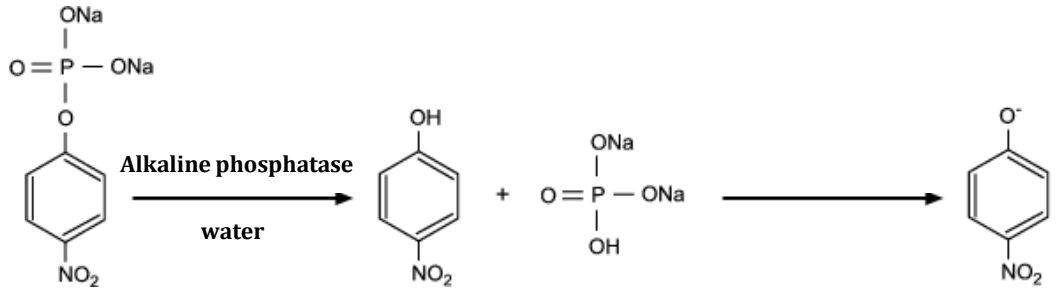
2.2.9 Bioactivity of BNP1-32 and BNP-metabolites *in vitro*

2.2.9.1 Bioactivity Measurements of BNPs in cells

Determination of bioactivity of BNP1-32 and BNP-metabolites was performed in transfected HEK293 cells or VSMC, HDMEC, and MC seeded in 24-well plates using the cGMP complete ELISA Kit for cells (Enzo Life Sciences, Loerrach, Germany) for the determination of cGMP levels. Medium was removed and vehicle or the appropriate solutions of the natriuretic peptides have been gently applied in each well without destroying the monolayer. Cells were incubated for 5 minutes at 37 °C. Afterwards, solutions were removed and a mixture of 150 µL of 0.1 M HCl and 0.1% Triton X100 was applied for the lysis of the cells. The plate has been incubated for another 10 min at 37°C. After harvesting the cells and centrifugation at 600 *g*, cGMP has been measured in the supernatants (see section 2.2.10.2).

2.2.9.2 Detection of cGMP in cells

Concentrations of cGMP were measured with the cGMP complete ELISA Kit for cells (Enzo Life Sciences, Loerrach, Germany) according to the instructions of the manufacturer. In brief, after neutralization of the samples with a neutralizing reagent provided in the Kit, samples have been pipeted into a goat anti-rabbit IgG-coated microtiter plate. A conjugate of alkaline phosphatase and cGMP was added. Afterwards a rabbit polyclonal antibody to cGMP was added to bind in a competitive manner either cGMP of the conjugate or the samples. After a 2 hour incubation time at room temperature and three washing steps with a buffered saline solution containing detergents, p-nitrophenyl phosphate was added. The remaining conjugated phosphatase bound by the antibody forms p-nitrophenol, which reacts under alkaline conditions forming p-nitrophenolate as follows:



p-nitrophenyl phosphate **p-nitrophenol** **p-nitrophenolate**

P-nitrophenolate has a yellow color. The intensity of the color is inversely proportional to the concentration of cGMP initially present in the samples. The absorbance was detected at 405 nm with a correction at 570 nm and 590 nm.

2.2.10 Bioactivity of BNP1-32 and BNP metabolites *ex vivo*

Investigations on isolated vessels using the myograph system have been performed in teamwork with Dr. Yong Wang in the ECCPS, Giessen. Mesenteric resistance arteries from male Sprague Dawley rats (4 month old) were used for all experiments. The arteries were dissected from the surrounding connective tissue and placed in Krebs-Henseleit solution (KHS). Second order artery segments, approximately 1 mm in length, were cut and mounted in a myograph chamber (Danish Myo Technology, Denmark). Each of the four myograph units is attached to the force transducer on one side and to a micrometer on the other side. All segments have individually controlled gas inflow and temperature control permitting the preparations in all four chambers to be kept under 37°C, in the constantly bubbled (95% O_2 plus 5% CO_2) KHS ensuring an examination under isometric conditions. Each vessel ring was placed under optimal stretch of approximately 100 mmHg by determining passive length-tension relationships using normalization software (ADInstruments, Germany).

The rings were precontracted with an enriched KHS (125 mM KCl) to determine the maximum contractility and then washed with normal KHS several times. Endothelium integrity was assessed by contracting the segments with noradrenalin, followed by stimulation with acetylcholine. After washing to restore tension to baseline level the rings where allowed to stabilize for 30 min.

2.2.11 Bioactivity of BNP1-32 and BNP metabolites *in vivo*

2.2.11.1 Acute infusion of BNP1-32 and BNP metabolites in mice

Male C57BL/6 mice of about 16 weeks were initially anesthetized with 4% isoflurane and were intubated for artificial ventilation with a mixture of 20% oxygen and 80% room air through a rodent ventilator, to which 2% to 2.5% isoflurane was added for continuous anesthesia. During surgery, animals were kept on a homeothermic blanket to maintain body temperature. The right common carotid artery and the right internal jugular vein have been dissected. A Mikro-Tip® mouse pressure catheter (Millar instruments, Texas) was inserted into the carotid artery and a cannula has been inserted into the jugular vein. Baseline blood pressure and heart rate were monitored and recorded for 10 minutes with a pressure transducer system and a blood pressure monitoring software (TSE systems GmbH, Germany). Through the cannula a single bolus injection of BNP1-32, BNP1-30, BNP1-29, BNP7-32, BNP7-30, BNP1-45 (100 µl/30 g body weight of a $5x10^{-6}$ M solution), or saline has been performed. The hemodynamic parameters were measured for 4 min after injection. Subsequent to the measurement, EDTA blood was taken by puncturing of the left ventricle. Blood samples were centrifuged, and the plasma was snap frozen in liquid nitrogen and stored at -80°C until further processing. Plasma cGMP levels were measured using the cGMP complete ELISA Kit for blood (Enzo Life Sciences, Loerrach, Germany) according to the manufacturer's instructions (see section 2.2.12.2). Animal numbers used are mentioned in the appropriate sections (see Results section 3.7).

2.2.11.2 Detection of cGMP in plasma

Concentrations of cGMP in plasma were measured with the cGMP complete ELISA Kit for blood (Enzo Life Sciences, Loerrach, Germany) according to the instructions of the manufacturer. In brief, cGMP has been extracted from the plasma by adding 2 vol % of 95% ethanol to the collected plasma. After incubation for 5 min, samples have been centrifuged for 10 min at 600 *g*. The supernatant has been dried down in a concentrator (Eppendorf, Germany) and reconstituted in assay buffer provided in the Kit. Subsequently, the samples have been pipeted into a goat anti-rabbit IgG-coated microtiter plate. A conjugate of alkaline phosphatase and cGMP was added. Afterwards a rabbit polyclonal antibody to cGMP was added to bind in a competitive manner either cGMP of the conjugate or the samples. After a 2 hour incubation time at

room temperature and three wash-steps with a buffered saline solution containing detergents, p-nitrophenyl phosphate was added. The remaining conjugated phosphatase bound by the antibody forms p-nitrophenol, which reacts under alkaline conditions forming p-nitrophenolate having a yellow color. The intensity of the color is inversely proportional to the concentration of cGMP initially present in the samples. The absorbance is detected at 405 nm with a correction at 570 nm and 590 nm.

2.2.11.3 Continuous administration of BNP1-32 and BNP1-30 using the spontaneous hypertensive rat-model

Osmotic minipumps (Alzet, California; pump rate: 0.5 µl/h) have been put into sterile saline solution for three hours for preconditioning. After that, minipumps were filled each with either sterile saline, or filtered solutions of BNP1-32 or BNP1-30 to allow the administration of 300 ng/kg/min. Male spontaneously hypertensive rats (SHR) at the age of 12 weeks have been anesthetized through injection with ketamin/xylocaine/saline (3:0.5:6.5; 100 µl/100 g) and have been put under an infrared heating lamp to maintain body temperature. A small part of the fur on the backside was removed using depilatory crème (Reckitt Benckiser, England). Next, a primed osmotic minipump was implanted under the skin on the backside of the animal. Wounds have been closed with clips. After the animals woke up, they were kept in appropriate cages with normal housing and food for three days. For the determination of hemodynamic parameters, animals have been anesthetized with 4% isoflurane and were kept ventilated with a mixture of 20% oxygen and 80% room air through a rodent ventilator, to which 2% to 2.5% isoflurane was added for continuous anaesthesia. Animals were kept on a homeothermic blanket to maintain body temperature. The right common carotid artery has been dissected. A Mikro-Tip® rat pressure catheter (Millar instruments, Texas) was placed into the right carotid artery to measure arterial blood pressure and heart rate. Parameters were recorded for 10 minutes with a blood pressure monitoring software (TSE systems GmbH, Germany). The experimental procedure was performed in accordance to similar procedures described previously[91, 229].

Finally, EDTA blood was taken by puncturing of the left ventricle. Blood samples were centrifuged, and the plasma was snap frozen in liquid nitrogen and stored at -80°C until further processing. Plasma cGMP levels were measured using the cGMP

complete ELISA Kit for blood (Enzo Life Sciences, Loerrach, Germany) according to the manufacturer's instructions (see section 2.2.12.2). Animal numbers used are mentioned in the appropriate sections (see Results section 3.8).

2.2.12 Liver perfusion *in situ*

After anesthesia through injection with ketamin/xylocaine/saline (3:0.5:6.5; 10 µl/10 g), a male C57BL/6 mouse has been placed on a homeothermic blanket to maintain body temperature. The abdominal cavity has been opened and the *vena portae* and into the *vena cava inferior* have been dissected. The recirculation into the heart was prevented by the ligation of the *vena cava inferior* in front of the heart. A cannula has been inserted into *vena portae* and into the *vena cava inferior*. The pre-perfusion solution has been infused manually through the *vena portae* trying to maintain a constant pressure. The escaped blood and pre-perfusion solution has been collected through the *vena cava inferior*. This pre-perfusion allowed the removal of all blood in the liver. After the pre-perfusion phase, 3 ml of a pre-perfusion solution supplemented with 10^{-5} M BNP1-32 was infused through the *vena portae* into the liver and fractions of 150 µl each have been collected from the *vena cava inferior*. After the whole BNP1-32 solution was infused, the pre-perfusion solution was infused again and additional fractions have been collected to ensure that all infused quantities of BNP1-32 were eluted from the liver. All perfusion solutions have been heated to 37°C beforehand. All elution fractions collected were qualitatively and quantitatively analyzed with HPLC and MALDI-TOF-MS.

2.2.13 Experiments on animals

Experiments on animals, blood collection, and organ explanations were performed in accordance with the Animals (Scientific Procedures) Act 1986 of the Parliament of the United Kingdom, the Federal Law on the Use of Experimental Animals in Germany and were approved by the local authorities (Home Office London or Regierungspraesidium Giessen).

2.2.14 Statistics

For statistical comparisons, Student's *t* test, One-way ANOVA or Two-way ANOVA with Bonferroni's Multiple Comparison (Graph Pad Prism 5.01; Graph Pad Software Inc., San Diego, California) were performed (mean ± SEM). Significance was considered at a value of $P < 0.05$. It has been highlighted in the text or in the figure legends which of the statical tests have been used and which significances were calculated.

3 RESULTS

3.1 Metabolism of BNP1-32

BNP1-32 is synthesized in the heart and partly in other organs[26]. Moreover, it is transported to various organs within the blood stream. Thus, BNP1-32 is permanently exposed towards the catalytic activity of extracellular and membrane-bound enzymes.

It was aimed to investigate the metabolism of BNP1-32 and the formation of corresponding BNP metabolites by this catalytic activity. Firstly, the metabolism of BNP1-32 by membrane-bound enzymes of different organs highly supplied with blood has been determined and compared.

3.1.1 Metabolism of BNP1-32 by the proteolytic activity of different organs

3.1.1.1 Comparison of the proteolytic activity of organs towards BNP1-32

BNP1-32 has been synthesized as recombinant peptide via solid-phase synthesis (see Method section 2.2.1). The quality and purity of the synthetic peptide has been proven by HPLC and mass spectrometry. Recombinant BNP1-32 was incubated with murine membrane preparations of different organs harboring intact peptidase activities (see Method section 2.2.2). In particular, preparations of heart, lung, liver, kidney, and spleen were used. The remaining concentration of BNP1-32 in these incubations was quantified by HPLC and compared between the different organs at one distinct point of time. The experiment has been designed to get a qualitative overview about the proteolytic activity of the organ preparations towards BNP1-32.

Figure 9 illustrates that proteolytic activity towards BNP1-32 is very divergent between the organs investigated. Although protein concentration of the membrane preparations has been analyzed before and was normalized for each sample, the proteolysis of BNP1-32 was unequal. Hardly any proteolysis of BNP1-32 occurred in incubations with heart and spleen preparations, while only a small part of the initial peptide concentration was left when incubated with kidney preparations. The proteolysis of BNP1-32 observed in lung and liver preparations was similar. However, both organ preparations showed higher proteolytic activity towards BNP1-32 compared to heart and spleen but much less compared to kidney.

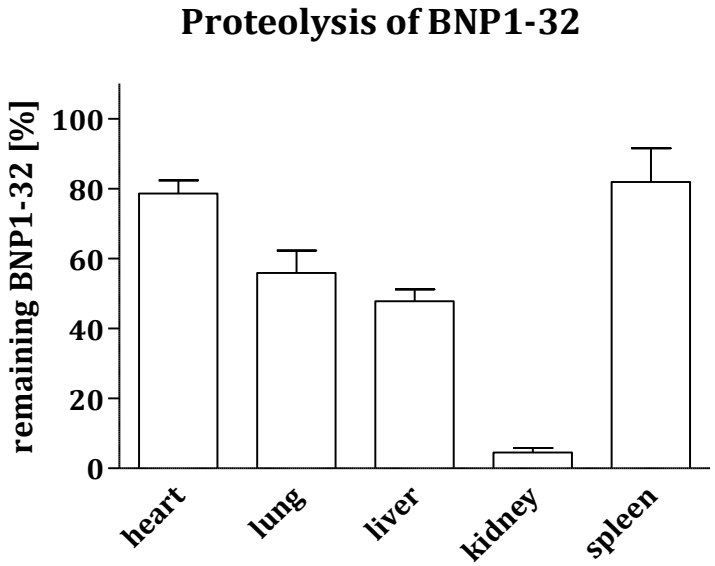


Figure 9: Quantification of the remaining concentration of BNP1-32 in percent after incubation with membrane preparations of heart, lung, liver, kidney and spleen for 90 min. The initial BNP1-32 concentration before incubation was set to 100%. Membrane preparations were normalized to protein concentration.

This first dataset highlights that proteolytic activities towards BNP1-32 diversifies between the organs indicating that the amount of proteolyzing enzymes differs from organ to organ. However, at the same time it is also likely that each organ does harbor different peptidases responsible for BNP1-32 proteolysis. Thus, emerging products of BNP1-32 proteolysis might vary between the preparations due to different enzymatic activity present in these organs. Such BNP products have been determined in each organ preparation in the following investigations.

3.1.1.2 Investigation of initial proteolytic products of BNP1-32

The formation of initial products emerging from the proteolysis of BNP1-32 has been investigated for each organ. The peptide has been incubated with the membrane preparations until proteolysis of 20 to 25 % was achieved. The emerging BNP1-32 cleavage products have been separated by HPLC (**Figure 10**).

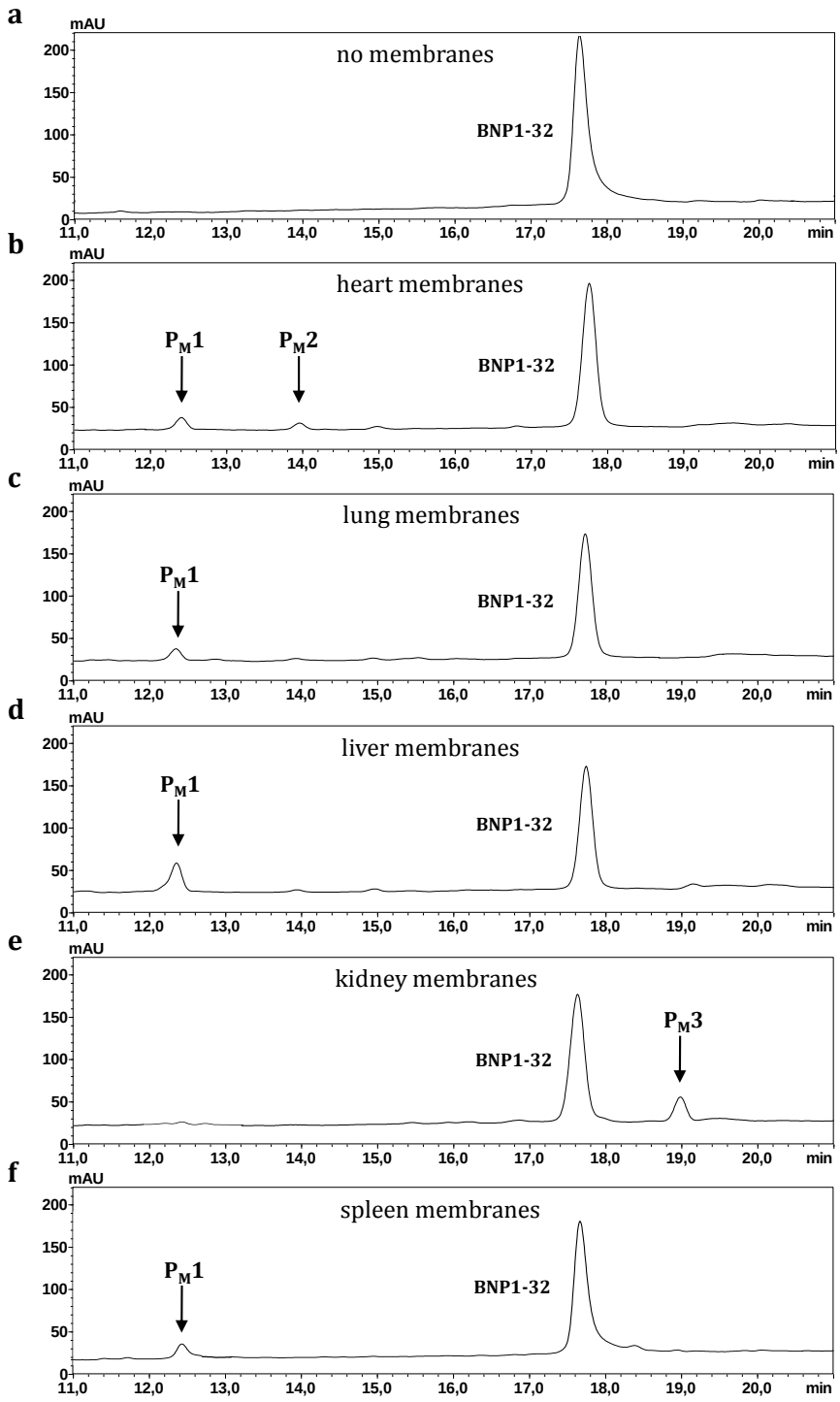


Figure 10: HPLC chromatograms of **(a)** BNP1-32 alone, and BNP1-32 incubated with membrane preparations of **(b)** heart, **(c)** lung, **(d)** liver, **(e)** kidney, and **(f)** spleen until 20-25% of the initial BNP1-32 concentration was proteolyzed. According to their retention times, initial cleavage products are labeled with P_M1, P_M2 and to P_M3. HPLC method 1 was used to separate peaks.

An optimal HPLC method has been established beforehand to allow distinct peak separation (see Methods section 2.2.3). For the identification of significant products, an automatic threshold for the integration of the peaks was set.

Figure 10a shows the peak of pure BNP1-32. Its retention time (rt) emerging from its interaction with the column with the set conditions is 17.7 min. Besides this peak, new peaks with different retention times occurred in the chromatograms obtained from the incubations with the different organ preparations (**Figure 10b-f**). These peaks represent cleavage products of BNP1-32. Two peaks were generated through incubation with heart ($P_M1\&2$), and one each in incubations with lung (P_M1), liver (P_M1), kidney (P_M3) and spleen (P_M1).

Among these six product peaks, three had the same retention time. Peak P_M1, eluting first, (rt_{PM1} = 12.3 min) occurred in all approaches except for kidney. Conversely to P_M1, peak P_M3 (rt_{PM3} = 19.0 min) occurred in kidney only. Peak P_M2 (rt_{PM2} = 13.9 min) was found in heart only.

It could be revealed that the generation of P_M1 was highest in liver compared to other organ preparations used. A comparison of the amount of P_M1 generated in the organs within 90 min, is shown in **Figure 11**, in which the peakarea of P_M1 in liver was set to 100%. The absolute peakarea of P_M1 in liver approximates 40% of the initial peak of BNP1-32.

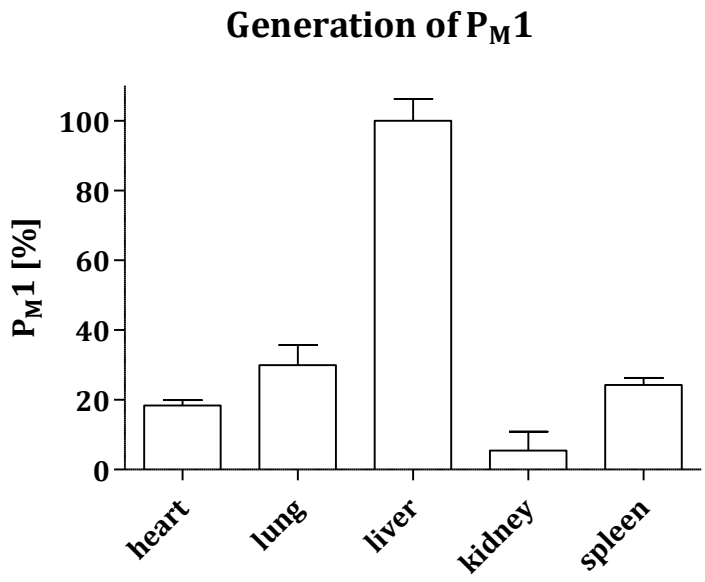


Figure 11: Quantification of the generation of P_M1 from BNP1-32 in percent after incubation with membrane preparations of heart, lung, liver, kidney and spleen for 90 min. Membrane preparations were normalized to protein concentration. The highest amount of P_M1 generation was set to 100%.

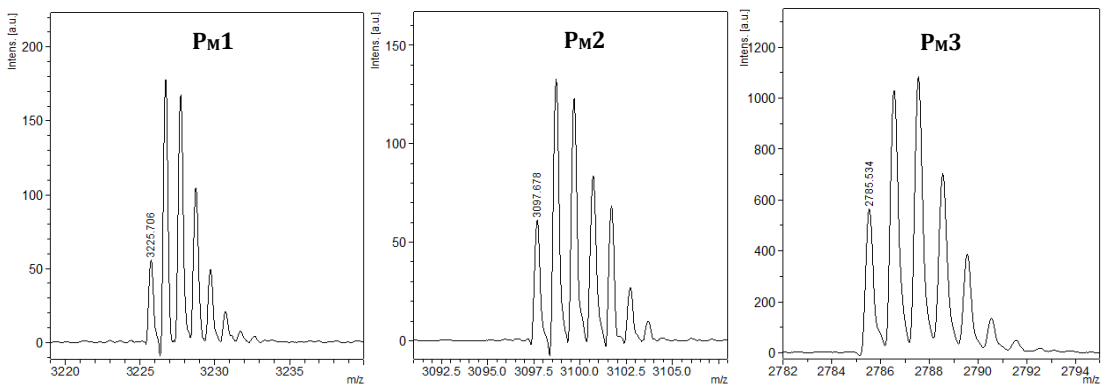
The occurrence of these different product peaks indicates that the enzymatic processing of BNP1-32 varies between the organs. The identification of the amino acid sequence of these products and hence the cleavage sites of BNP1-32, is described in the next chapter.

3.1.1.3 Identification of the initial proteolytic products of BNP1-32

Each peak has been manually fractionated after chromatographic separation for analysis with MALDI-TOF MS to obtain masses (see Methods section 2.2.4).

As expected, the six peaks could be identified as three different BNP peptide fragments varying in their sequence lengths. **Figure 12a** shows the MALDI-TOF spectra of the three products with their corresponding masses.

a



b

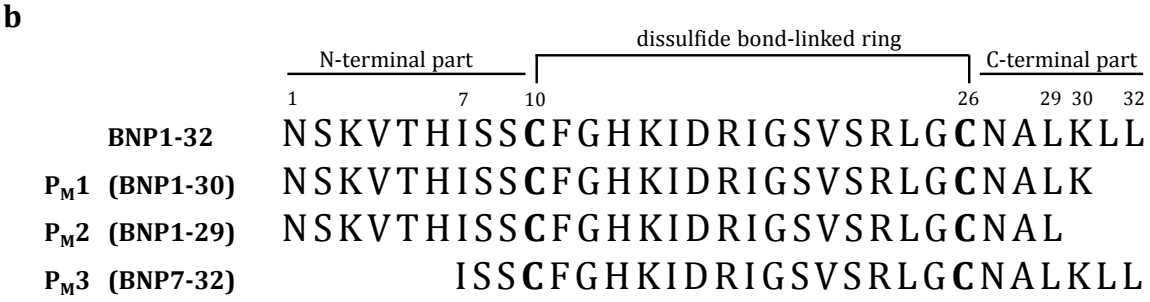


Figure 12: a) MADLI-TOF-MS spectra of the three different BNP products generated through incubation with organ membrane preparations. Each spectrum shows the isotope distribution of the mass peaks; **b)** Amino acid sequences of BNP1-32 and the three identified BNP products P_M1, P_M2 and P_M3 conducted from their masses. Relevant amino acids are numbered according to their order, beginning with the N-terminus of the mature BNP1-32.

Figure 12b displays the peptides' amino acid sequences conducted from their masses. For comparison also BNP1-32 is shown. The BNP fragments represented by the peaks P_M1 and P_M2 are C-terminally truncated, while the peptide of peak P_M3 is N-terminally truncated compared to the mature BNP1-32. P_M1, which occurred in all

incubations except for the one with kidney, is truncated by the two C-terminal leucine (L), decreasing the number of amino acids to 30. Subsequently, this peptide is named BNP1-30. The peptide represented by P_M2 is additionally shortened by lysine (K), reducing the number of amino acids to 29. Thus, the peptide is named BNP1-29. The third product, P_M3, is truncated by six amino acids at the N-terminal side and is named BNP7-32. BNP7-32 has been described before in 2007 by an approach of Pankow *et al.* as the renal meprin A (EC 3.4.24.18) cleavage product of BNP1-32[160].
According to the monoisotopic peptide masses obtained from MALDI-TOF-MS and the comparison with the linear forms of the peptides, it was revealed that the two cysteines at positions 10 and 26 are present in a non-reduced form and are involved in a disulfide bridge (C^{10}-C^{26}). Thus, all truncated forms obtained in this approach still harbor the disulfide-linked ring structure, which is the prerequisite for biological activity of natriuretic peptides[39] (see Introduction section 1.1.2). Hence, the three identified BNP products are referred to as metabolites rather than degradation products.

3.1.1.4 Overview of the proteolysis of BNP1-32 by organ membrane preparations

The investigation of the initial metabolism of BNP1-32 revealed that proteolytic activities towards the peptide vary between the organs. Kidney preparations had the highest proteolytic activity against BNP1-32, whereas the peptide was relatively stable in heart and spleen preparations (**Figure 9**). Moreover, the formation of three BNP metabolites was observed (**Figure 10**). The C-terminally truncated BNP1-30 was generated through the proteolytic activity of all organs except kidney. Comparing all organs, BNP1-30 was accumulating the most using liver membrane preparations (**Figure 11**). BNP7-32, the N-terminally truncated BNP metabolite was emerging in kidney only. The third BNP metabolite, BNP1-29 was generated in heart only. These results highlight that BNP metabolism is organ-dependent.
Moreover, it is possible that the BNP metabolites are further metabolized. To complement the results, the metabolites found have been further investigated for their metabolism.

3.1.1.5 Metabolism of the identified BNP metabolites

The identified metabolites BNP1-30, BNP1-29, and BNP7-32 have been synthesized via solid-phase synthesis and were incubated with the organ preparations to see if they are further processed.

Table 13 summarizes the results of this approach. Incubation of BNP1-30 led to a dominant formation of BNP1-29 in all organ preparations except for kidney. BNP1-29 has also been present in heart preparations incubated with BNP1-32, thus, BNP1-30 might function as an intermediate product for the formation of BNP1-29. The significant accumulation of BNP1-29 in heart which is initially not present in other organs also generating BNP1-30 might be due to an increased concentration of the peptidase responsible for the generation of BNP1-29 or a decreased concentration of the peptidase degrading BNP1-29.

Proteolysis of BNP1-29 led to the formation of another new BNP metabolite, BNP7-29, occurring in heart, lung and kidney but not in liver and spleen. Moreover, also BNP1-30 was metabolized to another new BNP metabolite, BNP7-30, in the same tissues, indicating that a proteolytic activity is able to remove the six N-terminal amino acids from both substrates similar to the formation of BNP7-32 in these tissues.

Incubation of BNP7-32 with organ preparations led to the generation of BNP7-30 in lung, heart, liver, and spleen. In kidney itself, the C-terminally truncation forming BNP7-30 was, similarly to BNP1-30, not happening.

Table 13: Summary of BNP metabolites occurring after incubation of the initial BNP metabolites BNP1-30, BNP1-29 and BNP7-32 with membrane preparations of heart, lung, liver, kidney and spleen.

	Formation of metabolites with membrane preparations of:				
	heart	**lung**	**liver**	**kidney**	**spleen**
BNP1-30	1-29 7-30	1-29 7-30	1-29 -	- 7-30	1-29 -
BNP1-29	7-29	7-29	-	7-29	-
BNP7-32	7-30	7-30	7-30	-	7-30

In conclusion, BNP1-32 and BNP metabolites can be C-terminally and N-terminally truncated in heart and lung. On the contrary, in kidney, only an N-terminal truncation is possible, whereas in liver and spleen only a C-terminal truncation of the peptides is

observed. This evolves the hypothesis that BNP metabolism is organ-specific and that full proteolysis of BNP1-32 needs the coactions of different organs and different peptidases.

To get a more physiological insight in the metabolism of BNP1-32 and to confirm the above results, it was decided to perform an organ perfusion *in situ*, exemplarily done in liver.

3.1.2 Metabolism of BNP1-32 during liver perfusion *in situ*

3.1.2.1 Formation of BNP1-32 products during liver perfusion

Murine liver was selected as organ of choice, since the generation of BNP1-30, which was found in nearly all tissues, was highest in this organ. After setting up the experiment and performing a pre-perfusion of the organ, the mature BNP1-32 was infused through the *vena portae* into the liver (see Method section 2.2.12). Twenty four fractions of the eluting BNP1-32-solution have been collected from the *vena cava inferior* over time and have been quantitatively and qualitatively analyzed with HPLC (Method 1) and MALDI-TOF-MS.

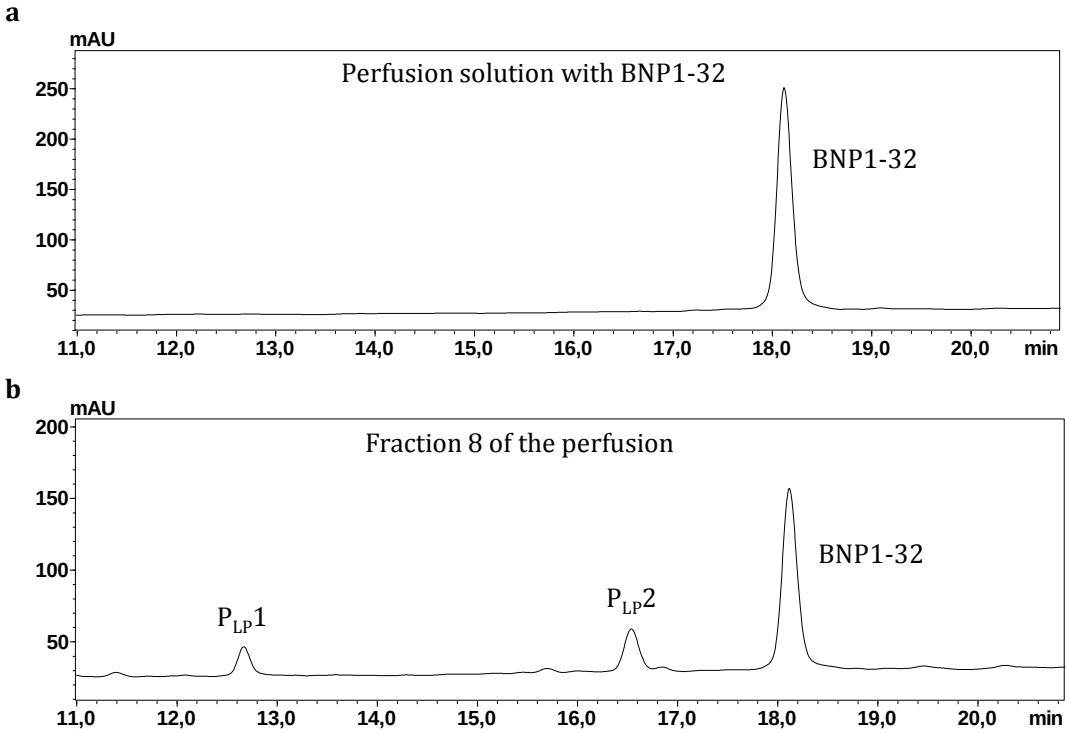


Figure 13: a) HPLC chromatogram of the perfusion solution containing BNP1-32. BNP1-32 occurs as a distinct peak with a retention time of 18.2 min; **b)** HPLC chromatogram of fraction 8 obtained during perfusion of the liver displaying the initial BNP1-32 peak and two BNP products, $P_{LP}1$ and $P_{LP}2$. HPLC method 1 was used.

The chromatogram in **Figure 13a** shows the distinct peak of BNP1-32 in the perfusion solution before passing the organ. In the elution fractions collected after the perfusion, exemplarily shown for the fraction 8 in **Figure 13b**, three different peaks occurred in the chromatogram, whereby one of these peaks (rt = 18.2 min) represents the initially infused BNP1-32. BNP1-32 concentration was reduced by approx. 40% compared to the pure perfusion solution. The additional two peaks $P_{LP}1$ (rt = 12.7 min) and $P_{LP}2$ (16.7 min) are BNP products generated by peptidase activities in this organ. The products and the initial BNP1-32 have been quantified in all fractions (**Figures 14a-c**)**.** The first three fractions after beginning of the perfusion contained the pre-perfusion solution and no peptide peaks. Thus the volume of these fractions, which was about 450 µl, represents the dead volume of the liver.

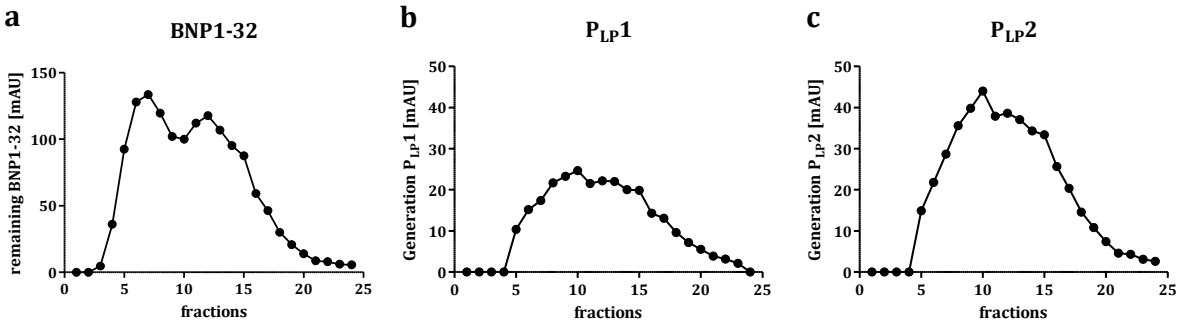


Figure 14: Quantification of **a)** BNP1-32 and BNP1-32 products **b)** $P_{LP}1$ and **c)** $P_{LP}2$ in the fractions obtained during liver perfusion in mAU.

From fraction 4 on, BNP1-32 levels began to rise (**Figure 14a**) followed by the appearance of two product peaks $P_{LP}1$ and $P_{LP}2$ (**Figure 14b&c**), indicating that BNP1-32 was successfully perfused trough the organ and that peptidase activities were present in the liver model. Summing up the peak areas of the two products and the remaining BNP1-32, less than 10% of the peptide concentration disappeared by e.g. retaining in the organ or by leakage, respectively. From fraction 10 on, the infusion of the BNP1-32-solution was stopped and exchanged with the pure perfusion solution. The slope seen in the peptide concentrations around this time was caused by the handling. From fraction 15 on the concentration of the peptides strongly started to decline, since peptide infusion was stopped and remaining peptide amounts were eluted.

3.1.2.2 Identification of BNP products formed during perfusion

With MALDI-TOF-MS the masses of the two products have been examined. Product 1 ($P_{LP}1$) could be assigned to the peptide BNP1-30, whose generation was already observed using the membranes of liver and also other organ preparations *in vitro*. The slight drift in the retention time compared to the approach using organ membrane preparations occurred due to the progressed life-time of the HPLC column.

Additionally a C- and N-terminally truncated peptide could be assigned to the mass of product 2 ($P_{LP}2$, 2313.6 Da). This peptide is truncated on the N- and C-terminus and was named according to its amino acid sequence BNP4-24 (**Figure 15**). BNP4-24, is very likely a biologically inactive peptide fragment since the cleavage site 24'-25' lies within the ring and destroys this structure. Thus, BNP4-24 is a degradation product rather than a metabolite and did not receive further attention in the following investigations.

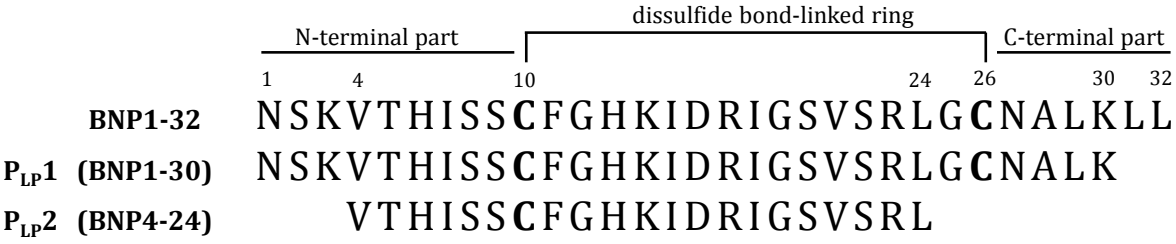


Figure 15: Amino acid sequences of BNP1-32 and the two identified BNP products $P_{LP}1$ and $P_{LP}2$, conducted from their masses. Relevant amino acids are numbered according to their order beginning with the N-terminus of the mature BNP1-32.

The data confirm above results using liver membrane preparations and demonstrate that BNP1-30 is a major cleavage product of BNP1-32.

Beside the exposure of BNP1-32 to membrane-bound enzymes, BNP1-32 is also in contact with extracellular proteolytic activity present in the circulation. In the following chapter, metabolism of BNP1-32 by proteolytic activity of blood has been investigated.

3.1.3 Metabolism of BNP1-32 by proteolytic activity of serum and plasma

3.1.3.1 Comparison of proteolytic activity towards BNP1-32 of different blood preparations

BNP1-32 is circulating in the blood and is thus transported to and through different organs. Chapter 3.1.1 demonstrated how BNP1-32 is metabolized by the proteolytic activity of distinct organs initially generating BNP1-30, BNP1-29, and BNP7-32. The blood itself contains major protein sources and high peptidase activities, e.g. for the breakdown of peptides. Thus, its proteolytic activity towards BNP1-32 was investigated.

The blood was taken from mice and diluted preparations of serum or plasma by adding heparin or citrate for anticoagulation have been made from it (see Method section 2.2.2). EDTA was not used as an anticoagulant in this approach to avoid inhibition of metallopeptidase activities in the blood that might influence BNP metabolism. However, citrate binds calcium ions, which decreases activity of enzymes using Ca^{2+} as cofactors.

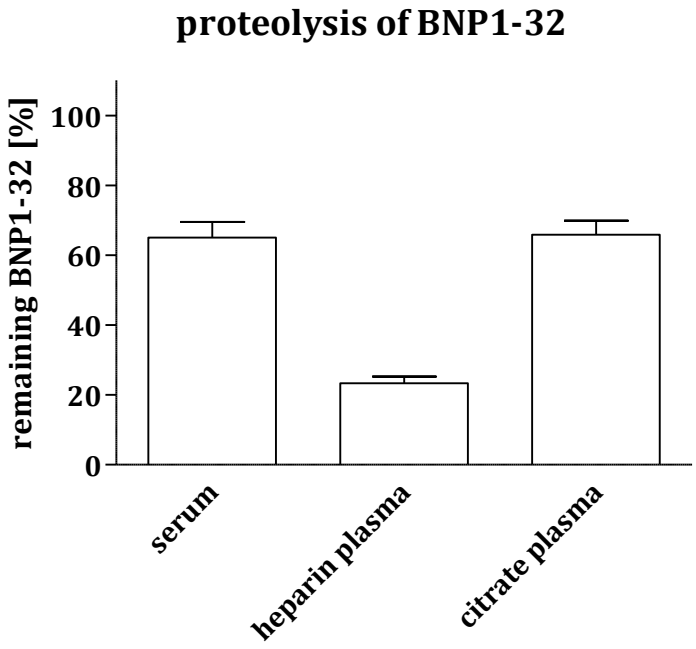


Figure 16: Quantification of the remaining concentration of BNP1-32 in percent after incubation with diluted serum, heparin plasma, or citrate plasma for 90 min. The initial concentration of BNP1-32 was set to 100%.

After incubation of BNP1-32 with the different blood preparations, about 40% of BNP1-32 has been proteolyzed in serum and citrate blood (**Figure 16**). However, in heparin blood preparations 80% of the peptide has been removed in the same time. It might be possible that heparin does not interfere as much as citrate with the proteolyzing enzymes of the blood and thus, more BNP1-32 is proteolyzed. However, in serum where no anticoagulant was added, some enzymatic activity might be lost caused by coagulation and thus, BNP1-32 proteolysis is less pronounced compared to proteolysis in heparin plasma. To reveal initial emerging BNP products, the chromatograms of the incubations have been analyzed in detail.

3.1.3.2 Investigation of proteolytic products of BNP1-32 in blood preparations

Figure 17 shows representative chromatograms of BNP1-32 incubated with diluted serum, heparin, or citrate plasma (red line). For comparison the chromatograms of serum, citrate, and heparin plasma alone have been displayed in the same figure (black line). This allows discrimination between peaks naturally occurring in the blood and peaks generated through BNP1-32 cleavage. For better peak separation, the HPLC method was optimized (Method 2) as such that the full spectrum of eluting peaks can be displayed in the chromatogram. The changes in the methods led to a shift of retention times for all peptides compared to those emerging from the method used in section 3.1.1. (Method 1). The intransparent peak areas in the beginning of the chromatograms represent compounds in the buffer and the sample that do not bind to the column but directly rinse through.

In the chromatograms of serum, citrate, and heparin blood alone (black line), two or three naturally occurring peaks were found. Two peaks with the retention times of 5.8 min and 7.9 min occurred in all preparations while an additional peak with the retention time of 6.6 min occurred in the serum only. Another very dominant peak with a retention time of 11.5 min occurred in the heparin blood preparations. The peak represents the compound heparin. All other peaks have been fractionated and analyzed with MALDI-TOF-MS. It was not aimed to identify these compounds, but to determine, if the peaks are related to BNP. Although it is very unlikely to see endogenous BNP fragments in unpurified blood samples with UV-detection, the analysis was performed to rule out any doubts. As expected it was revealed that none

of the peaks referred to a truncated BNP sequence. To investigate proteolytic products of BNP1-32, the peptide was spiked into the diluted blood preparations.

BNP1-32 can be seen as a clear peak with a retention time of 9.9 min in all chromatograms (red line). After incubation for 90 min, two peaks P_B1 and P_B2 were observed in the chromatograms. P_B1 has a retention time of 7.9 min. Coincidentally, the peak had the same retention time as an endogenous peak of the blood preparations and the overlay led to an increase of the whole peak area. As stated before, the endogenous peak was not related to any BNP metabolite. P_B1 was only marginally occurring in citrate blood incubations, which might indicate that it was formed by a Ca^{2+}-dependent enzyme.

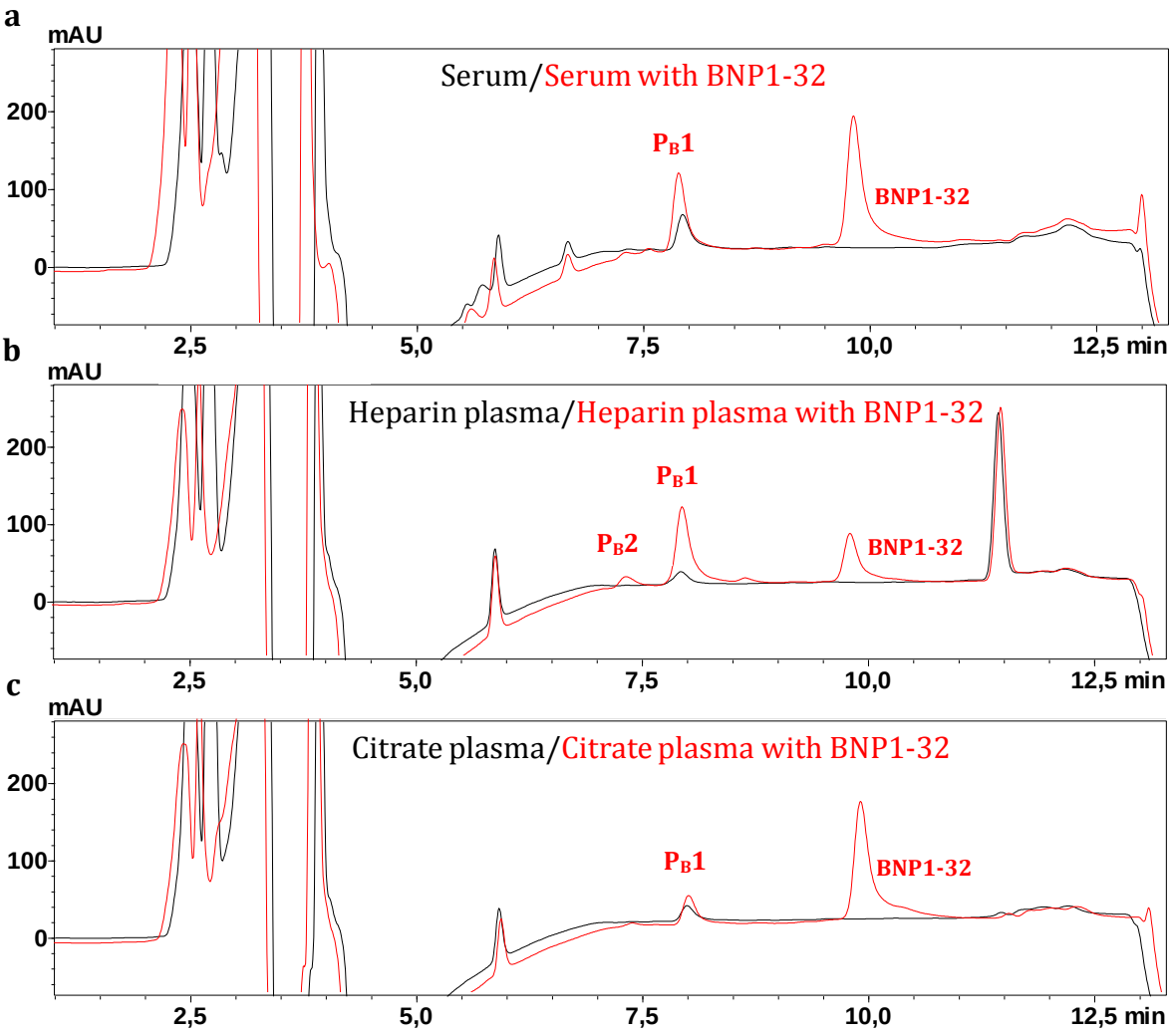


Figure 17: Overlay of chromatograms of diluted **a)** serum, **b)** heparin plasma or **c)** citrate plasma alone (black lines) and incubated with BNP1-32 for 90 min (red lines). Emerging peaks from the incubations are labelled with P_B1 and P_B2. Unlabeled peaks are present in the blood samples itself at 5.8 min and 6.6 min. Intransparent peaks with a retention time of 2 to 4 min represent compounds with low binding affinity to the column. HPLC method 2 was used to separate peaks.

Moreover, a second small peak (P_B2) with a retention time of 7.3 min was occurring in the heparin preparations. This peak occurred marginally also in the other two chromatograms. It might have been larger in heparin preparations compared to the others, since here the reduction of the BNP1-32 peak was also highest and thus more of the product was generated. An additional peak occurred in chromatogram **a)** and **c)** right at the end of the elution phase. This peak is an artefact caused by the method. In the following chapter, the BNP products behind the peaks P_B1 and P_B2 have been investigated.

3.1.3.3 Identification of BNP1-32cleavage products in blood

The new peaks have been fractionated and analyzed with MALDI-TOF-MS. The mass of the dominant peak P_B1 corresponds to a new truncated BNP metabolite harboring 31 amino acids. This metabolite is shortened by one leucine at the C-terminus compared to BNP1-32 and was subsequently named BNP1-31 (**Figure 18**). Peak P_B2 could be identified as BNP1-29, which was initially found in membrane preparations of the heart (see section 3.1.1.3) and was moreover formed from BNP1-30 in lung, liver and spleen (see section 3.1.1.5).

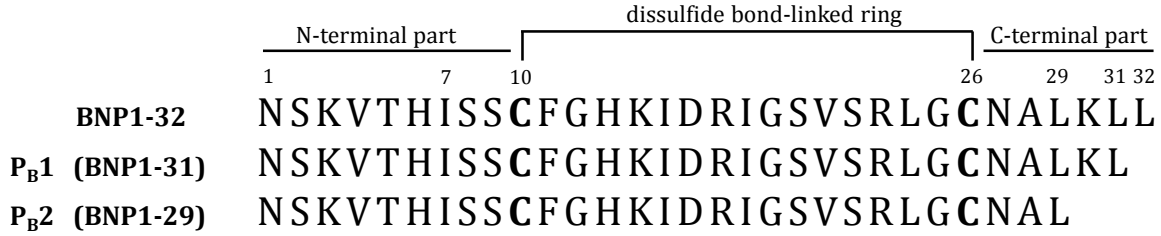


Figure 18: Amino acid sequences of BNP1-32 and the two BNP products P_B1 and P_B2 identified in blood, conducted from their masses. Relevant amino acids are numbered according to their order, beginning with the N-terminus of the mature BNP1-32.

The renal metabolite BNP7-32 and also the new metabolite BNP1-30, which were generated in lung, liver, spleen and heart incubations, did not occur in the chromatogram of the blood samples. However, this does not necessarily mean that these metabolites were not generated. Especially the generation of BNP1-30 is very likely since its potential precursor BNP1-31 and its subsequent cleavage product BNP1-29 were discovered. The result might indicate that BNP1-30 has reduced proteolytic stability compared to other metabolites in blood. In this case, BNP1-30 would not occur in the chromatogram, since the *de novo* generated peptide would directly be metabolized or degraded further, and a distinct peak cannot accumulate.

To test this hypothesis, the stability of the different BNP metabolites in blood preparations was tested.

3.1.3.4 Proteolytic stability of BNP1-32 and BNP metabolites

Since the identified BNP metabolites expose different C- or N-termini, their proteolytic stability can be altered compared to the mature BNP1-32. In this approach, BNP1-32 and the initial BNP metabolites BNP1-30, BNP1-29 and BNP7-32 have been incubated with serum, citrate, or heparin plasma to reveal their proteolysis. Since with all blood preparations the same results have been achieved, the data are only shown exemplarily for serum incubations (**Figure 19**).

After first testing it was observed, that BNP1-30 has dramatically reduced stability in the blood preparations and subsequently the serum has been additionally diluted to highlight this difference as seen in **Figure 19**. While BNP1-32, BNP1-29, and BNP7-32 were stable over 40 min in the diluted blood preparations, BNP1-30 had an approximate half-life of only 5 min. The exposure of the C-terminal lysine in BNP1-30 is obviously unfavorable for its stability. However, the additional removal of the C-terminal lysine forming BNP1-29 seems to stabilize the peptide again against proteolytic activity of the blood.

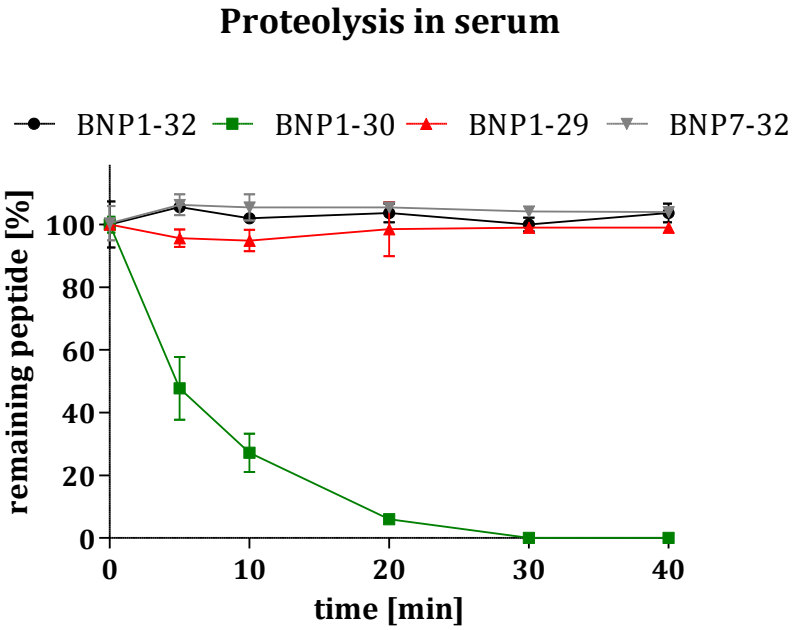


Figure 19: Quantification of the remaining concentrations of BNP1-32, BNP1-30, BNP1-29 and BNP7-32 in percent after incubation with serum over time (use of plasma results in the same values). In contrast to the approaches before, serum has been additionally diluted by factor 10 to enable the demonstration of the rapid proteolysis of BNP1-30.

The N-terminal truncation in BNP7-32 does also not decrease its stability in the time investigated.

Analysis of BNP1-30 proteolysis revealed that the peptide is one-to-one cleaved into the metabolite BNP1-29, which is accumulating over time (**Figure 20**). In an additional experiment it could be observed that once BNP1-29 is generated from BNP1-30, it is as stable as BNP1-32 over time in the diluted serum.

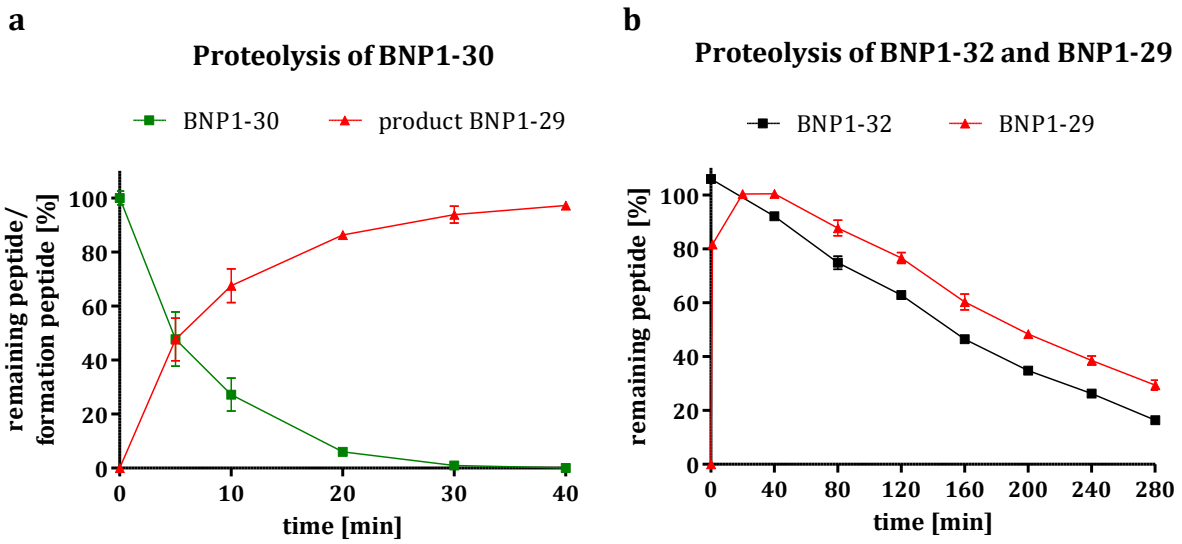


Figure 20: a) Quantification of the remaining concentration of BNP1-30 (green line) and the generation of its cleavage product BNP1-29 (pink line) after incubation with serum over time; **b)** Quantification of the remaining concentration of BNP1-32 in comparison to BNP1-29 (pink line) after its generation from BNP1-30.

It is likely, that the metabolite BNP7-30 which is a cleavage product from BNP1-30 or BNP7-32 has also the same reduced stability as BNP1-30 has, since it exerts the same C-terminal lysine. In a subsequent experiment, proteolysis rate BNP7-30 has been investigated. Moreover it was revealed which cleavage product emerges from the proteolysis of the peptide (**Figure 21**).

Congruent with the above hypothesis, BNP7-30 (blue line) was similarly unstable as BNP1-30. Obviously, the C-terminal removal of the two leucine seems to be decisive for the proteolysis rate. Notably, it was revealed that BNP7-30 was converted completely into into BNP7-29 (red line) which was accumulating over time. The removal of the C-terminal lysine at position 30 as in BNP1-29 and BNP7-29 seems to stabilize the peptide against cleavage.

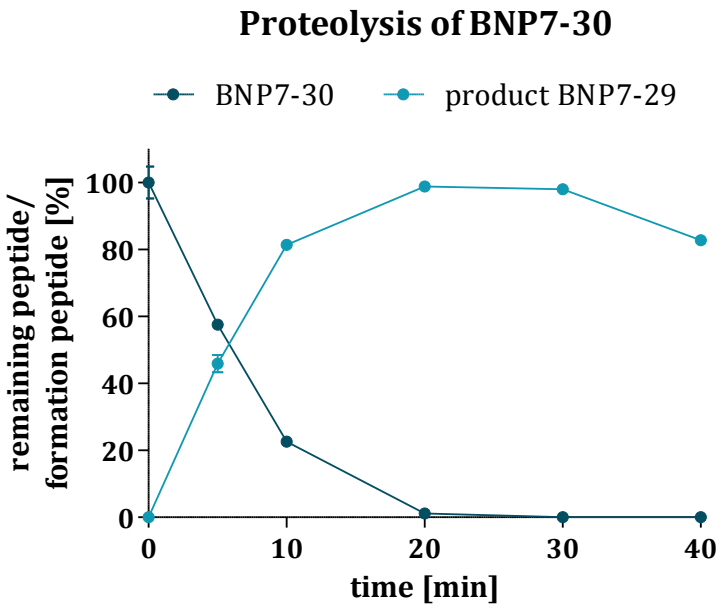


Figure 21: Quantification of the remaining concentration of BNP7-30 (blue line) and the generation of its cleavage product BNP1-29 (red line) after incubation with serum over time.

The metabolite BNP7-29 and also BNP1-31 have not been available as synthetic peptides and could thus not been tested for their stability. However, BNP7-29 was after its generated from BNP7-30 accumulating within 30 min (**Figure 21**). Thereafter, proteolysis of the peptide started which indicates that the peptide is less stable compared to BNP1-29 (**Figure 20 a**). BNP1-31 was accumulating through incubation of BNP1-32 with serum or plasma. Thus, it can be concluded that BNP1-31 is at least more stable as BNP1-30.

3.1.3.5 Overview about the metabolism of BNP1-32 in blood

Proteolysis of BNP1-32 in blood preparations led to the formation of the BNP metabolites BNP1-31 and BNP1-29. However, it is very likely that BNP1-30 is also formed. Since its proteolytic stability in blood was drastically reduced compared to other BNPs it did not accumulate in the incubations and was thus not visible in the chromatograms. It might be possible that carboxypeptidase activity in the blood led to a successive removal of C-terminal amino acids from BNP1-32. All BNP peptides discovered still contain the disulfide-linked ring structure.

3.1.4 Summary of the metabolism of BNP1-32

Figure 22 displays the secondary structures of BNP1-32 and the BNP products identified by performing experiments with membrane preparations (see section 3.1.1), blood preparations (see section 3.1.3), and liver perfusion (see section 3.1.2).

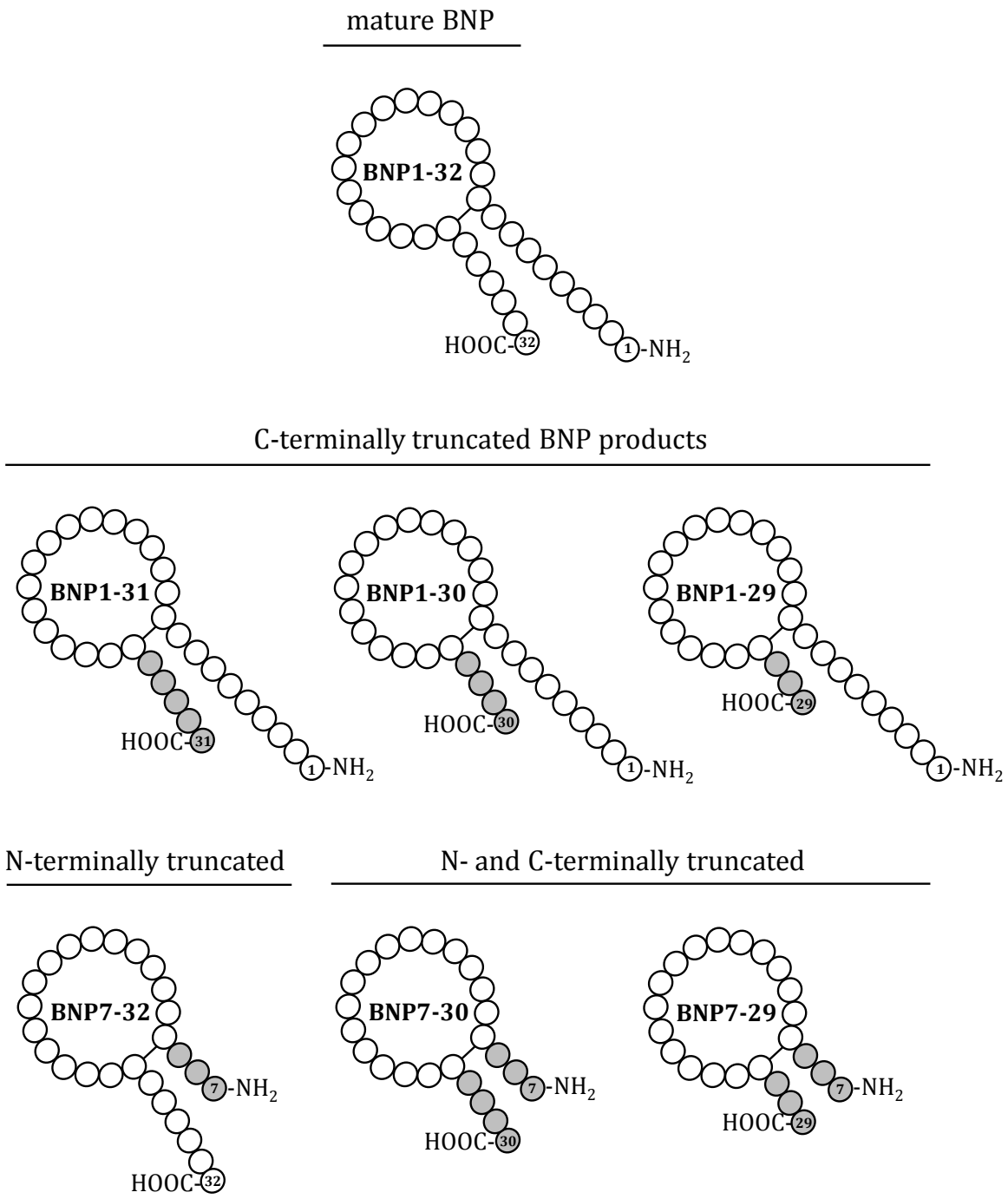


Figure 22: Secondary structures of BNP1-32 (mature BNP) and the BNP metabolites BNP1-31, BNP1-30, BNP1-29, BNP7-32, BNP7-30, and BNP7-29.

In summary, four different metabolites have been found initially. Three metabolites namely BNP1-31, BNP1-30 and BNP1-29 are C-terminally truncated, whereas the fourth metabolite BNP7-32 is N-terminally truncated. Metabolism of the initial metabolites led to the formation of BNP7-30 and BNP7-29 (see section 3.1.1.5).
The BNP metabolite BNP1-30 was found in most approaches. Besides its formation in all membrane preparations except kidney and its formation during liver perfusion it was most likely also formed in blood. Thus, it was aimed to investigate the enzymes responsible for its generation.

3.2 Identification of enzymes generating BNP1-30

3.2.1 Strategy for the identification of enzymes from membrane preparations generating BNP1-30

To identify the BNP1-30-generating enzyme(s) present in the membrane preparations of each organ generating BNP1-30, enzyme inhibitors have been added to the incubations (see Method section 2.2.2.5 and **Table 14**). These inhibitors can diminish the activity of their corresponding enzyme within the membrane preparations and thus reduce or decline the generation of BNP1-30 from BNP1-32. On the one hand, general enzyme inhibitors inhibiting a class of enzymes e.g. EDTA as a metallopeptidase inhibitor or aprotinin as serine-type peptidase inhibitor were used to reveal the peptidase(s) family. On the other hand, specific inhibitors, inhibiting distinct peptidases, have been used.

Table 14: List of peptidase inhibitors and their specificity used to reveal the peptidases generating BNP1-30.

Inhibitor	Inhibited peptidases /peptidase families
Actinonin	Meprin A
Alliskiren	Renin
Aprotinin	Serine-type peptidases
Candoxatrilat	Neprilysin (NEP)
Captopril	Angiotensin-converting enzyme (ACE)
Carboxypeptidase inhibitor (CP inhib.)	Carboxypeptidases, mostly A, B and C

Chymostatin	Serine peptidases and most cysteine-peptidases
Diprotin A	Dipeptidyl-peptidase IV (DPPIV)
EDTA	Metallopeptidases
F480	Angiotensin-converting enzyme 2 (ACE2)
GEMSA	Carboxypeptidase B-like enzymes
Kallistop	Kallikrein
Leupeptin	Serine- and cysteine-type peptidases
Pepstatin	Aspartylproteases (e.g. renin)
Roche cocktail [without EDTA]	Serine peptidases, cysteine peptidases
SM19712	Endothelin-converting enzyme 1 (ECE-1)
Trypsin inhibitor (trp.inhib.)	Trypsin-like peptidases
Z-pro-prolinal (ZPP)	Prolylcarboxypeptidase (PRCP), prolylendopeptidase (PREP)

3.2.2 Identification of BNP1-30-generating enzymes in heart

Figure 23 shows the amount of BNP1-30 accumulated through incubation with membrane preparations of the heart in the presence of enzyme family-specific inhibitors (white bars) or enzyme-specific inhibitors (grey bars). The amount of BNP1-30 generated with the membranes in the absence of peptidase inhibitors was set to 100% (black bar). One-Way ANOVA was used to determine statistical differences, which was rather insensitive for small changes in BNP1-30 accumulation caused by the multitude of groups analysed in parallel. However, this was thought reasonable, since the enzyme inhibitors can also influence BNP metabolism of cleavage products of BNP1-30. Thus, inhibitors can have influence on accumulation of BNP1-30, which is not caused by the inhibition of the particular peptidase generating the metabolite but on other peptidases e.g. proteolyzing BNP1-30. It was anticipated that rather big decreases of BNP1-30 concentrations are caused mainly by the inhibition of peptidases generating the metabolite. The generation of BNP1-30 using membrane preparations of heart could be strongly reduced by two enzyme inhibitors. The serine-type peptidase inhibitor aprotinin decreased the generation of BNP1-30 by 2/3 and the carboxypeptidase inhibitor (CP inhib.) by 80%. Other family-specific inhibitors and specific inhibitors for enzymes known to be involved in the generation

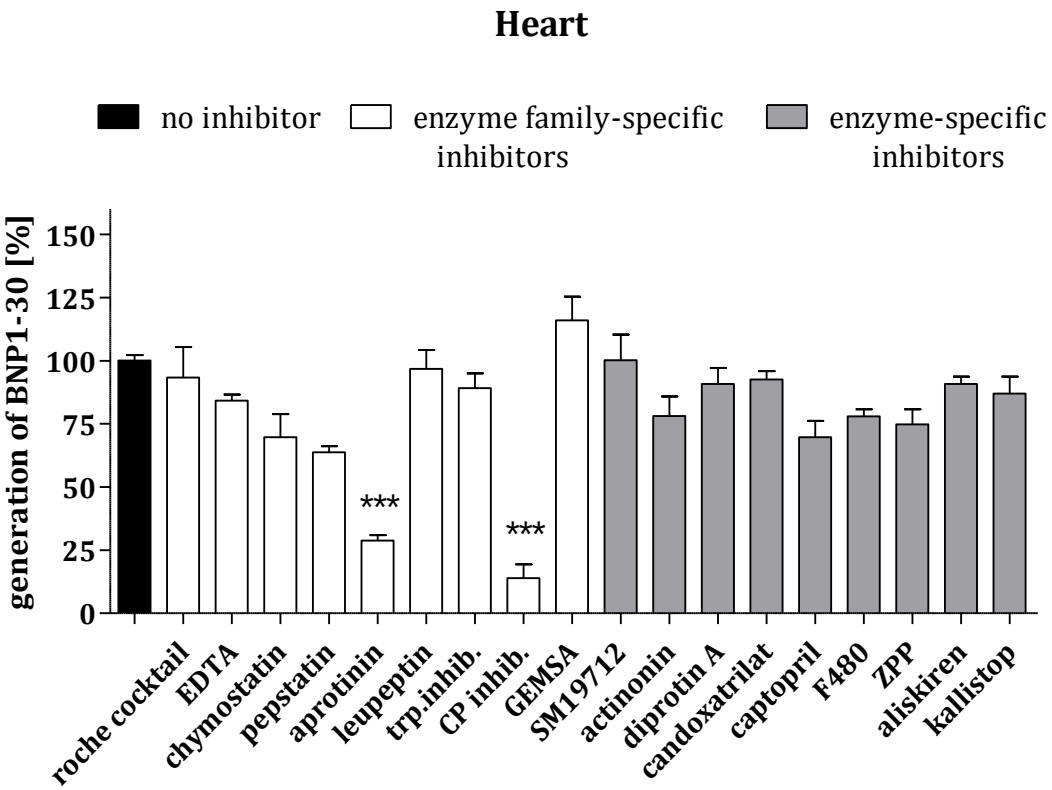


Figure 23: Quantification of the accumulation of BNP1-30 in percent in the presence of family-specific enzyme inhibitors (white bars) and enzyme-specific inhibitors (grey bars) using heart membrane preparations. The initial concentration of BNP1-30 generated in the incubations with heart membrane preparations without the use of inhibitors was set to 100% (black bar). For abbreviations of the inhibitors see Table 2. Significances were calculated with One-way ANOVA; *** $P < 0.001$ vs. no inhibitor.

of vasoactive peptides like ACE or ACE2, and peptidases known to cleave BNP (Meprin A, DPPIV, NEP) did not show significant effects on BNP1-30 accumulation.

To get a more detailed insight into the metabolism of BNP1-32 in the heart, atrium and ventricle have been investigated separately.

The use of the inhibitors showed a slightly different profile for the two heart tissues (**Figure 24a&b**). Whereas the carboxypeptidase inhibitor decreased the formation of BNP1-30 by 80% in the heart, it showed a much higher effect in the ventricle alone, where the generation of BNP1-30 was completely abolished. Vice versa, the effect in atrium was less pronounced. Aprotinin had approximately the same effect in both tissues. Since aprotinin and also the carboxypeptidase inhibitor reduced BNP1-30 generation, it was anticipated that a serine-carboxypeptidase is responsible for the generation of BNP1-30 in heart membranes.

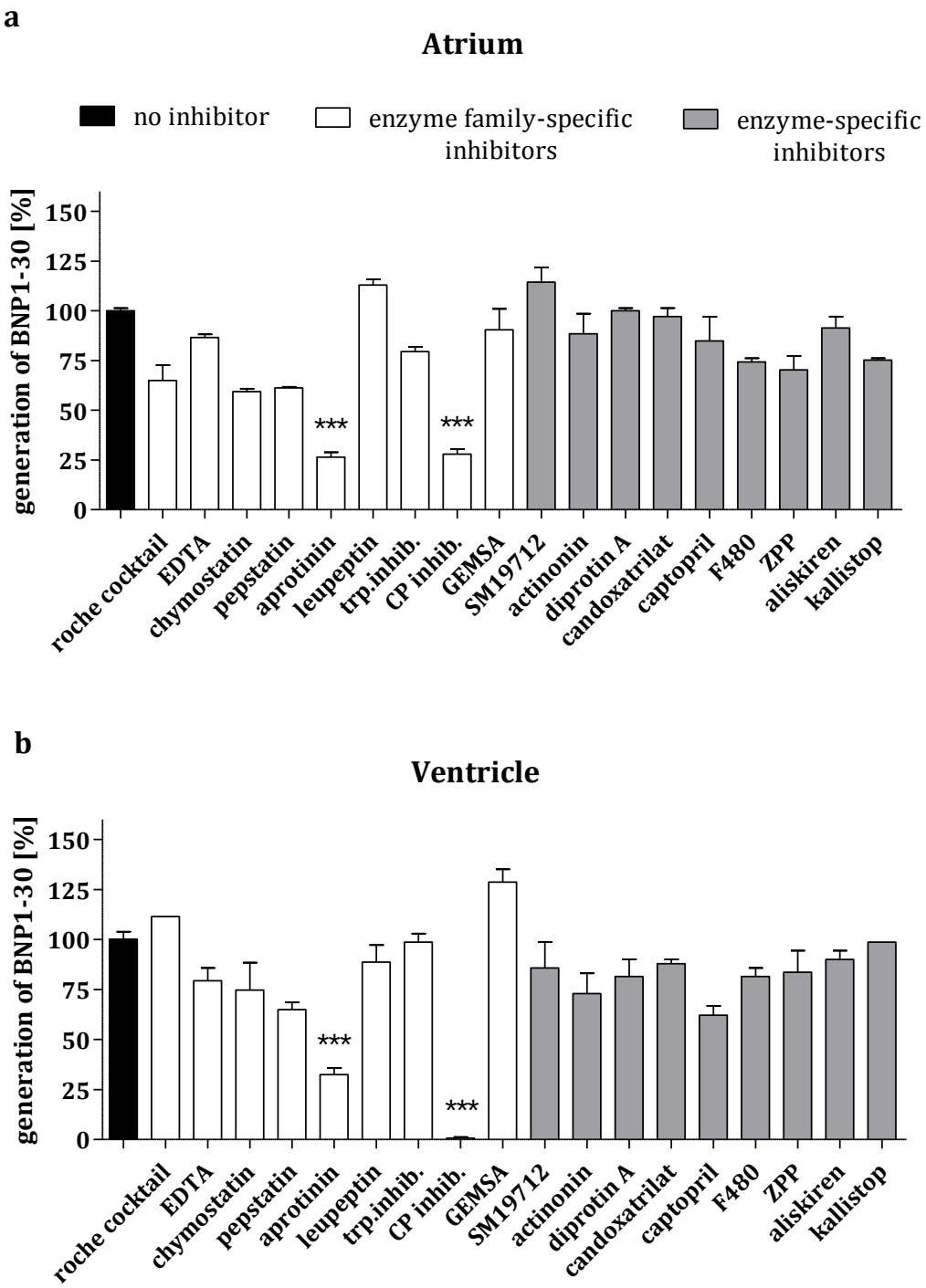


Figure 24: Quantification of the accumulation of BNP1-30 in percent in the presence of family-specific enzyme inhibitors (white bars) and enzyme specific inhibitors (grey bars) using **a)** membrane preparations of atria or **b)** membrane preparations of ventricles. The initial concentration of BNP1-30 generated in the incubations with heart membrane preparations without the use of inhibitors was set to 100% (black bar). For abbreviations of the inhibitors see Table 2. Significances were calculated with One-way ANOVA; *** $P < 0.001$ vs. no inhibitor. When no error bar is seen, variations are within the bar.

In the *numerical classification scheme for enzymes* four different serine-carboxypeptidases are described. One of them, the serine-type D-Ala-D-Ala carboxypeptidase (EC 3.4.16.4) can be found in bacteria only and cleaves

preferentially after alanin[230]. The second candidate, Prolylcarboxypeptidase (Angiotensinase C, PRCP, EC 3.4.16.2), belongs to the S28 class of serine proteases firstly isolated from swine kidney lysosomal fractions[231]. It cleaves bradykinin and angiotensin II[231]. Because of its actions, PRCP is considered to be a cardioprotective enzyme[232], also since PRCP polymorphism is linked to hypertension and pre-eclampsia[233, 234] and its depletion leads to vascular dysfunction and hypertension in mice[235]. PRCP favorably proteolyzes penultimate C-terminal prolines and is thus not an optimal candidate to cleave BNP1-32 which harbors a penultimate leucine. Moreover, the prolyloligopeptidase inhibitor Z-Pro-Prolinal (ZPP), which is also described as a PRCP inhibitor[235] had no effect on BNP1-30 generation (**Figure 24**). Additionally, recombinant PRCP has been incubated with BNP1-32 and no formation of BNP1-30 could be observed (**Figure 25a**).

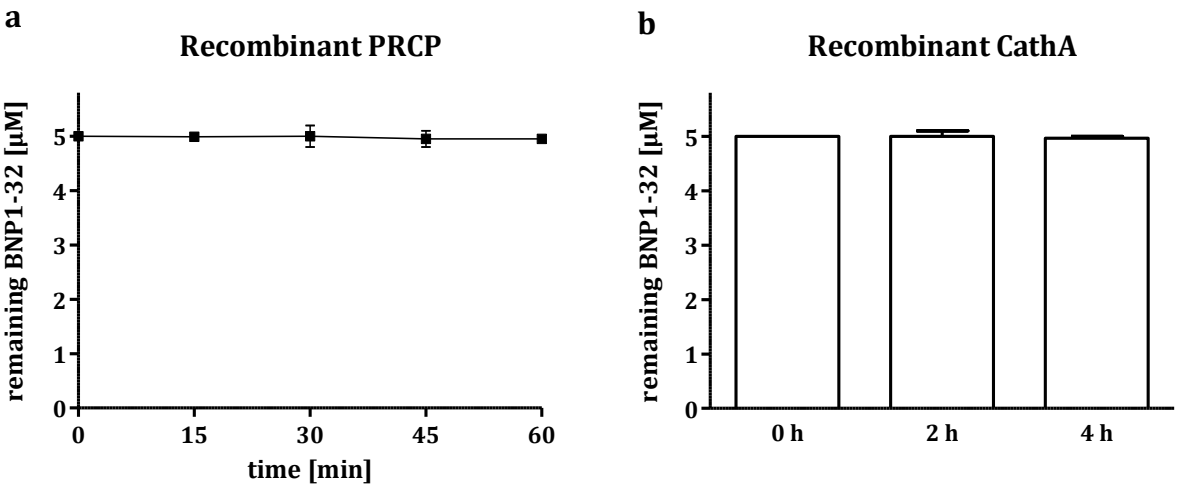


Figure 25: Quantification of the remaining concentration of BNP1-32 after incubation of 5 µM BNP1-32 with **a)** 2.5 mU Prolylcarbocypeptidase (PRCP) and **b)** 0.15 mU Cathepsin A (CathA) over time.

The other two candidates are Carboxypeptidase C and D. CPD (EC 3.4.16.6) is involved in the processing of growth factors and hormones in Drosophila[236] and human[237], however, it preferentially releases C-terminal arginines or lysines, which are not present at the C-terminus of BNP1-32. The CPC or better known as Cathepsin A (CathA, EC 3.4.16.5) is widely distributed in mammalian tissues on the cell surface but is also present in lysosome and is secreted from the cell[238]. CathA has a broad substrate spectrum, since this enzyme works multifunctional as deamidase, esterase, and carboxypeptidase with a preference for substrates with hydrophobic amino acid residues at the C-terminus[239]. CathA can hydrolyse several regulatory peptides,

including substance P, met-enkephalin, and oxytocin and has a high activity against the vasoactive peptide endothelin-1[240] and also against angiotensin (Ang) I by releasing a C-terminal leucine and converting it to Ang-(1-9)[241]. The mouse model of CathA deficiency shows significantly increased arterial blood pressure[240]. To test its actions on BNP1-30 formation, recombinant CathA was incubated with BNP1-32. No formation of BNP1-30 was observed (**Figure 25b**).

Since none of the serine-carboxypeptidases investigated was able to generate BNP1-30 from BNP1-32, it was hypothesized that the sought after peptidase might be a carboxypeptidase that is not a serine peptidase but is nevertheless in part inhibitable by aprotinin. Aprotinin is a single-chain polypeptide with a molecular weight of 6.5 kDa. It is a typical Kunitz-type protease inhibitor and inhibits especially trypsin and related proteolytic enzymes by the tight binding of its lysine in the active site of such peptidases. Aprotinin gained special importance in the treatment of influenca, since it targets trypsin-like proteases responsible for influenza virus hemagglutinin cleavage and virus activation in infected organism[242]. Moreover, it has a long history in medical practice in the treatment of pancreatitis and post-operative bleeding[243, 244], and several licensed compounds of aprotinin are available including Trasylol™ (Bayer AG, Germany) or Antagosan™ (Sanofi Aventis, France)[245]. Since aprotinin seems to be involved in many pathways it might be possible that it can interfere with a carboxypeptidase not being a typical serine peptidase. For this purpose other recombinant carboxypeptidases have been tested for their ability to form BNP1-30, subsequent to this chapter. However, prior, enzymes generating BNP1-30 in lung, liver and spleen have been investigated.

3.2.3 Identification of BNP1-30-generating enzymes in lung

Other than for the heart, aprotinin and carboxypeptidase inhibitor had no significant effects on BNP1-30 generation in lung, although its concentration was in trend reduced compared to other incubations (**Figure 26**). However, the metallopeptidase inhibitor EDTA reduced BNP1-30 generation by approx. 50% indicating the involvement of a peptidase from the family of metallopeptidases. The group of metallopeptidases includes a variety of enzymes and specific metallopeptidase-inhibitors for enzymes well known to catabolize natriuretic peptides or vasoactive peptides e.g. meprin A (actinonin), DPPIV (diprotin A), NEP (candoxatrilat), and ACE

(captopril) have been investigated in detail. None of them showed an inhibition on BNP1-30 formation.

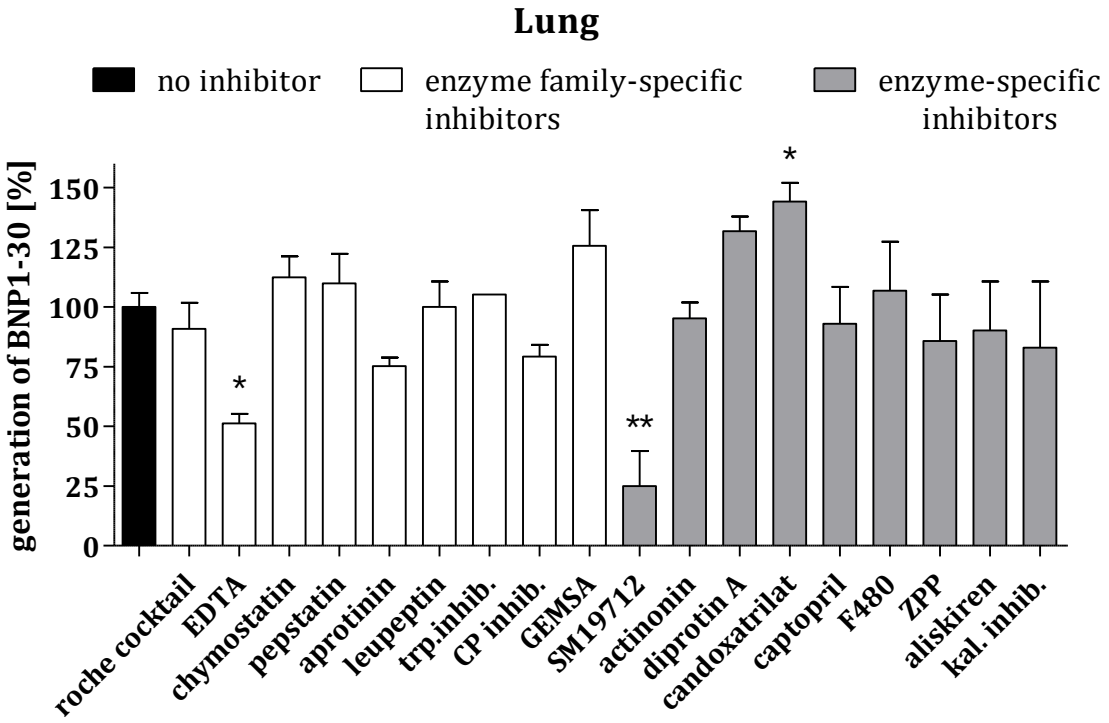


Figure 26: Quantification of the accumulation of BNP1-30 in percent in the presence of family-specific enzyme inhibitors (white bars) and enzyme specific inhibitors (grey bars) using lung membrane preparations. The initial concentration of BNP1-30 generated in the incubations with lung membrane preparations without the use of inhibitors was set to 100% (black bar). For abbreviations of the inhibitors see Table 2. Significances were calculated with One-way ANOVA; * $P < 0.05$; ** $P < 0.01$ vs. no inhibitor. When no error bar is seen, variations are within the bar.

The administration of the NEP inhibitor candoxatrilat even increased the peak area of BNP1-30 indicating that the degradation of BNP1-30 or of one of its own cleavage products is performed by NEP. Since EDTA also inhibits NEP, and NEP inhibition leads to an increase of the BNP1-30 peak, the inhibitory effect of EDTA on BNP1-30 formation might be even higher than seen.

Interestingly, instead of the inhibitors known to be involved in BNP proteolysis, another inhibitor named SM19712 led to a pronounced and significant reduction of BNP1-30 generation by about 75%. SM19712 is a selective inhibitor of endothelin-converting enzyme 1 (ECE-1; EC 3.4.24.71)[246]. ECE-1 belongs to the family of neutral metallopeptidases, which also includes NEP, and was first isolated and purified from aortic endothelial cells[247, 248]. ECE-1 is involved in the cleavage of beta-amyloid, bradykinin, and substance P[249] and generates endothelin-1 from Big-endothelin-1[248].

Since the inhibitor of ECE-1 had a strong effect on BNP1-30 generation, the degradation rate of BNP1-32 in lung was investigated in the presence of this inhibitor to reveal, if ECE-1 inhibition increases BNP1-32 concentration compared to non-treated samples. Notably, the presence of the inhibitor did not change BNP1-32 proteolysis rates significantly over time, however in trend the proteolysis was decreased (**Figure 27**). This data indicates that also other peptidases than ECE-1 are involved in the degradation of BNP1-32 in lung.

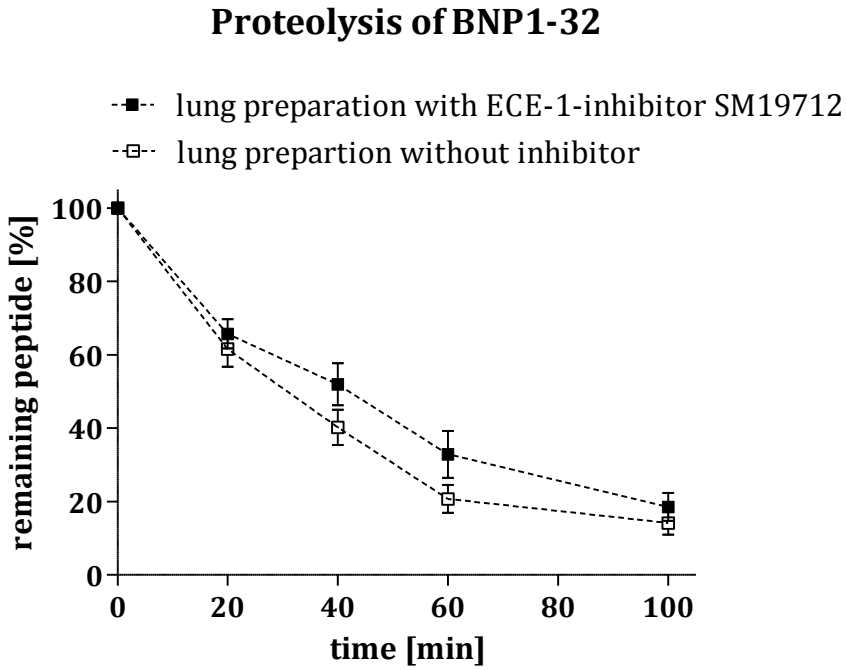


Figure 27: Quantification of the remaining concentration of BNP1-32 in percent after incubation with lung membrane preparations with (black symbols) or without (clear symbols) the presence of the specific ECE-1 inhibitor SM19712 over time.

3.2.4 Identification of BNP1-30-generating enzymes in liver

Similar to the situation in lung but less pronounced, EDTA and also the ECE-1 inhibitor SM19712 suppressed the formation of BNP1-30 in the incubations of liver membranes and BNP1-32 **(Figure 28**). The carboxypeptidase inhibitor and also the trypsin inhibitor increased the formation of BNP1-30 indicating that BNP1-30 was further cleaved by carboxypeptidases and trypsin in liver.

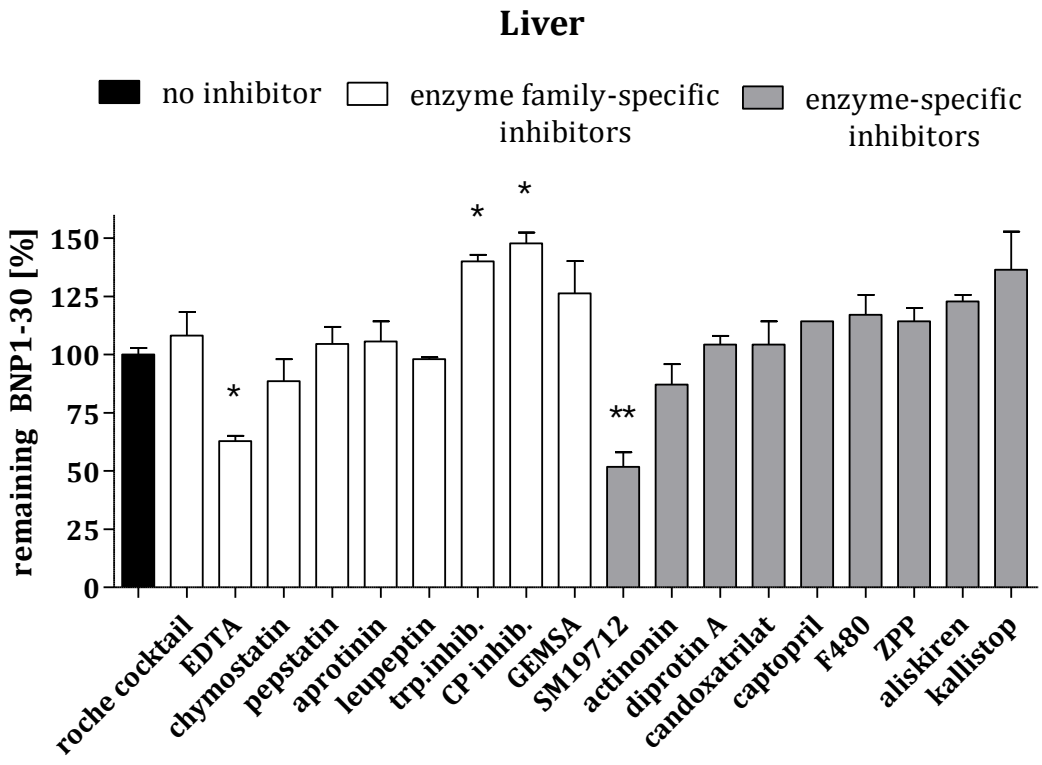


Figure 28: Quantification of the accumulation of BNP1-30 in percent in the presence of family-specific enzyme inhibitors (white bars) and enzyme specific inhibitors (grey bars) using liver membrane preparations. The initial concentration of BNP1-30 generated in the incubations with liver membrane preparations without the use of inhibitors was set to 100% (black bar). For abbreviations of the inhibitors see Table 2. Significances were calculated with One-way ANOVA; * $P < 0.05$; ** $P < 0.01$ vs. no inhibitor. When no error bar is seen, variations are within the bar.

3.2.5 Generation of BNP1-30 in spleen

Similar to the heart membranes, aprotinin and the carboxypeptidase inhibitor inhibited BNP1-30 generation using spleen membranes (**Figure 29**). However, the effect was less pronounced compared to the heart. EDTA, SM19712, and other inhibitors had no significant influences, although EDTA seemed to increase BNP1-30 formation, which is in full contrast to the situation in lung and liver where EDTA reduced BNP1-30 formation. Most likely, EDTA was inhibiting an enzyme in spleen proteolyzing BNP1-30 and thus more peptide was accumulated compared to non-inhibition.

Moreover, ZPP seems to decrease BNP1-30 generation. The One-way ANOVA analysis did not show any significance, however, the student's t-test showed $P < 0.01$. As stated before, ZPP is inhibiting PRCP, but it was revealed that recombinant PRCP is not able to form BNP1-30 from BNP1-32 (see section 3.2.2). Besides PRCP, ZPP is inhibiting prolylendopeptidase (PREP). However, similar to the PRCP, PREP requires

a proline next to the peptide bond it is cleaving[250] and is thus, not able to cleave BNP1-32, respectively.

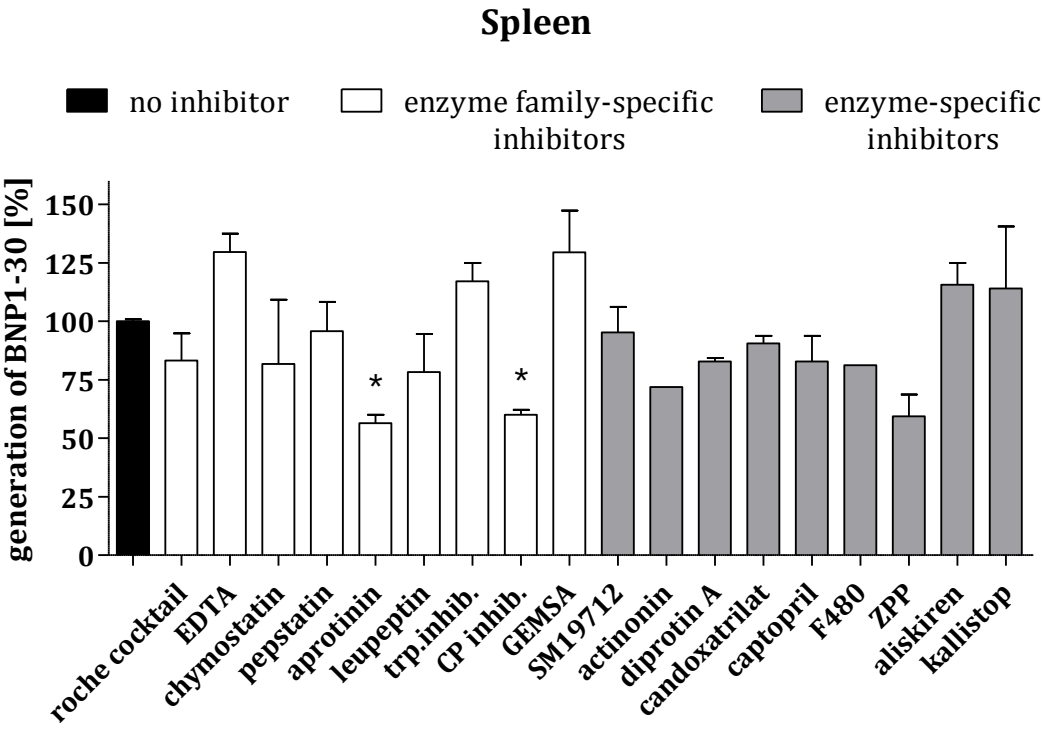


Figure 29: Quantification of the accumulation of BNP1-30 in percent in the presence of family-specific enzyme inhibitors (white bars) and enzyme specific inhibitors (grey bars) using liver membrane preparations. The initial concentration of BNP1-30 generated in the incubations with liver membrane preparations without the use of inhibitors was set to 100% (black bar). For abbreviations of the inhibitors see Table 2. Significances were calculated with One-way ANOVA; * $P < 0.05$ vs. no inhibitor. When no error bar is seen, variations are within the bar.

3.2.6 Overview of BNP1-30-generating enzymes present in membranes of organs

In the first chapters it was described that both, proteolysis rates of BNP1-32 and subsequent BNP metabolite formation is different in each organ. However, it was observed that BNP1-30 formation was abundant among the majority of organs. Thereupon, it has been investigated which peptidase or rather which peptidases are responsible for its generation. Interestingly, the above results disclosed that the proteolytic activity responsible for the formation of BNP1-30 is not equal in each organ. In lung and liver ECE-1 is responsible for BNP1-30 formation, whereas in heart and spleen other carboxypeptidases generate the peptide. The data indicate that the generation of BNP1-30 plays an overriding role, since different enzymes with different localisations appear to catalyze BNP1-30 formation.

To confirm the just revealed results, recombinant ECE-1 has been investigated for its ability to form BNP1-30. Moreover, other recombinant carboxypeptidases despite the ones that have been tested already (PRCP, CathA; see section 3.2.2) have been investigated for their ability to form BNP1-30. Also recombinant meprin A, which is catalyzing the formation of BNP7-32 from BNP1-32[160] has been used to reveal its further role in BNP metabolism.

3.3 Particular recombinant enzymes generating BNP metabolites

3.3.1 BNP metabolism with endothelin-converting enzyme-1

According to the above results performing incubations with membrane preparations of lung and liver in combination with enzyme inhibitors, ECE-1 could be identified as one enzyme cleaving BNP1-32 into BNP1-30. To confirm this data, recombinant ECE-1 has been incubated with BNP1-32. The overlay of two chromatograms in **Figure 30** shows that the incubation with the enzyme led to the formation of BNP1-30 (red line) similarly as during incubation of BNP1-32 with liver membrane preparations (black line). Additionally, the product peak has been fractionated and analyzed with MALDI-TOF-MS, revealing, that the emerging mass matched with the mass of the metabolite BNP1-30. Thus, it was confirmed that recombinant ECE-1 is able to generate BNP1-30 from BNP1-32.

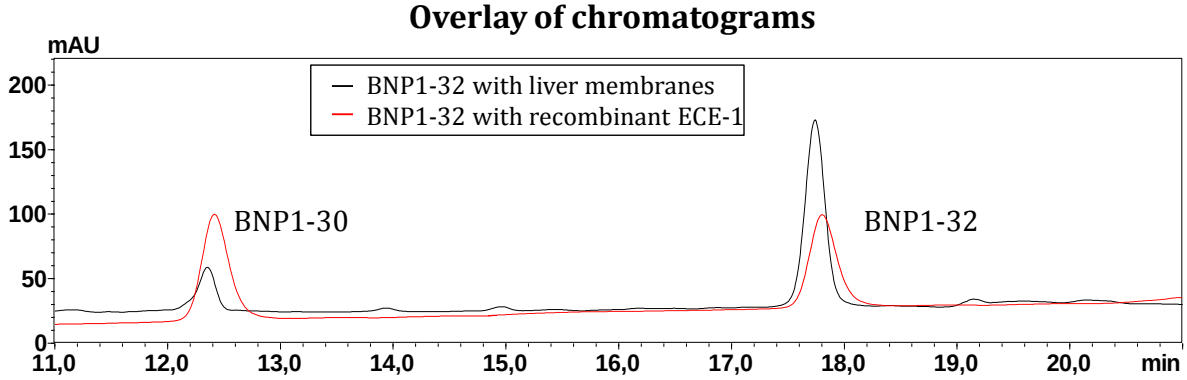


Figure 30: Overlay of HPLC chromatograms of the incubation of BNP1-32 with recombinant endothelin-converting enzyme 1 (ECE-1, red line) and BNP1-32 incubated with liver membrane preparations (black line); HPLC method 1 was used.

Moreover, ECE-1 was incubated with other BNP metabolites including BNP7-32, which has the same C-terminus as BNP1-32. **Figure 31** shows a comparison of all incubations. BNP7-32 was cleaved by ECE-1 to a similar extend as BNP1-32.

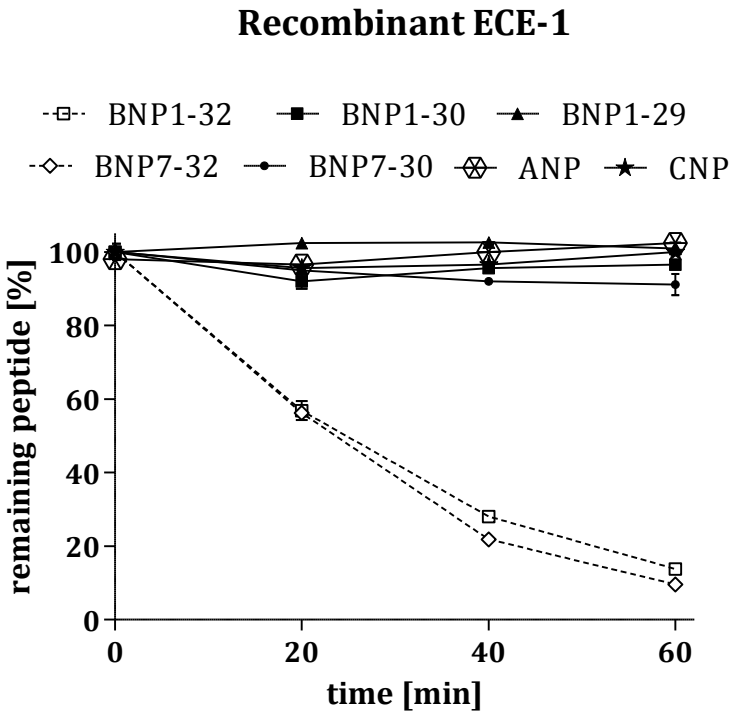


Figure 31: Quantification of the remaining concentration of BNP1-32 and the BNP metabolites BNP1-30, BNP1-29, BNP7-32, and BNP7-30 as well as ANP and CNP after incubation with 0.1 mU recombinant endothelin-converting enzyme 1 (ECE-1) over time.

Analysis of the product peak with MALDI-TOF-MS revealed that ECE-1 was forming BNP7-30 from BNP7-32. Other C-terminally truncated BNP metabolites like BNP1-29 and also the peptides BNP1-30 and BNP7-30 were not further processed by the enzyme. Additionally, it has been tested whether ECE-1 is able to truncate other natriuretic peptides. Thereby it was revealed that ANP and CNP were not cleaved by the enzyme (**Figure 31**).

It has been shown that BNP1-30 is also formed by other enzymes than ECE-1 in heart and spleen. PRCP and CathA have been excluded to be able to catalyze the reaction (see section 3.2.1). Thus, additional recombinant carboxypeptidases like the angiotensin-converting enzymes and the carboxypeptidases A and B, which are also inhibitable by the carboxypeptidase inhibitor used in the incubations in section 3.2 have been investigated as described in the next chapter.

3.3.2 BNP metabolism with recombinant carboxypeptidases

3.3.2.1 BNP metabolism with angiotensin-converting enzymes

Angiotenin-converting enzyme (ACE; EC 3.4.15.1) and ACE2 (EC 3.4.17.23) are classical carboxypeptidases of the renin-angiotensin system metabolizing and catabolizing vasoactive peptides[251].

The renin-angiotensin system plays an important role in the pathophysiology of hypertension[252] and heart failure[253]. Its activity is controlled by renin, an aspartyl peptidase, which cleaves angiotensinogen to generate AngI[254]. ACE, a zinc-metallopeptidase, mediates the crucial step in which the vasoconstricting peptide AngII (Ang-(1-8)) is formed from the inactive AngI (Ang-(1-8)) by cleaving two amino acids at the C-terminus[255, 256]. Its homologue ACE2 cleaves AngII, releasing Ang-(1-7), which counteracts many of the detrimental effects of AngII[257].

It was shown before that inhibitors of both enzymes did not show significant effects in organ incubations (see section 3.2). However, since both enzymes are carboxypeptidases and especially ACE as a dipeptidylcarboxypeptidase is able to cleave a C-terminal dipeptide that can by hydrophobic[258], they have been tested for their ability to form BNP1-30 as well as to cleave BNP metabolites. However, the proteolytic activity of ACE towards the BNPs was compared to its ability to cleave AngI. **Figure 32a** shows that while 70% of AngI were cleaved by ACE, all BNP metabolites were left unaltered.

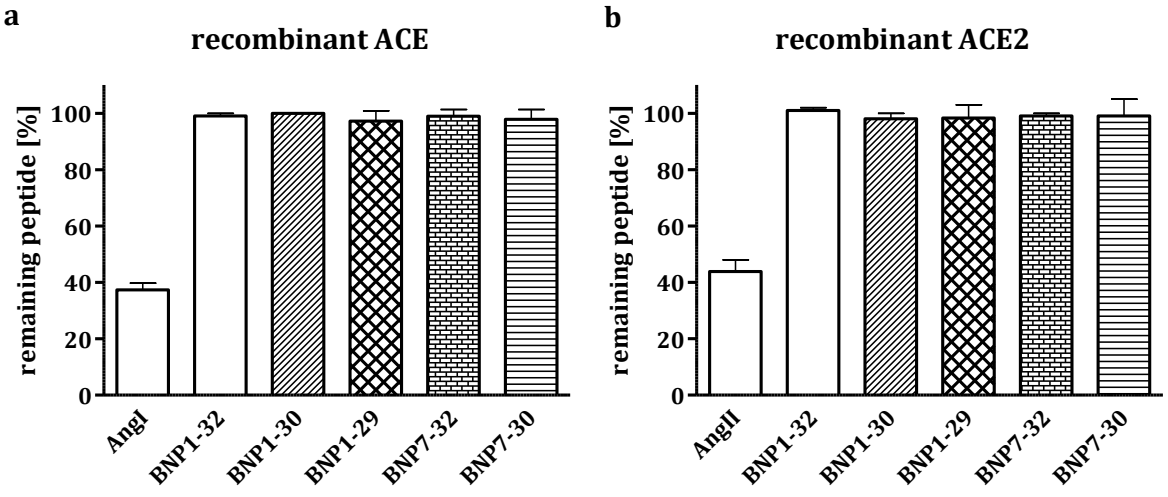


Figure 32: a) Quantification of the concentration of Angiotensin I (AngI), BNP1-32, BNP1-30, BNP1-29, BNP7-32 and BNP7-30 after incubation with 0.1 mU recombinant angiotensin-converting enzyme (ACE) for 20 min; **b)** Quantification of the concentration of AngII BNP1-32, BNP1-30, BNP1-29, BNP7-32 and BNP7-30 after incubation with 0.08 mU recombinant ACE2 for 20 min. Where error bars are not indicated, SEM is within the bars.

Similar to ACE, ACE2 did not show any proteolytic effect on BNP1-32 or BNP metabolites using the same specific activity of the enzyme that was able to cleave AngII (**Figure 32b**).

3.3.2.2 Metabolism with carboxypeptidase A

Recombinant carboxypeptidase A (CPA, EC 3.4.17.1) has been described before to mediate the formation of Ang1-9 form AngI[259] by the removal of the C-terminal leucine. Since BNP1-32 also harbors a C-terminal leucine it was hypothesized that this enzyme can cleave the peptide. Incubation of recombinant CPA with the peptides indeed revealed that BNP1-32 but also BNP1-29 and BNP7-32 were proteolyzed by the enzyme (**Figure 33**). As expected, BNP1-30 and BNP7-30 were stable since CPA is not able to cleave the C-terminal lysine[260].

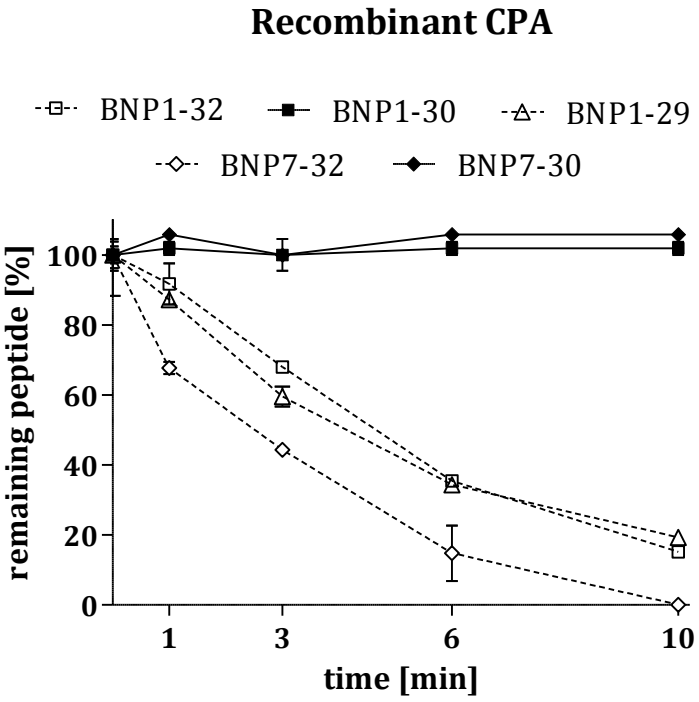


Figure 33: Quantification of the remaining concentration of BNP1-32 or the BNP metabolites BNP1-30, BNP1-29, BNP7-32, and BNP7-30 after incubation with 0.025 mU recombinant carboxypeptidase A (CPA) over time.

Analysis with HPLC and MALDI-TOF-MS revealed that CPA successively cleaved BNP1-32 leading to the generation of BNP1-31 and BNP1-30 (**Figure 34**). Since the C-terminal lysine of BNP1-30 cannot be further cleaved by the enzyme, the metabolite is accumulating over time through the ongoing metabolism of BNP1-31 (red and blue line).

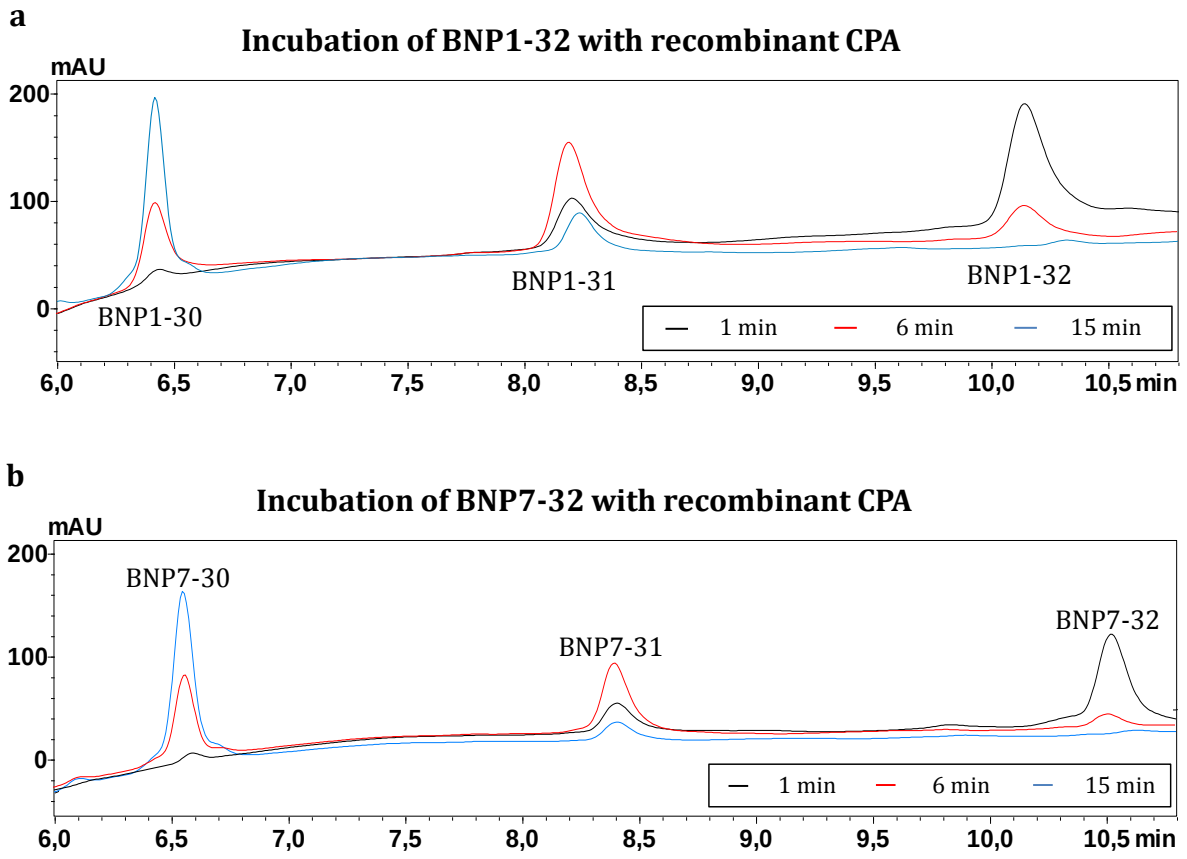


Figure 34: Overlay of HPLC chromatograms of the incubation of **a)** BNP1-32 and **b)** BNP7-32 with recombinant carboxyepeptidase A (CPA) at 1 min (black line), 6 min (red line), and 15 min (blue line). HPLC method 2 was used.

Similarly, it was revealed that BNP7-32 is cleaved into BNP7-30 forming the intermediate product BNP7-31 (**Figure 34b**). Also here, BNP7-30 was accumulating over time, since CPA was not able to further metabolize the peptide.

3.3.2.3 Metabolism with carboxypeptidase B

Recombinant CPB (EC 3.4.17.2) preferentially hydrolyzes C-terminal arginine and lysine[261]. Thus, BNP1-32 is an unfavorable substrate for this enzyme, however BNP1-30 and BNP7-30, which harbour a C-terminal lysine, were hypothesized to be substrates for CPB. As shown in **Figure 35a**, CPB was indeed able to cleave BNP1-30

and BNP7-30. In parallel, BNP1-32, BNP1-29 and BNP 7-32 were not cleaved by the enzyme.

Analysis of the cleavage products with HPLC and MALDI-TOF-MS revealed that BNP1-30 is cleaved in a one-to-one ratio into BNP1-29 and similarly BNP7-30 is cleaved in a one-to-one ratio into BNP7-29 (**Figure 35b**).

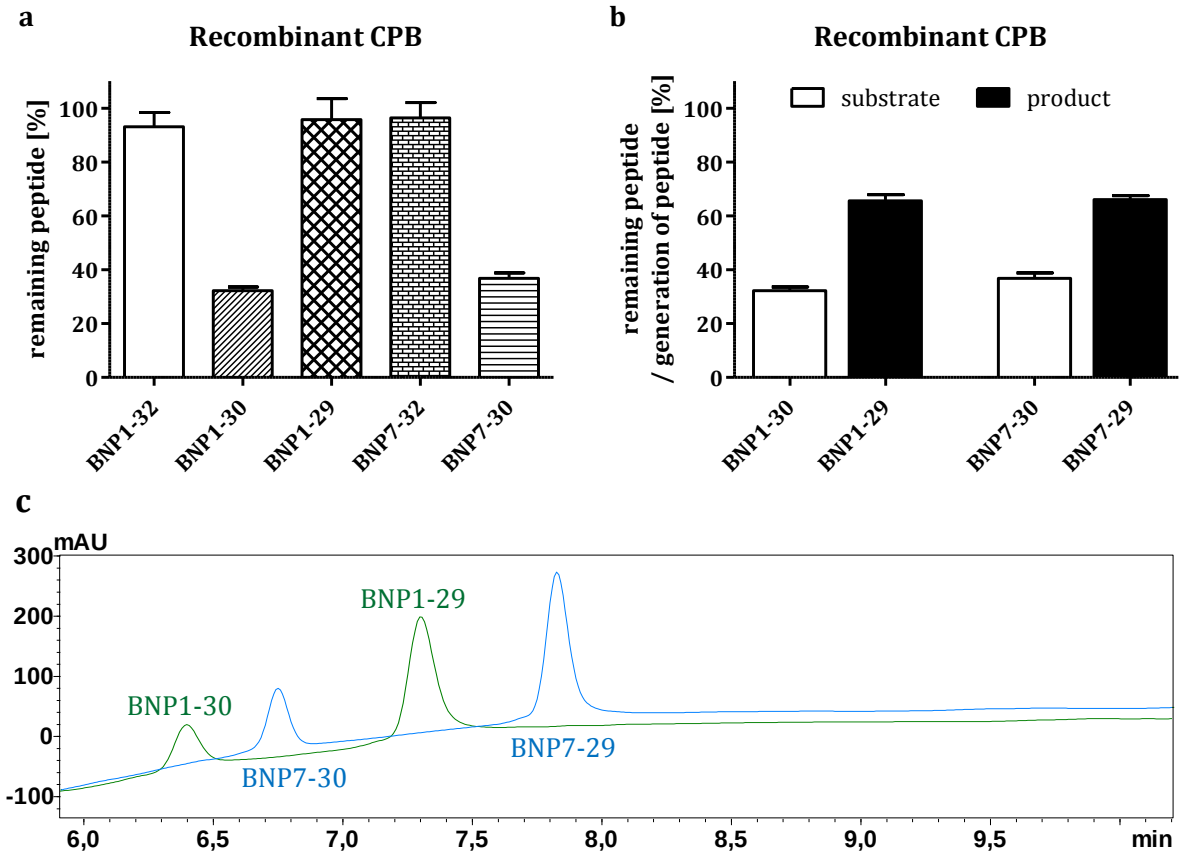


Figure 35: a) Quantification of the remaining concentration of BNP1-32 and of the BNP metabolites BNP1-30, BNP1-29, BNP7-32, and BNP7-30 after incubation with 0.1 mU recombinant carboxypeptidase B (CPB) for 10 min; **b)** Quantification of the remaining concentration of BNP1-30 and BNP7-30 after incubation with 0.1 mU recombinant CPB for 10 min (white bars). Next to the substrate-bars the concentration of the corresponding CPB cleavage products BNP1-29 and BNP7-29 are displayed (black bars); **c)** overlay of chromatograms displaying the separation of peaks after incubation of BNP1-30 with recombinant CPA forming BNP1-29 (green line) or incubation of BNP7-30 with recombinant CPA forming BNP7-29(blue line). HPLC method 2 was used.

Interestingly, these results reflect the situation observed in the incubations of the substrates in serum (see section 3.1.3.4). In these experiments, BNP1-30 and BNP7-30 were also completely cleaved into BNP1-29 and BNP7-29, whereas other BNP metabolites have not been metabolized in the same time. Thus, it was aimed to reveal if CPB is the enzyme in serum responsible to catalyze these reactions. Therefore, GEMSA, an inhibitor of CPB-like enzyme activity that was specifically

shown to inhibit CPB[262] has been supplemented into the incubations of the peptides with serum. Product formation has again been analysed with MALDI-TOF-MS and peaks have been quantified with HPLC. Results are plotted in **Figure 36.** Importantly this experiment disclosed that enzyme activity forming BNP1-29 and BNP7-29 was nearly completely inhibited by GEMSA.

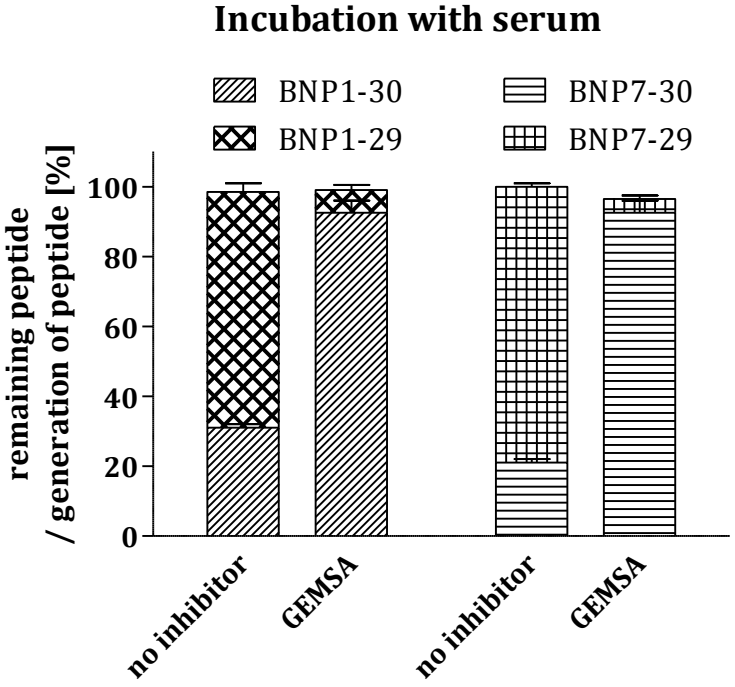


Figure 36: Quantification of the remaining concentration of BNP1-30 (fascinated) or BNP7-30 (horizontal stripes) after incubation with serum for 10 min with or without the presence of the carboxypeptidase B (CPB) inhibitor GEMSA. The stacked bars show in parallel the generation of their corresponding cleavage products BNP1-29(fascinated grid) or BNP7-29 (horizontal grid) with or without the presence of GEMA.

3.3.3 Metabolism with meprin A

As shown before by an approach of Pankow *et al.*[160], BNP1-32 is metabolized by renal meprin A into the metabolite BNP7-32. Here, it was furthermore investigated if besides BNP1-32 also the newly identified metabolites BNP1-30 and BNP1-29 are substrates for meprin A. Therefore, the peptides have been incubated with recombinant meprin A and cleavage products have been analysed with MALDI-TOF-MS as well as quantified with HPLC. As shown in **Figure 37**, recombinant meprin A catalyzes the formation of BNP7-32 from BNP1-32. Similarly, the incubation with BNP1-30 led to the formation of BNP7-30 and the incubation with BNP1-29 led to the formation of BNP7-29. Since the proteolysis rates between the peptides did not

significantly differ, it can be anticipated that the enzyme has similar substrate specificity towards the three peptides. Thus, it is likely, that renal meprin A is also be able to form the metabolites BNP7-30 and BNP7-29 under physiological conditions if the corresponding substrates are present in the kidney.

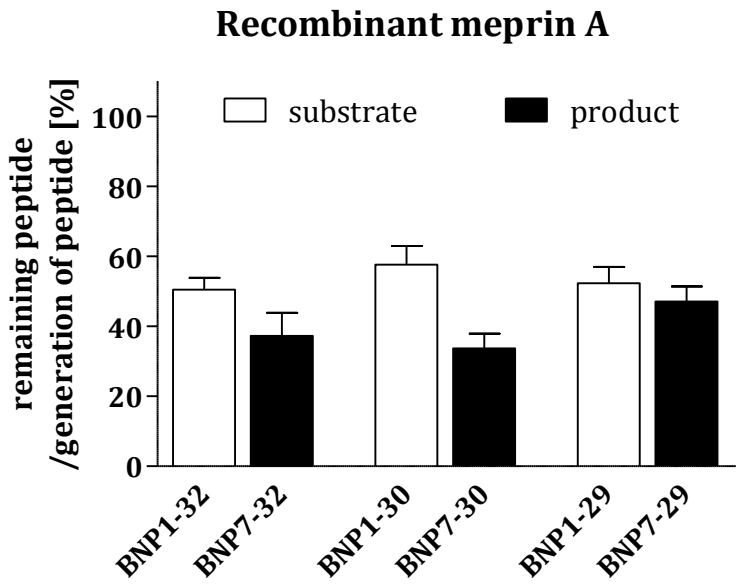


Figure 37: Quantification of the remaining concentration of BNP1-32, BNP1-30 and BNP1-29 after incubation with 0.1 mU recombinant meprin A for 20 min (white bars). Next to the substrate-bars the concentration of the corresponding meprin A proteolysis products BNP7-32, BNP7-30, and BNP7-29 are displayed (black bars).

3.3.4 Overview of enzymes metabolizing BNP1-32

The above results confirm that recombinant ECE-1 is able to generate BNP1-30 from BNP1-32, indicating that ECE-1 activity present in lung and liver as revealed by the use of the specific ECE-1 inhibitor is likely responsible for the generation of BNP1-30 in these tissues. Moreover, it could be disclosed that CPA is also able to generate BNP1-30 through the intermediate product BNP1-31. Importantly, the enzyme CPB was discovered being able to cleave BNP1-30 and BNP7-30 forming their corresponding C-terminal cleavage products BNP1-29 and BNP7-29 similar to the situation seen in blood preparations. It was moreover shown, that other carboxypeptidases like CathA, PRCP, and the key enzymes of the renin-angiotensin system, ACE and ACE2, did not show activity towards BNP1-32 or investigated BNP metabolites with the specific activity used. Meprin A, which generates BNP7-32, is also able to proteolyze other BNP metabolites, such as BNP1-30 and BNP1-29,

forming BNP7-30 and BNP7-29. In summary, for each BNP metabolite, discovered during incubation with membrane preparations or blood preparation, at least one enzyme was identified being responsible for its formation. Results are summarized in **Table 15**.

Table 15: Overview about enzymes cleaving BNP1-32 and BNP metabolites forming other BNP metabolites.

Substrate	Enzyme	Product
BNP1-32	Endothelin-converting enzyme-1	BNP1-30
	Carboxypeptidase A	BNP1-31 and BNP1-30
	Meprin A	BNP7-32
BNP1-30	Carboxypeptidase B	BNP1-29
	Meprin A	BNP7-30
BNP1-29	Carboxypeptidase A	not defined
	Meprin A	BNP7-29
BNP7-32	Endothelin-converting enzyme-1	BNP7-30
	Carboxypeptidase A	BNP7-31 and BNP7-30
BNP7-30	Carboxypeptidase B	BNP7-29

This collection of data strongly indicates that many enzymes are involved in the metabolism of BNP1-32. Finally, it was of particular interest if NEP is able to inactivate BNP metabolites.

3.4 Degradation of BNP metabolites by NEP

NEP was thought to be the main enzyme catabolizing and thus inactivating natriuretic peptides[120, 122]. Structural principles were shown previously to this work making clear that the amino acid sequence and especially the lengths of the natriuretic peptides are decisive for the ability of NEP to inactivate them[20]. Thus, it was tested if the newly identified BNP metabolites are better substrates for NEP in contrast to BNP1-32. For comparison, ANP and CNP degradation by NEP has been displayed too.

The incubation with recombinant NEP revealed no statistical difference between degradation of BNP1-32 and BNP1-30 within 20 min, whereas CNP was already degraded by 75% (**Figure 38**).

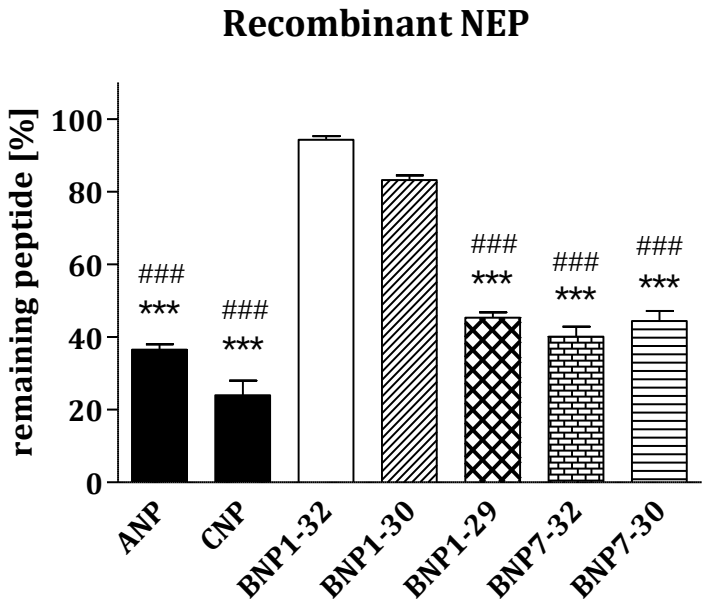


Figure 38: Quantification of the remaining concentration of ANP, CNP, BNP1-32, BNP1-30, BNP1-29, BNP7-32, and BNP7-30 in percent after incubation with 0.25 mU recombinant neprilysin (NEP) for 20 min. Significances have been calculated with One-way ANOVA: *** $P < 0.001$ vs. BNP1-32; ### $P < 0.001$ vs. BNP1-30.

However, the trend indicates that BNP1-30 degradation might be faster compared to BNP1-32 with longer incubations times. The metabolites BNP1-29, BNP7-32 and BNP7-30 were degraded in a much faster way compared to BNP1-32 and BNP1-30, reaching similar values as ANP. The missing lysine at the C-terminus of BNP1-29 seems to have a very favorable influence on the peptides' degradability by NEP.

It can be confirmed that NEP is not directly degrading BNP1-32, but favors shorter BNP metabolites. Thereby, the loss of the C-terminal lysine or the loss of the six N-terminal amino acids achieved by meprin A are increasing degradation levels to be comparable to ANP. The data also indicate that BNP metabolism is an important prerequisite for BNP clearance by NEP.

Since the opening of the ring structure of the natriuretic peptides leads to their inactivation, it is anticipated that the BNP metabolites, which still harbor this structure, exert biological activity. This hypothesis was investigated in different approaches described in the next chapters.

3.5 Biological activity of BNP1-32 and BNP metabolites *in vitro*

BNP1-32 and the BNP metabolites BNP1-30, BNP1-29, BNP7-32, and BNP7-30 were tested for their biological activity by determining their ability to stimulate natriuretic peptide receptors (see Method section 2.2.9). The bioactivity of the metabolites is positively correlating to the amount of cGMP being generated in the cells upon natriuretic peptide receptor stimulation. The emerging cGMP concentrations have been measured by immunosorbant assays (see Method section 2.2.9.2).

3.5.1 Natriuretic peptide receptor-transfected HEK293 cells

3.5.1.1 Functionality of the receptors

HEK293 cells have been transfected with the receptors NPRA or NPRB. First of all, the transfection and functional expression of the receptors was proven by stimulating the transfected cells with ANP, BNP1-32, and CNP and determining the cGMP generation in comparison to that of cell culture medium (DMEM)-stimulated cells, which served as controls.

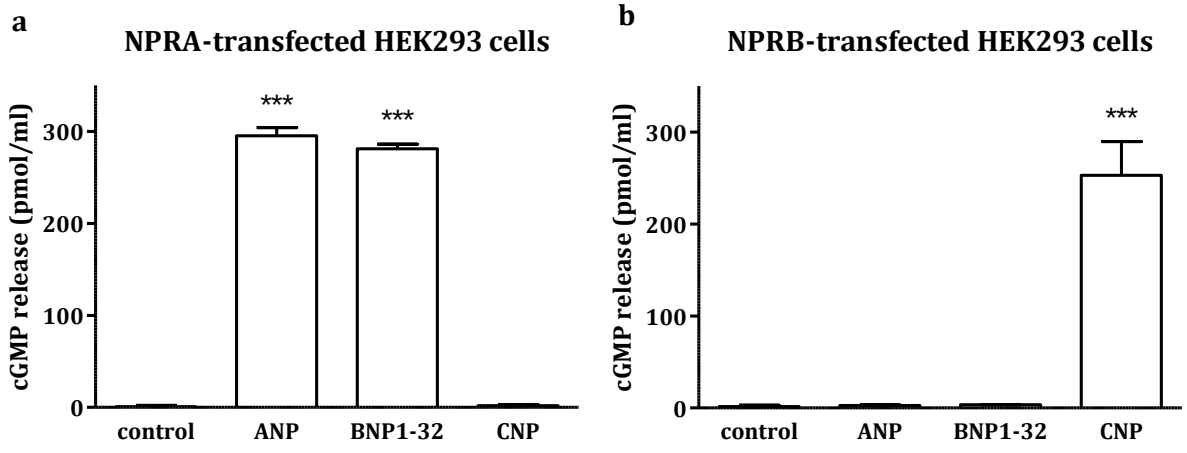


Figure 39: Quantification of cGMP levels after stimulation of **a)** NPRA- and **b)** NPRB-transfected HEK293 cells with cell culture medium (control), or ANP, BNP1-32 or CNP with a peptide concentration of 10^{-7} M. Significances were calculated with One-way ANOVA: *** $P < 0.001$ vs. control [n = 3, in triplicates].

Figure 39 shows the quantification of the cGMP amount generated by the stimulation of such transfected cells. Stimulation of NPRA-transfected HEK293 cells with ANP and BNP1-32 led to the generation of about 300 pmol/ml cGMP (**Figure 39a**). Stimulation

with CNP did not lead to cGMP formation, since CNP only has a marginal affinity towards NPRA, but has a high affinity towards the NPRB (see Introduction section 1.1.3). Thus, conversely, CNP stimulation of NPRB-transfected HEK293 cells led to the generation of about 250 pmol/ml cGMP, whereas ANP and BNP1-32 stimulation did not lead to a noteworthy generation of the second messenger (**Figure 39b**).

3.5.1.2 Stimulation of NPRA-transfected HEK293 cells

Since functionality of the receptors has been proven, NPRA-transfected HEK293 cells have been stimulated with BNP1-32 and BNP metabolites. **Figure 40** summarizes the amount of cGMP generated after such stimulation.

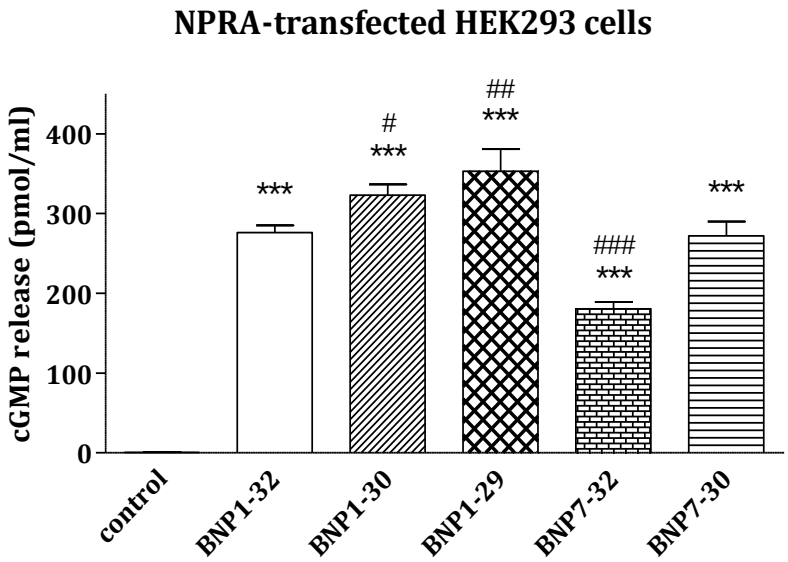


Figure 40: Quantification of cGMP levels after stimulation of NPRA-transfected HEK293 cells with cell culture medium (control), BNP1-32, BNP1-30, BNP1-29, BNP7-32, and BNP7-30 with a peptide concentration of 10^{-7} M. Significances were calculated with One-way ANOVA: *** $P < 0.001$ vs. control; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ vs. BNP1-32 [n = 4, in triplicates].

All metabolites were able to stimulate significant cGMP generation compared to the control confirming the hypothesis that, although the new BNP metabolites are truncated on different positions, they are still bioactive.

Notably, only the renal metabolite BNP7-32 was less active compared to BNP1-32, while all other metabolites showed similar activities or were even more efficient in stimulating cGMP formation. In fact, the C-terminally truncated metabolites BNP1-30 and BNP1-29 were more bioactive compared to BNP1-32. The shortest metabolite BNP7-30 was in contrast to the N-terminally truncated peptide BNP7-32 similarly bioactive compared to BNP1-32. Obviously, the negative influence of an N-terminal

truncation like in BNP7-32 seems to be buffered by an additional C-terminal truncation in BNP7-30.

3.5.1.3 Stimulation of NPRB-transfected HEK293 cells

It was demonstrated before that BNP1-32 does stimulate NPRA but not NPRB (**Figure 39**). To investigate if the C- and N-terminal truncations of the peptide do influence receptor specificity, NPRB-single-transfected HEK293 cells have been stimulated (**Figure 41**).

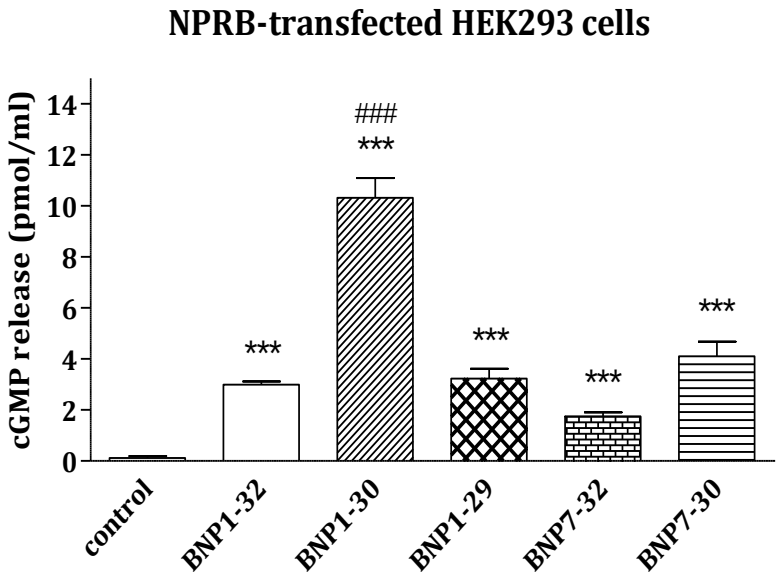


Figure 41: cGMP levels after stimulation of NPRB-transfected HEK293 cells with cell culture medium (control), BNP1-32, and BNP metabolites with a peptide concentration of 10^{-7} M. Significances were calculated with One-way ANOVA: # $P < 0.05$; *** $P < 0.001$ vs. BNP1-32 [n = 4, in triplicates].

As stated before, BNP1-32 is only marginally able to stimulate cGMP generation in comparison to CNP. As seen in **Figure 41**, the stimulation with the peptide led to the generation of approx. 3 pmol/ml cGMP. Surprisingly, BNP1-30 showed much higher effects on NPRB-transfected cells compared to BNP1-32. Although cGMP levels were still low compared to those arising from NPRA-stimulation, the amount of cGMP generated is tripled in comparison to usual levels achieved by stimulation with BNP1-32. Notably, other metabolites did not show this increased effect on NPRB.

3.5.1.4 Stimulation of NPRA & NPRB double-transfected HEK293 cells

Since the experiments on the NPRB revealed that BNP1-30 stimulation led to a significantly higher cGMP generation compared to BNP1-32, it was tested whether the stimulation of both receptors in parallel would lead to an additional increase in

cGMP formation upon stimulation with BNP1-30. Thus, to complete the investigations, the bioactivity of BNP1-32 and the BNP metabolites was investigated in NPRA&B-double-transfected HEK293 cells.

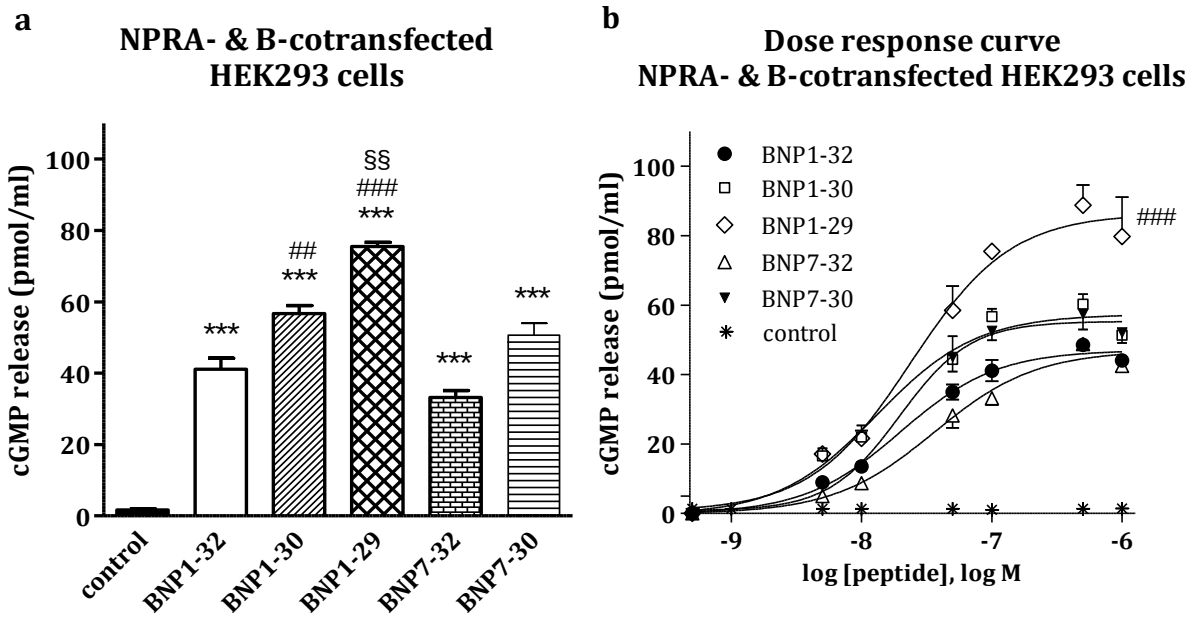


Figure 42: a) Quantification of the cGMP levels after stimulation of NPRA & B-cotransfected HEK293 cells with BNP1-32, BNP1-30, BNP1-29, BNP7-32, and BNP7-30 with a peptide concentration of 10^{-7} M .Significances were calculated with One-way ANOVA: *** $P < 0.001$ vs. control; ## $P < 0.01$; ### $P < 0.001$ vs. BNP1-32; §§ $P < 0.01$ vs. BNP1-30 [n = 3 in triplicates] **b)** dose response curves obtained from the stimulation of NPRA & B-cotransfected HEK293 cells with BNP1-32, BNP1-30, BNP1-29, BNP7-32, and BNP7-30. Significances were calculated with Two-way ANOVA: ###$P < 0.001$ vs. BNP1-32 (although not indicated, all curves are *** $P < 0.001$ vs. control) [n = 3 in triplicates].

As shown in **Figure 42a**, all metabolites were able to stimulate significant cGMP generation compared to the control. The graph shows the same pattern as for the NPRA-single transfected cells, since this receptor is the major effector for cGMP formation by BNPs. Notably, cGMP values were lower in comparison to those measured in NPRA-single transfected cells, although same plasmid concentration was used in both approaches. Very recently, it was discovered in our working group, that subunits of NPRA and NPRB can interact with each other forming heterodimers with altered ligand affinities[263].Thus, cGMP values from single-transfected cells might not necessarily summarize. To provide insight into the potency of the peptides, experiments with different concentrations of the peptides have been made and dose response curves have been calculated. **Figure 42b** shows the different curves for each metabolite applied. As revealed before, BNP1-29 showed highest efficacy with a top value of 86 pmol/ml, whereas BNP7-32 had the lowest with a top value of

47 pmol/ml with a peptide concentration of 5 x 10^{-7} M (**Table 16**). The EC_{50} values were similar for all peptides (also **Table 16**). Significance towards BNP1-32 was reached only for BNP1-29 using Two-way ANOVA caused by the high number of groups being compared. In a comparison using only one curve in comparison to that of BNP1-32, BNP1-30 and BNP7-30 are also significantly different to BNP1-32.

Table 16: Summary of EC_{50} and Top values from dose response curve.

	BNP1-32	**BNP1-30**	**BNP1-29**	**BNP7-32**	**BNP7-30**
EC_{50}	1.979e-008	1.312e-008	2.389e-008	3.620e-008	1.955e-008
Top	47.03	57.36	86.37	46.92	55.32

Besides using receptor-transfected cells, bioactivity of the BNP metabolites was also tested using cells endogenously expressing natriuretic peptide receptors. Cell types, which are known to be essential in blood pressure homeostasis, have been utilized for such investigations. Results are described in the next chapter.

3.5.2 Cells endogenously expressing natriuretic peptide receptors

Vascular smooth muscle cells (VSMC), expressing both, NPRA and NPRB, have been isolated from murine aortas and cultured until passage 2 to 3 (see Method section 2.2.9.5). Since the cell number varied between the single experiments due to methodological reasons, cGMP generation upon BNP stimulation has been calculated as percentage and is illustrated as a summary of all experiments in **Figure 43**. Also here, BNP1-30 and BNP1-29 were more efficient in stimulating the receptors compared to BNP1-32. However, the bioactivity of BNP1-29 was comparable to BNP1-30 and not higher as seen in NPR-double transfected cells. BNP7-30- and BNP7-32-mediated cGMP generation was similar to BNP1-32.

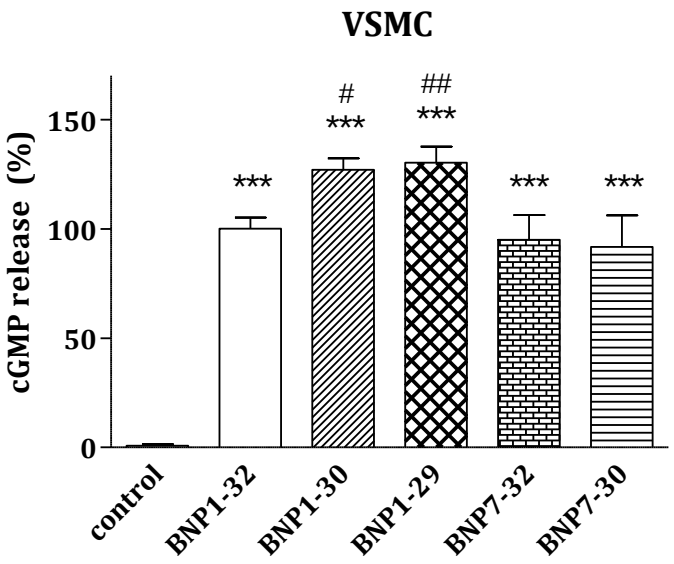


Figure 43: Quantification of the cGMP levels after stimulation of vascular smooth muscle cells (VSMC) with BNP1-32, BNP1-30, BNP1-29, BNP7-32, and BNP7-30 with a peptide concentration of 10^{-7} M. Significances were calculated with One-way ANOVA: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs. BNP1-32 [n = 3 in triplicates].

Stimulation of primary human dermal microvascular endothelial cells (HDMEC), dominantly expressing endogenous NPRA, revealed a similar stimulation pattern compared to NPRA-transfected HEK293 cells (**Figure 44a**). The metabolites BNP1-30 and BNP1-29 exerted stronger effects on the cells compared to BNP1-32, with BNP1-29 being again most active. BNP7-32 and BNP1-30 were similarly active compared to BNP1-32.

Next, the stimulation of mesangial cells (MC), primarily expressing NPRB and influencing small resistance vessels in the kidney under physiological conditions, was performed **(Figure 44b**). Since the culture of these cells has been delicated and cell numbers were low, investigations were limited to BNP1-32 and the two most bioactive metabolites BNP1-30 and BNP1-29.

BNP1-30 was again significantly more effective than BNP1-32 in stimulating cGMP formation. In contrast, BNP1-29 stimulation led to the generation of similar cGMP amounts compared to BNP1-32. Notably, this reflects what was observed in NPRB-transfected HEK293 cells (see section 3.5.1.3), however here the increased effects of BNP1-30 are less pronounced.

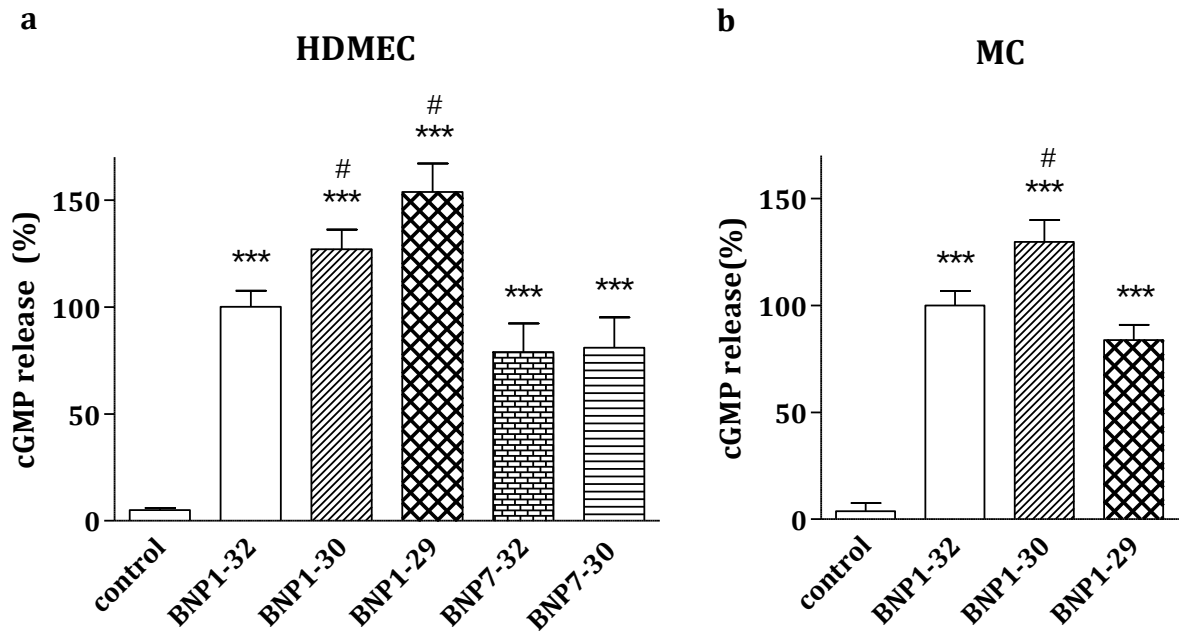


Figure 44: Quantification of the cGMP levels after stimulation of **a)** human dermal microvascular endothelial cells (HDMEC) or **b)** mesangial cells (MC) with BNP1-32, BNP1-30, BNP1-29, BNP7-32 and BNP7-30 with a peptide concentration of 10^{-7} M. Significances were calculated with One-way ANOVA: # $P < 0.05$ vs. BNP1-32 [n = 3 in triplicates].

In all previous experiments bioactivity of the BNP metabolites has been determined by their ability to stimulate cGMP generation. To get a closer insight into the bioactivity of BNP1-32 and BNP metabolites, their vasoactivity has been investigated by determining their ability to induce vasorelaxation on isolated vessels in an *ex vivo* approach.

3.6 Biological activity of BNP1-32 and BNP metabolites *ex vivo*

Mesenteric arteries have been isolated from rats, mounted into a myograph system and stimulated with the peptides (see Method section 2.2.10). Since the myograph system allows a measurement of four vessel preparations in parallel, stimulations with BNP1-32, BNP1-30, BNP1-29, and water as a control have been performed simultaneously and were compared to each other.

Experiments showed that all peptides were able to stimulate vasorelaxation of the vessels to a higher extend than natural vasorelaxation caused by water over time (**Figure 45a**). BNP1-29 was in trend more effective in relaxing the vessels compared

to the other two metabolites however, without reaching statistical difference (Two-way ANOVA: $P = 0.78$ vs. BNP1-32 and P = 0.25 vs. BNP1-30).

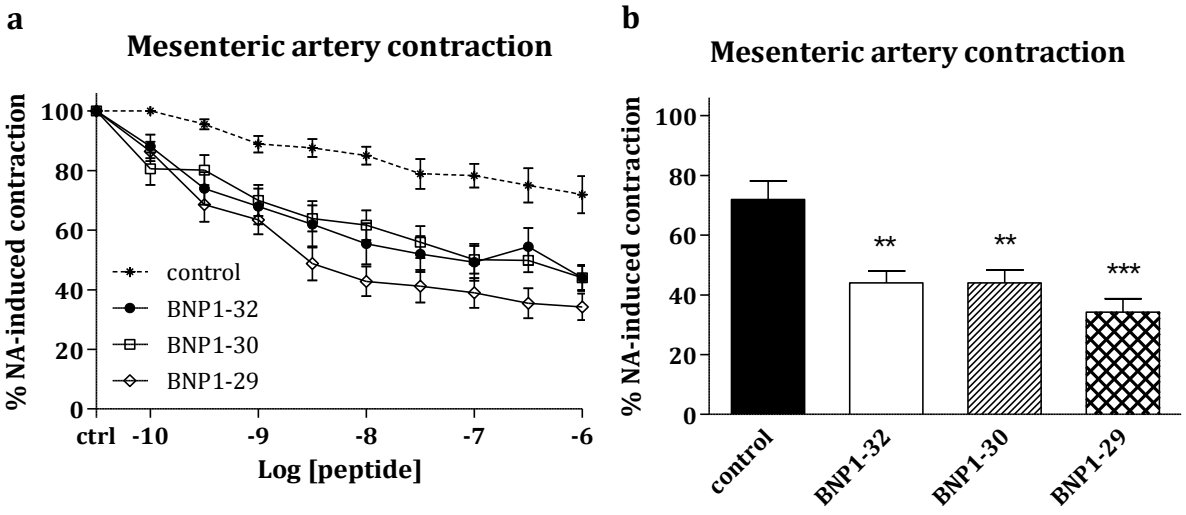


Figure 45: **a)** Stimulation of isolated mesenteric arteries with different concentrations of BNP1-32, BNP1-30, and BNP1-29. Water stimulation functions as control; **b)** Summary of the stimulation of isolated mesenteric arteries with BNP1-32, BNP1-30 and BNP1-29 at a concentration of 10^{-6} M per peptide. Significances were calculated with One-way ANOVA: ** $P < 0.01$; *** $P < 0.001$ vs. control [n = 7].

Figure 45b illustrates the difference in vasorelaxation with a peptide concentration of 10^{-6} M. BNP1-32 and BNP1-30 achieved a relaxation of about 72%, whereas BNP1-29 was with a value of 62% slightly more effective.

The investigations revealed that the newly identified BNP metabolites BNP1-30 and BNP1-29 are able to induce vasorelaxation in isolated vessels similarly to BNP1-32. Thus, it is likely that BNP metabolites are also able to induce vasorelaxation under *in vivo* conditions, which would be indicated by a decrease in blood pressure. In the next chapter such experiments are described.

3.7 Biological activity of BNP1-32 and BNP metabolites under normotensive conditions *in vivo*

3.7.1 Rationale of the studies *in vivo*

The bioactivity of the BNP metabolites has been characterized *in vitro* and *ex vivo,* whereby it was shown that all BNP metabolites investigated are bioactive. The two

metabolites BNP1-30 and BNP1-29 were similarly or in the majority of experiments even more bioactive compared to BNP1-32. Based on these findings, investigations to the effects of BNP metabolites under *in vivo* conditions have been considered. Therefore, an animal model has been utilized. In the following approaches, BNP1-32 or BNP metabolites have been independently administered to mice in similar study designs in which their acute influence on blood pressure under normotensive conditions has been revealed.

3.7.2 Acute effects of BNP metabolites on blood pressure in normotensive mice

2.7.2.1 Study design

In this approach, BNP1-32, the BNP metabolites, and saline, as a control, have been injected into the jugular vein of ventilated normotensive mice under anesthesia and hemodynamic parameters e.g. mean arterial blood pressure (MAP) and heart rate have been recorded by a millar catheter inserted into the left carotid artery (see Method section 2.2.11.1). The experiment was separated into three sets. Set1 included the C-terminally truncated metabolites BNP1-30 and BNP1-29 in comparison to BNP1-32. Set2 included BNP7-32 and BNP7-30 compared to BNP1-32. Set3 included BNP1-32 and the peptide BNP1-45, an alternative variant of BNP found in rodents only (see Introduction section 1.2.1).

3.7.2.2 BNP1-30 and BNP1-29 – Set1

In the first experimental set the two new metabolites BNP1-30 and BNP1-29, which have been the most bioactive peptides in the *in vitro* studies, were characterized and compared with the actions of the mature BNP1-32.

Figure 46 shows representative blood pressure curves for the infusion of either (**a**) saline, (**b**) BNP1-32, (**c**) BNP1-30, or (**d**) BNP1-29.

Heart rate of mice under isoflurane anesthesia averaged between 450 to 600 bpm (grey scale). All peptide and saline injections did not change heart rate compared to the mean heart rate before injection. **Figure 46a** shows the changes in the MAP after injection of the peptides or saline. Saline injection did not change MAP compared to the baseline proving that the influence of volume expansion on the blood pressure is negligible (bp: +0.3 ± 1.4 mmHg, n = 5). In contrast, every peptide infusion resulted in

a transient decrease in blood pressure within the first minute after injection compared to their baselines. Interestingly, comparing the peptides, the newly identified metabolite BNP1-30 caused a two-fold higher decrease in the MAP (-6.8 ± 0.8 mmHg, n = 7) compared to BNP1-32 (-3.4 ± 0.65 mmHg, n = 7) without changing the action time.

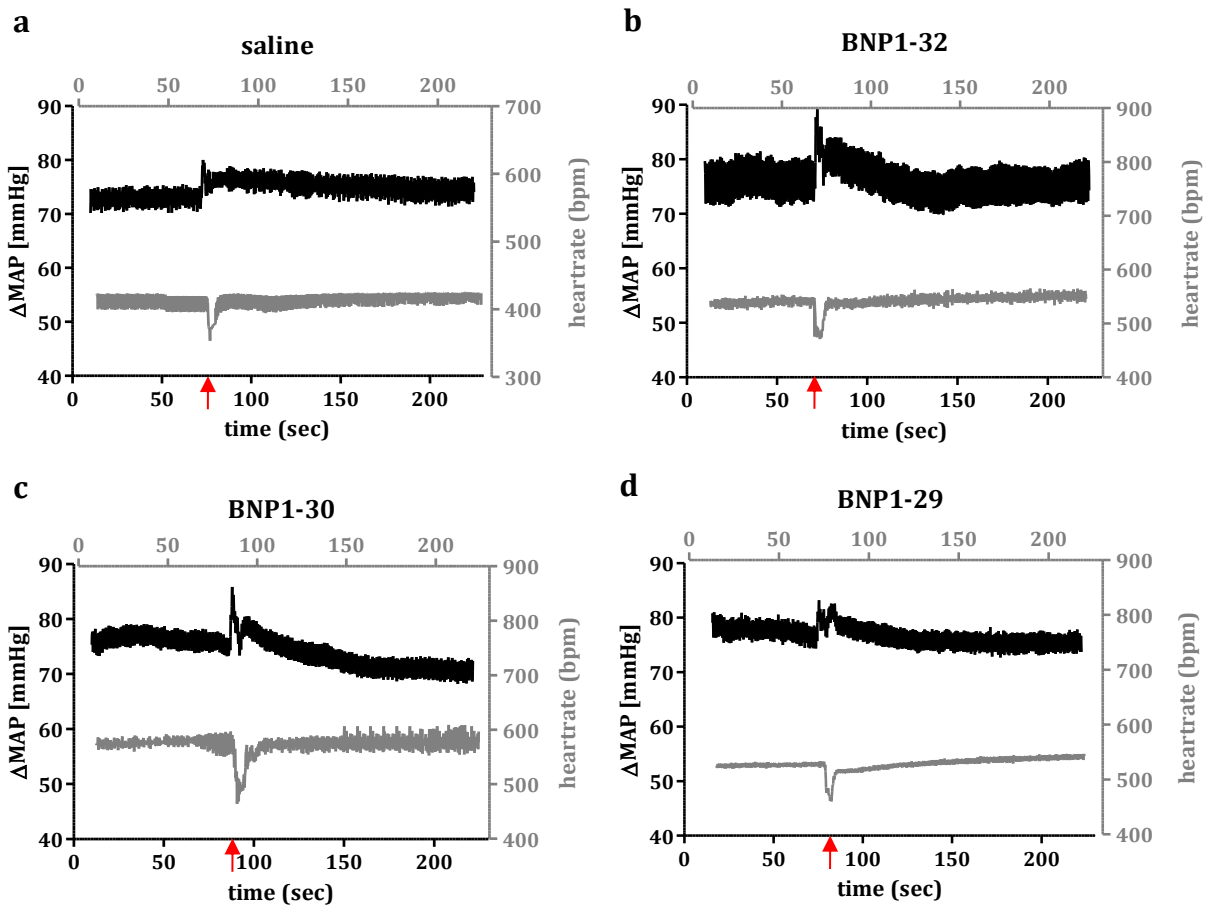


Figure 46: Representative blood pressure curves after **a)** saline, **b)** BNP1-32, **c)** BNP1-30 and **d)** BNP1-29 injection. Saline or peptides have been infused to normotensive mice after a stable baseline blood pressure was established (infusion indicated by red arrows). The left black scale shows the mean arterial blood pressure (MAP) in mmHg, the right grey scale shows the corresponding heart rate in beats per minute (bpm).

This stronger effect of BNP1-30 confirms the data obtained *in vitro,* where BNP1-30 was more potent compared to BNP1-32 in generating cGMP. However, BNP1-29, which was also more potent in NPRA-transfected cells compared to BNP1-32 had only comparable effect to BNP1-32 (-3.4 ± 0.5 mmHg, n = 7). BNP1-29 and BNP1-32 injections decreased the MAP by 6% whereas BNP1-30 decreased MAP by 12% compared to baseline blood pressure.

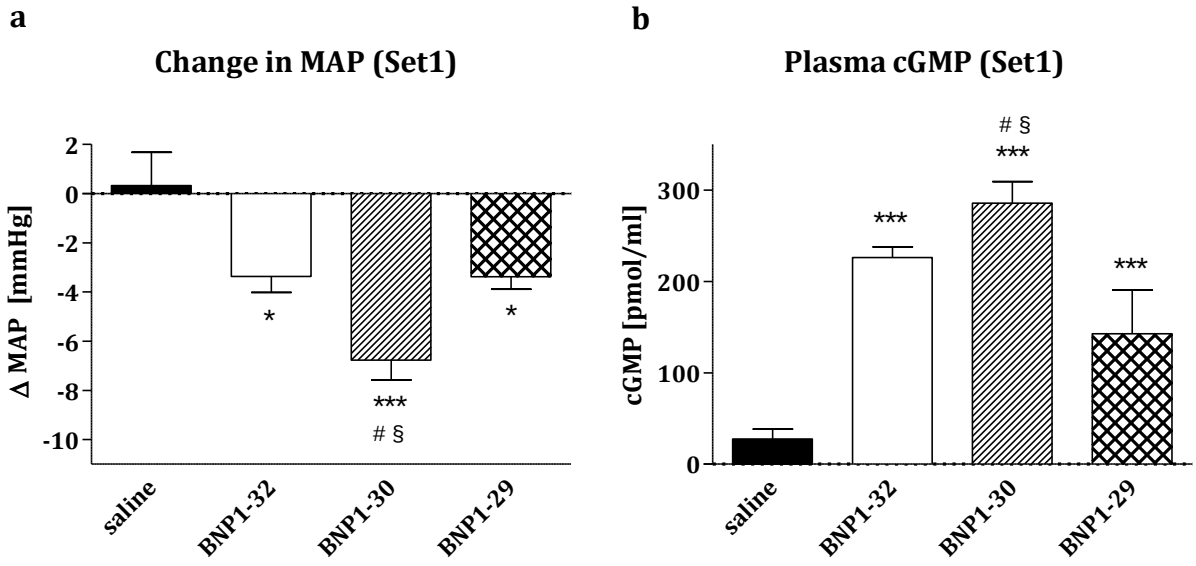


Figure 47: a) Change in the mean arterial blood pressure (ΔMAP) in mmHg after bolus injection of BNP1-32, BNP1-30 or BNP1-29 in comparison to saline in an acute phase experiment in a normotensive mouse model; **b)** Quantification of cGMP levels in plasma samples obtained from the same mice after the injection of BNP1-32, BNP1-30, BNP1-29 and saline (in fold change). Significances have been calculated with One-way ANOVA:* $P < 0.05$, *** $P < 0.001$ vs. saline; $^{\#}P < 0.05$ vs. BNP1-32; $^{\S}P < 0.05$ vs. BNP1-29 [n = 7 per peptide, n = 5 for saline].

Figure 47b shows the cGMP levels in the blood taken by cardiac puncture after hemodynamic characterization. Since, the BNPs mediate their blood pressure effects trough NPR-stimulation, cGMP values should reflect their actions. Indeed it could be revealed that the values were elevated in the peptide-infused mice compared to the saline-infused animals. Moreover, cGMP values for BNP1-30-infused mice were significantly higher compared to the other BNP-infused groups, being congruent with the blood pressure data.

3.7.2.3 BNP7-32 and BNP7-30 – Set2

In a second set of normotensive animals, bioactivities of BNP7-32 and BNP7-30 have been compared to BNP1-32 and saline injections. Saline and BNP1-32 injections led to a very similar decrease in blood pressure as obtained in the first set proving reliability of the method (saline: +0.5 ± 0.8 mmHg, n = 5; BNP1-32: -3.4 ± 1.1 mmHg, n = 8; **Figure 48a**). Moreover, the data make clear that the BNP metabolites BNP7-32 and BNP7-30 were both not able to significantly lower the blood pressure (BNP7-30: -1.6 ± 1.3 mmHg, n = 8; BNP7-32: -0.3 ± 0.4 mmHg, n = 8). These results are in part reflected by cGMP values measured in plasma of the same mice (**Figure 48b**). While the cGMP levels of the BNP1-32-injected mice were strongly

elevated compared to the saline-injected mice, tha levels in the BNP7-32-injected mice did not significantly differ from that of the control animals. The cGMP levels of BNP7-30-infused mice however were significantly elevated compared to saline and BNP7-32- infused mice but were lower compared to BNP1-32. Looking again on the blood pressure data and taking into account that since working with animals the SEM of parameters between different animals is naturally relatively high, the in trend decreased blood pressure and the elevated cGMP values after peptide injection indicate that BNP7-30 has some ability to decrease blood pressure. However, apparently BNP1-32 is more efficient in reducing the blood pressure compared to both BNP metabolites, BNP7-30 and BNP7-32.

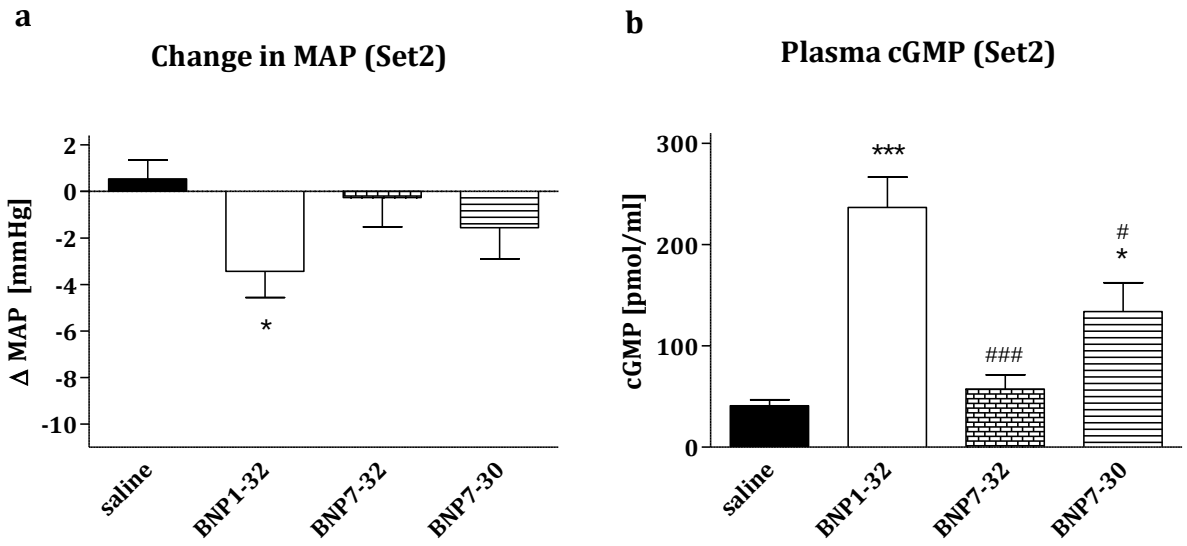


Figure 48: a) Change in the mean arterial blood pressure (MAP) in mmHg after bolus injection of BNP1-32, BNP7-32 or BNP7-30 in comparison to saline in an acute phase experiment in a normotensive mouse model; **b)** Quantification of cGMP levels in plasma samples obtained from the same mice after the injection of BNP1-32, BNP7-32, BNP7-30and saline (in fold change). Significances were calculated with One-way ANOVA: * $P < 0.05$, *** $P < 0.001$ vs. saline; # $P < 0.05$, ### $P < 0.001$ vs. BNP1-32 [n = 8 per peptide, n = 5 for saline].

3.7.2.4 BNP1-30 and BNP1-45 – Set3

In a third independent set of animals BNP1-30, which was revealed as the most potent BNP metabolite *in vivo* so far was compared to BNP1-45, a BNP metabolite generated by an alternative way of BNP processing found in rodents only (see Introduction 1.2.1).

Both peptide injections caused a significant decrease of the blood pressure compared to the saline injection, whereby also here BNP1-30 was the more efficient peptide

(BNP1-30: -6.3 ± 1.0 mmHg, n = 7 vs. BNP1-45: -3.6 ± 0.5 mmHg, n = 7 **Figure 49a**). BNP1-45 was similar effective compared to BNP1-32 in Set1 and Set2. The cGMP levels in blood samples of BNP1-30 and BNP1-45-infused animals were elevated compared to that of the control blood (**Figure 49b**). Although there was no significant difference between the cGMP levels of the two peptides, the concentrations in BNP1-30-infused mice were in trend higher compared to BNP1-45-infused mice.

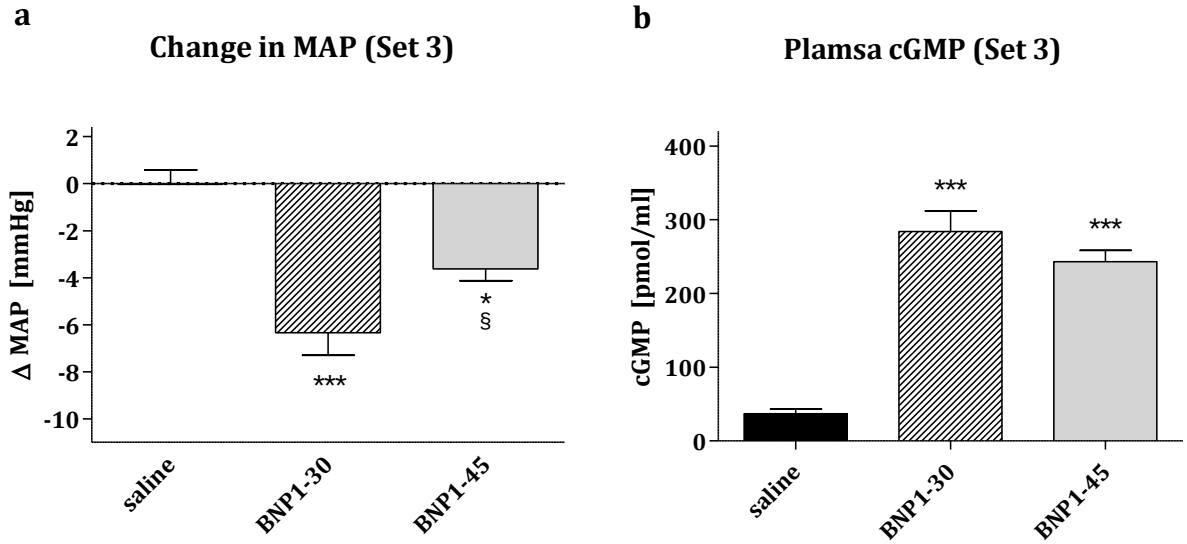


Figure 49: a) Change in the mean arterial blood pressure (MAP) in mmHg after bolus injection of BNP1-30 and BNP1-45, a BNP metabolite generated by an alternative way of BNP processing found in rodents only, in comparison to saline in an acute phase experiment in a normotensive mouse model; **b)** Quantification of cGMP levels in plasma samples obtained from the same mice after the injection of BNP1-30, BNP1-45 and saline. Significances were calculated with One-way ANOVA: * $P < 0.05$, *** $P < 0.001$ vs. saline; § $P < 0.05$ vs. BNP1-30 [n = 7 per peptide, n = 5 for saline].

3.7.3 Summary of the metabolites' bioactivity under normotensive conditions

As suggested on the basis of the data *in vitro* and *ex vivo*, it could be shown that BNP metabolites BNP1-30, BNP1-29, and BNP7-30 exert biological activity under *in vivo* conditions. On the contrary, BNP7-32 did, if at all, only showed marginal influences on the blood pressure in this animal model. Importantly, it was revealed that the metabolite BNP1-30 is more effective in decreasing the blood pressure compared to BNP1-32 and other BNP metabolites. Apparently, the same concentration of the peptide even doubled the effect on the MAP compared to BNP1-32. Since BNP1-30 showed these strong effects under normotensive conditions, it was decided to test its

actions also under pathophysiological conditions. The study design and the emerging results are described in the next chapter.

3.8 Biological activity of BNP1-32 and BNP metabolites under hypertensive conditions

3.8.1 Rationale of the approach

Recombinant BNP1-32 (Nesiritide®) is approved for the treatment of cardiovascular diseases[177]. Since the previously performed approach *in vivo* (section 3.7) highlighted that the newly discovered BNP metabolite BNP1-30 exerts a stronger blood pressure-lowering effect than BNP1-32 under normotensive conditions, it was aimed to compare the effects of both peptides under hypertensive conditions. An animal model of severe hypertension, the spontaneously hypertensive rat-model, has been chosen for such approach.

3.8.2 The spontaneously hypertensive rat -model

3.8.2.1 Study design

Spontaneously hypertensive rats (SHR) develop hypertension without pharmacological intervention or surgical procedures around 5-6 weeks of age, reaching systolic pressures between 180 and 200 mmHg in adult age[264]. Due to this very high pressure, structural alterations like hypertrophy and fibrosis occur in the heart of these animals at the age of 6 month[265]. The model is similar to the etiopathology of essential hypertension in humans and is applicable to determine antihypertensive actions of drugs[266, 267]. The aim of this approach was to investigate the antihypertensive effects of the newly identified BNP1-30 in comparison to BNP1-32.

In the experiment, minipumps filled with either saline or solutions of BNP1-32 or BNP1-30, have been implanted subcutaneously into 12-week-old animals and blood pressure has been measured after three days of continuous peptide administration (see Method section 2.2.11.2).

3.8.2.2 Influence on the blood pressure

Animals in the control group that received saline had a systolic blood pressure (SBP) of 199.8 ± 2.1 mmHg (n = 6) indicating a state of severe hypertension in these animals (**Figure 50**). Administration of BNP1-32 and BNP1-30 over three days caused different alterations in the blood pressure. BNP1-32 administration did reduce SBP by approx. 13.5 mmHg (BNP1-32: 186.3 ± 4.4 mmHg, n = 6) compared to that of saline-treated animals without reaching statistical difference in the One-way ANOVA, but being equal to a *P*-value of 0.03 in the student's t-test vs. saline. However, the treatment with the metabolite BNP1-30 led to a profound blood pressure drop-down. On average, SPB was reduced by 36.5 mmHg (BNP1-30: 163.3 ± 6.7 mmHg, n = 6). Thus, the reduction was almost 3-fold higher compared to that achieved with BNP1-32 treatment.

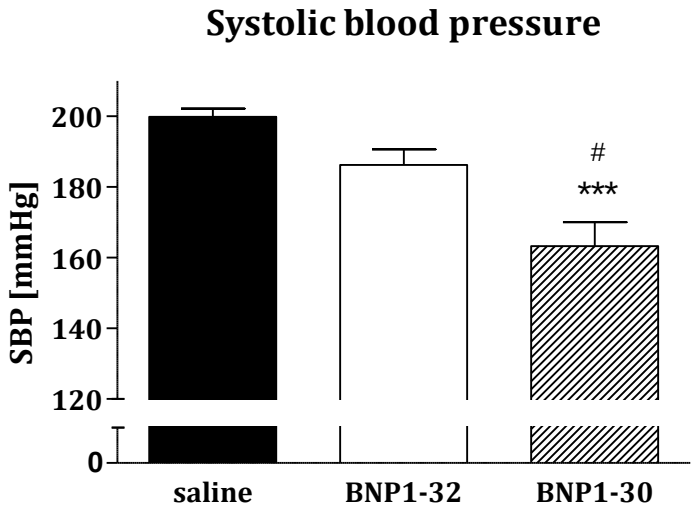


Figure 50: Systolic blood pressure (SBP) in mmHg of spontaneously hypertensive rats (SHR) treated with either saline, BNP1-32, or BNP1-30 by the administration through osmotic minipumps for three days; Significances have been calculated with One-way ANOVA: *** $P < 0.001$ vs. saline; # $P < 0.05$ vs. BNP1-32 [n = 6 per peptide or saline].

The cGMP levels measured in the blood of the animals did not differ among the groups, but showed in trend increased values for both peptide-treated animals, whereby levels were highest in the BNP1-30-treated SHR (**Figure 51**). However, cGMP values of all groups have been very low and near the detection limit of the assay. Thus, variations in the cGMP levels appear large. Low cGMP levels have been reported before in these animals, likely caused by the cGMP-degrading enzyme phosphodiesterase 5 (PDE-5), which was shown to be up-regulated in SHR[268]. In

parallel this indicates that the effects observed in these animals might not be solely mediated by a cGMP response.

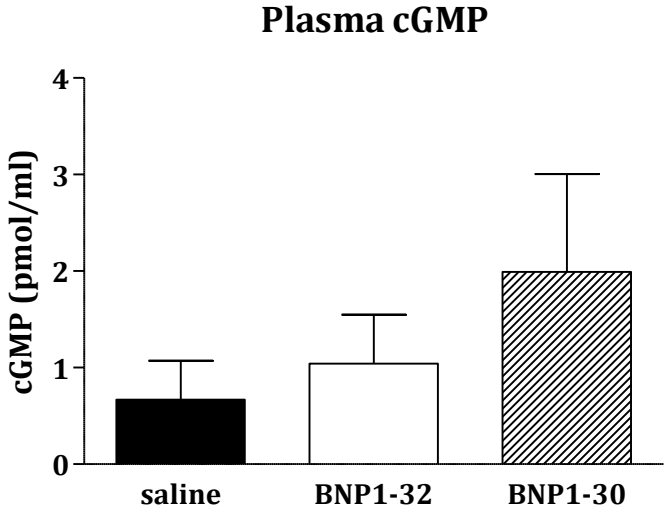


Figure 51: cGMP levels measured in blood of spontaneously hypertensive rats (SHR) treated with either saline, BNP1-32, or BNP1-30 by the administration through osmotic minipumps for three days; Significances have been calculated with One-way ANOVA [n = 6 per peptide or saline].

3.8.2.3 Analysis of cardiac markers

Collagen 1 production was analysed by real-time polymerase chain reaction (rt-PCR, see Method section 2.2.5) in the hearts of the SHR by determining cardiac levels of COL1A1 mRNA, which is encoding for the pro-α1 chain of the collagen 1 precursor. COL1A1 mRNA levels were down-regulated in trend in both peptide-treated groups indicating that first improvements in cardiovascular remodeling took place within the three days (**Figure 52a**).

Moreover, cardiac ANP and BNP mRNA levels have been measured by rt-PCR to reveal alterations of the cardiovascular status of the animals. ANP mRNA levels in the heart were down-regulated in both peptide-treated groups, but the effect was doubled through BNP1-30 administration (**Figure 52b**). Since the primary stimulus for ANP generation is wall stretch, and obviously this wall stretch was reduced through the decrease in blood pressure, the results of the ANP measurements are confirming the blood pressure data.

For BNP1-32-treated SHR, there was no alteration of BNP mRNA levels compared to saline-treated animals. However, a significant down-regulation by around 40% in BNP1-30-treated animals compared to saline-treated rats was observed (**Figure 52c**).

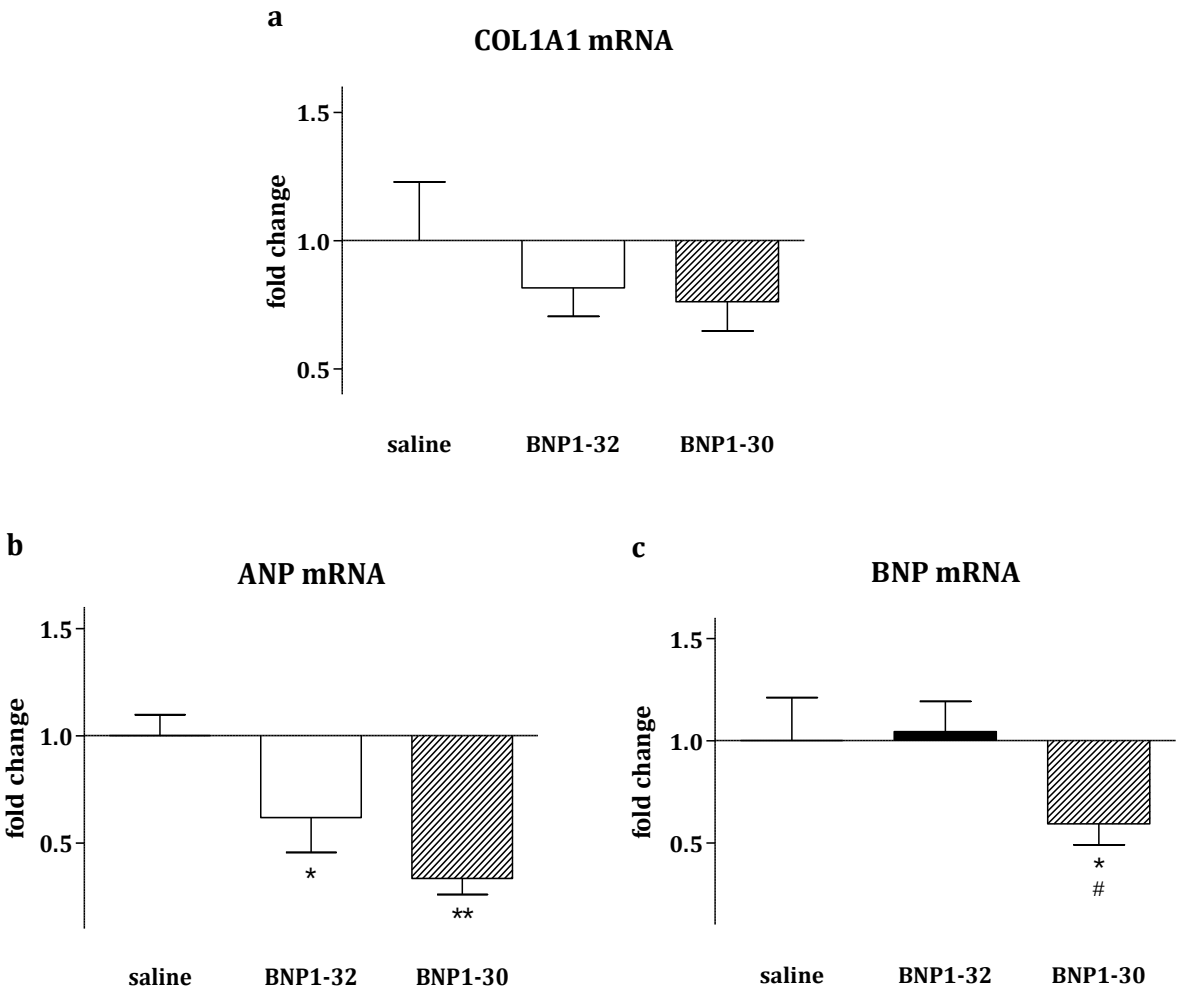


Figure 52: a) cardiac COL1A1 mRNA levels in fold change, **b)** cardiac ANP mRNA levels in fold change, and **c)** cardiac BNP mRNA levels measured in fold change of spontaneously hypertensive rats (SHR) treated with either saline, BNP1-32, or BNP1-30 by the administration through osmotic minipumps for three days; Significances have been calculated with One-way ANOVA: * $P < 0.05$, ** $P < 0.01$ vs. saline; # $P < 0.05$ vs. BNP1-32 [n = 6 per peptide or saline].

Similar to ANP, BNP generation is induced by wall stretch of the myocardium. Thus, the difference in BNP mRNA levels between the peptide-treated groups also here confirms the blood pressure data. A possible negative feedback mechanism on BNP generation through the administration of BNP seems not to be present, since BNP levels are unaltered in BNP1-32-treated animals. Since BNP is used as a biomarker for

the progression of cardiovascular diseases[186-189], the decrease of BNP mRNA through the treatment of BNP1-30 indicates an improvement of cardiovascular health of the animals. Obviously this improvement was not achieved with the conventional BNP1-32-treatment.

3.8.3 Summary of the actions of BNP1-32 and BNP1-30 under hypertensive conditions

In a model of severe hypertension, it could be highlighted that the newly discovered BNP metabolite BNP1-30 was more efficient in reducing hypertension compared to BNP1-32. This effect was almost 3-fold higher than that achieved by the treatment of the conventional BNP1-32. This finding and also the results obtained from other *in vivo* experiments described in this thesis, strongly indicate that the newly discovered BNP metabolites and especially BNP1-30 are not just intermediate products in the metabolism of BNP on the way to BNP inactivation, but might represent comparable or even superior treatment options for hypertension and possible other blood pressure-associated diseases compared to BNP1-32.

4 DISCUSSION

4.1 Significance of peptide metabolism

Peptide metabolism plays an essential role for the efficiency of peptide systems. Such peptide metabolism has been investigated in major peptide systems like the renin-angiotensin system, another important player in blood pressure homeostasis besides the natriuretic peptide system[269, 270]. A key peptide of the renin-angiotensin system, the octapepide AngII, is the bioactive form of angiotensinogen formed by the actions of renin[271] and ACE[174]. AngII has been found to be metabolized into many truncated, but bioactive peptides. Such metabolites are e.g. AngIII (2-8)[272], AngIV (3-8)[273] and Ang-(1-7)[274]. But, they are not only biologically active, they also exert their biological effects in part through different receptors. While AngII and AngIII have similar affinities towards the AngII receptor subtypes (AT) AT_1 and AT_2[275, 276], Ang-(1-7) was observed to be associated with Mas signaling[277]. The metabolite AngIV binds to the AT_4, which is a transmembranal enzyme, the insulin-regulated membrane aminopeptidase (IRAP), indicating that AngIV is involved in glucose uptake[278, 279] and thus has a different physiological function than its precursor AngII. Moreover, metabolites of AngII are formed by different peptidases as e.g. ACE2 (Ang-(1-7) from AngII)[280] and aminopeptidase A (AngIII from AngII)[281].

Peptide metabolism appears also in other peptide systems. Bradykinin (BK), a vasoactive nonapeptide of the kinin-kallikrein system with cardioprotective effects, was shown to be metabolized in circulation forming BK(1-5)[157] and BK(1-7)[282]. Big-endothelin-1 (Big-ET-1) from the endothelin system, which is another important system in the homeostasis of blood pressure, is despite its activation to ET-1 with greater vasoconstrictive potency than any known substance[283], also cleaved forming ET-1(1-31)[158]. This metabolite is amongst other properties also a potent vasoconstrictor.

Metabolism within the natriuretic peptide system has not been described intensively. In this thesis BNP metabolism has been investigated comprehensively and it was disclosed that BNP is, similarly to other vascoactive peptides, metabolized by different enzymes forming a variety of BNP metabolites and that this metabolism plays an essential role in many ways.

4.2 The newly disclosed metabolism of BNP1-32

4.2.1 The metabolism of BNP1-32 is organ-specific

Processing of BNP starts with the formation of pro-BNP from pre-proBNP[140]. This precursor is than cleaved by the actions of corin and furin into the N-terminal fragment NT-proBNP and the bioactive mature fragment BNP1-32[19]. The work of this thesis revealed, that against the common view that BNP1-32 represents the endpoint of BNP processing, the peptide is further metabolized into BNP metabolites in a complex process. In fact, the very first approach described in this thesis already indicated that this metabolism must be complex. This first experiment showed that the velocity of BNP proteolysis is divergent among the organs although protein concentrations of all membrane preparations used had been normalized (see Results section 3.1.1.1). Furthermore, it was revealed that proteolysis of BNP1-32 is accompanied by the generation of a variety of BNP metabolites. Six novel BNP metabolites were discovered namely BNP1-31, BNP1-30, BNP1-29, BNP7-31, BNP7-30, and BNP7-29. Most BNP metabolites were only generated in particular organs, while others were not formed there. For example, it was disclosed that the BNP metabolite BNP1-30 was generated in heart, lung, liver, and spleen, but not in kidney (see Results section 3.1.1.2). Vice versa, it was shown that BNP7-32 is generated in kidney, but not in other organs investigated. Similarly, it was disclosed that BNP1-29 is initially generated in heart only and BNP1-31 was solely identified using blood preparations (see Results section 3.1.3.3). Thus, the first main conclusion from the work of this thesis is that BNP metabolism is organ-specific.

4.2.2 The coactions of different organs leads to the metabolism of BNP1-32

Investigations revealed that some BNP metabolites generated in particular organs can be further metabolized in other organs. For example, it was observed that the BNP metabolite BNP7-32 formed in kidney can undergo a C-terminal truncation in lung and spleen to form BNP7-30 (see Results section 3.1.1.5). Furthermore, it was disclosed that BNP1-29 formed in heart can be truncated into BNP7-29 in kidney. In general it was observed that while in kidney mainly an N-terminal truncation takes place, in liver and spleen mostly a C-terminal truncation of BNP metabolites appears. Through the use of different organ membrane preparations it was however possible to successively generate every newly identified BNP metabolite including the shortest identified BNP metabolite BNP7-29. Only this stepwise truncation leads to full proteolysis of BNP1-32. Thus, according to these results, the second main conclusion from this work is that full proteolysis of BNP1-32 needs the involvement of several organs.

All organs are connected to each other through the circulation, which allows the transport of dissolved compounds from organ to organ. It is thus conceivable that some BNP metabolites are specifically produced in one organ and are then transported through the circulation to another organ, in which further metabolism can be realized, whereby also the blood exerts its own proteolytic activity towards the peptides. Therefore, it is hypothesized that the connection of different organs through the circulation allows the formation of a network for full metabolism and degradation of BNP1-32. Such a network has been illustrated in **Figure 53.**

4.2.3 Enzymes involved in BNP metabolism

It was revealed that the generation of BNP metabolites is organ-specific and that full metabolism needs the coactions of different organs, since they harbor the different peptidase activities responsible for the formation of different BNP metabolites. To develop a new overview about BNP processing, the newly identified pathways in the formation of BNP metabolites including the role of particular enzymes are shortly summarized here. A variety of potential candidates being responsible for BNP1-32 metabolism have been identified. Notably, the formation of every single BNP

metabolite identified during incubations with membrane preparations of organs and blood preparations could be reproduced with one or more recombinant enzymes.

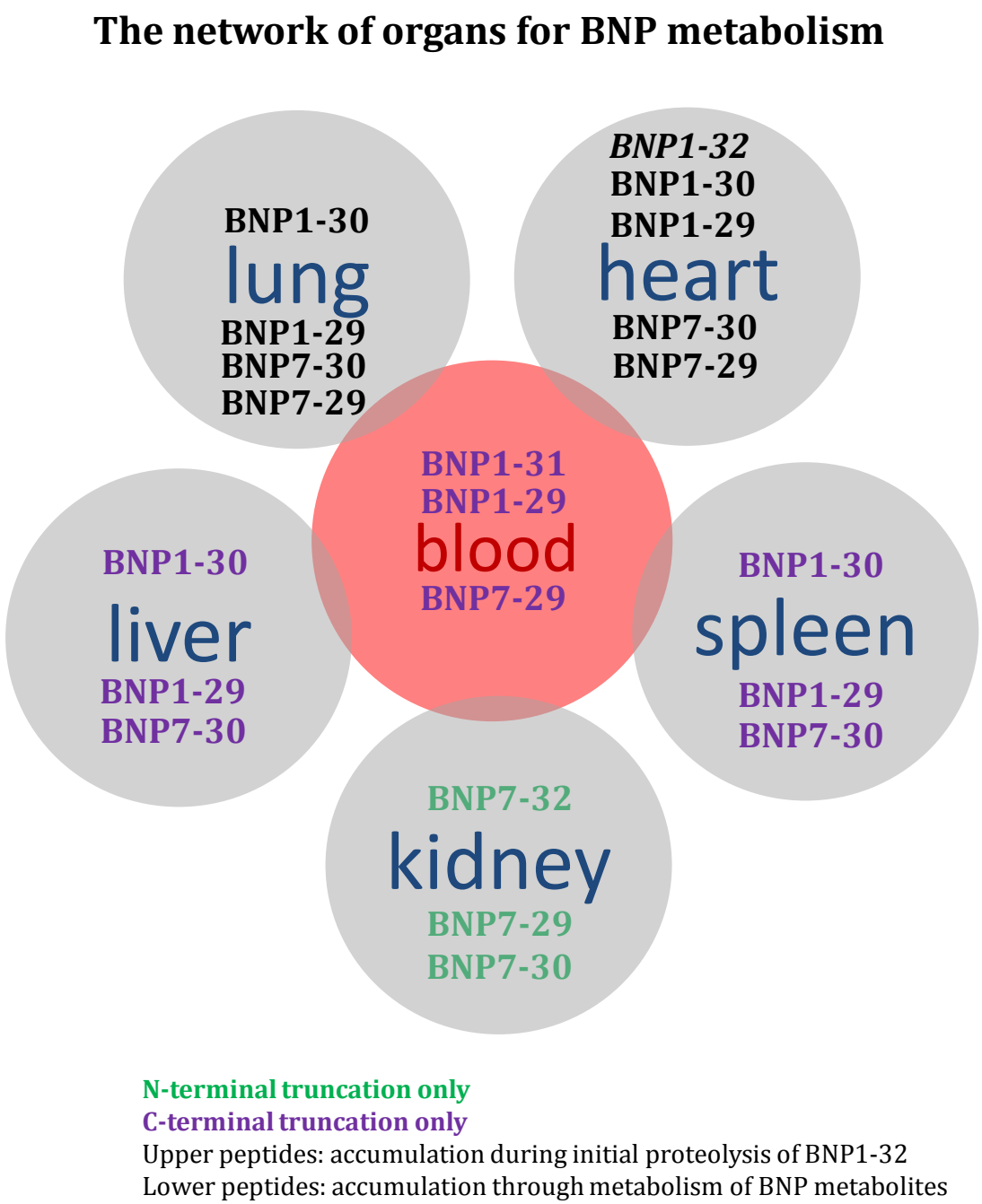


Figure 53: Overview about the organs and the blood forming a network for the metabolism of BNP1-32 and BNP metabolites. All organs are connected to each other through blood circulation and thus, the transport of BNP metabolites to each organ is possible. All metabolites actually detected through the incubation of BNP1-32 or initial BNP metabolites with membrane preparations or blood preparations have been displayed.

Two of these enzymes are involved in the generation of BNP1-30. Recombinant ECE-1 is able to hydrolyze the C-terminal dipeptide from BNP1-32 generating BNP1-30. Similarly, ECE-1 hydrolyzed the same dipeptide at the C-terminus of BNP7-32 generating BNP7-30. A second peptidase, CPA, was identified to catalyze the formation of BNP1-30 via the intermediate product BNP1-31. Similarly, CPA was able to form BNP7-30 from BNP7-32 through the intermediate product BNP7-31. In parallel it was shown that other recombinant carboxypeptidases like ACE, ACE2, CathA, and PRCP were not able to cleave BNP1-32. Furthermore, it could be disclosed that CPB is able to metabolize BNP1-30 into BNP1-29 and similarly BNP7-30 into BNP7-29. Finally, it was shown that meprin A is besides its role in the formation of BNP7-32 from BNP1-32 also able to hydrolyze substrates as BNP1-30 and BNP1-29 forming BNP7-30 and BNP7-29.

Notably, it could be shown that inhibitors of these enzymes supplemented into the incubations with membrane preparations or blood preparation were able to decrease BNP metabolite formation. The ECE-1 inhibitor SM19712 diminished the formation of BNP1-30 in lung and liver, while the carboxypeptidase inhibitor, inhibiting CPA, was attenuating BNP1-30 formation in heart and spleen. Furthermore, it was observed that the presence of the CPB inhibitor GEMSA completely abolished the formation of BNP1-29 and BNP7-29 in blood preparations. Since it could be proven that enzyme inhibitors were able to diminish BNP metabolite formation in the incubations, it can be hypothesized that these particular enzymes are likely responsible for BNP metabolite formation under physiological conditions. As a third main conclusion it can be stated that a variety of enzymes are involved in the formation of BNP metabolites framing a much more complex picture of BNP processing than described in the literature.

4.2.4 The role of BNP metabolism for the degradation by NEP

NEP was thought to be the main enzyme inactivating natriuretic peptides[120, 122]. However, in an approach of Walther *et al*. in 2004 it was shown that inhibition of NEP in tissues like kidney and heart, using the specific inhibitor candoxatrilat, does decrease proteolysis rates of the natriuretic peptides ANP but not of BNP1-32[125]. In the same work it was shown that proteolysis rate of BNP1-32 was unchanged in tissues from NEP knockout mice compared to wild type controls. Thus it was revealed

that BNP1-32 is not a primary substrate for NEP[125]. In own work prior to this thesis a model has been established showing that N-and C-terminal length are decisive for the degradability of natriuretic peptides[20]. The results of this thesis confirm this model, since it was revealed that shorter BNP metabolites are better substrates for NEP. Additional data of the work might give new insight into the mechanism of NEP. It was revealed that the newly identified metabolite BNP1-30 with a C-terminal truncation by two amino acids is still not degradable by NEP (see Results section 3.4). However, the additional truncation by the C-terminal lysine in the newly discovered BNP1-29 markedly increased the degradability similar to that of the N-terminally truncated BNP7-32 and the likewise newly discovered BNP7-30. Obviously, the C-terminal lysine plays a decisive role in the adjustment of the peptide in the catalytic center of NEP. To date, a concrete model showing the interaction of particular amino acids of BNP1-32 with binding sites of NEP is not available. It might be possible that the missing lysine, which is positively charged, decreases the repulsion between the peptide and the peptidase and thus the peptides' adjustment in the catalytic cavity of NEP is favored. The new metabolites BNP7-31 and BNP7-29 have not been available as recombinant peptides, however, according to the derived model for the degradation of natriuretic peptides by NEP (see Introduction section 1.1.5.3) it was anticipated that they are degradable by the enzyme.

The increased degradability of the majority of the BNP metabolites in comparison to BNP1-32 highlights the importance of the BNP metabolism for the clearance of circulating BNP.

4.2.5 A new model of BNP processing

Based on the results of this thesis a new overview about BNP processing is drawn in **Figure 54.** The upper part of the scheme shows the conventional pathway of BNP processing as described in the literature and leading to the formation of NT-proBNP (76 aa) and the bioactive BNP1-32 (32 aa), which has been designated as the mature BNP[19, 284]. All newly disclosed pathways leading to BNP metabolism have been added to this overview. All enzymes discovered being able to metabolize BNP1-32 or BNP metabolites are displayed. NEP cleavage sites have been indicated at the respective BNP metabolites.

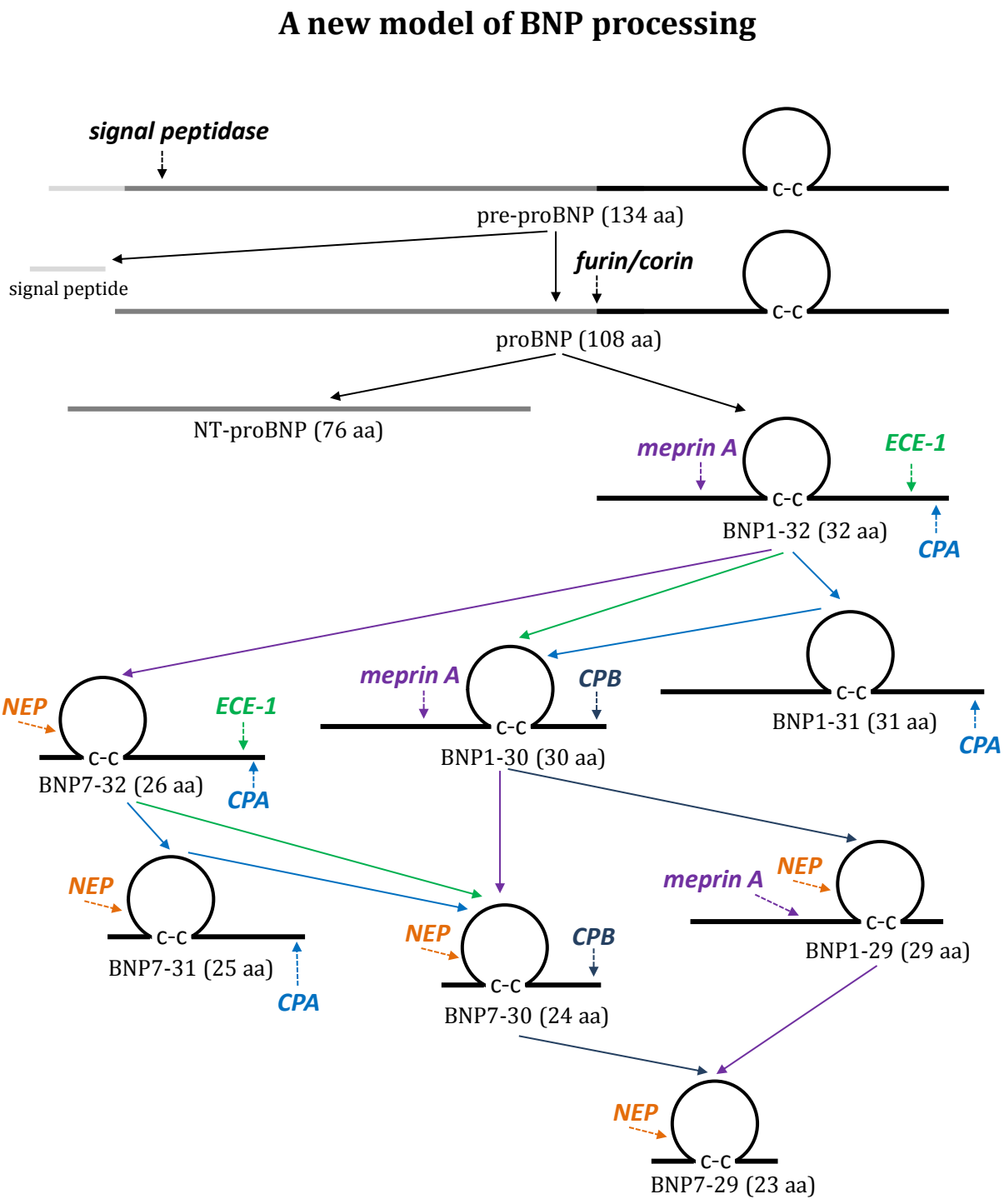


Figure 54: Overview about the possible processing of BNP in the murine system starting from the precursor pre-pro BNP towards the metabolite BNP7-29. Known enzymes able to catalyze the cleavages towards BNP metabolites are named. The NEP cleavage site in particular BNP metabolites that are substrates for the enzyme is highlighted in the respective peptides. Abbreviations: B-type natriuretic peptide (BNP); disulfide bridge (C-C); amino acids (aa); endothelin-converting enzyme-1 (ECE-1); carboxypeptidase A (CPA); carboxypeptidase B (CPB), neprilysin (NEP)

4.2.6 Limitations of the incubation studies

It might be possible that more metabolites emerge from BNP processing in other organs. Moreover, it is possible that metabolites like BNP7-30 or BNP7-29 are, despite their inactivation by NEP, further metabolized leading to the generation of more BNP metabolites. However, there are methodical reasons that might have lead some BNP metabolites undetected in the approaches performed. The presence of a peak in the chromatograms is dependent on the generation, but also on the proteolysis of the peptide behind that peak. A peak can only be seen in the chromatogram, when its generation is faster than its proteolysis. The preparations harbor a multitude of enzymatic activities. In case a newly formed peptide is directly further proteolyzed and this reaction has faster kinetics than its generation, the peak will not rise in the chromatogram. Such phenomena might be seen in blood incubations. It was revealed, that BNP metabolites have different stability in serum compared to BNP1-32 due to their truncations on the N-and C-termini (see Results section 3.1.3.4). While BNP1-29 was similarly stable as BNP1-32, BNP1-30 has drastically reduced stability. The same was true for BNP7-30, while its subsequent cleavage product BNP7-29 was more stable. Thus, only precursors or proteolysis products of these peptides can be seen in the chromatogram.

Furthermore, all peptidase activities present on blood cells could not be considered, since cells have been removed during preparation of serum and plasma. Thus, it might be that more BNP metabolites are formed under physiological conditions. Additionally, it needs to be noted, that the blood preparations have been diluted to perform the experiments. The reason for this was on the one hand, that proteolysis of e.g. BNP1-30 in undiluted blood is so fast that minimal differences (e.g. pupating) in the handling time of repeated measurements cause very big changes in proteolysis time. On the other hand, undiluted serum samples would massively contaminate the HPLC system and lead to a blockage of the column. The dilution of the blood was done with water, which causes changes in the colloid osmotic pressure in the blood samples. Thus, proteolysis times revealed in the experiments cannot be extrapolated, and thus exact kinetics could not be determined. To reveal real half-life times of the metabolites, an approach *in vivo* could be performed. An overview about such an experiment is given in section 4.2.8.

4.2.7 The different impact of proteolytic activities in BNP1-32 metabolism

As stated above it is anticipated that metabolism of BNP needs the coactions of enzymes of different organs including blood. It remains to be clarified which of the peptidase activities has the highest impact on BNP metabolism. Certainly, the blood represents a major proteolytic activity towards BNP1-32, however, perfusion of huge organs like lung and liver surely plays a significant role in BNP metabolism. In contrast to heart and spleen preparations, faster proteolysis of BNP1-32 was observed in lung, liver, and kidneys (see Results section 3.1.1.1). In parallel, these organs represent the organs with the highest perfusion rates in the body as measured by their percentage of bodymass[285]. The lung itself has the highest perfusion rate and measured by this, the perfusion of the heart is less than 10%. Thus, BNP processing might be, despite in blood, most significant in these organs. Moreover, it is possible that more metabolites are generated in other organs and tissues of the body that have not been examined during this study. Organs in which BNP processing might be significant could be thyroid, adrenal, and small intestine where small quantities of BNP transcripts have been found[26] and a local processing might be conceivable.

Moreover, it is essential to investigate BNP metabolism in a gender-dependent manner. The presence of gender-dependent physiological characteristics e.g. sexual organs could be accompanied with a qualitatively and quantitatively altered expression on gene and protein-level, subsequently leading to a differentially regulated BNP metabolism. Different expression profiles of enzymes have been documented in males and females e.g. higher ACE2 activity in males than females[286], or different expression of CPB in aged males and females[287]. Moreover, BNP transcripts have been specifically discovered in ovary and uterus[26], indicating that a specific mode of BNP processing might have been developed in females but not in males. A gender-dependent regulation of BNP processing might be a prerequisite for the significant differences observed in the development of cardiovascular diseases in males and females, evidenced by numerous reports[288-291]. Thus, processing of BNP1-32 should be investigated apart from an approach targeting BNP metabolism in the body as a whole proteolytic system also in a gender-dependent manner.

4.2.8 The complexity of BNP metabolism - a future study

The investigation of BNP metabolism as a whole could be realized by a targeted infusion of BNP1-32 into female and male animal models. This kind of approach would allow the distribution of the peptide to all organs and would thus give indications about BNP metabolism under physiological conditions including all proteolytic activity present towards BNP1-32 and also towards BNP metabolites. The experimental setting of such an approach needs to include precise calculations of the time BNP would need to pass the system. In theory, in an adult mouse with an approx. blood volume of 2.5 ml, a typical ejection fraction of 55 µl[292] and a heart rate of approx. 500 bpm, the total volume would be pumped within approx. 12 seconds through the body. Thus, after such time period, blood samples for the identification of emerging BNP metabolites need to be taken. The correct handling of these blood samples is essential. As revealed in this thesis, some BNP metabolites are quickly degraded within the blood. Thus, blood samples need to be collected in the presence of enzyme inhibitors to avoid further metabolism of BNPs in the test-tube. Such enzyme inhibitors should be aprotinin, EDTA, and carboxypeptidase inhibitors especially the CPB inhibitor, since CPB was suggested to be the enzyme tremendously fast metabolizing BNP1-30 and BNP7-30 in serum (see Results section 3.3.2.3). Finally, a method needs to be assessed to allow identification of BNP metabolites present in these blood samples. The identification of those will however be the most challenging step. Since peptide quantities infused must not be too high to allow reasonable investigations under physiological conditions, concentrations of the single emerging BNP metabolites will be too low for the detection with UV-light as used in the HPLC approaches before analyzing BNP metabolites in the incubation studies. An alternative approach would be a targeted labeling of the peptide being infused, to increase the sensitivity of its detection. Such labeling could be performed with fluorescent- or radioactive compounds, whereby the position of the label plays a decisive role. A C- or N-terminal labeling would not make sense, since the formation of BNP metabolites through truncations on their C and N-termini would lead to a loss of the signal. Thus, a labeling must be realized within the ring-structure of the peptide. Such labeling must not influence the tertiary structure of the peptide as such, that peptidases are no longer able to cleave the peptide. Other than for the fluorescent labeling which often utilizes large compounds such as fluorescein, which

was also shown to influence detection methods like electrophoresis[293], radiolabeling with the e.g. very small tritium would be more suitable[294, 295]. The detection of radiolabeled peptides requires the use of a liquid scintillation counter (LSC) when using an HPLC approach. Otherwise, the use of radio immune assays or autoradiography with primary separation of the BNP metabolites with gel electrophoresis could be considered. Since there are no specific antibodies for the single BNP metabolites, a standard radio immune assay cannot be performed. The use of autoradiography would require a sufficient separation of BNP metabolites by gel electrophoresis beforehand. Since BNP metabolites mostly differ just in one or very few amino acids, such a method needs to be optimized first. Appropriate preliminary experiments separating BNP metabolites with gel electrophoresis have been performed using tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE) according to Schaegger *et al*. as a start point[226]. Through an optimization of the method it was possible to separate BNP1-32 from BNP7-32 and even BNP1-30 (see supplements **Figure S1**).

In summary, such approach would disclose the BNP metabolism resulting from the actual network of organs and circulation under physiological conditions. A simultaneous infusion of enzyme inhibitors inhibiting peptidases responsible for BNP metabolism, would moreover allow the identification of the impact of distinct enzymes such as ECE-1 in BNP metabolism.

Besides this reasonable approach it is however of overriding interest to establish a method for the detection of endogenously occurring BNP metabolites. The development of such a method is very challenging, since endogenous metabolite quantities are potentially very small. A description of possible methodogical procedures can be found in section 4.7.

4.3 Bioactivity of the newly discovered BNP metabolites

The bioactivity of the BNP metabolites has been characterized *in vitro* (see Results section 3.5), *ex vivo* (see Results section 3.6) and *in vivo* (see Results section 3.7 and 3.8). It was shown that all BNP metabolites investigated are bioactive. Notably, the two metabolites BNP1-30 and BNP1-29 were in the majority of experiments even

more bioactive compared to BNP1-32. The metabolite BNP7-32 was less bioactive compared to BNP1-32 and all other BNP metabolites. The additional truncation on the C-terminus in BNP7-30 seems to have a favorable effect on bioactivity, since its activity was in the majority of experiments higher compared to BNP7-32 or even comparable to BNP1-32.

The N- and C-termini of the peptides obviously play an important role in the stimulation of the natriuretic peptide receptors. The C-terminal truncation of two amino acids like in BNP1-30 increases the formation of cGMP by the NPRA. The truncation of one more amino acid like in BNP1-29 seems to have an additional favorable effect on cGMP generation via the NPRA. Labrecque *et al.* hypothesized a model in which the dimerization of NPRA takes place either in a tight and loose conformation[296]. Thereby, binding of natriuretic peptides on the receptor tightens the contact of both receptor subunits. The tight state exerts high guanylyl cyclase activation. It was hypothesized that binding of BNP is less efficiently tightening the receptor subunits than the smaller ANP. Since the C-terminal-truncated BNP metabolites show increased stimulation of the NPRA it might be possible that the effects seen are caused by an increased induction of tight NPRA dimerization.

Notably, BNP1-30 increased cGMP generation on the NPRB compared to BNP1-32 and all other BNP metabolites. Also during acute infusion of BNP metabolites into normotensive mice, BNP1-30 was by far the most effective metabolite (see Results section 3.7). The reduction of blood pressure by this peptide was doubled compared to BNP1-32. Notably, the increased bioactivity of BNP1-29 compared to BNP1-32 *in vitro* was not seen in this *in vivo* study. Although the peptide has higher activity on NPRA- and NPRA & NPRB-double-transfected HEK293 cells, on smooth muscle and endothelial cells, it is not more potent in blood pressure reduction during acute infusion compared to BNP1-32. In contrast BNP1-30, which was also more potent, compared to BNP1-32 in the just specified *in vitro* experiments, was more potent in reducing the blood pressure. However, BNP1-29 shows less bioactivity compared to BNP1-30 in two experiments: the stimulation of NPRB-transfected cells and the stimulation of mesangial cells, both showing the actions of the peptides on NPRB. This indicates that NPRB might play a distinctive role during blood pressure regulation in the acute infusion experiment. It might also be possible, that an additional unknown receptor of BNP is present, mediating the observed effects. Some groups hypothesized the existence of an unknown receptor for BNP[56, 297]. Such a receptor

must have a higher binding affinity towards BNP1-30 compared to BNP1-32 and BNP1-29.

Concrete binding studies of the metabolites towards their receptors should be performed to reveal the specific effects of the missing amino acids on the binding affinities towards NPRA and NPRB.

4.4 The newly identified metabolite BNP1-30

4.4.1 The generation of BNP1-30 is an additional activation of BNP1-32

The generation of BNP1-30 seems to be a common pathway of BNP processing. The metabolite is formed in lung, liver, heart, and spleen preparations. Moreover, it was proven that it is formed in an intact liver after perfusion with BNP1-32 (see Results section 3.1.2.1). Although the metabolite is abundantly formed, the enzymes responsible for its generation differ among the organs. Both ECE-1 and CPA are able to form BNP1-30 from BNP1-32 and it might be that an additional peptidase in heart and spleen is able to catalyze its generation (see Results section 3.2.2 and 3.2.5). The multitude of enzymes involved in the generation of the metabolite and its abundant formation in many organs is highlighting that generation of BNP1-30 must be a volitional and essential step in the processing of BNP1-32. This is supported by the fact that BNP1-30 is more bioactive compared to BNP1-32, especially under *in vivo* conditions and even under pathological circumstances. This increased bioactivity is summating from an increased activity on the NPRA and in parallel from an increased activity on the NPRB. Thus, it can be concluded that the formation of BNP1-30 represents a further activation of BNP1-32.

4.4.2 The altered receptor profile of BNP1-30

4.4.2.1 Implications for the design of recombinant natriuretic peptides

BNP1-30 represents an exceptional case among the BNP metabolites including BNP1-32. It was discovered, that the generation of cGMP via the NPRB is about 3-fold higher for this newly identified metabolite compared to all others (see Results

3.5.1.3). The removal of the two C-terminal amino acids enables the peptide to better stimulate the receptor than its precursor BNP1-32 or its subsequent cleavage product BNP1-29. Thus, BNP1-30 is besides its ability to strongly induce cGMP generation via the NPRA axis, also able to stimulate the NPRB axis and represents a unique receptor profile among all BNPs investigated and moreover among all endogenously expressed natriuretic peptides known. A similar receptor profile, although more pronounced for the NPRB, was observed for the chimeric peptide ACNP consisting of the ring structure of CNP and the N-and C-terminus of ANP[129]. However here, the C-terminus of the peptide is not truncated but the incorporation of fragments of peptides either stimulating NPRA or NPRB seems to be decisive for the receptor profile (see Introduction section 1.1.6). Apart from that, it might also be conceivable that metabolites of other natriuretic peptides like ANP and CNP, which have not been investigated so far, do have altered receptor affinities. Thus, it would be highly interesting to measure binding affinities of recombinant natural or chimeric natriuretic peptides towards the natriuretic peptide receptors and to reveal the particular amino acids that interact with the receptors. It might be conceivable that a chimeric peptide of the CNP ring-structure and the C- and N-terminal arms of BNP1-30 can stronger activate both receptors. Own appropriate experimental studies following this approach have been initiated.

4.4.2.2 Implications for pathophysiology

The increased activity of BNP1-30 towards the NPRB might be an explanation for a physiological phenomenon observed in a model of BNP-overexpression. Suda *et al.* described that animals having more than 3-fold higher copy numbers of the BNP transgene, have long bone growth including an elongation of limbs, paws, and tails and other skeletal malformations like kyphosis[108] (see Introduction 1.2.3). Notably, ANP-overexpressing mice with plasma ANP concentrations elevated at least 8-fold, as compared with nontransgenic littermates, do not show skeletal alterations[298]. Interestingly, BNP-overexpressing mice that are additionally knocked down for the NPRA, the receptor for ANP and BNP1-32, continue to exhibit marked longitudinal growth of vertebrae and long bones comparably to the BNP-overexpressing mice with normal NPRA expression[297]. Originally it was observed, that the CNP/NPRB axis is responsible for the skeletal development and that NPRB knockout mice develop a severe phenotype of dwarfism and bon malformation[93]. Assuming that BNP-

overexpressing mice also have higher levels of the metabolite BNP1-30, which is able to markedly stimulate the NPRB, this additional action on the NPRB axis possibly explains the observed effects. In early years a report has been published suggesting a correlation between scoliosis and congenital heart diseases[299] and Liu *et al.* reported recently high incidences of cardiac abnormality in Asian patients with adolescent idiopathic scoliosis[300]. Since BNP levels are markedly upregulated during certain cardiac diseases there might be a significant influence of BNP on this malformation, although very little reports can be found investigating the correlation between skeletal and cardiac disorders, respectively.

4.4.3 BNP1-30 under hypertensive conditions

Since BNP1-30 had a profound and much stronger effect on blood pressure reduction under normotensive conditions compared to BNP1-32 and other BNP metabolites (see Results section 3.7) a model of severe hypertension, the SHR model, has been used to reveal the actions of BNP1-30 in comparison to BNP1-32 under pathological conditions. Within the study it was mainly focused on their actions on blood pressure but first insights into their effects on fibrosis have also been revealed. It was decided to use minipumps continuously administering the peptides rather than performing repeated injections, since dosing by injections can increase plasma levels exceeding the drugs' effective concentration, resulting in toxicity or simply wasting of the compound. Likewise, the therapeutic effect of the injected drug can vanish between the injections because of its decrease below effective doses.

4.4.3.1 Effects of BNP1-30 on blood pressure under hypertensive conditions

A massive difference in the blood pressure measured after three days was observed between the groups. While there was no significant difference between the saline and the BNP1-32-treated animal groups, BNP1-30 reduced the blood pressure by more than 30 mmHg (see Results section 3.8.2.2). Several reasons for this substantial difference between the effects of the peptides are conceivable.

It was revealed that both, BNP1-32 and BNP1-30, show the same vasorelaxing effects on isolated mesenteric arteries of rats in the *ex vivo* approach performed during the work of this thesis (see Results section 3.6). Thus, the more potent effects of BNP1-30

on blood pressure seen in the SHR might not be related to its actions on mesenteric arteries. However, these experiments have been performed on mesenteric arteries from healthy animals. It might be that effects of the peptides are different in pathologically altered mesenteric arteries from SHR. It has been reported that smooth muscle cells in mesenteric arteries of the SHR have a slower relaxation rate and contract more and faster[301]. *Ex vivo* experiments using arteries from SHR might shed light on possible differential effects of BNPs under pathological conditions compared to normal conditions. However, it has been shown in this thesis that BNP1-30 has increased bioactivity on isolated primary VSMC from aortas and on mesangial cells isolated from kidneys compared to BNP1-32. It has been reported that mesangial cells from SHR show a significantly higher growth rate than those from Wistar Kyoto rats (WKY) probably being involved in the development of hypertension in this animal model[302]. It might be that the increased vasorelaxing activity of BNP1-30 in comparison to BNP1-32 on these cells is of special importance in the reduction of blood pressure in SHR.

It has been described in the literature that NPRA is downregulated during cardiovascular diseases but NPRB is not[77]. It might be that NPRB-levels are also downregulated in SHR due to their severe hypertension and the accompanied adverse effects on heart function. Thus, the strong effects seen for BNP1-30-treatment might be in part mediated by its unique effects on the NPRB. However, no significant increases in cGMP levels have been observed in the blood of the BNP1-30-treated SHR (see Results section 3.8.2.2). In general cGMP levels found in blood of the untreated SHRs and peptide-treated animals were similarly low. The accumulation of cGMP is dependent of its generation and clearance. The clearance of cGMP is realized by phosphodiesterase-5 (PDE5) that degrades cGMP intracellularly[303] and by the multidrug resistance protein 5 (MRP5/ABCC5), which functions as a cellular export pump for cGMP[304]. Thus, the potential effects of the second messenger are limited to its generation, export and degradation. Generation of cGMP is on the one hand mediated by the stimulation of natriuretic peptides on their receptors and the subsequent activation of receptor-bound guanylyl cyclase catalyzing the formation of cGMP from GTP[305]. On the other hand, cGMP production can be derived by nitric-oxide stimulation of soluble guanylyl cyclase[306]. PDE5 was shown to be responsible for the hydrolysis of cGMP derived from the latter mentioned mechanism[307]. However, recently, evidence was generated for a functional retargeting of PDE5 from

one cellular compartment to another, revealing a role for natriuretic peptide-derived cGMP hydrolysis by this esterase in diseased heart myocardium[308]. Since PDE5 expression is highly increased in the hearts of SHR[309], cGMP degradation is likely highly increased in these animals. Thus, less cGMP can be exported through the cGMP transporter, which could at least in part explain the low cGMP values measured in blood. Moreover, there is indication that the ABCC5 transporter expression is differently regulated in cardiovascular diseases[310] probably also in hypertension. Measurement of cGMP levels in tissues of the SHR might give an explicit insight in the effects of BNP-treatment on cGMP homeostasis.

4.4.3.2 Effects of BNP1-30 on fibrosis under hypertensive conditions

It was shown before, that BNP1-32 exerts antifibrotic activity by antagonizing the production of collagen I through TGF-β stimulation[311]. Collagen I is the most abundant collagen in the body and forms thin fibrils that cross-link to one another in the spaces around cells[312]. Already in 1956, it was discovered that extensive production of collagen yields in the formation of fibrosis[313]. In the thesis mRNA of COL1A1 was determined. The gene encodes for the pro-α1 chain which forms together with the pro-α2 chain the type I procollagen[312]. Procollagen I is processed forming collagen I. COL1A1 mRNA was slightly reduced in BNP1-32 and also BNP1-30-treated SHR showing that both peptides are exerting antifibrotic effects (see Results section 3.8.2.3). Impressingly, this effect was seen already after three days of treatment. Cardiac fibrosis is developing in early age in the SHRs. The results revealed that the peptides might be able to reverse also existing fibrosis as indicated by a decreased production of pro-α1 mRNA. Interestingly, both peptides had similar effects in this manner although their ability to reduce blood pressure was divergent. It is thus indicated that both peptides have direct, blood pressure-independent effects on the development of cardiac fibrosis. It has been suggested, that BNP possibly mediates its anti-proliferative effects in cardiac fibroblasts also through the NPRC axis[101]. It is thus conceivable that BNP1-32 and BNP1-30 have similar affinities towards this receptor just as observed for all classical natriuretic peptides[50].

4.4.4 Is BNP1-30 a better drug than Nesiritide®?

In chronic heart failure, circulating natriuretic peptide concentrations are increased but their effects are attenuated due to several reasons, for example the increased local natriuretic peptide degradation through NEP[314], reduced NPRA activity due to receptor degradation[315] and downregulation[77, 316]. The latter mentioned process was described by Potter *et al*. in 2007[77], where they showed that NPRA is downregulated in failing hearts of mice but NPRB is not. They found that CNP exerted twice as much activity as ANP due to dramatic reductions in NPRA activity without changes in NPRB activity. Thus, NPRB accounts for the majority of the natriuretic peptide-dependent activity in the failing heart. Recently, they disclosed an upregulation of NPRB in human failing hearts[317]. Since NPRB seems to play an essential role in failing hearts, a drug targeting both natriuretic peptide receptors may be a more beneficial treatment option than a drug like Nesiritide® (= BNP1-32) that targets NPRA only. BNP1-30 was shown to be three times more effective on the NPRB, although its actions were still low compared to that of CNP. However, since BNP1-30 is not only more active on the NPRB, but in parallel, is also more active on the NPRA it might be better targeting the altered receptor profile in failing hearts than Nesiritide®. Further parameters including its effects on natriuresis and diuresis need to be assessed. Since it has been reported the NPRB axis rather acts anti-natriuretic[87] it might be that the diuretic effects of BNP1-30 are reduced compared to Nesiritide®. Dose-response curves should moreover reveal if the peptide exerts adverse effects on renal function, which was observed during treatment with Nesiritide® in some studies[182].

4.4.5 Possible role of endogenous BNP1-30 during anti-hypertensive treatment in SHR

In September 2012 a paper was published describing the effects of an combined inhibitor of NEP and ECE-1, namely SOL1, for the treatment of hypertension in the SHR-model[318]. The authors revealed that the inhibitor had, opposing to their expectations, no effect on blood pressure in these animals. The data revealed from this thesis might give an explanation for the results observed in that study. In conclusion of this data it is likely that the NEP inhibitor and the ECE-1 inhibitor have opposing effects and thus SOL1 shows no effect on hypertension. ECE-1 has sequence

homologies to NEP, which is also a zinc-dependent metallopeptidase[319] and both enzymes have similarities in their substrate specificity. However, their specificity towards natriuretic peptides is different. As revealed in this thesis, ECE-1 is converting BNP1-32 towards BNP1-30, whereas NEP has very low affinity towards BNP1-32, but can inactivate shorter BNP metabolites. Thus, the two enzymes have opposing effects in the processing of BNP. Whereas ECE-1 is additionally activating BNP1-32 by forming the more bioactive BNP1-30, NEP is inactivating BNP metabolites. Thus, on the one hand, NEP inhibition could increase BNP activity[i], since levels of BNP metabolites such as BNP1-29 would increase. Indeed, it was observed that blood pressure was reduced upon NEP inhibition in SHR[320] and other hypertensive animal models[321]. On the other hand, treatment with an ECE-1 inhibitor *in vivo* should in part decrease the formation of BNP1-30. Since it was shown in this thesis that BNP1-30 has very pronounced effects of blood pressure compared to BNP1-32 in the SHR-model, inhibition of BNP1-30 formation would decrease BNP activity[i]. Thus, the decrease of BNP activity[i] by an ECE-1 inhibitor and the increase of BNP activity[i] by an NEP inhibitor might be compensatory and explain the ineffectiveness of SOL1 in decreasing blood pressure. The same missing effect on blood pressure was seen with the ECE-1/NEP inhibitor SLV 338 in stroke prone SHR[322]. However, the use of the triple inhibitor CGS 35601, inhibiting NEP, ECE-1 and additionally ACE reduced blood pressure in SHR significantly[323]. Also the dual ACE/NEP inhibitor, omapatrilat significantly decreases blood pressure in SHR[324].

The use of enzyme inhibitors represents a common treatment option for the therapy of cardiovascular diseases. In the next chapters the impact of several enzymes involved in BNP metabolism for the treatment of cardiovascular diseases is discussed. Thereby the inhibition of these enzymes and the accompanied effects on BNP metabolism and thus cardiovascular health is of special interest.

[i] BNP activity = activity of the whole BNP pool including all metabolites

4.5 The role of particular BNP-metabolizing enzymes in cardiovascular diseases

4.5.1 Endothelin-converting enzyme-1 (ECE-1)

In this thesis it could be revealed that inhibition of ECE-1 in lung and liver tissues strongly decreases BNP1-30 accumulation (see Results section 3.2.3 and 3.2.4). ECE-1 is an integral membrane zinc peptidase present in endothelial cells[325] and was specifically also purified from lung[326]. There is a second isoform of ECE, ECE-2, having 59% homology with ECE-1[327]. However, its pH optimum is 5.5 with virtually no activity at neutral pH indicating that ECE-2 is involved in intracellular processes rather than extracellular ones. ECE-1 is the main enzyme catalyzing the conversion of Big ET-1 to the very potent vasoconstrictor ET-1[325]. Notably, ECE-1 is also involved in the reduction of levels of BK[282], substance P[249], neurotensin[328], and AngI[328].

The decrease of BNP1-30 concentration through ECEC-1 inhibition under normotension could have two opposing effects. On the one hand biological BNP activity[i] could be decreased, since the concentrations of BNP1-30 are reduced. On the other hand, biological BNP activity[i] could be increased, since the half-life of BNP pool would be increased because BNP metabolites like BNP1-29 generated from BNP1-30 are quickly degraded by NEP. Supporting the latter hypothesis, Ruvanova *et al.* reported about a decrease of blood pressure after acute heart failure by administration of the ECE-1 inhibitor PP36, which could be caused by less generated ET-1. Interestingly, the concentration of ET-1, did not change between the treated and untreated groups[329]. It might be conceivable that the inhibition of ECE-1 led to an increased half-life of BNP activity[i], which subsequently could have caused blood pressure reduction. Supporting the first statement, Telemaque *et al.* demonstrated that overexpression of ECE-1 does not lead to systemic hypertension although levels of the vasoconstrictor ET-1 are increased[330]. A possible explanation for this might be that increased ECE-1 activity also increases the formation of BNP1-30 with higher biological activity acting against hypertension.

[i] BNP activity = activity of the whole BNP pool including all metabolites

4.5.2 Carboxypeptidase A (CPA)

Since it could be revealed that the carboxypeptidase inhibitor decreased BNP1-30 formation in heart and spleen (see Results section 3.2.2 and 3.2.5), different carboxypeptidases have been evaluated for their ability to form BNP1-30. ACE and ACE2 as the key players of the renin-angiotensin system were not able to cleave BNP1-32 (see Results section 3.3.2.1). ACE is a dipeptidylcarboxypeptidase, being able to cleave dipeptides at the C-terminus that do not harbor a penultimate proline or an asparagine and glutamine at the very last position. Although, BNP1-32 does not harbor such amino acids in these positions it is not cleaved by the enzyme with the specific activity used. Its classical substrate AngI harbors 10 amino acids and is thus much smaller compared to BNP1-32. The size might be an exclusion factor for the inability of the enzyme to metabolize the peptide. The same might be true for ACE2. However, it could be shown that recombinant CPA was forming BNP1-30 through the intermediate product BNP1-31. CPA has been described before to mediate the formation of Ang(1-9) form AngI[259]. In this step, the C-terminal leucine is removed, similarly to the reactions forming BNP1-31 and BNP1-30, where also the C-terminal leucine is removed. CPA expression was described in the heart but was not found in lung, liver and kidney[331]. An isoform of CPA the CPA-6 is expressed in brain and spleen also having a prevalence for the removal of a C-terminal leucine[332]. Thus, CPA or an isoform of CPA is highly likely also the enzyme generating BNP1-30 in spleen. Notably, CPA is a metallopeptidase but EDTA was not able to inhibit BNP1-30 formation in heart and spleen, but it was observed that aprotinin has a significant effect. However, it was implicated before that CPA inhibition by EDTA might be a slow reaction[333]. In subsequent experiments, recombinant CPA was incubated with EDTA but no inhibition in its activity has been observed while the carboxypeptidase inhibitor diminished its activity by 90% (see supplementary, **Figure S2**). In the same approach, it was shown that aprotinin, which had a significant inhibitory effect on BNP1-30 formation in the heart, did not inhibit CPA. This indicates that at least one more peptidase might be responsible for the formation of BNP1-30 in heart and spleen, which is sensitive to aprotinin.

The inhibition of CPA was investigated over the last decade, since it is used as a model enzyme for developing design strategies of inhibitors that restrain the catalytic activity of zinc proteases[334-336]. A specific inhibitor of CPA should be tested for its

ability to decrease BNP1-30 formation in heart and spleen incubations. Although, as described before, it cannot be anticipated that formation of BNP1-30 is diminished completely by this inhibitor, since it is likely that another peptidase, being sensitive to aprotinin, is able to form BNP1-30. According to a literature search in Pubmed, CPA inhibition *in vivo* has not been performed yet. It is likely that inhibition of CPA *in vivo* will have effects on blood pressure homeostasis, since the enzyme exerts both, effects on the renin-angiotensin system by catalyzing the formation of Ang1-9[259] and likely also effects on the natriuretic peptide system by catalyzing the formation of BNP1-30.

4.5.3 Carboxypeptidase B (CPB)

Recombinant CPB was revealed to catalyze the formation of BNP1-29 and BNP7-29 from BNP1-30 and BNP7-30 (see Results section 3.3.2.3). CPB is a component of the coagulation pathway that protects blood clots from fibrinolysis[337]. Its alternative name is thrombin-activatable fibrinolysis inhibitor [TAFI]. It is produced mainly by the liver as the zymogen proCPB, which is activated through the removal of the N-terminus during thrombotic events[337, 338]. It was shown that plasmin catalyzes this activation i*n vitro*, but especially the thrombin/thrombomodulin complex is considered as the physiological activator of proCBP[339]. CPB is regarded as a procoagulant, since it removes C-terminal lysines from fibrins and thereby supports the maintenance of fibrin clots[340]. CPB is able to cleave BK[341] and osteopontin, a proinflammatory cytokine that plays an important role in the pathogenesis of rheumatoid arthritis[342]. Thus, CPB is regarded as a regulator of vascular inflammation[341, 343]. The presence of activated CPB in serum samples might explain the fast degradation seen for BNP1-30 and BNP7-30 in comparison to other BNP metabolites. However, this also indicates that both metabolites might not be this fast degraded under *in vivo* conditions, when CPB is rather inactive. It was shown that homozygous CPB-deficient mice do not show any differences in intravascular coagulation, nor survival or fertility[344]. However, no cardiovascular parameters such as blood pressure or cardiac function have been determined in these studies, respectively. It was published before that CPB activity is increased in a canine model of myocardial infarction[338]. From the perspective of this work it would be interesting to evaluate the influence of CPB deficiency in a model of myocardial infarction (MI). An increase of CPB would lead to a decrease of BNP1-30 levels in the circulation.

BNP1-30 was shown to be more potent in decreasing blood pressure compared to BNP1-32 in this thesis. Thus, it is conceivable that CPB deficient mice undergoing MI experience some beneficial effects in comparison to their wild-type litter mates undergoing MI, since the metabolism of BNP1-30 by this enzyme would at least be decelerated and thus circulating BNP1-30 levels would be increased.

During BNP1-32 proteolysis in heparin plasma it was revealed that BNP1-31 and BNP1-29 were formed but no formation of BNP1-30 was observed (see section 3.1.4). It was however hypothesized that BNP1-30 is formed in blood preparations but significant quantities cannot accumulate, since it was disclosed that its stability in blood samples is dramatically reduced by its fast metabolism into BNP1-29. This metabolism was completely inhibited by the CPB inhibitor GEMSA (see Results section 3.3.2.3). Thus, in a subsequent experiment, GEMSA should be supplemented into the incubation of BNP1-32 and serum to disclose whether the inhibition of BNP1-30 metabolizing activity would lead to an accumulation of BNP1-30 during BNP1-32 proteolysis as a prove that BNP1-30 is indeed generated in serum. However, it might be that over time other peptidases than CPB proteolyze BNP1-30 similarly as seen for BNP1-32 proteolysis when inhibiting ECE-1 in lung tissues. Thus, it needs to be assessed first, if BNP1-30 in the presence of GEMSA is similarly stable as BNP1-32 over time.

4.5.4 Meprin A

Meprin A is another enzyme responsible for BNP1-32 metabolism. Meprin A is a multimeric metalloendopeptidase that is expressed mainly in mammalian kidney and intestine[345]. The recombinant enzyme is beside the formation of BNP7-32 also able to form BNP7-30 and BNP7-29 from BNP1-30 and BNP1-29. It can be anticipated that BNP1-31 is also a substrate for meprin A.

It could be shown before that infusion of actinonin, an inhibitor of meprin A, increases circulating immunoreactive BNP levels[160]. However, no data investigating blood pressure or cardiac function has been published, and thus it is not clear whether the increase of circulating immunoreactive BNP indeed increases BNP activity[i]. However, the latter scenario is highly likely, since meprin A inhibition should increase the half-life of meprin A substrates as BNP1-32, BNP1-30, and BNP1-29,

which moreover have increased biological activity compared to the cleavage products of meprin A.

4.5.5 Main conclusion

Inhibition of relevant enzymes metabolizing BNP1-32 might change circulating concentrations of BNP metabolites and thus BNP activity[i]. Notably, depending on the enzyme being inhibited, two opposing effects can occur. On the one hand, the inhibition of BNP-metabolizing enzymes e.g. of ECE-1 might lead to a decrease of BNP activity[i], since the formation of BNP metabolites with higher biological activity compared to BNP1-32 is decreased. On the other hand, BNP activity[i] might be increased e.g. by inhibition of CPB, since the generation of BNP metabolites, which are in part degradable by NEP, is blunted and thus clearance of BNP is reduced. Thus, the discovery of the particular enzymes being able to metabolize BNP1-32 and form distinct BNP metabolites with unique bioactivity and degradability represents an essential gain of knowledge for the development of pharmaceutical drugs on the basis of enzyme inhibition, e.g. dual enzyme inhibitors (see section 4.4.5). Due to the disclosure of the different pathways in BNP metabolism involving distinct enzymes it might be possible to balance possible beneficial and adverse effects of drugs like enzyme inhibitors on e.g. blood pressure regulating systems and thus for the treatment of cardiovascular diseases.

4.6 Implications for BNP metabolism in other species

In this thesis BNP metabolism has been investigated representatively for the murine system. The availability of cells, tissues, and different genetically modified animal models is tremendous, which enabled research from scratch towards the approvement in the whole living system. Primary because of the good availability, a multitude of different applications including murine cells (primary cells like VSMC and MC), murine tissues (membrane preparations) and blood, vessel preparations (*ex vivo* experiments), and the animals as physiological system itself (liver perfusion, peptide administration during normo- and hypertension) could be utilized to

[i] BNP activity = activity of the whole BNP pool including all metabolites

characterize BNP metabolism. The identification of BNP metabolism is also seen as a substantial gain of knowledge for the use of the murine system, being the basic animal model in so many research applications. Several examples have been discussed in this thesis were BNP metabolism might play a role in these animals models.

Although differences in amino acid sequence are present between the BNPs of all mammalian species, it can be anticipated that BNP metabolism is similarly important to that revealed in the murine system. Indeed, some own preliminary experiments showed that BNP metabolism is similarly complex in human organ preparations. Human BNP exerts a similar N-terminus compared to murine BNP1-32. Human BNP1-32 harbors a positively charged arginine at position 30 similar the positively charged lysine in murine BNP1-30. Moreover, both peptides harbor the leucine at position 29, thus formation of human BNP1-29 and effects on natriuretic peptides receptors might be highly similar to murine BNP1-29. Own unpublished work and work by others[219] indicate that the generation of human BNP metabolites like BNP3-32, BNP1-30 and BNP1-29 is present in the human system too. Thus, this work also represents a template for the investigation of BNP metabolism in humans and is according to own preliminary studies applicable to it. Ongoing studies will be resolved soon.

Besides the investigation of BNP metabolism in different species, it is of special interest to develop a method for the detection of endogenously generated BNP metabolites. In the next chapters strategies as well as preliminary results are introduced and clinical implications of the detection of distinct BNP metabolites are discussed.

4.7 Strategies for the detection of endogenous BNP metabolites

Normal concentrations of the total BNP pool in healthy subjects range in the lower picomolar field[171, 346, 347]. Thus, the concentration of distinct BNP metabolites can be expected to be 10 to 100 fold lower reaching the femtomolar range. Despite the very low concentrations, the detection of endogenous BNP fragments in such a complex biological fluid like blood increases the complicacy of such an approach.

Own strategies for the detection of endogenous BNP metabolites have been designed and preliminary studies have been made. Such efforts included, besides the work on blood sample preparation including C18 extraction and size exclusion filters, the use of analytical methods like MALDI-TOF-MS in collaboration with the Protein Analytics Group at the Institute of Biochemistry, Giessen, and of the triple-quadroupole mass spectrometry in collaboration with the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Graz, Austria. First preliminary investigations revealed that detection of endogenous BNP metabolites might require the use of antibodies enabling a prior purification of all BNP fragments from blood.

Niederkofler *et al.*[219] developed an automated method utilizing antibody derivatived affinity pipettes for the specific binding of BNP fragments from blood samples followed by the elution of those on a MALDI-target. Such approach revealed the occurrence of some BNP metabolites in blood of heart failure patients. However, despite the use of an antibody binding within the ring-structure of BNP1-32, the authors used a second antibody in parallel binding to the amino acids 27 to 32 within the peptide. Thus, the detection of all fragments emerging from a C-terminal truncation of BNP1-32 was not given. Notably, the quantification of the found metabolites revealed that their concentration in blood was higher compared to that of BNP1-32.

Hawkridge *et al.*[348] developed a dual-antibody immunoaffinity purification assay to purify BNP1-32 from plasma. In solid-phase extraction, they used a primary antibody against human BNP1-32 to capture BNP1-32 and a secondary antibody to isolate and purify the antibody-antigen complex. For the analysis of extracted samples they used nano-liquid chromatography (LC) electrospray ionization (ESI) Fourier transform (FT) ion cyclotron resonance (ICR) mass spectrometry (MS). As a control they compared levels of BNP1-32 measured with their method with BNP levels obtained from a commercially available BNP test (Triage® test, Biosite). Notably, they found, that levels of BNP1-32 were 50% lower in the samples compared to immunoreactive BNP levels detected with the Triage® test.

4.8 Detection of endogenous BNP metabolites – clinical implications

Mass spectrometry tests revealed first insights into the qualitative composition of the BNP pool in plasma[219, 348]. As stated above, Hawkridge *et al.* compared BNP levels measured by the Triage® test with levels of BNP1-32 determined by their developed method using nano-LC-ESI-FT-ICR MS in plasma of patient with severe heart failure and found that levels of BNP1-32 in these samples were much lower compared to immunoreactive BNP levels found with the Triage® test[348]. They suggested the occurrence of 'cross-reactive species' detected by the antibodies of the test contributing to the measured values. Niederkofler *et al.* who detected also only small quantities of endogenous BNP1-32 but revealed the occurrence of other BNP fragments suggested that immunoreactive BNP assays might overrepresent the actual presence of biological BNP activity[i] [9]. Both conclusions do not give consideration to the possible biological activity of these BNP fragments. According to the results presented in this thesis, the occurrence of bioactive BNP metabolites like BNP1-30, BNP1-31, BNP1-29, BNP7-32, BNP7-30, and BNP7-29 extremely expands the pool of immunoreactive BNP. Commercially available kits like the Triage® BNP kit are based on the detection of BNP by a monoclonal antibody directed against an epitope within the 17 aa ring structure of the peptide. Thus, the antibody binds the mature BNP1-32 and all BNP fragments with an intact ring structure, respectively (see Introduction section 1.2.6). All new metabolites still harbor the intact ring structure, which is the requirement of biological activity of these peptides. Assumedly, a test with an antibody directed against an internal epitope will detect all BNP fragments. Thus, the result of such a test will be the quantification of all BNP fragments rather than the specific quantification of BNP1-32 only.

On the one hand, the detection of the whole BNP pool by these assays has proven to be clinically relevant in the diagnosis and prognosis of cardiovascular diseases and does not change the general conclusion made by the results of such assays. However, the concentrations determined in such assays do not reflect the actual presence of bioactive and thus beneficial BNP. Assuming that a value of 100 pg/ml of BNP measured with a commercially available BNP test in a murine blood sample equates

[i] BNP activity = activity of the whole BNP pool including all metabolites

100% of the bioactivity of BNP1-32. However, the composition of the BNP pool measured with this test does not only include BNP1-32, but highly likely a variety of other BNP metabolites. Assuming that the measured BNP pool is composed of equal parts of BNP1-32, BNP1-30, and BNP1-29, the theoretical bioactivity of this pool would be significantly higher compared to a BNP pool composed of BNP1-32 only. Calculating this excess of bioactivity on the basis of their ability to stimulate NPRA & NPRB-double transfected cells (see Results section 3.5.1.4), such bioactivity would reach a value of 135.2%. Vice versa, this value can also be decreased assuming that the majority of the BNP pool is composed of metabolites like BNP7-32 or BNP7-30 which exert less biological activity compared to BNP1-32. Thus, it can be concluded that the BNP bioactivity present in a sample is depending on the composition of different BNP metabolites.

4.9 Specific diagnosis of cardiovascular diseases with BNP metabolites

The early diagnosis of cardiovascular pathologies through the general practitioner before the manifestation of a cardiac event or in the clinical setting, when heart failure is often already present, is pivotal. A variety of biomarkers have been utilized during the last decades to make diagnosis faster and more efficient[349-351]. However, the limitation of many tests determining biomarkers including the conventional BNP-test is their lack in the differentiation between specific cardiovascular diseases[220, 352, 353].

Many cardiovascular diseases have in common that gene and protein expression is altered, which in turn is depending on the specific pathology. It was found that gene expression is altered during heart failure. Heidecker *et al.* found forty-six genes that are overexpressed in patients with heart failure having a good outcome compared to patients with heart failure having a poor outcome[354]. Alexander *et al.* found the concentration of sixty-one metabolites significantly different between patients with primary dilated cardiomyopathy and control individuals[355], indicating that also levels of peptidases generating these metabolites are altered. Viral infection (e.g., parvovirus) or nonviral infection (e.g. Chagas disease) might lead to an altered peptidase expression. For example it was found that mRNA levels of ECE-1 are

elevated in Chagas Disease[356]. Furthermore higher activity of ECE-1 is associated with pulmonary arterial hypertension[357]. Meprin is markedly downregulated in chronic renal dysfunction[358], whereby renal dysfunction is often associated with cardiac dysfunction[359]. NEP expression associates with cardiometabolic risk and plasma levels of NEP increase with obesity, a disease which is frequently accompanied by cardiac pathologies[360]. Circulating levels of DPPIV, the peptidase generating the human BNP3-32, is associated with left ventricular dysfunction.

This up or down-regulation of peptidases due to specific cardiovascular diseases will subsequently also change the concentrations of their substrates and products, e.g. that of BNP1-32 and BNP metabolites. An up-regulation of e.g. ECE-1 would increase concentrations of BNP1-30 a down-regulation of Meprin A would decrease levels of BNP7-32. Besides altered metabolite-generation it might also be that metabolite-degradation is specifically altered in a disease. It was for example shown in patients with idiopathic cardiomyopathy, that degradation of natriuretic peptides is markedly changed even in early stages of the disease[361]. Thus, the composition of the BNP pool is depending on the generation or degradation of BNP metabolites. Generation and degradation is in turn depending on enzymes whose expression level or activity is potentially altered during the development and etiopathology of a distinct cardiovascular diseases. It can therefore be hypothesized that patients with specific cardiovascular diseases have specific BNP metabolite profiles. The collection of such BNP metabolite profiles for every cardiovascular disease including different stages of the disease would allow the establishment of a database, which could be used for a rapid and specific diagnosis once the BNP metabolite profile of a patient has been assessed. In contrast to that, a conventional BNP assays will only determine the concentration of the whole BNP pool in a patients' blood sample. Thus, increases in levels of the BNP pool allow the discrimination between healthy and diseased patients. If BNP levels of a patient are above a certain cut-off level the patient will have to undergo more examinations to specify the cardiovascular disease. Applying a BNP-test being able to distinguish between the different BNP metabolites and to quantify them opens the avenue for a more specific diagnosis of heart diseases and might even make further clinical assessment unnecessary. Taking metabolite-specific antibodies as a basis of this test, such an assay would only require a blood sample for the analysis. Such a non-invasive test would be usable in the clinic, e.g. emergency room, but might also be helpful in a pre-clinical setting e.g. in the physician's office.

The compilation of a BNP metabolite profile as a standard method during medical check-ups could contribute to risk stratification and early diagnosis of developing diseases, since a significant proportion of cardiovascular pathologies have a genetic background or develop without symptoms. For instance, a coronary artery disease might be present for years with symptoms occurring only late in the etiology, however, peptidase and metabolite levels might be already changed in an early onset. Just as well, the determination of BNP metabolite profiles during the individual treatment of a patient with a cardiovascular disease could be used for the monitoring of etiopathology and guidance of therapy. Thus, the collection of BNP metabolite profiles throughout the life span of patients could contribute to the health status of the individual. An overview about the possible usage of the compilation of a BNP metabolite profile in the diagnosis and risk stratification of cardiovascular diseases has been pictured in **Figure 55.**

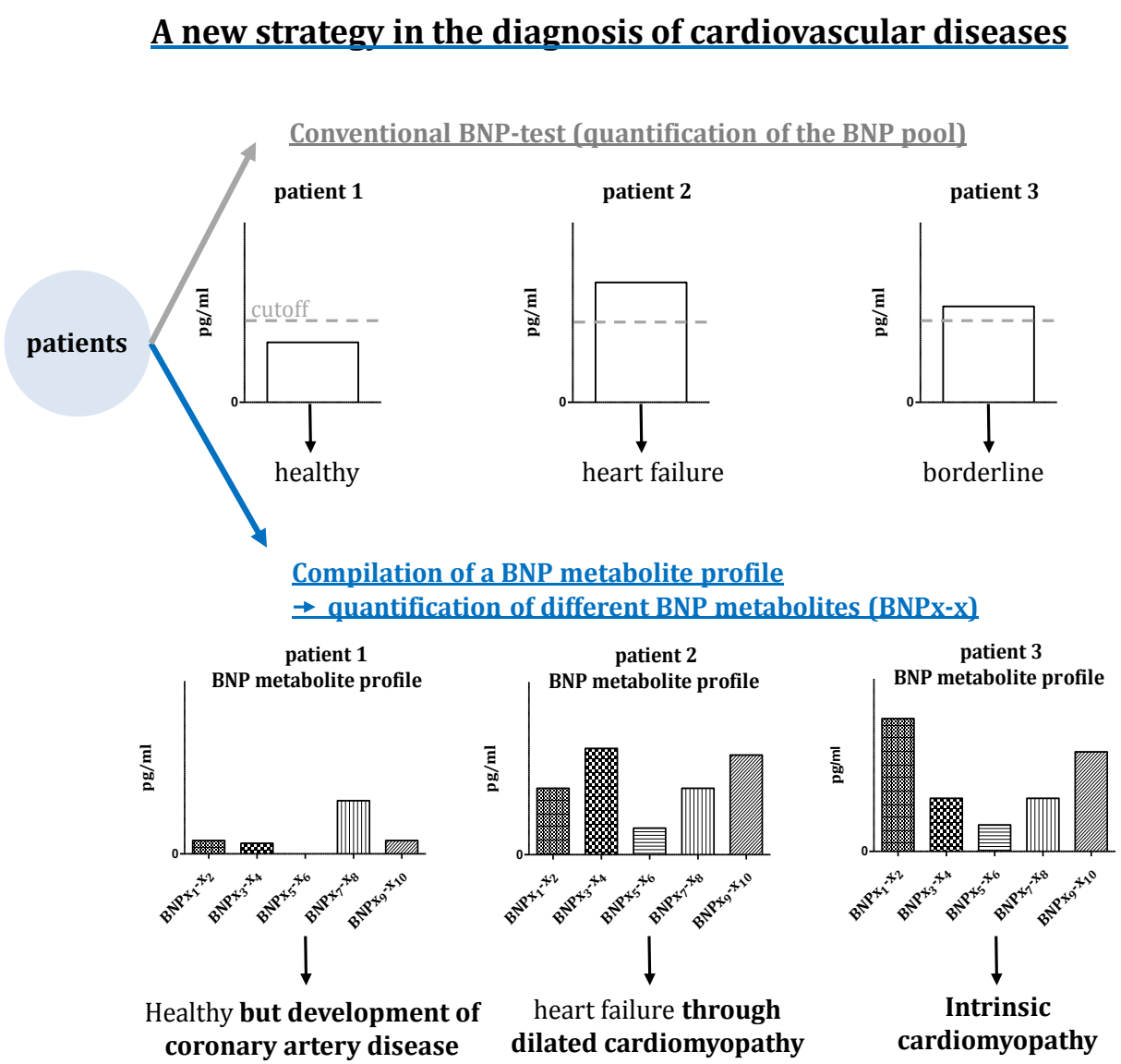
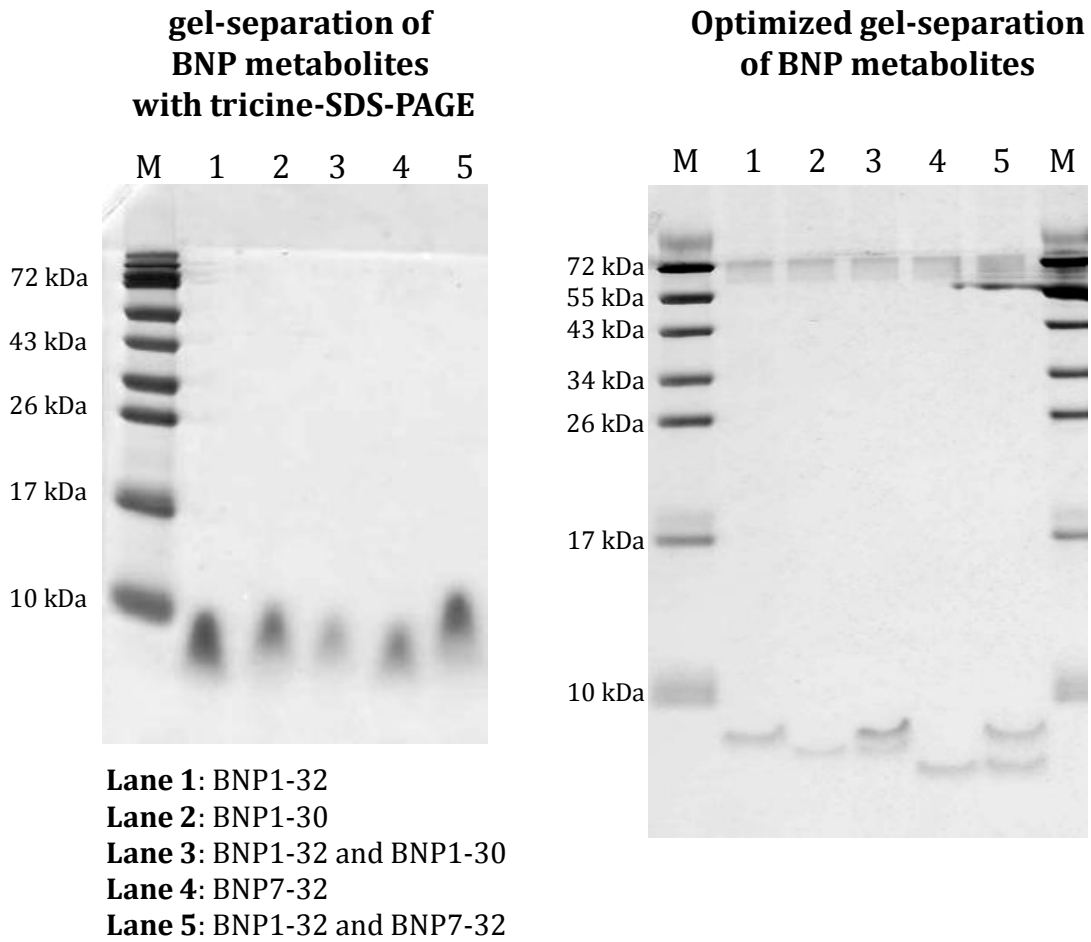


Figure 55: Comparison of two diagnostic strategies for the determination of BNP in patients. The upper part of the figure shows the quantification of the whole BNP pool with a conventional BNP-test. The lower part shows a new strategy for the diagnosis of specific cardiovascular diseases by identifying and quantifying BNP metabolites leading to a compilation of a specific BNP metabolite profile for each patient. Abbreviations: B-type natriuretic peptide (BNP), B-type natriuretic peptide metabolites (BNPx-x)

5 SUPPLEMENTARY FIGURES

Figure S1



Lane 1: BNP1-32
Lane 2: BNP1-30
Lane 3: BNP1-32 and BNP1-30
Lane 4: BNP7-32
Lane 5: BNP1-32 and BNP7-32

Figure S1: optimized trycine-gel after electrophoresis displaying single BNP metabolites as well as their separation from each other. In particular, lane 3 shows the separation of BNP1-32 (upper band) and BNP1-30 (lower band). Lane 5 shows the separation of BNP1-32 (upper band) and BNP7-32 (lower band). M = marker. For method see section 2.2.7

Figure S2

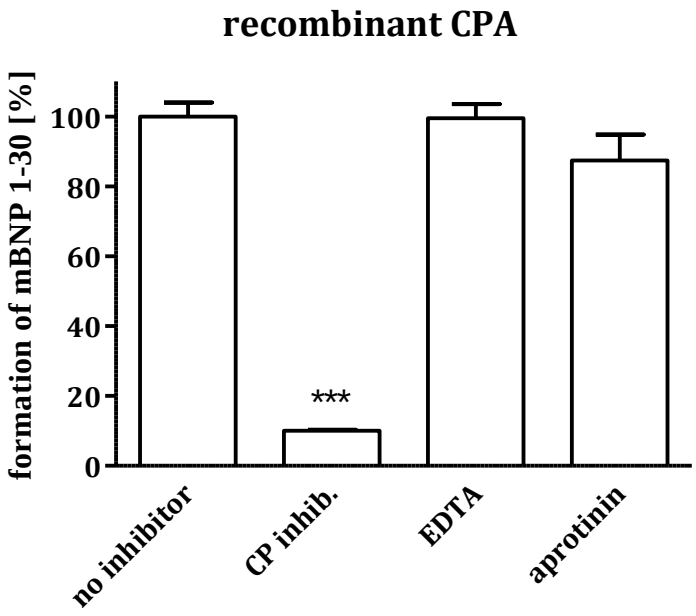


Figure S2: Quantification of the generation of BNP1-30 with recombinant carboxypeptidase A (CPA) in the presence of the carboxypeptidase inhibitor (CP inhib), EDTA, or aprotinin.

6 SUMMARY

The B-type natriuretic peptide (BNP1-32) is secreted in large amounts upon increasing blood pressure and other cardiopathological conditions to protect the body by its vasoactive and antifibrotic effects. It is routinely measured in the clinic as a biomarker for the diagnosis and prognosis of heart failure and is used as a drug (Nesiritide®) for the therapy of such. In circulation, BNP1-32 is constantly exposed towards proteolytic activity and it was thus hypothesised that, similar to other vasoactive peptides, BNP1-32 is truncated forming BNP metabolites with analog or altered bioactivity, before it is degraded.

Through this thesis it is possible to give a detailed overview about BNP1-32 metabolism including the discovery of different pathways and enzymes leading to the formation of a variety of BNP metabolites. The main findings are: firstly, BNP metabolism is organ-specific. Secondly, this organ-specific BNP metabolism leads to the generation of numerous different BNP metabolites, namely BNP1-31, BNP1-30, BNP1-29, BNP7-32, BNP7-31, BNP7-30, and BNP7-29. Thirdly, this generation of BNP metabolites involves a variety of different enzymes, of which one is the endothelin-converting enzyme-1 (ECE-1). Fourthly, full metabolism of BNP1-32 needs the concerted action of these enzymes. Fifthly, BNP metabolism also serves as a prerequisite for BNP clearance by neprilysin (NEP), since BNP metabolites such as BNP1-29 and BNP7-30 are degradable by the enzyme, whereas BNP1-32 is not. On the basis of these five main results, a comprehensive model of BNP processing was developed. As a sixth essential finding it was revealed that all newly identified BNP metabolites investigated exert biological activity *in vitro, ex vivo,* and *in vivo*. In particular, one of these BNP metabolites, BNP1-30, is able to reduce blood pressure substantially more strongly compared to BNP1-32 under normotensive and hypertensive conditions at least in part as a result of its altered receptor profile.

The work presented shows for the first time that the metabolism of BNP1-32 is complex and involves a variety of enzymes forming different BNP metabolites with

unique bioactivity and is thus representing an essential process for the cardioprotective actions of BNP. On the basis of this work it is implied that BNP processing is not purposed to generate BNP1-32 alone, but is aimed to generate BNP metabolites with similar and in part higher bioactivity compared to BNP1-32. It is thus concluded that the virtually mature BNP1-32 is rather a precursor for BNP1-30 or other BNP metabolites, a finding that suggests a fundamental revision and extension of the natriuretic peptide system. The experimental data also indicate that there might exist more metabolites of BNP, and surely also of other natriuretic peptides, exerting unique biological properties.

Furthermore, the results of this thesis can be translated into clinical applications. The newly identified BNP metabolites, but also the manipulation of enzymes being involved in their formation, represent new tools for the therapy of heart failure. In particular, the metabolite BNP1-30 might be a better treatment option of cardiovascular diseases than Nesiritide®. The identification of BNP metabolites also allows the development of new diagnostic strategies in the assessment of cardiovascular diseases. In this manner, the compilation of a specific BNP metabolite profile could, as opposed to the current BNP measurement in the clinic that does not distinguish between the various forms of BNP, give detailed information about risk stratification, etiopathology, and therapeutic success.

7 ZUSAMMENFASSUNG

Das B-Typ Natriuretische Peptid wird in Folge von erhöhtem Blutdruck und anderen kardiovaskulären Erkrankungen vermehrt sekretiert, um den Körper durch seine vasoaktiven und antifibrotischen Effekte zu schützen. In der Klinik wird es routinemäßig als Biomarker zur Diagnose und Prognose von Herzversagen, sowie als Medikament (Nesiritide®) zur Therapie solcher verwendet. Da zirkulierendes BNP konstant proteolytischer Aktivität ausgesetzt ist, wurde die Hypothese aufgestellt, dass BNP1-32, ähnlich wie andere vasoaktive Peptide, zu Metaboliten trunkiert wird, bevor es abgebaut wird.

Diese Arbeit ermöglicht die Aufstellung einer umfangreichen Übersicht über den Metabolismus des BNP1-32, welche die Entdeckung unterschiedlicher Wege und Enzyme, die zur Bildung einer Vielzahl an BNP Metaboliten führt, einschließt. Die wichtigsten Ergebnisse sind: Erstens, der Metabolismus von BNP ist spezifisch für jedes Organ. Zweitens, dieser organspezifische BNP-Metabolismus führt zur Generierung folgender BNP Metaboliten: BNP1-31, BNP1-30, BNP1-29, BNP7-32, BNP7-31, BNP7-30 und BNP7-29. Drittens, die Herstellung der BNP Metaboliten wird von mehreren unterschiedlichen Enzymen durchgeführt, zum Beispiel durch das Endothelin-konvertierende Enzym 1 (ECE-1). Viertens, der vollständige Metabolismus von BNP1-32 ist abhängig vom Zusammenwirken dieser Enzyme. Fünftens, der Metabolismus von BNP dient außerdem als Voraussetzung für den Abbau durch Neprilysin (NEP), da BNP Metaboliten wie BNP1-29 und BNP7-30 durch dieses Enzym degradiert werden können, jedoch nicht BNP1-32. Auf der Grundlage dieser fünf Ergebnisse wurde ein umfassendes Model für die Prozessierung von BNP entwickelt. Als sechstes Ergebnis kann formuliert werden, dass alle untersuchten und neu identifizierten BNP Metaboliten biologische Aktivität *in vitro, ex vivo* und *in vivo* aufweisen. Besondere Aufmerksamkeit kommt dem Metaboliten BNP1-30 entgegen, der den Blutdruck unter normotensiven und hypertensiven Bedingungen signifikant

stärker senken kann als BNP1-32, was zumindest teilweise durch seine veränderte Rezeptorspezifizität hervorgerufen wird.

Die Arbeit zeigt erstmalig, dass BNP1-32 einem komplexen Metabolismus unterliegt, der eine Vielzahl von Enzymen, die verschiedene BNP Metaboliten mit einzigartigen Aktivitäten generieren, einschließt, sodass er einen essentiellen Prozess darstellt, der zu den kardioprotektiven Eigenschaften des BNPs beiträgt. Die Arbeit impliziert, dass die Prozessierung von BNP nicht nur der Herstellung von BNP1-32 dient, sondern vielmehr die Generierung von BNP Metaboliten mit gleicher oder zum Teil sogar höherer Bioaktivität ermöglichen soll. Daraus wird gefolgert, dass das eigentliche, reife BNP1-32 eher ein Precursor für die Herstellung von BNP1-30 oder anderen BNP Metaboliten darstellt, was eine entscheidende Änderung und Erweiterung des Natriuretischen Peptid Systems zur Folge hat. Außerdem indizieren die experimentellen Daten das Vorkommen von zusätzlichen BNP Metaboliten sowie von Metaboliten anderer natriuretischer Peptide, die einzigartige biologische Funktionen aufweisen könnten.

Die Ergebnisse dieser Arbeit können außerdem klinische Anwendung finden. Die neu identifizierten BNP Metaboliten selbst, sowie die Beeinflussung von Enzymen, die diese herstellen, legen neue Behandlungsansätze in der Therapie von Herzversagen nahe. Im Besonderen ist der neue Metabolit BNP1-30 möglicherweise ein besseres Medikament zur Behandlung von kardiovaskulären Erkrankungen als Nesiritide®. Die Identifizierung von BNP Metaboliten ermöglicht außerdem die Entwicklung von neuen diagnostischen Strategien zur Bewertung von kardiovaskulären Erkrankungen. Beispielsweise könnte das Erstellen eines spezifischen BNP Metaboliten Profils im Vergleich zur konventionellen Messung von BNP, die nicht zwischen den einzelnen BNP Metaboliten unterscheidet, genauere Informationen über Risikostratifikation, Krankheitsverlauf sowie Therapieerfolg geben.

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9 ABBREVIATIONS

Amino acids and nucleic acids have been abbreviated according to the common one-letter code. Elements have been abbreviated as stated in the periodic system.

aa	amino acid
ABCC5	multidrug resistance protein 5
ACE	angiotensin-converting enzyme
ACN	acetonitrile
Ang	angiotensin
ANP	atrial natriuretic peptide
approx.	approximately
ATP	adenosine triphosphate
BK	bradykinin
BNP	B-type natriuretic peptide
BSA	bovine serum albumin
bpm	beats per minute
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanylyl monophosphate
cAMP	cyclic adenosine monophosphate
CathA	cathepsin A, carboxypeptidase C
CPA	carboxypeptidase A
CPB	carboxypeptidase B
C-C	disulfide bridge
CP inhib.	carboxypeptidase inhibitor
CNG	cGMP-dependent ion-gated channels
CNP	C-type natriuretic peptide
CT	threshold cycle
C-terminus	carboxyl-terminus
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DNP	dendroapsis natriuretic peptide
DPPIV	dipeptidyl-peptidase IV
DMSO	dimethyl sulfoxide
EC	European commission number
EC_{50}	half maximal effective concentration
ECE	endothelin-converting enzyme
EDTA	ethylenediaminetetraacetic acid

ELISA	enzyme-linked-immunosorbent assay
ET-1	endothelin-1
et al.	and others
FDA	Federal Drug Administration
FCS	fetal calf serum
GC	guanylyl cyclase
GDP	guanosine diphosphat
GIRK	G protein-gated inwardly rectifying K^+ channel
GTP	guanosine triphosphat
HEK293	hamster endothelial kidney cells 293
HDMEC	human dermal microvascular endothelial cells
HEPES	2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethansulfonic acid
HF	heart failure
HPLC	high performance liquid chromatography
HPRT1	hypoxanthine phosphoribosyltransferase 1
Ig	immunglobulin
Il	interleukin
i.p.	intraperitoneal
KHD	kinase homology domain
LB	Luria--Bertani
MALDI-TOF-MS	Matrix-assisted-laser-ionization time-of-flight mass spectrometry
MAP	mean arterial blood pressure
MAPK	mitogen activated protein kinase (MAPK)
MC	mesangial cells
MRP-5	multidrug resistance protein 5
MI	myocardial infarction
MS	mass spectrometry
mRNA	messenger ribonucleic acid
LC-ESI-FT-ICR MS	liquid chromatography electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry
n	number of experiments
NF-κB	renal nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NFAT	calcineurin-nuclear factor of activated T cells
NEP	neutral endopeptidase, neprilysin
N-Terminus	amino-terminus
NPR	natriuretic peptide receptors
NPRA	natriuretic peptide receptor A
NPRB	natriuretic peptide receptor B
NO	nitrogen monooxide
NOS	nitrogen monooxide synthase

P	p-value, probability
NPS	natriuretic peptide system
PBS	phosphate-buffered solution
RT-PCR	real-time polymerase chain reaction
PDE-5	phosphodiesterase-5
PIP_3	phosphatidylinositol (3,4,5)-trisphosphat
PKC	protein kinase C
PLC	phospholipase C
P_B	peak blood
P_{LP}	peak liver perfusion
P_M	peak membrane preparations
P/S	Penicillin / Streptomycin
PRCP	prolylcarboxypeptidase
PREP	prolylendopeptidase
RGS4	G protein signaling subtype 4
RAS	renin-angiotensin system
RNA	Ribonucleic acid
RT	room temperature
rt	retention time
Rpm	rotations per minute
SBP	systolic blood pressure
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
s.c.	subcutaneous
SEM	standard error of mean
SHR	Spontaneously hypertensive rats
t1/2	half-life
TAFI	thrombin-activatable fibrinolysis inhibitor
TFA	trifluoracetic acids
TGF-β	transforming growth factor
TNF-α	tumor necrosis factor alpha
Trp.inhib.	trypsin inhibitor
UV	ultraviolet
URO	urodilatin
vs.	versus
VSMC	vascular smooth muscle cells
WKY	Wistar Kyoto rats
ZPP	Z-pro-prolinal
Δ	Delta

Orders of magnitude

µ	micro (10^{-6})
c	centi (10^{-2})
f	femto (10^{-15})
k	kilo (10^{3})
m	milli (10^{-3})
n	nano (10^{-9})
p	pico (10^{-12})

Units of measurements

%	percent
°C	degrees of Celsius
AU	absorbance units
Da	dalton
g	gram
h	hour
L	liter
m	meter
M	molar
min	minute
mmHg	millimeter mercury
pH	hydrogen ion concentration
s	second

A ADDENDUM

A.1 ERKLÄRUNG

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

Gießen, Oktober 2012

Anja Schwiebs

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A.3 PUBLICATIONS

Full papers

Zhu X*, Wang Y*, **Schwiebs A**, Walther T. Chimeric natriuretic peptide ACNP stimulates both natriuretic peptide receptors, the NPRA and NPRB. *Mol. and Cell. Endocrin.* (*revised submission 23.10.2012, submission#:* MCE-D-12-00286R1*) *equally contributing first authors*

Schwiebs A, Wang Y, Zhu X, Pankow K, Siems WE, Walther T. The virtually mature BNP (BNP1-32) is the precursor for the more potent BNP1-30. *Circ. (submitted 15.08.2012, submission#:* 137851*).*

Nahmod K, Walther T, Cambados N, Fernandez N, Meiss R, Tappenbeck N, Wang Y, Raffo D, Simian M, **Schwiebs A**, Pozner RG, Fuxman Bass JI, Pozzi AG, R Geffner JR, Kordon EC, Schere Levy C. AT1 receptor blockade delays post-lactational mammary gland involution: A novel role for the renin angiotensin system. (2012) *FASEB J*.,26: 1982-94.

Bruesehaber E*, **Schwiebs A***, Schmidt M, Boettcher D, Bornscheuer UT. Production of pig liver esterase in batch fermentation of E. coli Origami. (2010) *Appl Microbiol Biotechnol,*. 1337-1344. **equally contributing first authors*

Pankow K*, **Schwiebs A***, Becker M, Siems WE, Krause G, Walther T. Structural substrate conditions are required for neutral-endopeptidase mediated natriuretic peptide degradation. (2009) *J Mol Biol*., 393(2), 496-503. **equally contributing first authors*

Abstract publications

Schwiebs A, Wang Y, Zhu X, Siems WE, Walther T. The B-Type Natriuretic Peptide Metabolite BNP1-30 is a More Potent Vasorelaxant Peptide Than The Mature BNP1-32. (2011) *Hypertension, 58*, E91-E91.

Zhu X, Wang Y, **Schwiebs A**, Walther T. Designed Natriuretic Peptide ACNP Stimulates Natriuretic Peptide Receptors A and B: A Promising Treatment Option for Cardiovascular Diseases? (2011) *Hypertension*, 58, E162-E163.

Schwiebs A, Wang Y, Pankow K, Siems WE, Walther T. Metabolism of Murine BNP1-32: Biochemistry and Consequences for (Patho) Physiology. (2010) *Hypertension*, 56, E147-E147.

Patents

Walther & **Schwiebs**: German Patent: Application No: DE 10 2010 047 583 A1 Verfahren und Vorrichtung sowie deren Verwendung zur Diagnostik von spezifischen kardiovaskulären Erkrankungen. (Day of filing: 07.10.2010)

Walther & **Schwiebs**: PCT Patent: Application No: PCT/EP2011/062655 Stoffe und deren Verwendung zur Beeinflussung natriuretischer Peptid-Rezeptoren. (Day of filing: 22.07.2011)

Presentations

July 2012	**Poster presentation** at the Cardiovacsular Research Sessions of the AHA Conference 2012, New Orleans, USA
Mar. 2012	**Poster presentation** at the awardee session of the German Endocrinology meeting in Mannheim, Germany
Nov. 2011	**Oral presentation** at the German Hypertension Society at the German Hypertension Association Meeting (Deutsche Hochdruckliga), Cologne, Germany.
Sept. 2011	**Poster Presentation** at the High Blood Pressure Research Conference 2011, Orlando, USA.
Jun. 2011	**Poster Presentation** at the 36th FEBS meeting 'Biochemistry for tomorrow's medicine' in Torino, Italy.
May 2011	**Oral presentation** at the Heart Failure Congress 2011 from the European Society of Cardiology in the Young Investigator Award Session for Basic Science in Gothenburg, Sweden.
Oct. 2010	**Poster Presentation** at the Giessen Graduate School of Life Sciences annual meeting in Giessen, Germany.
Oct. 2010	**Poster Presentation** at the High Blood Pressure Research Conference 2011, Washington D.C., USA.
Mar. 2010	**Oral Presentation** at the Northern Cardiovascular Research meeting in Manchester, UK.
Nov. 2009	**Poster Presentation** at the German Hypertension Society at the German Hypertension Associaton Meeting (Deutsche Hochdruckliga), Luebeck, Germany.

Awards/Promotions

May 2012	Travel Grant from the German Academic Exchange Service (DAAD) for the internship at the medical University Graz, Austria

Jan. 2012	Awardee (second-best) of the Novartis price „Young endocrinology" 2012
Nov. 2011	Young Investigator Award from the German Society of Hypertension (Deutsche Hochdruckliga)
Aug. 2011	Travel Grant from the German Academic Exchange Service (DAAD) for the High Blood Pressure Research Conference 2011 in Orlando, USA
May 2011	Travel Grant from the European Society of Cardiology for the Heart Failure Congress 2011 in Gothenburg, Sweden
May 2011	Travel Grant from the German Society for Biochemistry and Molecular Biology (GBM) for the 36th FEBS meeting in Torino, Italy
Nov. 2009	Young Investigator Award from the German Society of Hypertension (Deutsche Hochdruckliga)