Molecular mechanism of natriuretic peptide secretion and adaptation to disease in animal model and clinical practice

Doctoral dissertation

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I. Abbreviations

ACE: angiotensin II convertase enzyme
ANP: atrial natriuretic peptide
BMI: body mass index
BNP: brain (B-type) natriuretic peptide
CNP: C-type natriuretic peptide
ECE: endothelin converting enzyme
ELISA: enzyme-linked immunoassay
ET-1: endothelin-1
ET_A: endothelin type A receptor
FITC-FPR-CMK: fluorescein-isothiocyanate-Phe-Pro-Arg-chloromethyl ketone
GC: guanylyl cyclase
GFR: glomerular filtration rate
HF: heart failure
LV: left ventricle
MAb: monoclonal antibody
NEP: neutral endopeptidase
NO: nitric oxide
NP: natriuretic peptides
NPR: natriuretic peptide receptor
NPR-A: natriuretic peptide receptor type A
NPR-B: natriuretic peptide receptor type B
NPR-C: natriuretic peptide receptor type C
NSTEMI: non-ST elevation myocardial infarction
PBS: phosphate buffered saline
RIA: radioimmunoassay
SNAP-25: synaptosomal-associated protein of 25kDa
SNARE: soluble N-ethylmaleimide-sensitive attachment protein receptors
Syn2: syntaxin 2
Syn4: syntaxin 4
Syn6: syntaxin 6
TSH: thyroid-stimulating hormone
VAMP: vesicle-associated membrane protein

II. Introduction

1. Heart as an endocrine organ

Since W. Harvey's discovery in the early XVIIth century [1] we know that the heart is the mechanical center of the circulation. Apart from this vital function, later comes the observation that the heart is also an endocrine organ. In 1956 Kisch et al. [2] identified atrial granules by electromicroscopy, and at the same time Henry and Gauer published [3] that diuresis is induced by atrial distention (the so-called Henry-Gauer reflex). These findings were followed by de Bold's observation in 1981 [4] that infusion of atrial extract in rat caused natriuresis, diuresis and hypotension. Finally, in 1983 the responsible bioactive substance was identified: several laboratories purified from different species the first member of the natriuretic peptide family, atrial natriuretic peptide (ANP) [5-8]. Interestingly, as so many times before, historical description/literature had forecasted what science proved much later: more than 2000 years ago, Flavius, a Roman historian named the workers who built the harbor of Caesarea "urinatores", because of the observation that these underwater draftsmen urinated frequently. Research in the end of the XXth century revealed that this phenomenon is due to the increased ANP secretion caused by the elevated thoracic pressure during immersion.

Not long after the sequence of ANP was identified, subsequent studies discovered two other natriuretic peptides based on sequence and structural homology [9, 10]. Brain (or B-type) natriuretic peptide (BNP) was first detected from brain tissue [9], but later on was found to be mainly of cardiac origin [11] and to exert similar effect to those of ANP [12], while C-type natriuretic peptide (CNP) [10] is mainly produced by endothelial cells [13], and its main effect is smooth muscle relaxation in the peripheral vasculature [14].

2. Cardiac natriuretic peptides

A, Synthesis and structure

In healthy, adult hearts ANP is synthetised predominantly in atrial cardiomyocytes [15], while the main source of BNP is the ventricles [16]. However, in pathophysiological states resulting in augmented gene expression of natriuretic peptides (such as heart failure), both the atria and the ventricles secrete significant amount of ANP [17, 18]. This expression pattern is otherwise typical for the developing heart [19]. During embryonic and neonatal development, ANP is produced both by atrial and ventricular cardiomyocytes. After birth, ventricular ANP expression is repressed [20], until it reaches the insignificant amount typical of healthy adult ventricles.

In the endoplasmic reticulum ANP and BNP are synthesized as pre-propeptides [21]: the pre-prohormone contains 151 amino acid residues in the case of human pre-proANP [22], and 134 amino acids in human pre-proBNP [23]. After splitting off the N-terminal signal sequence and additional posttranslational processings the pro-hormone pro-ANP (126 amino acids) and pro-BNP (108 amino acids) are stored in membrane-coated specific secretory granules. Upon secretion, proteolytical cleavage by corin [24], a serine protease, yields the active peptide C-terminal ANP (28 aa) and the biologically inactive N-terminal fragment NT-proANP. Studies indicate that corin also acts as the pro-BNP convertase, similarly resulting in active peptide BNP (32 aa) and the N-terminal NT-proBNP [25]. Natriuretic peptides also share a common structural motif consisting of a 17-amino-acid disulphide-bound ring at the carboxy-terminal region [21]. Human pro-ANP shares strong homology with other mammalian pro-ANPs (around 80%), with considerably high homology in the carboxy-terminal residues (exceeds 90%) [26], and the corin cleavage site is conserved among mammalian species in 100% [25]. However, pro-BNP exhibits high species variation, even in the C-terminal sequence [27].

B, Corin, the pro-natriuretic peptide convertase

Corin is a transmembrane serine protease predominantly expressed in the heart (since it gained its name) [28]. Corin is a large polypeptide containing more than 1000 amino acids. It consists of a short cytoplasmic tail at the N-terminus, followed by an integral transmembrane domain and an extracellular fragment which contains a multi-domain extracellular stem-region and the trypsin-like serine protease domain. Its molecular mass is further increased by N-glycosylation, which has been shown to play an important role in recombinant corin zymogen activation [29]. Corin is synthesized as a zymogen, pro-corin, and requires proteolytic cleavage at a conserved activation site between the stem-region and the protease domain to become an active enzyme [30, 31]. After cleavage, the stem and the protease domains remain connected by a disulfide bond. The biological role of corin has been investigated in vitro and in vivo. In in vitro assays, recombinant corin converts pro-ANP into biologically active ANP in a highly sequence-specific manner [24, 32]. Mice lacking the corin gene have spontaneous hypertension and cardiac hypertrophy [33]. Recent studies have implicated corin in experimental heart failure [34, 35], and reparative fibrosis of the infarcted heart [36]. A very recent study reported associations between single nucleotide polymorphisms in the corin gene and hypertension and cardiac hypertrophy in humans [37]. These studies highlight the biological significance of corin.

C, Regulation of natriuretic peptide level

Until secretion, cardiac natriuretic peptides stored in membrane-coated specific granules, which mainly exhibit a perinuclear subcellular localization [38]. Atrial granules are abundant, and have been shown to contain predominantly pro-ANP alone, but granules containing both pro-ANP and pro-BNP have been identified as well [39]. The presence of the secretory apparatus in adult ventricular cells was mainly recognized in pathological states causing increased natriuretic peptide expression/secretion, but even in these conditions in a much lesser amount than that of atrial cardiomyocytes [40]. The

explanation behind this observation might rely on the rather different secretory pattern of the two cardiac natriuretic peptides. Though both ANP and BNP are secreted in a constitutive as well as a triggered manner, regulation of ANP secretion is mainly controlled by triggered secretion of vesicle-stored ANP from the atria. Radiolabeling studies have shown that under unstimulated conditions ~60% of the newly synthesized ANP is intended for storage, and only the remaining 40% is secreted upon synthesis [41]. On the contrary, BNP secretion is almost entirely achieved in a constitutive manner, and increased plasma BNP level is a result of elevated protein expression rather than of triggered exocytosis of stored vesicles. This is supported by the observation that whereas 60% of the circulating BNP is of ventricular origin, the mature BNP tissue concentration in the ventricles is only 1% of that in the atria [20, 21].

a, Regulators of natriuretic peptide synthesis and secretion

Although there are numerous studies focusing on the mechanism of natriuretic peptide secretion, there are still several questions remained to be answered, especially about the factors governing BNP release. However, while describing the regulation of natriuretic peptide secretion, there are three characteristics to keep in mind: 1) as in other specific secretory cells, the amount of secreted hormone is determined by the rate of biosynthesis and exocytosis; 2) since BNP is predominantly secreted in a constitutive manner, the determinative factor for BNP plasma level is peptide synthesis. ANP secretion is regulated by stimulated exocytosis in the acute settings, whereas altered gene expression is responsible for chronic ANP level changes. 3) Factors that induce gene expression typically trigger secretion as well, and vice versa [40].

Shortly after the discovery of ANP, *mechanical stretch* of the atria emerged as a physiologic regulator of ANP release [42]. Though since then several neurohumoral factors have been implicated in the regulation of natriuretic peptide release, atrial distention (for ANP), as well as ventricular volume load (for BNP) have been confirmed as the major determinant of NP secretion (for review see [43, 44]. Mechanical stretch induces short-

term secretion of preformed vesicles in the acute setting, while chronic hemodynamic overload causes re-expression of the cardiac fetal gene program including increased NP expression in both the atria and the ventricles. A simple demonstration that atrial wall stretch rather than increased pressure causes elevated ANP secretion comes from the observation that in cardiac tamponade increased atrial pressure does not result in increased ANP secretion, however, pericardiocentesis leads to acute ANP release [45]. Despite the numerous studies and the importance of wall stretch on NP secretion, the molecular mechanism behind is still not fully understood. The most probable explanation is that short-term atrial stretch directly acts on cardiomyocytes through stretch-activated ion channels linked to $G_{i/o}$ regulatory protein [46], but the signal transduction leading to actual vesicle translocation, docking and release remains to be elucidated. The exact mechanism of chronic volume overload on altered NP expression is similarly still enigmatic. Furthermore, the role of distention in increased NP expression is not as clear as in acute ANP release. However, the level of ANP expression in the ventricles is correlated with the degree of hypertrophy [47, 48]. Long standing strain induces dramatic phenotypical changes in cardiomyocytes. Activation of proto-oncogenes activates other genes, including contractile-protein coding and natriuretic peptides genes [49], leading to hypertrophy and increased NP secretion. In addition to the increased expression, the localization of NP synthesis changes as well; fetal distribution of ANP synthesis reappears [50, 51], causing significant amount of ventricular ANP expression.

Another potent stimulus of ANP secretion is *hypoxia/ischemia* [52]. Nocturia associated with sleep apnea might be explained based on this theory [53]. However, increased ANP secretion as a response to hypoxia might be attributed, at least partially, to paracrine factors such as endothelin [54, 55].

In cell culture studies, *endothelin* is one of the strongest secretagogue of ANP and one of the most powerful agonist of NP gene expression [56-58]. In ex vivo and in vivo experiments, effect of endothelin is additional to that of mechanical stretch: atrial stretch induced ANP secretion is further augmented by endothelin-1 (ET-1) [59, 60]. This synergistic effect might be explained by the partially different signal transduction route: ET-1 binds to endothelin type A receptor (ET_A) [61] of cardiomyocytes which is linked to

G_q protein, a different G protein than the one involved in stretch-induced ANP secretion (the latter is most likely linked to G_o regulatory protein) [46]. ET-1 has been shown to induce the expression of proto-oncogenes, which in turn activate other genes, such as contractile-protein coding genes resulting in hypertrophic changes, as well as the genes of the natriuretic peptides [62].

Vasoconstrictor hormones, such as *angiotensin II, norepinephrine, epinephrine, vasopressin* were extensively studied for their effect on NP expression and secretion (for review see [63] and [43]). Although there are still controversies, the impact of these hormones on NP secretion appears to be mainly indirect, mediated by altered venous return and cardiac afterload.

Early cell culture studies found that *steroid hormones* increase NP expression [64, 65]. This observation is supported by the later finding, that the rat ANP gene contains two corticoid-binding sequences [66]. Experiments conducted on perfused atria showed that *corticotropin releasing factor* stimulates ANP secretion, supposedly in a direct receptor-mediated way [67].

Furthermore, *opioids* have been shown to increase ANP secretion both in vivo and in isolated atria [68].

As a counterbalance for the several stimulatory regulators described above, *nitric oxide* (NO) [63] and *C-type natriuretic peptide* [69] has an inhibitory effect on ANP secretion mediated by cGMP. *Negative feedback* is also present in natriuretic peptide secretion regulation: atrial natriuretic peptide has been shown to inhibit its own secretion [70].

Taken together and comparing the cardiac secretion of natriuretic peptides to other endocrine systems, there are a few remarks that need to be emphasized: 1) there is no tropic hormone regulating natriuretic peptide secretion; 2) there is no disease caused by natriuretic peptide overproduction; 3) similarly, there is no known disease caused by insufficient natriuretic peptide production; 4) the main trigger for natriuretic peptide secretion is mechanical stimulus; 5) there are several endogen bioactive agents regulating natriuretic peptide secretion, (as it was discussed earlier), however, angiotensin II, endothelin and catecholamines are the main hormonal regulators of natriuretic peptide secretion.

b, Molecular machinery behind the secretory process: the SNARE-hypothesis

In neurons and other specialized secretory cells secretion (or exocytosis) is mediated through the molecular interaction of SNAREs (soluble N-ethylmaleimide-sensitive attachment protein receptors) [71]. The SNAREs are composed of members from 3 different gene families: the syntaxins, the SNAP-25 family (synaptosomal-associated protein of 25kDa), and the VAMPs (for vesicle-associated membrane proteins) (reviewed in [72-74]. SNARE molecules form ternary complexes which are required for membrane fusion. Along with the SNARE proteins, members of the Sec1/Munc18 gene family also play essential role in exocytosis [75]. Deletion of the Sec1/Munc18 proteins in most cases led to lethal phenotype. In mammalian cells three Munc18 isoforms have been identified: Munc18a, which is most abundantly expressed in neurons, while Munc18b and Munc18c are primarily found in non-neuronal tissues. Although the exact function of the Munc18 proteins is still not fully understood, observations suggest that Munc18 proteins regulate SNARE complex formation by binding to the closed conformations of syntaxins and thereby preventing the formation of a ternary SNARE complex [76]. Apart of the interaction with the SNARE proteins, Munc18 reacts with several molecules involved in vesicle trafficking or signal transduction [75]. This characteristic makes Munc18 an ideal candidate to be responsible for the fine tuning of the secretory machinery.

D, Natriuretic peptide receptors and effects of natriuretic peptides

Natriuretic peptides exert their actions on the target cells by binding to transmembrane receptors (for a review see [27]. There are three natriuretic peptide receptors (NPR): NPR-A, NPR-B and NPR-C. The two former, NPR-A and NPR-B possess a guanylyl cyclase (GC) intracellular domain, while the latter, NPR-C is lacking the GC domain. NPR-A and NPR-B are mediating NP effects by increasing intracellular cGMP concentration. NPR-C

mainly functions as a clearance receptor for all three natriuretic peptides. NPR-A has the highest affinity for ANP binding, but it specifically binds BNP as well, while it has a very low affinity for CNP. NPR-B preferentially binds CNP, therefore it considered as the specific CNP receptor. Apart the endocytotic removal by the specific NP clearance receptor, NPR-C, circulating NPs are also removed from the circulation via enzymatic degradation through neutral endopeptidase (NEP). In human, clearance of BNP from the plasma is more reduced by both mechanisms compared to that of ANP (plasma half life of ANP is 2-4 min, BNP: ~22min.) [40]. The inactive N-terminal fragments have a longer half life for both atrial and brain natriuretic peptide.

NPR-A is expressed in several tissues, however, the principal localizations are the vascular smooth muscle, kidneys, adrenals, heart and lung. NPR-A is responsible for mediating the actions of ANP and BNP. The most important among those are lowering the blood pressure, increasing renal sodium and water excretion, and the antimitogenic effect [21, 27, 40] Due to NPR-A's binding preference to ANP (enhanced by the possibility of prompt ANP secretion), ANP has a stronger, though shorter effect, while BNP's effect is more sustainable.

a, Cardiovascular actions of natriuretic peptides

The natriuretic peptides' blood pressure lowering effect relies on several compounds. ANP causes reduction in preload by increasing permeability of the capillaries [77, 78] and by increasing venous capacitance [79]. This effect is supported by the renal actions, resulting in enhanced natriuresis and diuresis [80]. BNP lowers blood pressure by predominantly decreasing the afterload [81]. The natriuretic peptides' main cardiac actions are increased lucitropy [82] and lowering the threshold for vagal afference [83]. The latter results in suppressed reflex tachycardia and vasoconstriction, which otherwise would follow the reduced blood pressure.

The antiproliferative activity [84] of natriuretic peptides gets more and more attention. Its clinical importance relies on its role in cardiovascular remodeling in different

cardiovascular disorders, such hypertension, postangioplasty restenosis, and after myocardial infarction

b, Renal actions

The renal actions of natriuretic peptides, namely, the natriuretic and diuretic effects gave the hormones their names.

The natriuretic and diuretic effects are consequences of both renal hemodynamics and direct tubular actions [80], though the latter has a dominant role. Dilatation of the afferent arterioles and constriction of the efferent arterioles result in increased glomerular filtration rate (GFR). Relaxation of the mesangial cells leads to increased effective area for filtration, further increasing the GFR.

ANP is antagonizing angiotensin II–stimulated Na^+ and water transport in the proximal convoluted tubule. Furthermore, ANP and BNP directly reduce plasma renin – angiotensin II – aldosterone concentrations [85].

c, Actions in other organs

Natriuretic peptides, especially ANP, appear to have an integrating role in embryo- and organogenesis [19]. Its importance has been implicated in the development of the skeletal system [86], immune system [87] and reproduction [88].

Natriuretic peptides are also produced in the brain and might act as non-conventional neurotransmitters [89]. Although circulating natriuretic peptides basically cannot cross the blood-brain barrier [90], but they can reach the central nervous system outside the barrier, and can express central effects. Natriuretic peptides have been shown to centrally decrease water drinking [91] and salt appetite [92]. ANP also inhibits vasopressin secretion [93] and decreases the sympathetic tone [94]. ANP has also been shown to lower intra-ocular pressure [95], and this effect seems to be independent of its blood pressure lowering effect.

d, Phenotype of knockout mice

Genetically modified animal models are a very powerful tool for studying the function of different hormones and receptors *in vivo*. It also allows examining the long term and the developmental effects of the protein in question.

Studies with ANP transgenic and knockout animals [96] supported the role of ANP in the long-term blood pressure regulation, and cardiovascular and renal adaptation to elevated dietary salt intake. Homozygous *ANP gene knock out* mice [97] have no detectable ANP, and their blood pressure is elevated. The heterozygous have normal basal plasma ANP and normotension, but on high salt diet they become hypertensive. The *ANP transgenic animals* [98] have lifelong hypotension.

Deletion of the BNP gene [99] results in cardiac fibrosis, however, it does not cause hypertension or ventricular hypertrophy. *Overexpression of BNP* manifests in arterial hypotension [100] and skeletal overgrowth [101].

There are two *NPR-A receptor knockout* mouse models, developed by two independent groups [102, 103]. The two models are similar in the reported salt resistant hypertension, however, only Oliver et al. [103] observed marked cardiac hypertrophy with interstitial fibrosis and early death. *Overexpression of NPR-A gene* results in proportional decrease of the arterial blood pressure, and this effect is more expressed in animals on high salt diet [104].

NPR-C gene deleted mice [105] have increased plasma ANP half-life, but their ANP plasma concentration does not differ significantly from the wild-type controls. However, there is a small, but significant decrease in their blood pressure, due to increased urinary output. Homozygous NPR-C deleted mice also express skeletal deformities associated with a considerable increase in bone turnover.

3. Pathophysiology

As it was mentioned earlier, there is no known disease caused by overexpression or insufficient secretion of natriuretic peptides. However, natriuretic peptides have an important and extensively studied role in maintaining cardiovascular homeostasis (for a recent review see [106]. In several cardiovascular diseases, secretion and expression rate and pattern of natriuretic peptides is altered, which beneficially changes the pathophysiology of the given disease, as well as provides a biomarker for better judgement of diagnosis and prognosis. Apart of the well-known cardiovascular pathological changes, recent observations describe their modulatory effects on asthma and inflammation [107].

A, Pathophysiology of natriuretic peptides in cardiovascular diseases

a, Heart failure

Among cardiovascular diseases with increased natriuretic peptide level, heart failure is one of the most extensively studied. NP level starts to rise before symptoms develop, and continues to rise as the disease progresses, resulting in a close correlation between cardiac impairment and plasma level of BNP [108]. From the diagnostic point of view, it is worth to note that NP level is elevated in both systolic and diastolic dysfunction [109, 110], as well as in left ventricular and right ventricular dysfunction [111]. This is not so surprising considering the mechanism behind the increased NP secretion, but from the clinical point of the sometimes challenging diagnosis of diastolic heart failure or acute pulmonary embolism, it becomes easy to recognize its significance.

In compensated heart failure, increased natriuretic peptide level successfully ameliorates cardiac and systematic functions by its volume-contracting, vasodilatative and antiproliferative properties [112]. However, overt heart failure is characterized by very

high concentration of natriuretic peptides, which are seemingly inefficient in controlling the symptoms. As a result, overt heart failure patients suffer of natriuretic peptide deficiency, despite of the measured high natriuretic peptide plasma concentration [113]. Results from several studies suggest that the explanation behind this finding is multifactorial: increased secretion of the biologically less active pro-BNP instead of the active form BNP_{1-32}, as well as abnormal processing of proBNP into inactive BNP fragments might contribute to the pathology of decompensated heart failure [114]. In addition, recent animal studies have shown that up-regulation of phosphodiesterase in heart failure impairs cGMP signaling of natriuretic peptide receptors [115], resulting in diminished natriuretic peptide effects even with normal/high hormone concentration.

b, Hypertension

Hypertensive patients with normal heart geometry have a slightly elevated natriuretic peptide level. If hypertension is associated with left ventricular hypertrophy, a well-known risk factor for all the cardiovascular complications of hypertension, natriuretic peptide level is further increased [116]. This correlation is strongest with concentric hypertrophy, which is in particular linked to increased morbidity and mortality [117].

c, Acute coronary syndrome

Hypoxia is a strong trigger of natriuretic peptide secretion [52]. In acute coronary syndrome, BNP is elevated regardless of detectable contractile dysfunction, and in the presence of normal ECG and undetectable troponin [106]. The elevation of natriuretic peptides in response to hypoxia can be viewed as an important homeostatic mechanism, since their vasodilatative effects might increase blood flow in the coronaries, as well as result in beneficial reduction of the cardiac oxygen demand due to the reduced preload and afterload.

d, Arrhythmias

Arrhythmias themselves probably do not cause changes in natriuretic peptide secretion/expression. However, if the arrhythmia results in wall distention, NP levels can rise significantly. Several studies reported elevated natriuretic peptide level in atrial fibrillation [118, 119].

B, Pathophysiology of natriuretic peptides beyond cardiovascular diseases

Female gender and older age causes higher natriuretic peptide levels [120, 121]. Although these are certainly not pathological reasons, but the explanation for it is yet unknown.

Renal impairment causes increased natriuretic peptide level, which is at least in part due to decreased natriuretic peptide clearance. NPR-C is primarily localized in the kidneys, while neutral endopeptidase expressed in several tissues, among those the kidneys contain a significant amount [27]. Since clearance of NT-proBNP is not mediated by NPR-C nor neutral endopeptidase, it might be more sensitive to reduced renal filtration and clearance [122]. Both BNP and NT-proBNP values requires adjustment for correct interpretation [123].

BNP level is decreased in *obesity* [124]. Several studies have found that BNP plasma concentration is inversely related to BMI (Body Mass Index) [123]. Although the pathophysiological mechanism behind this observation is unknown, more hypotheses exist to explain this correlation. Increased concentration of the NPR-C receptor on adipocytes emerged as a possible explanation [125], however, other studies contradicted to it by the finding that BNP level was correlated with greater lean mass, but not greater fat mass [126]. Others have found that decreased natriuretic peptide production might be the responsible mechanism [127].

High output states –such as *cirrhosis, hyperthyroidism* – might cause increased natriuretic peptide level, however, the pathophysiological mechanism classifies these cases as elevated NP level due to cardiac dysfunction [128].

Similarly, *amyloidosis* might entail increased natriuretic peptide level due to cardiac dysfunction [129].

Increased BNP level in *sepsis* is due to myocardial dysfunction [130].

ANP has been reported to directly affect the innate (macrophages and polymorphonuclear leukocytes) and the adaptive (dendritic cells and lymphocytes) *immune system* [131]. N-terminal pro-ANP derivatives has been shown to exert antiinflammatory effect [132].

In the lung, ANP causes vasodilatataion, bronchodilatation, increased vascular permeability and surfactant production. During *asthma* exacerbations plasma concentration of ANP is increased, with highest concentration measured in the pulmonary arteries [107].

4. Clinical use

Although the two cardiac natriuretic peptides are very similar in their biological effects and properties, clinical data have proved that BNP is preferential for most of the clinical applications. Unlike ANP, which is stored in granules and secreted with even minor triggers (e.g. snack-induced ANP release, [133]) BNP has a minimal storage, and plasma BNP level is rather determined by the rate of protein expression. This, in concert with BNP's longer plasma half life due to its slower clearance by both NPR-C and NEP, results in a more stable hormone level [106]. Free of the clinically unimportant fluctuations, BNP has been proved to be a more reliable and sensitive marker for several cardiovascular diseases [134]. Furthermore, several studies have found that measurement of NT-proBNP, the biologically inactive N-terminal fragment, with its even longer plasma half life, is advantageous over BNP for diagnostic purposes [123].

Most of the recent studies examining the use of natriuretic peptides with diagnostic and screening intentions originated from the publication of the Framingham Heart Study.

Wang et al. [134] examined BNP and NT-proANP levels in more than 3000 asymptomatic subjects. They found that increased BNP levels were independently predictive of the risk of death, heart failure, atrial fibrillation, stroke and transient ischemic attack. Similar

results were obtained for NT-proANP, however, BNP outperformed NT-proANP levels with respect to most outcomes.

A, Screening

In general, the diagnostic usefulness of a screening test depends largely on the prevalence of the disease. Left ventricular (LV) hypertrophy is a common complication of hypertension, however, up to 50% of the patients might be asymptomatic and unaware of their conditions [123, 135]. Although echocardiography is the gold-standard for its diagnosis, the cost and limited unavailability might be prohibitive for screening purposes. Measuring BNP or NT-proBNP levels in populations where the prevalence of LV hypertrophy exceeds 1% (e.g. diabetics, elderly, hypertonics) is an efficient and cost-effective screening method. In populations with relatively low prevalence of LV hypertrophy, the strong negative predictive value of low natriuretic peptide level can be used to 'rule out' the disease [109].
A recent study examined the use natriuretic peptide measurement to screen participants in athletic events [136].

B, Diagnostic use

After 2000, assays for the measurement of plasma BNP and NT-proBNP became commercially available and approved for helping the diagnosis of *heart failure* [106, 137]. Since then, several multicenter studies have been published, evaluating the importance of natriuretic peptides in the diagnosis of heart failure. Although specificity and sensitivity highly dependent on the cut-off values, BNP level was highly sensitive and specific for the diagnosis of heart failure (e.g. a study by Wieczorek et al. [108] reported 82% sensitivity and 99% specificity for distinguishing control patients and patients with congestive heart failure at a threshold of 100pg/ml). BNP and NT-proBNP measurements – especially the

latter – had fairly good results for the diagnosis of *pre-clinical ventricular impairment* as well. In primary care practices, NT-proBNP values improved diagnostic accuracy by 21% [138]. In the differential diagnosis of *acute dyspnea*, an early study found that BNP more accurately predicted the final diagnosis of heart failure than left ventricular ejection fraction [139]. Furthermore, a more recent study [140] found that NT-proBNP was even 17% more specific for the diagnosis of heart failure in dyspneic patients, than BNP.

In *valvular disease,* symptom onset overlaps with the elevation of natriuretic peptide level [141-143]. In aortic stenosis, this correlation appeared to be more precise (with adequate cut-off values) than the assessment of valvular area by echocardiography [141].

Measurement of BNP level might be particularly useful in the diagnosis of *acute coronary syndrome,* especially in unstable angina and non-ST elevation myocardial infarction (NSTEMI). Elevated BNP has a diagnostic accuracy over 90% for acute coronary syndrome, even when there are no ECG changes or detectable troponin [106].

Knowing BNP plasma concentration might help the differential diagnosis of *syncope*. A recent study found that moderately increased BNP level identified cardiac syncope by 82% sensitivity and 92% specificity [144].

C, Prognostic use

NP levels have a strong prognostic value both in acute and chronic *congestive heart failure,* predicting sudden death, exercise capacity, cardiac and all cause mortality more accurately than NYHA class or left ventricular ejection fraction [106].

Several studies have shown the strong predictive value of both BNP and NT-proBNP for all-cause mortality and heart failure in the management of *acute coronary syndrome* and *stable coronary artery disease* [106]. In stable coronary artery disease, NT-pro-BNP is a marker of long-term mortality and a better prognostic factor than conventional cardiovascular risk factors [145]. After myocardial infarction, persistent elevation of NT-proBNP is associated with chronically impaired LV function [123].

In *hypertension,* NT-proBNP level predicts future cardiovascular events, even with normal left ventricular function [146].

In *cardiac and vascular surgery,* preoperative BNP level helps to predict post-operative outcome [147]. Recent studies reported promising results with BNP measurement in the assessment of allograft function and prognosis of *cardiac transplantation* [148].

Natriuretic peptides have a clinically significant predictive value in *valvular diseases.* In mitral regurgitation, aortic regurgitation and stenosis, BNP level correlates with the severity of disease [141-143]. Mitral stenosis causes only moderate increase in NP level due to underfilling of the left ventricle, however, NP level decreases after valvulotomy [149].

In *atrial fibrillation* both pre- and post-cardioversion natriuretic peptide levels predict recurrent atrial fibrillation after cardioversion [106]. Knowledge of the probability of postcardioversion reversion to atrial fibrillation might help to select patients who benefit more from rate control and anticoagulation therapy.

In *pulmonary embolism*, elevated BNP level, especially with concomitant echocardiographic signs of right ventricular dysfunction has a strong prognostic value for worse outcome [150].

D, Monitoring therapy

Monitoring BNP level in *heart failure* patients has been found helpful in tailoring therapy both in the inpatient and outpatient settings. Since plasma BNP levels can largely vary between patients with similar signs and symptoms, changes in BNP level during the course of the disease of an individual patient can inform more precisely about the efficacy of the therapy; clinical improvement should be associated with significant changes in BNP level. While serial BNP levels might help to guide the therapy, acquiring BNP measurement upon admission and before discharge strongly predicts outcome [123].

In the outpatient treatment of congestive heart failure, BNP level-guided therapy reduced hospital admissions and cardiac death [151]. Knowledge of the ‘steady-state’ BNP level of

a HF patient might help early recognition of need for further evaluation or starting a more aggressive therapy.
Measuring preimplantation BNP levels predicts response for *cardiac resynchronization* therapy [152], and thus aids in selecting the appropriate candidates, while monitoring BNP levels informs about the efficacy of the therapy [153].

E, Therapeutic use

For the therapeutic modification of the natriuretic peptide system, two main approaches were applied: administration of ANP/BNP or inhibition of (enzymatic) clearance of NP.
Nesiritide, the human recombinant BNP, was approved in 2001 for the treatment of acute decompensated heart failure. In a preceding clinical study [154] conducted on patients hospitalized with symptomatic heart failure, nesiritide infusion was found to reduce dyspnea and fatigue, decrease pulmonary wedge pressure, systemic vascular resistance, plasma aldosterone level, and overall improved clinical status. Its main side effect was dose-related hypotension. Subsequent studies compared nesiritide with other standard treatments. Compared to dobutamine, nesiritide proved to be effective in improving the clinical status [155]. Nesiritide was lacking the pro-arrhythmic effect of dobutamine, but equally reduced hospital stay and re-admission rate compared to dobutamine. Nesiritide was found to be safe with adjunctive beta-blocker therapy [156], as well as in renal impairment [157] and acute coronary syndrome [158].
Recently, its subcutaneous administration is under investigation with promising results [159], and there are new efforts to develop an orally active form [160].
Carperitide, the human recombinant ANP participated in several clinical studies, however, it is not approved for treatment [161].

Inhibition of the clearance enzyme neutral endopeptidase (NEP) is another promising way for increasing natriuretic peptide level. However, since NEP is involved in the degradation of other hormones [162], namely, the vasoconstrictor endothelin-1 and angiotensin II, as

well as the vasodilator bradykinin, NEP's inhibition increases vasoactive hormone levels from both the vasodilator and vasoconstrictor sides. Fortunately, this drawback can be overcome by simultaneous inhibition of angiotensin convertase (ACE). A new class of drugs, so-called vasopeptidase inhibitors, met this requirement. Studies examining the efficacy and safety of omapatrilat, the prototype of the vasopeptidase inhibitors, reported favourable results [163, 164]. However, the relative frequency of related angioedema restricted its application. Another therapeutic combination under clinical trials is the neutral endopeptidase – endothelin-converting enzyme inhibitor (NEP/ECE inhibitor). Finally, the triple vasopeptidase inhibitors (ACE/NEP/ECE) are presently investigated in animal trials [162].

III. Aims

1. In vitro and ex vivo studies: physiologic mechanism of ANP secretion from cardiomyocytes

Despite the numerous studies (there are more than 20000 articles published on natriuretic peptides to date) and the importance of natriuretic peptides in health and disease, there are still several questions about the molecular mechanisms controlling natriuretic peptide secretion and processing that remained unanswered.

A. Regulation of ANP secretion from cardiomyocytes

Studies on the trafficking of ANP-containing vesicles in cardiomyocytes, mechanism of fusion of the granules with the plasma membrane and its regulation has just been recently started [165]. SNARE molecules and SNARE related peptides, such as the Munc18 molecules are widely studied regulators of vesicle trafficking and membrane fusion in other secretory cells [71, 75]. Our aim was to investigate whether ANP secretion is proceeded through a SNARE-dependent mechanism, and whether Munc18c, a well-known regulator of SNARE complex formation, controls ANP secretion from cardiomyocytes.

B. Characterization of corin, the pro-ANP convertase

Specific granules of cardiomyocytes contains the pro-hormonal forms of ANP, *pro-ANP*, while the main form found in the circulation is the biologically active peptide ANP [21]. Corin, the enzyme responsible for pro-ANP activation by a specific proteolytic cleavage, was recently identified [28]. Although corin has been shown to be abundantly expressed in the heart, its cellular localization was unknown. Corin cDNA sequence had also predicted

that corin is synthesized as a zymogen, pro-corin, and needs proteolytical cleavage for activation. To address these issues, our aim was to investigate the cardiac localization and posttranslational modification and molecular forms of corin.

2. In vivo studies: natriuretic peptide secretion in animal model of diabetes combined with cardiac hypertrophy

Diabetes mellitus is associated with several cardiovascular diseases [166]. Natriuretic peptides and endothelin are counterregulatory hormones involved in the cardiovascular complications of diabetes mellitus. Although there are numerous reports on these changes in the basal state, little is known about the responses to increased acute hemodynamic burden in diabetes. The aim of our experiments was to characterize the changes of ANP and endothelin secretion in response to acute hemodynamic load in experimental diabetes.

3. Clinical practice: natriuretic peptide secretion in bradycardia

Increased natriuretic peptide secretion due to tachycardia-related cardiomyopathy is a well-known phenomenon in clinical practice [106, 167, 168]. The cellular mechanism behind the increased secretion is myocardial stretch. However, not only tachycardia, but bradycardia might lead to increased cardiac wall tension. Our aim was to investigate whether bradycardia might manifest in clinically significant elevation of natriuretic peptide serum level, and whether cessation of the arrhythmia results in a reduction of the NP level.

IV. Material and methods

1. Cloning, bacterial expression, and purification of bacterial proteins

Recombinant mouse corin fragments, stem domain (residues 134-865 [30]) and protease domain with activation peptide (residues 854-1113), were generated in bacterial systems (Fig. 7A). The coding regions of corin were cloned by PCR from a total mouse heart cDNA library (Clontech Lab. Inc., Palo Alto, CA) into pCR^{TM} II vector (Invitrogen, Carlsbad, CA) using primers: the stem domain (residues 134-865), 5'-GAATTCATGCCTTTGTGGGAACATTAA-3'(sense) and 5'-GCGGCCGCATTCGGGCAGCAGG-3'(antisense); the protease domain with activation peptide (residues 854-1113), 5'-GGATCCAAGCAAGACTGTGGC -3' and 5'-GCGGCCGCTCCTTGGGATTTCTTTTG-3'; (Sigma-Genosys, The Woodlands, TX). The sequences of all clones were confirmed by sequencing both DNA strands. The confirmed cDNAs were ligated into the pGEX-4T-2 (Amersham Biosciences, Piscataway, NJ) or the pProEX-HTb vector (Invitrogen). Restriction enzymes were from New England Biolabs Inc. (Beverly, MA). Rapid DNA Ligation kit was from Roche Diagnostics (Mannheim, Germany).

Corin protease domain fragment, residues 869-1113, was amplified by PCR using pProEX-HTb-protease domain fragment 854-1113 cDNA as template. The sense and antisense primers used were 5'-GAATTCATGATCCTTGGGGGTCG-3' and 5'-GCGGCCGCTCCTTGGGATTTC-TTTTG-3'. The PCR products were digested with EcoR1 and Not1, and cloned into the pGEX-4T-2 vector. The constructs were verified by DNA sequencing and restriction digestion

Two GST-tagged proteins representing the stem domain and the protease domain construct (residues 869-1113) were expressed in *Escherichia coli* strain BL21. The proteins were solubilized from inclusion bodies with 6 M guanidine HCl and 10 mM dithiothreitol in TE

buffer (50 mM Tris-HCl,10 mM EDTA, pH 8.0), refolded in 55 mM MES, pH 6.5 buffer with 0.56 mM NaCl, 0.44 mM KCl, 1.1 mM EDTA, 440 mM sucrose, and 550 mM L-arginine and dialyzed against TE buffer. The GST tag was cleaved with thrombin. The protease domain construct (residues 854-1113) was expressed as a His_6-tagged protein. The protein was solubilized from inclusion bodies, refolded in 55 mM Tris-HCl, pH 8.2 buffer with 264 mM NaCl, 11 mM KCl, 2.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 440 mM sucrose and 550 mM L-arginine, purified on Ni-Sepharose column and dialyzed against TE buffer. The proteins were analyzed by SDS-PAGE and Western blotting using mouse anti-GST-tag (Santa Cruz Biotech., Inc., Santa Cruz, CA) or anti-His-tag antibody (QIAGEN Inc., Valencia, CA).

2. Antibodies

Anti-ANP serum directed against c-ANP was purchased from Phoenix Pharmaceuticals (Belmont, CA, USA), polyclonal anti-syntaxin 2 and syntaxin 4 were obtained from Synaptic Systems (Göttingen, Germany), monoclonal anti-syntaxin 4 was purchased from BD Biosciences (San Jose, CA, USA), and monoclonal anti-syntaxin 6 was obtained from Abcam (Cambridge, MA, USA).

A, Polyclonal antibody generation

Rabbit polyclonal antibodies were raised against two recombinant mouse corin fragments from the GST-tagged proteins – corin stem domain (residues 134-865) and corin protease domain (residues 869-1113). Rabbit polyclonal antibodies directed against residues unique to Munc18c (residues 269-277) and r-syntaxin 4 (cytoplasmic domain) were also generated. After obtaining preimmune serum, New Zealand white rabbits were subcutaneously immunized with 50 to 100 μg of purified proteins emulsified in Freund's adjuvant every 2 weeks over 3-4 months, and blood samples were taken to monitor

antibody production. The specificity of the polyclonal serums was tested against the original immunizing antigens and mouse heart tissue and other tissues extracts by Western blotting.

B, Monoclonal antibody generation

Female BALB/c mice (Charles River, Wilmington, MA) were immunized intraperitoneally with 50 μg of recombinant His_6-tagged corin protease domain (residues 854-1113) antigen in complete Freund's adjuvant and two weeks later were given booster injections of 50 μg with incomplete Freund's adjuvant. The antibody titer was determined by solid phase radioimmunoassay binding against protein antigen and BSA. Spleenocytes were fused with murine SP2/0 cell line in polyethylene glycol (PEG) containing medium. Hybridomas supernatants were tested for the production of anti-protease domain MAbs by solid phase RIA in COBRA II gamma counter, using (^{125}I)-sheep anti-mouse antibody (American Diagnostica Inc.) as a secondary antibody. The positive clones were analyzed for their ability to bind (^{125}I)-corin protease domain (854-1113) protein. Hybridomas were tested for the production of corin-specific antibody by immunoblotting using recombinant corin protease-domain or control proteins, and mouse heart or other tissues extracts as an antigen. Hybridomas were cloned by limiting dilution and sub-cloned. MAbs have been classified and sub-classified by ELISA (Zymed Lab. Inc., San Francisco, CA). MAbs, 12G8, 14H1, and 19C10 were identified as IgM-K class and 16E6 as IgG-2b class.
Monoclonal antibodies against recombinant Munc18c were also produced as described, and their specificity was established by immunoblotting against purified recombinant Munc18a, Munc18b, and Munc18c proteins.

3. Protein extraction

A, Protein extraction from tissue

Hearts were obtained either from isoflurane-anesthetized Sprague-Dawley rats or harvested from BALB/c mice following CO_2 inhalation and thoracotomy. Briefly, tissue was quickly washed in ice-cold phosphate buffered saline (PBS) pH 7.4, snap-frozen in liquid nitrogen, and homogenized in 1mM TRIS buffer pH 7.4 supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) in a ratio of 0.1 g tissue per 1 ml of buffer. Tissue homogenates were spun down at 10,000 rpm, 10 min, 4 ^{o}C at a Beckman J2-MI rotor. Supernatants rich in cytosolic proteins were saved at -80 ^{o}C. Pellets were washed in 5 ml ice-cold 1 mM TRIS buffer pH 7.4 supplemented with complete protease inhibitor cocktail (Roche Diagnostics), and centrifuged as above. After washing, pellets rich in membrane proteins were homogenized in a detergent-containing high ionic concentration buffer (1% Triton-X100 buffer, containing: 50 mM TRIS-HCl pH 7.4, 125 mM NaCl, 1% Triton-X100, 0.1% complete protease inhibitor cocktail) in a ratio of 0.1 g tissue per 1 ml buffer. Tissue homogenates were incubated for 1 hr at 4 ^{o}C with shaking, followed by centrifugation at a Beckman J2-MI rotor at 10,000 rpm, 10 min, 4 ^{o}C. Supernatant was collected and saved at -80^{o}C until further analysis. Protein concentration of extracts was measured by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Total mouse tissue (brain, liver, lung, skeletal muscle and heart) extracts were obtained by extraction of homogenized tissue with Trizol reagent (Invitrogen) according to the manufacturer's protocol. The protein concentration of the extracts was determined by the Protein Assay kit (Pierce).

In separate experiments, contracting mouse hearts (left ventricle) were injected *in situ* with 1 μM FITC-Phe-Pro-Arg-chloromethyl ketone (FITC-FPR-CMK, Haematologic Technologies Inc., Essex Junction, VT) in PBS, incubated for 5 min and harvested. Membrane-bound proteins were extracted according to the protocol above.

B, Protein extraction from cultured cells

Cultured neonatal atrial cardiomyocytes and HL-1 cells were incubated for 1 h at 4 ^{o}C in 50 mM Tris-HCl, pH.7.4 buffer containing 125 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail and centrifuged at 10,000 *g* at 4 ^{o}C. The protein concentration of the supernatant was determined by the Protein Assay kit (Pierce, Rockford, IL).

4. Plasma peptide extraction and radioimmunoassay

Acidified plasma (1.5 mL) was applied to Sep Pack C18 cartridges previously activated by methanol and 0.1 % trifluoroacetate, and eluted with 80% acetonitrile in 0.1% trifluoroacetate buffer. Eluates were evaporated and reconstituted with radioimmunoassay buffer. Immunoreactive peptide concentrations (NT-proANP, ET-1) were determined with radioimmunoassays using polyclonal antibodies and a goat secondary antibody. Intra-assay and interassay coefficients of variation were lower than 10% and 15%, respectively.

5. Concanavalin A-Sepharose binding assay and cell surface labeling

Cell lysates (5.5 μg/ml) were incubated with one-fifth volume of Concanavalin A-Sepharose (Amersham Bioscience) for 1.5 h at 4 ^{o}C according to the manufacturer's protocol.

HL-1 cells were incubated in PBS containing 1 mg/ml EZ-Link^{TM} Sulfo-NHS-LC-Biotin (Pierce) at 4 ^{o}C for 40 min with swirling. The excess of EZ-Link^{TM} Sulfo-NHS-LC-Biotin was captured by incubation with 50 mM Tris HCl, pH 7.4 at 4 ^{o}C for 30 min. After washing, cells were lysed, and lysates were incubated with Immobilized Streptavidin (Pierce) for 2 h at room temperature. Bound and unbound fractions were analyzed by Western blotting.

6. Western-blotting

Following denaturation by 5 minutes boiling in Laemmli sample buffer, samples were subjected to 10% SDS-PAGE and electroblotted to Immobilon-P transfer membrane (Millipore Corp., Bedford, MA, USA). Non-specific binding sites were blocked by incubation with 5% skim milk (ICN Biomedical Inc., Costa Mesa, CA, USA) powder in 50 mM Tris-HCl, pH 7.4, with 150 mM NaCl, and 0.1% Tween-20 buffer. After incubation with various primary antibodies (as indicated), secondary goat anti-rabbit/or goat anti-mouse (IgG + IgM) alkaline phosphatase-labeled antibody (Amersham Biosciences, Piscataway, NJ, USA) was used for detection. Immunoreactivity was visualized with ECF (enhanced chemifluorescence) substrate (Amersham Biosciences) by fluorescence/phosphoimager Storm 840 (Amersham Biosciences). The blots shown are representative of at least three independent experiments. To analyze other components, immunoblots were sometimes stripped of initial antibodies by incubation in stripping buffer (100 mM β-mercapto-ethanol, 62.5 mM TRIS-HCl, 2% sodium-dodecyl-sulfate) for 30 minutes at 50 ^{o}C with occasional gentle shaking. Following incubation, membranes were washed five times in water, and once in 0.1% Tween-20 in Tris buffered saline (0.1% Tween-TBS, contains: 50 mM Tris, 125 mM NaCl, 0.1% Tween-20), until traces of β-mercapto-ethanol was removed. Then membranes were blocked in 5% skim milk (ICN Biomedical Inc.) powder in 0.1% Tween-TBS. Prior to additional immunoblot analysis, the blots were shown to be without immunoreactivity after developing them with enhanced chemifluorescence detection system and analysis by phosphorimaging.

7. Immunoprecipitation

For the immunoprecipitation of syntaxin proteins, heart extracts rich in membrane-bound proteins were pre-cleared by incubation with protein A agarose (Pierce) or protein G Sepharose 4B (Zymed Laboratories Inc., South San Francisco, CA, USA) beads for 1 hr at

4°C with gentle shaking. Purified polyclonal anti-syntaxin 2 or anti-syntaxin 4 antibodies (5 µg), or rabbit IgG (5 µg, control) were coupled to 50 µl protein A agarose slurry by incubation for 90 minutes at room temperature with rotation. Similarly monoclonal anti-syntaxin 6 (5 µg) anti-digoxin antibody (5 µg, control) were coupled to 50 µl rec-protein G Sepharose 4B slurry under the same conditions. Following incubation, beads were washed three times with PBS. Pre-cleared protein extract (2 mg) was immunoprecipitated by incubation with antibody-coupled beads for 4 hr at 4°C with gentle shaking. Immunoprecipitates were washed four times with ice-cold PBS, boiled in sample buffer, and centrifuged at 10.000 rpm for 2 minutes at room temperature on a tabletop centrifuge.

Corin protein immunoprecipitation was done according to the following protocol: the murine anti-corin protease domain MAbs or control monoclonal anti-mouse IgM and IgG were preincubated with protein G-Sepharose beads (Pierce) for 60 min at room temperature. Beads were washed with RIPA buffer (12.5 mM sodium mono-phosphate, 1% (w/w) Nonidet P40, 5mM sodium fluoride, 5mM sodium vanadate, 100 units/ml aprotinin, 1% Triton X-100, pH 7.2) and 0.1 M sodium acetate buffer, pH 5.0 and incubated with mouse heart extract (membrane fraction) for 60 min at room temperature. After washing with RIPA buffer, immuno-precipitated proteins were solubilized in SDS-PAGE sample buffer.

Supernatants from each experiment were analyzed by SDS-PAGE followed by immunoblotting as described above.

8. Cardiomyocytes and HL-1 cell culture

Neonatal rat atrial and ventricular cardiomyocytes were isolated and cultured according to a modified protocol (Worthington Biochemical Corp., Lakewood, NJ, USA). Briefly, atria and ventricles were dissociated under the microscope and processed separately. Following overnight trypsinization at 4°C cells were released by repeated collagenase digestion at 37 °C. Cells were plated at high density (10^5/well) on fibronectin/gelatin-coated 24-well plates and incubated overnight in a 4:1 mixture of Dulbecco's Modified Eagle Medium (DMEM)

low glucose (Invitrogen, Carlsbad, CA, USA) and Medium199 (Invitrogen), containing 10% fetal bovine serum. The following day non-adherent cells were removed and the medium was replaced with a modified complete serum-free media (CSFM [64]), that contains: DMEM/F12 (Invitrogen), 25 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA), 1.7 μM insulin (Sigma-Aldrich), 1 μM transferrin (Sigma-Aldrich), 10 nM sodium-selenite (Sigma-Aldrich), 1 nM tri-iodo-thyronine (Sigma-Aldrich), 0.2% BSA (Sigma-Aldrich), 190 nM dexamethasone (Sigma-Aldrich), 1 mg/l arachidonic acid (Sigma-Aldrich), 0.5 mg/ml docosahexanoic acid (Sigma-Aldrich), and 100 Unit/ml penicillin/streptomycin (Invitrogen)). Cells were maintained in CSFM media at 37 ^{o}C in 95%/5% O_2/CO_2.

The murine cardiomyocyte-like cell line HL-1 was kindly provided by Dr. William C. Claycomb (Louisiana State University Medical Center, New Orleans, LA). HL-1 cells were cultured according to the published protocol [169] in Claycomb medium (JRH Biosciences, Lenexa, KA) supplemented with 10% fetal bovine serum (JRH Bioscience), 100 μg/ml penicillin/streptomycin, 0.1 mM norepinephrine (Sigma) and 2 mM L-glutamine (Life Technologics).

9. Immunofluorescence staining

Cardiomyocytes were grown on an 8-well glass chamber slide (Nalge Nunc International, Rochester, NY, USA) and prepared for immunofluorescence by washing with phosphate–buffered saline (PBS) followed by fixation with Cytofix buffer (BD Biosciences) for 20 min at room temperature. Cells were permeabilized with 0.1% Triton-X100 for 1 minute or 0.1% saponin for 4 min at room temperature. Non-specific binding sites were blocked by incubation with 1% BSA for 30 min at room temperature and primary antibodies were applied for 1 hr. After repeated washing in PBS, primary antibodies were detected by 1 hr incubation with Alexa Fluor 488-labeled goat anti-mouse or Alexa Fluor 594-labeled goat anti-rabbit secondary antibodies (Invitrogen). Nuclei were visualized by staining with 4',6'-diamidino-2-phenylindole hydrochloride (5 min). Slides were mounted with Prolong Antifade Kit (Invitrogen) and analysed with an LSM 510 Meta Confocal Microscope with

an Argon-488, He-Neon 543, 633 lasers, with 63x oil immersion lens. Localization of labeled proteins was analyzed using Axion vision software (Zeiss LSM Image Examine, Version 3.1.0.99).

10. Cell permeabilization, secretion assay and ANP measurement

Atrial or ventricular neonatal cardiomyocytes plated in 24-well plates (10^5 cells/well) were used for secretion assay on culture day 7-9. Streptolysin-O (SLO; Sigma) was dissolved in 0.05% BSA at a concentration of 25,000 unit/ml, reduced with 2 mM dithiothreitol on ice for 30 min, aliquoted and stored at -80 ^{o}C. Cells were briefly rinsed with an intracellular Ca^{2+}-buffering solution (KG buffer containing 100 nM free Ca^{2+}: 140 mM K-glutamate, 5 mM PIPES, 12.5 mM Mg-Acetate, 2.5 mM EGTA, 2.5 mM EDTA, 5 mM D-glucose, 0.7 mM $CaCl_2$; pH 7.2 adjusted with KOH) then incubated for 20 min at 37^{o}C with 190 µl KG buffer containing 100 unit SLO and various antibodies (0.15 mg/ml). Secretion was induced by priming the samples for 5 min at 37 ^{o}C with Na_2ATP (2 µl, 500 mM; Sigma), Na_2GTP (2 µl, 200 mM; Calbiochem, San Diego, CA, USA) and increasing the free Ca^{2+} concentration up to ~100 µM by adding 6.2 µl 0.1M $CaCl_2$. The free Ca^{2+} concentration was determined by the WinMaxc v2.05 software ([170] see also http://www.stanford.edu/~cpatton/maxc.html) and the titration curves described by Knight and Scrutton [171]. Supernatants were collected on ice and then stored at -80^{o}C until samples were analysed. Cells were lysed in 1% Triton-X100 followed by 5 min centrifugation at 12000 rpm. Supernatants were collected and saved at -80^{o}C until further evaluation. Immunoreactive ANP measurement was measured with a c-ANP ELISA kit (Phoenix Pharmaceuticals). The percentage of permeabilization was assessed with a colorimetric lactate dehydrogenase (LDH) assay kit (Sigma-Aldrich).

11. Flow cytometry analysis

HL-1 cells were cultured in 6-well plates under conditions described above. Non-permeabilized cells were washed with ice-cold PBS containing 3% fetal bovine serum, collected, centrifuged at 2000 *g* for 2 min, resuspended at a density of $4x10^6$ cells/ml and stained with anti-corin pooled (12G8/16E6/14H1, 10 μg/ml) or non-specific control pooled (IgM/IgG/IgM, 10 μg/ml) MAbs in culture medium for 30 min. After repeated washing and incubation for 15 min with PBS containing 1% fetal goat serum, cells were stained with Alexa Fluor 488-labeled goat anti-mouse IgG/IgM (0.5 μg/ml) for 35 min. Cells were washed with PBS and fixed with BD Cytofix buffer. Fluorescence was measured in a flow cytometer (Becton Dickinson FACSCalibur) with CellQuest software for acquisition and analysis**.**

Statistical analysis was performed by paired *t*-test. Statistically significant differences between groups were defined as $P<0.05$.

12. Diabetes induction and arterio-venous shunt preparation in dogs

Mongrel dogs from each sex were randomly assigned for the diabetic or the metabolically healthy group. Diabetes was induced by a single infusion of alloxan-monohydrate (560μmol/kg; Sigma-Aldrich). Dogs were investigated 8 weeks after the induction of diabetes.

Arterio-venous shunt was prepared as a model of acute hemodynamic load in each group. General anaesthesia was induced and maintained by sodium pentobarbital (133 μmol/kg Nembutal; Phylaxia, Sanophi, Budapest, Hungary). A section of 3-4cm shunt was placed in both distal femoral regions, between the femoral vein and artery. Control animals were sham operated. Dogs were sacrificed on postoperative day 1 or 3. Samples of peripheral

and coronary sinus blood and pericardial fluid were collected, and plasma was separated and frozen at -25^{o}C until further evaluation. Blood pressure was measured in the abdominal aorta. Hearts were harvested and weighed.

13. Statistics

Differences between multiple groups were assessed by analysis of variance with a 1) Bonferroni-Dunn correction for multiple statistical inferences (in tissue culture studies); 2) Mann-Whitney test (in animal models of cardiac load and diabetes). In corin studies, statistical analysis was performed by paired *t*-test. A $P < 0.05$ was considered to be statistically significant. Error bars represent standard deviations. Results represent at least three independent experiments.

14. Animals

All studies were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and all procedures were approved by the responsible institutional animal care and use committee.

V. Results

1. Natriuretic peptide secretion from cardiomyocytes is regulated by Munc18c

A, Syntaxin 4 and Munc18c colocalize and interact at the plasma membrane of cardiomyocytes

Heart tissue is a complex cellular environment that contains cardiomyocytes, fibroblasts, endothelial and other cells. We used double immunostaining of primary rat cardiomyocytes to determine whether Munc18c, syntaxin 2, syntaxin 4 and syntaxin 6 were expressed by cardiomyocytes vs. other cell types. Cardiomyocytes were identified by the perinuclear, granular staining pattern of ANP antibody (Fig. 1A-C), as well as by the specific z-disc staining pattern of desmin antibodies (Fig. 1D). In contrast, control immunofluorescence studies using secondary antibodies only, or non-immune serum or an irrelevant monoclonal anti-digoxin antibody (data not shown) did not reveal fluorescence staining. Immunostaining by a monoclonal antibody specific for Munc18c predominantly labeled the plasma membrane of ANP-positive cardiomyocytes (Fig. 1A). Immunoblot analysis revealed that Munc18c was expressed in both atrial and ventricular tissues at the predicted relative molecular mass of 67 kDa (Fig. 2A). Furthermore, Munc18c proteins were detected in both the soluble and the membrane-bound protein extracts (data not shown).

Immunofluorescence studies showed that syntaxin 4 (Fig. 1B) was localized to the plasma membrane in ANP-positive cells. It was also readily detected by immunoblotting in both atria and ventricular lysates as a single band with a relative mass of ~36 kDa (Fig. 2A). Co-immunoprecipitation studies readily showed Munc18 – syntaxin 4 interaction when probed by a pan-anti-Munc18 antibody (Fig. 2B). Further investigation by a Munc18c specific antibody verified Munc18c – syntaxin 4 binding (Fig. 2C).

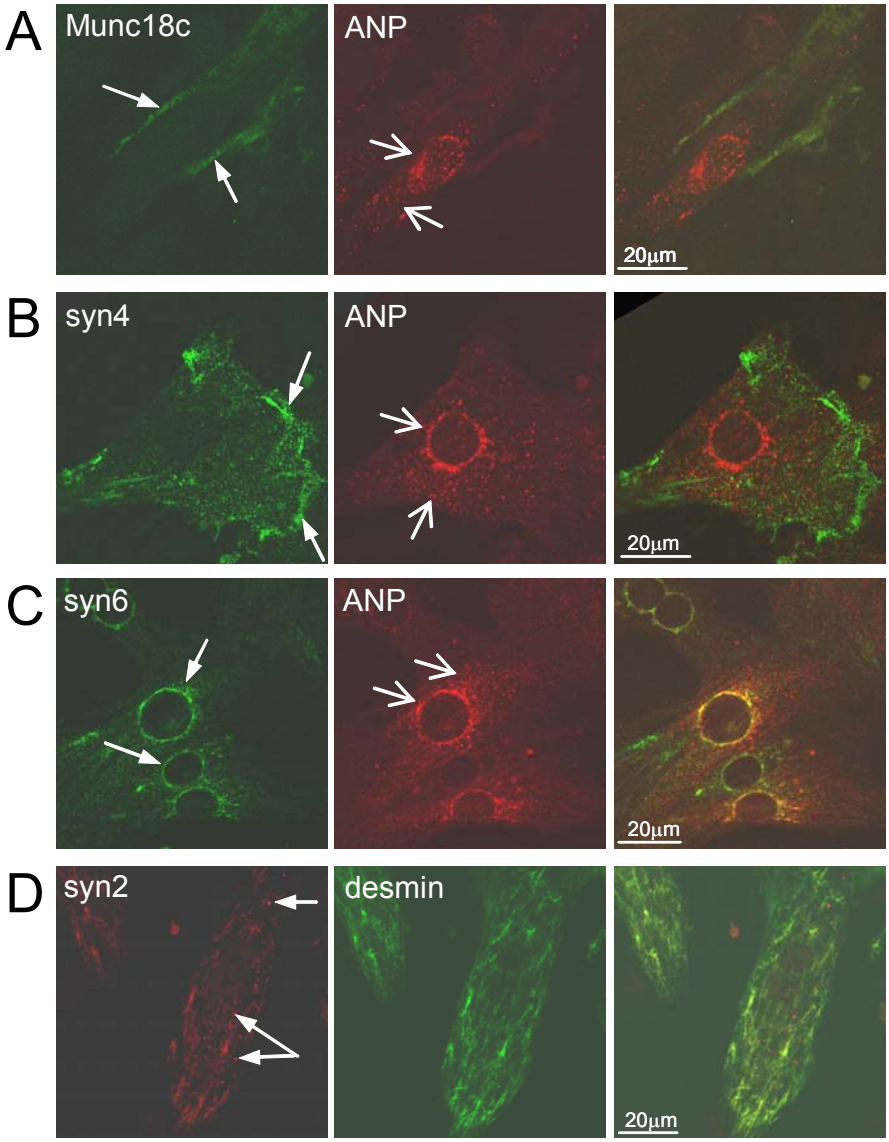

Fig. 1. Subcellular localization of Munc18c, syntaxin 4, syntaxin 6, and syntaxin 2 in cardiomyocytes by double immunofluorescence staining. *Left panel*: subcellular localization of Munc18c, syntaxin 4, syntaxin 6 and syntaxin 2 proteins in cells of cardiomyocyte culture. Arrows (↑) indicate specific staining pattern (see text). *Central panel*: simultaneous ANP or desmin staining. Arrows (↑) indicate specific pro-ANP containing granules. *Right panel*: merged images of the left and central panels display Munc18c, syntaxin 4 and syntaxin 6 and syntaxin 2 localization in cardiomyocytes identified by pro-ANP or desmin staining. **(A)**, Munc18c (green) and ANP (red); **(B)**, syntaxin 4 (green) and ANP (red); **(C)**, syntaxin 6 (green) and simultaneous ANP (red) staining, yellow indicates co-localization. **(D)**, syntaxin 2 (red) and desmin (green) staining.

Syntaxin 6 was detected in a perinuclear pattern that partially colocalized with ANP (Fig. 1C). However, ANP granules that were more peripherally distributed throughout the cell (towards the plasma membrane) did not exhibit syntaxin 6 labeling. In addition, ANP negative cells also displayed perinuclear syntaxin 6 staining. Immunoblotting studies demonstrated that syntaxin 6 was present in both atrial and ventricular tissues at an expected relative molecular mass of ~34 kDa (Fig. 2A). Co-immunoprecipitation probed by a pan-anti-Munc18 antibody demonstrated no Munc18 – syntaxin 6 interaction (Fig. 2B).

Syntaxin 2 (Fig. 1D) was detected in a diffuse granular pattern within the cytoplasm which differed from the cellular distribution observed with Munc18c, syntaxin 4 or syntaxin 6. Co-expression within cardiomyocytes was confirmed by double immunostaining with desmin (Fig. 1D). Syntaxin 2 was detected as a single band of relative mass ~34 kDa in both atrial and ventricular tissues, in contrast to the two immunoreactive bands with relative mass of ~36 and 34 kDa found in brain (Fig. 2A). Co-immunoprecipitation studies revealed only some Munc18 – syntaxin 2 interaction when immunoblotted by a pan-anti-Munc18 antibody (Fig. 2B). Furthermore, we could not detect syntaxin 2 – Munc18c interaction by a Munc18c specific antibody (data not shown).

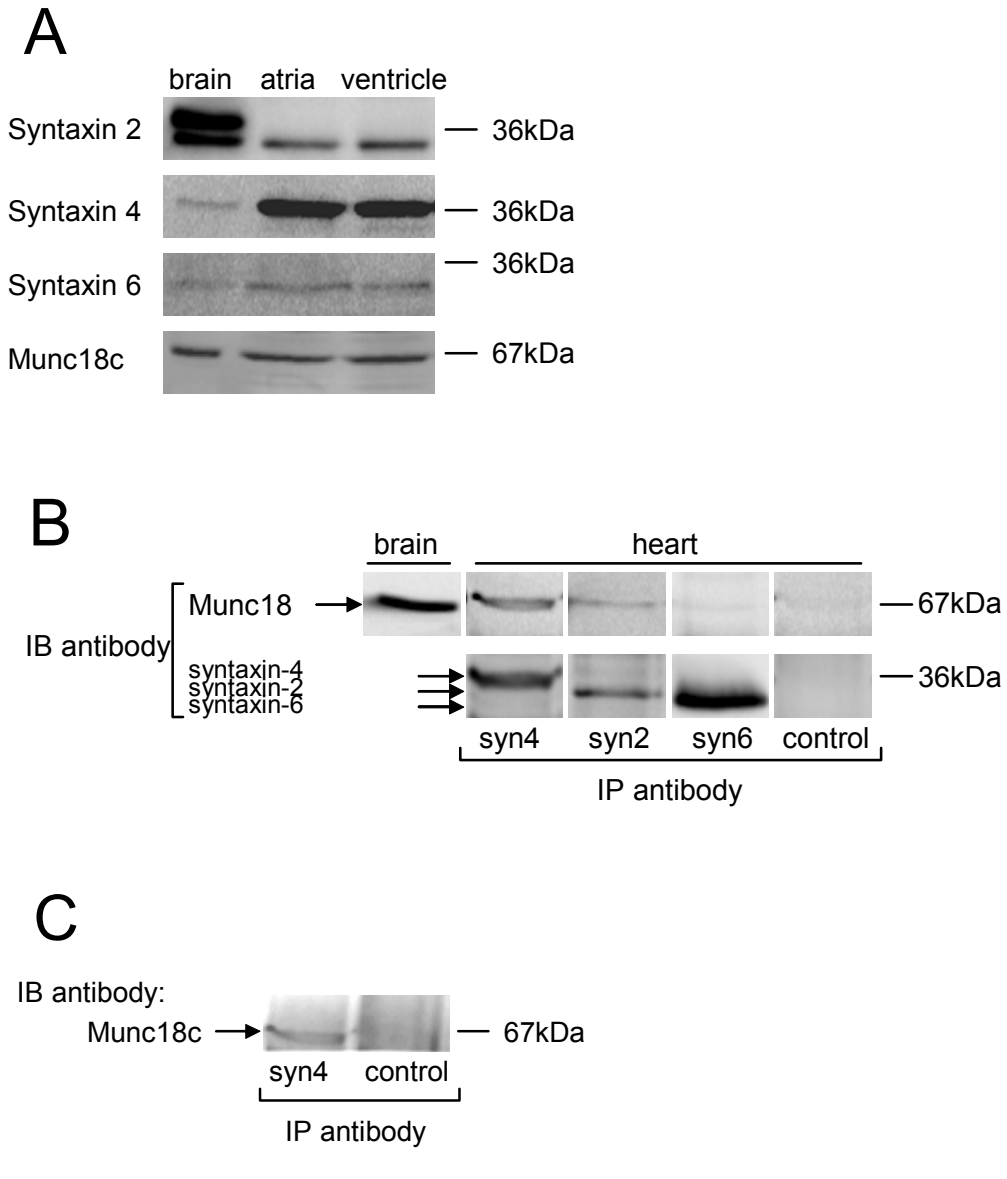

Fig. 2. Identification and interaction of Munc18c, syntaxin 4, syntaxin 2 and syntaxin 6 proteins in heart lysate. **(A)**, Detection of Munc18c, syntaxin 4, syntaxin 2 and syntaxin 6 proteins in heart lysate. Neonatal rat atria and ventricle lysates (70μg/lane) were subjected to 10% SDS-PAGE under reducing conditions followed by electroblotting. Blots were probed by specific antibodies as indicated. As a positive control, 10μg mouse brain protein extract was loaded. Note that anti-syntaxin 2 antibody detects double bands ~34 and 36kDa in brain lysate, whereas it identifies a single band ~34kDa in the heart lysates. A similar

immunoblot with adult tissue displayed similar results (not shown). **(B)**, Munc18 – syntaxin interaction in heart lysate. 2mg heart protein lysate was immunoprecipitated with either syntaxin 2, syntaxin 4, or syntaxin 6 antibody, as indicated. As a negative control, the same amount of heart proteins were immunoprecipitated with rabbit IgG, or anti-digoxin antibody (the latter not shown). After immunoprecipitation, samples were subjected to 10% SDS-PAGE under reducing conditions, transferred to PVDF membranes and analysed by immunoblotting with a pan anti-Munc18 antibody (upper panel). Lower panel shows immunoblots of the same membranes probed with an anti-syntaxin 2, anti-syntaxin 4 or anti-syntaxin 6 antibody according to the antibody used for immunoprecipitation. Experiment with adult heart demonstrated similar results (not shown). **(C)**, Munc18c – syntaxin 4 co-immunoprecipitation. Immunoprecipitation was performed with an anti-syntaxin-4 antibody or rabbit IgG (control) as described under panel B. Immunoblotting by a Munc18c specific antibody demonstrated *Munc18c* – syntaxin 4 interaction.

B, Ca^{2+}–induced ANP secretion in permeabilized, primary cardiomyocytes

In order to determine the functional role of Munc18c in cardiomyocytes we developed a system for examining triggered ANP secretion in permeabilized, neonatal cardiomyocytes. In these cells we found that constitutive or 'non-triggered' ANP secretion was different in atrial and ventricular cells. Atrial cardiomyocytes consistently secreted ANP about 1.5 ng/hr/10^5cells, while ventricular cardiomyocytes secreted a maximum of ~1.9 ng/hr/10^5cells at day 3 which decreased to a steady level of ~0.6 ng/hr/10^5cells on day 5 (Fig. 3A). After one week, baseline secretion stabilized in non-stimulated cells; therefore, secretion was typically studied in primary cardiomyocytes cultured for 7-9 days.

In order to introduce inhibitors of Munc18c into cardiomyocytes we used streptolysin-O (SLO) to selectively permeabilize the plasma membrane without disturbing the integrity or function of the secretory granules. In a dose-related fashion, SLO enhanced the permeabilization of the plasma membrane of cardiomyocytes, as measured by the leakage of cytosolic lactate-dehydrogenase into the media (Fig. 3B). At SLO concentrations of 500 units/ml there was a plateau in permeabilization of the plasma membrane as measured by LDH levels. In contrast, increasing SLO concentrations did not enhance the release of ANP from secretory granules, indicating that permeabilization of the membranes of the secretory granules did not occur (Fig. 3C). With increasing permeabilization of the plasma membrane, ANP secretion was reliably triggered by increasing the intracellular Ca^{2+}-concentration in the presence of ATP and GTP (Fig. 3C).

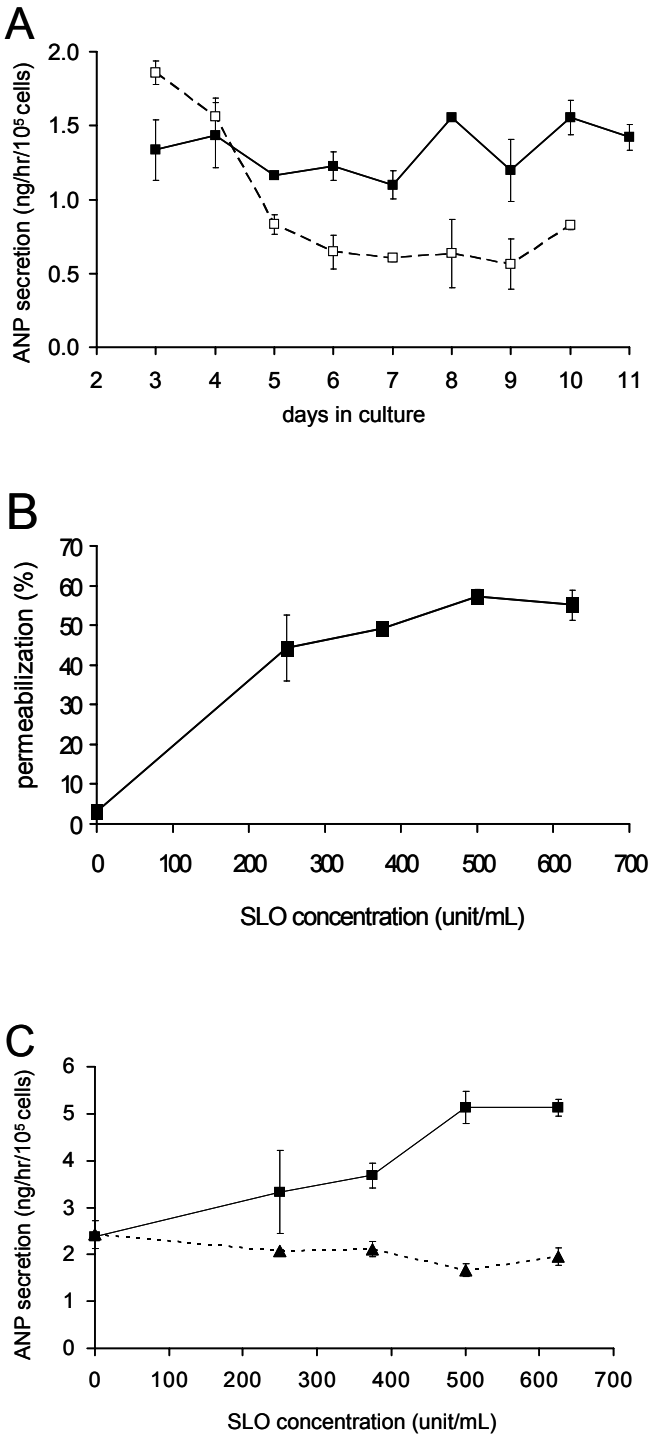

Fig. 3. Constitutive and triggered ANP secretion of intact and permeabilized cardiomyocytes. **(A)**, Constitutive ANP secretion in cultured atrial (close symbols) and ventricular (open symbols) cardiomyocytes. Equal amount (~10^5 cells) of atrial and

ventricular cardiomyocytes were plated on fibronectin/gelatin coated culture dishes (n=6). Cells were cultured in complete serum-free media and supernatant was collected in every 24 hours to determine ANP/pro-ANP content by enzyme-linked immunoassay against c-ANP. **(B)**, Permeabilization as a function of SLO-concentration. Atrial cardiomyocytes were incubated in intracellular (KG) media containing 100 nM free Ca^{2+} and increasing amount of streptolysin-O (SLO). After incubation, supernatant was collected and cells were lysed in 1% Triton-X100. The lactate dehydrogenase (LDH) content of the supernatant and cell lysate was measured and the percent of permeabilization was calculated as the ratio of total LDH released. **(C)**, Ca^{2+} -triggered and baseline ANP secretion in permeabilized cardiomyocytes. Atrial cardiomyocytes were permeabilized with increasing amount of SLO as above. The amount of ANP secreted in the presence (rectangular symbol) and absence (triangular symbol) of elevated Ca^{2+} (100 μM) is shown. Ventricular cardiomyocytes behaved similarly, except that the baseline ANP-secretion level was about half or one third of that of atrial cells (data not shown).

C, Munc18c inhibitors selectively enhance ANP secretion

This permeabilized cardiomyocyte system allowed us to examine the functional role of Munc18c on ANP secretion. When compared to baseline secretion in permeabilized atrial cardiomyocytes, increased Ca^{2+} triggered release of ANP (Fig. 4A, $p < 0.01$). Addition of a control monoclonal antibody had no effect on Ca^{2+}-triggered release of ANP. However, the introduction of the anti-Munc18c antibody significantly increased Ca^{2+}-triggered release of ANP from atrial cardiomyocytes ($p < 0.05$). A similar pattern was seen in ventricular cardiomyocytes. Ca^{2+}-triggered the secretion of ANP (Fig. 4B, $p< 0.05$). The anti-Munc18c antibody significantly enhanced Ca^{2+}-triggered secretion (Fig. 4B, $p <0.01$) but a control monoclonal antibody had no effect.

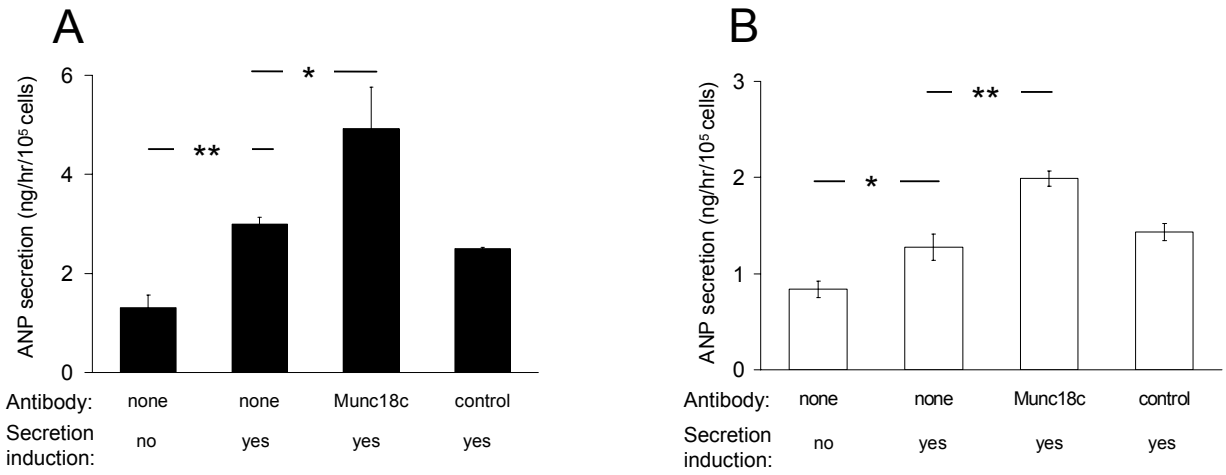

Fig. 4. Inhibition of Munc18c increases ANP secretion. Permeabilized cardiomyocytes were incubated in the absence ("none"), or in the presence of either a specific monoclonal anti-Munc18c ("Munc18c") or an unspecific monoclonal anti-digoxin ("control") antibody. Ca^{2+} (100 μM) was added to trigger secretion. The presence of the anti-Munc18c antibody significantly increased ANP secretion beyond that triggered by increased Ca^{2+}. This effect was specific, because the control monoclonal antibody of the same isotype did not alter ANP secretion. **(A)**, Culture of atrial cardiomyocytes. **(B)**, Ventricular cardiomyocytes. *p<0.05, **p<0.01

2. Pro-natriuretic peptide-convertase corin is localized on the cardiomyocyte surface

A, Anti–corin polyclonal and monoclonal antibody production and characterization

In order to investigate native corin on the protein level, anti-corin rabbit polyclonal antibodies (R34 anti-stem and R33 anti-protease domains) and mouse MAbs (against protease domain, 12G8, 14H1, 16E6, 19C10) were generated. Recombinant corin fragments corresponding to stem domain (residues 134-868) and protease domain fragments (residues 854-1113 and 869-1113) were cloned, expressed as GST-tagged and His_6-tagged fusion proteins and used as immunizing antigens (Fig. 5A). There is minimal cross-reactivity between the two polyclonal anti-corin antibodies, anti-stem domain (R34) and anti-protease domain (R33) (Fig. 5B). Anti-corin protease domain antibody specificity was tested against recombinant GST-tagged protease domain corin protein (residues 869-1113) using non-specific GST-tagged protein as a negative control (Fig. 5C). An abundant expression of corin mRNA previously was detected almost exclusively in heart [28, 30, 34], therefore polyclonal antibody specificity was confirmed by the ability to recognize corin in mouse heart extracts but not in extracts from other tissues (Fig. 5D). Western blotting of mouse heart extract under reducing conditions detected an immuno-reactive band with a relative mass of 205-210 kDa with anti-corin stem domain antibody (R34) and several low molecular weight fragments with anti-corin protease domain (R33) (Fig. 5D).

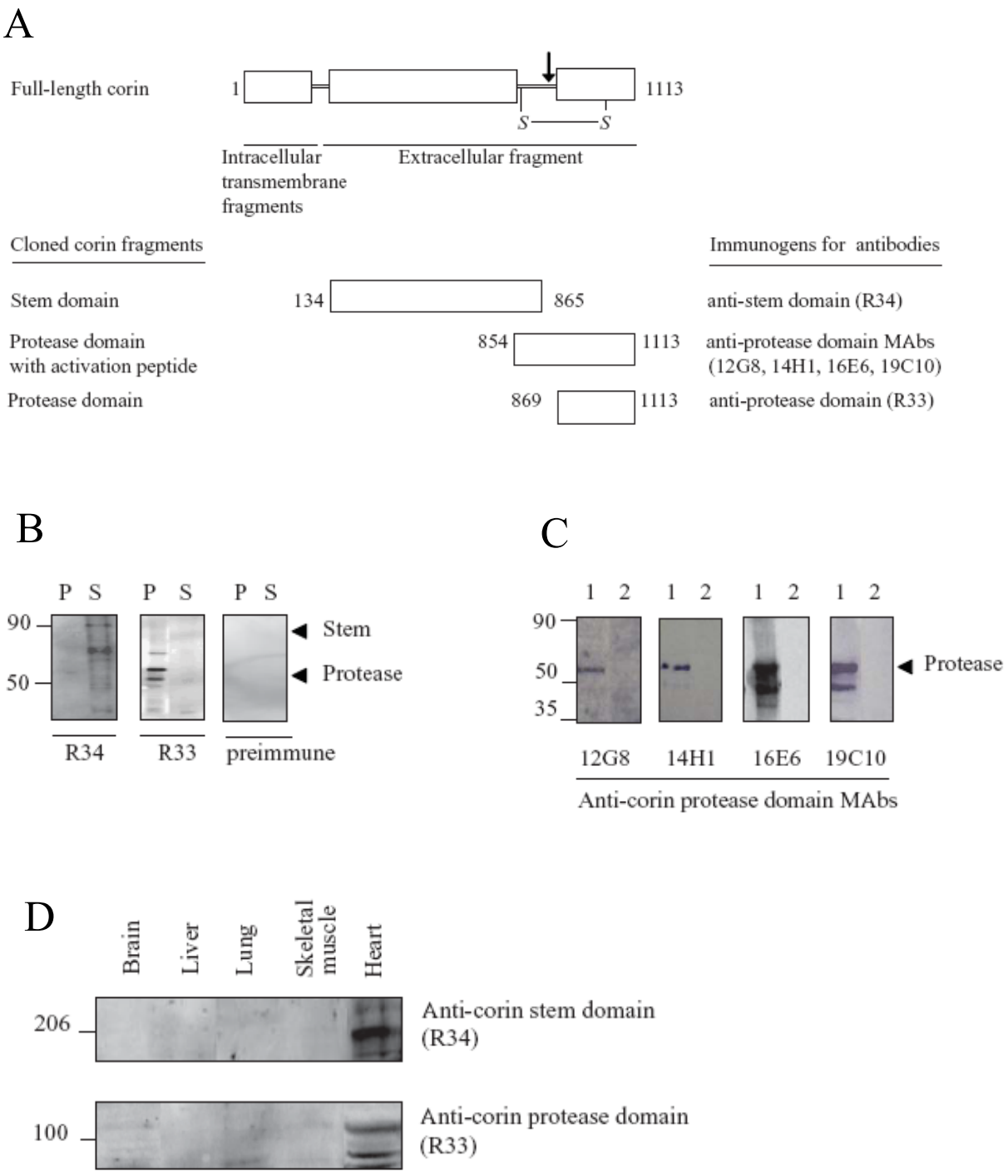

Fig. 5. (A) Schematic diagram of the full-length corin protein and corin structural fragments that were used for antibody production (as indicated). Residues corresponding to each fragment are shown. Full-length corin contains the intracellular and transmembrane

domain (1-133), stem domain (134-865) and protease domain (869-1113). The arrow indicates the activation (cleavage) site. The disulfide bond (*S*-*S*) connecting stem and protease domains after zymogen activation is shown. **(B)** Specificity of polyclonal anti-corin antibodies for recombinant corin fragments. Immunoblots loaded with equal amount of recombinant corin protease domain (P) and stem domain (S) were probed with anti-corin stem domain serum (R34), anti-corin protease domain serum (R33) or preimmune serum. **(C)** Specificity of anti-corin MAbs against immunizing agent. Western blots (reducing conditions) against recombinant corin protease domain (869-1113) (1) or non-specific recombinant protein (negative control) (2) were probed with anti-corin MAbs as indicated. Arrows indicate recombinant corin domains. **(D)** Western blot analysis of corin protein expression in murine tissue extracts (Trizol reagent) under reducing conditions. The blots were probed with polyclonal anti-corin antibodies as indicated. **(B-D)** Protein standards in kDa as indicated.

B, Expression and localization of native corin protein

Since previous report detects corin mRNA exclusively in cardiomyocytes [28], we analyzed the subcellular localization of native corin protein in these cells. Cultured non-permeabilized neonatal atrial cardiomyocytes were analyzed by immunofluorescence staining with anti-corin antibodies (Fig. 6A). Polyclonal anti-corin stem domain antibody (R34) and anti-corin protease domain (R33) antibody and MAbs (19C10 and 14H1) localized corin to the plasma membrane surface of cardiomyocytes. In contrast, non-specific control polyclonal IgG and monoclonal IgM did not recognize corin.

To examine whether native corin is expressed in cardiomyocytes that also express pro-ANP, permeabilized neonatal atrial cardiomyocytes were double stained with mouse anti-corin protease domain MAb (19C10) and rabbit polyclonal anti-pro-ANP antibodies and analyzed by confocal microscopy. Fig. 6B shows a typically perinuclear, granular staining pattern of pro-ANP (red) and membrane staining of corin (green).

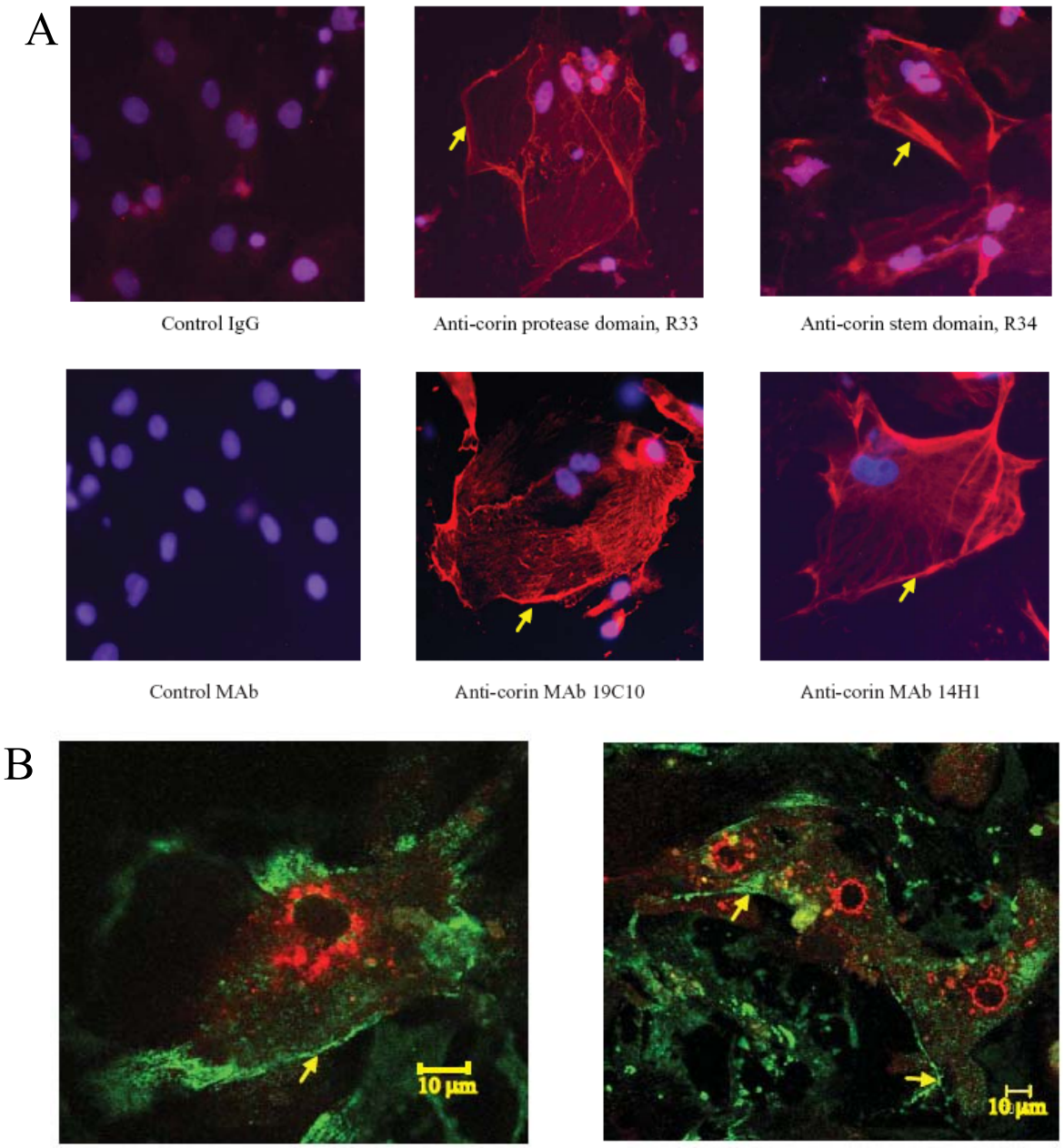

Fig. 6. Localization of native corin in cardiomyocytes and co-expression in cardiomyocytes expressing pro-ANP. Immunofluorescence staining of non-permeabilized **(A)** or permeabilized **(B)** neonatal rat atrial cardiomyocytes. **(A)** Cells were stained with anti-corin antibodies as indicated and detected with Alexa Fluor 594-labeled secondary antibodies. Rabbit pre-immune serum or mouse monoclonal IgM was used as a negative

control. Cells were visualized with a 20x lens. Images of membrane (red) and nuclei (blue) staining were layered, and merged images are shown. Arrows indicate membrane staining of corin. **(B)** Cells were double stained with anti-corin MAb 19C10 and rabbit polyclonal anti-pro-ANP antibody and detected with Alexa Fluor 488-labeled and Alexa Fluor 594-labeled secondary antibodies. Co-expression of corin (green) and pro-ANP (red) was analyzed with confocal microscopy, and merged images are shown.

C, Molecular size and structure of native corin in mouse heart tissue

The molecular size and structure of native corin was examined in mouse heart extracts in the presence of serine, cysteine and metalloprotease inhibitors to prevent proteolysis. To confirm that native corin is a 205-210 kDa membrane-bound protein, immunoprecipitation of the membrane fraction of mouse heart extract was performed. Anti-corin protease domain MAbs (12G8, 14H1 or 16E6) were able to precipitate corin, which was detected as a 205-210 kDA protein by Western blotting using anti-corin stem domain antibody (R34) for detection (Fig. 7A). Control antibodies (mouse IgG and IgM) and anti-corin MAb (19C10) were unable to immunoprecipitate corin. The 205-210 kDa immunoprecipitated band is consistent with the molecular size of corin specifically detected in mouse heart tissue with anti-corin stem domain antibody (R34) (Fig. 5B). Since a 205-210 kDa band was recognized under reducing conditions with both anti-corin stem domain antibody (R34) and anti-corin protease domain MAbs, it likely corresponds to full-length corin protein. The discrepancy seen between the predicted size, 123 kDa [30] and actual molecular mass of corin suggests that the mature corin undergoes post-translational modifications such as glycosylation (analyzed further).

The cDNA sequence predicts that corin is synthesized as a zymogen, pro-corin, and requires proteolytic cleavage to become an active enzyme. Furthermore, corin contains several putative disulfide bonds, upon activation the active corin would remain tethered to the cell-surface by a putative disulfide bond (located at Cys790 – Cys912 in human [28] or at Cys857 –Cys979 in mouse [30] sequence) that anchors the active protease domain to the

stem domain (Fig. 5A) [28, 30]. If corin is cleaved, lower molecular weight fragments should be observed upon treatment with a reducing agent that cleaves the disulfide bonds. To determine whether native corin protein is expressed on the plasma membrane surface as a zymogen and/or an active enzyme, Western blotting of the membrane fraction of mouse heart extracts was performed under reducing conditions. The anti-corin stem domain antibody (R34) detects corin fragments with relative masses of 180 kDA and 110 kDa (Fig. 7B). These fragments were not recognized by anti-corin protease domain antibodies and therefore might correspond to structures containing primarily stem domain. The anti-corin protease domain antibody (R33) detects corin fragments with relative masses of 45-50 kDa and 90 kDa (Fig. 7B). These fragments were not recognized by anti-corin stem domain antibody (R34) and might correspond to structures containing primarily protease domain. Immunoprecipitation of full-length corin (205-210 kDa) under reducing conditions (Fig. 7A) confirms that at least a portion of corin in cardiac tissue is the zymogen form. Detection of full-length corin and corin fragments in the membrane fraction of the mouse heart extract suggests that corin is present on the cardiomyocyte surface in both its zymogen (assessed by its inability to be cleaved by mercaptoethanol) and potentially active (cleaved by mercaptoethanol treatment) forms.

To further explore our finding that corin is present on the plasma membrane surface as a catalytically active enzyme, we used an irreversible protease inhibitor, FITC-FPR-CMK [172], which mimics the corin cleavage sequence in pro-ANP. When FITC-FPR-CMK is injected into the mouse heart, it forms a covalent complex with any active proteases that recognize its sequence. After FITC-FPR-CMK injection, mouse hearts were extracted and the membrane fraction was subjected to SDS-PAGE followed by electro-blotting. The fluorescent FITC-tag on the inhibitor allows for detection of protease-containing covalent complex. Two bands with molecular size around 50 and 90 kDa were detected after fluorescence scanning (Fig. 7C, right lane). The same membrane was then probed with anti-corin protease domain antibody (R33) confirming that the fluorescent bands corresponded to corin (Fig. 7C, left lane). The detected pattern (Fig. 7C) is consistent with patterns detected in mouse heart extract without FITC-FPR-CMK treatment (Fig. 7B).

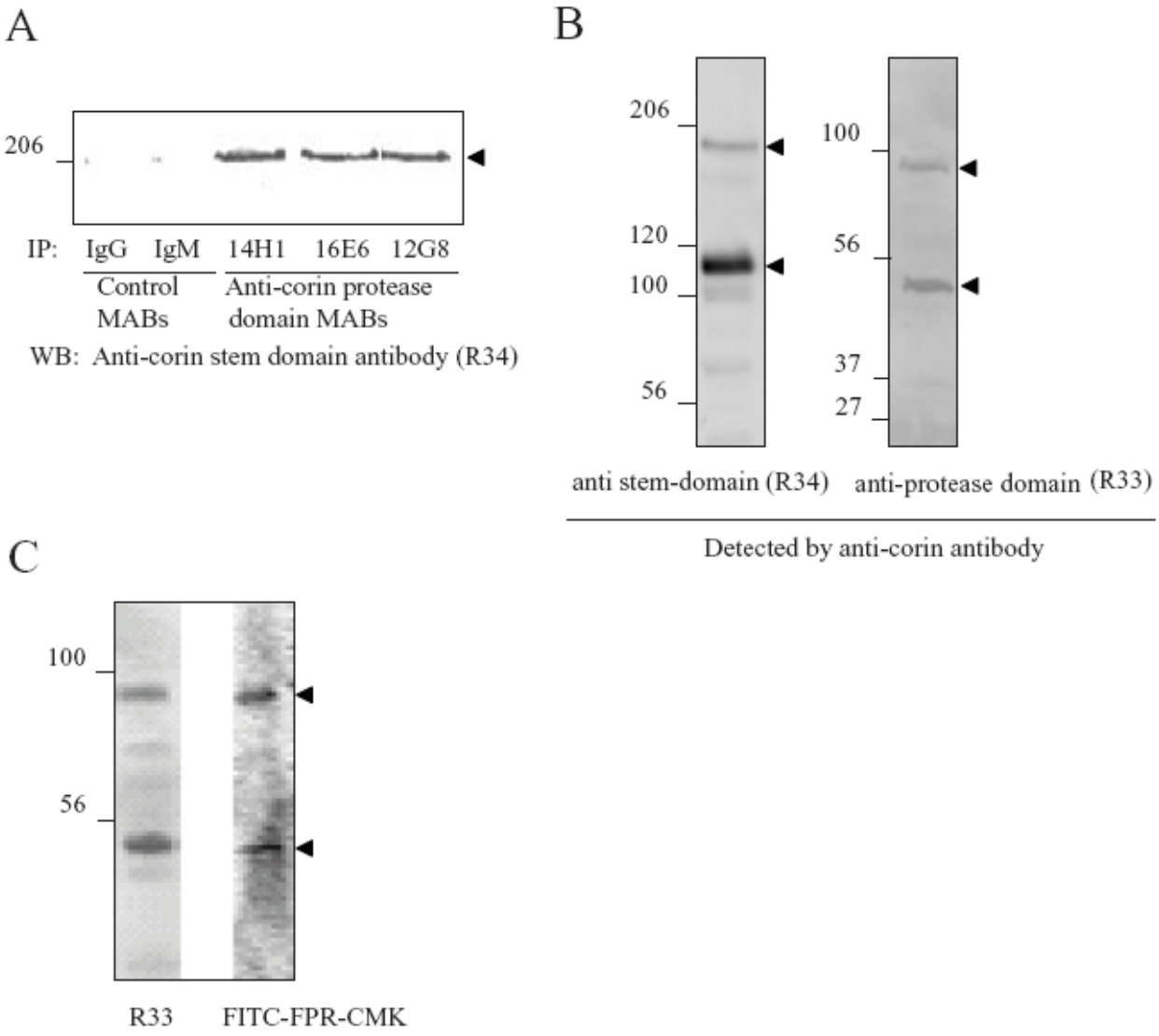

Fig. 7. Western blot analysis of mouse heart tissue extracts. **(A)** Immunoprecipitation of corin from the membrane fraction of mouse heart extract with anti-corin MAbs followed by Western blotting under reducing conditions with polyclonal anti-corin stem domain antibody (R34). Mouse monoclonal IgG and IgM were used as a negative control. **(B)** Western blotting of the membrane fraction of mouse heart extract under reducing conditions with anti-corin stem domain antibody (R34) and anti-corin protease domain

antibody (R33). **(C)** Mouse hearts were injected with 1 μM FITC-FPR-CMK and subjected to protein extraction. The membrane fraction was subjected to SDS-PAGE under reducing conditions followed by electro blotting. The membrane was scanned for fluorescence signal (right) and then probed with anti-corin protease domain antibody, R33 (left). Correlation between FITC-FPR-CMK and R33 signal is shown. Arrows indicate corin and corin fragments. The protein standards in kDa are as indicated.

D, Corin expression in cardiomyocyte-like HL-1 cells

The murine HL-1 cell line was derived from the AT-1 atrial cardiomyocyte tumor lineage and retains the phenotypic characteristics of the adult cardiomyocyte [169]. Therefore, HL-1 cells can be used as a convenient and reproducible culture system for the investigation of corin. The mRNA from a closely related cell line, HL-5, contains a corin transcript, and HL-5 cells are able to convert transfected pro-ANP to mature ANP [32]. To determine whether HL-1 cells express native corin protein, non-permeabilized HL-1 cardiomyocytes were visualized by immunofluorescence staining with anti-corin MAb. Anti-corin MAbs, but not control MAbs, localized corin to the plasma membrane surface of HL-1 cardiomyocytes (Fig. 8A). Western blot analysis of crude HL-1 cell extract under non-reducing conditions detects two bands with relative masses of 205-210 kDa and 110-115 kDa (Fig. 8B). The 205-210 kDa immuno-reactive band is consistent with molecular size of corin protein detected in mouse heart tissue (Fig. 7A).

The localization of corin on the external plasma membrane of HL-1 cells was confirmed by flow cytometry (Fig. 8C). Non-permeabilized cells have a significantly higher mean fluorescence signal when stained with anti-corin MAbs than negative control MAbs, demonstrating the presence of corin on the external plasma membrane. This is consistent with immunofluorescence staining results for non-permeabilized HL-1 cells (Fig. 8A). To further confirm the membrane-bound extracellular localization of corin, HL-1 cells were surface-labeled with biotin. The labeled cell lysate was separated on Immobilized Streptavidin followed by Western blotting with anti-corin MAbs. Results showed that 205-

210 kDa form of corin exhibits affinity toward Streptavidin–Agarose indicating its localization on the external plasma membrane of HL-1 cells.

To explain the discrepancy between the predicted and actual molecular mass of corin we evaluated the glycosylation status of corin. The HL-1 cell extract was separated on Concanavalin A-Sepharose, which binds only glycosylated proteins. Western blotting showed that the 205-210 kDa form of corin exhibits strong affinity toward Concanavalin A–Sepharose indicating its glycosylation. The 110-115 kDa form of corin had reduced glycosylation assessed by its inability to bind Concanavalin A-Sepharose (Fig. 8D). The glycosylation status of the 205-210 kDa form of corin was further confirmed by separation of the rat neonatal cardiomyocyte extract on Concanavalin A–Sepharose (Fig. 8D).

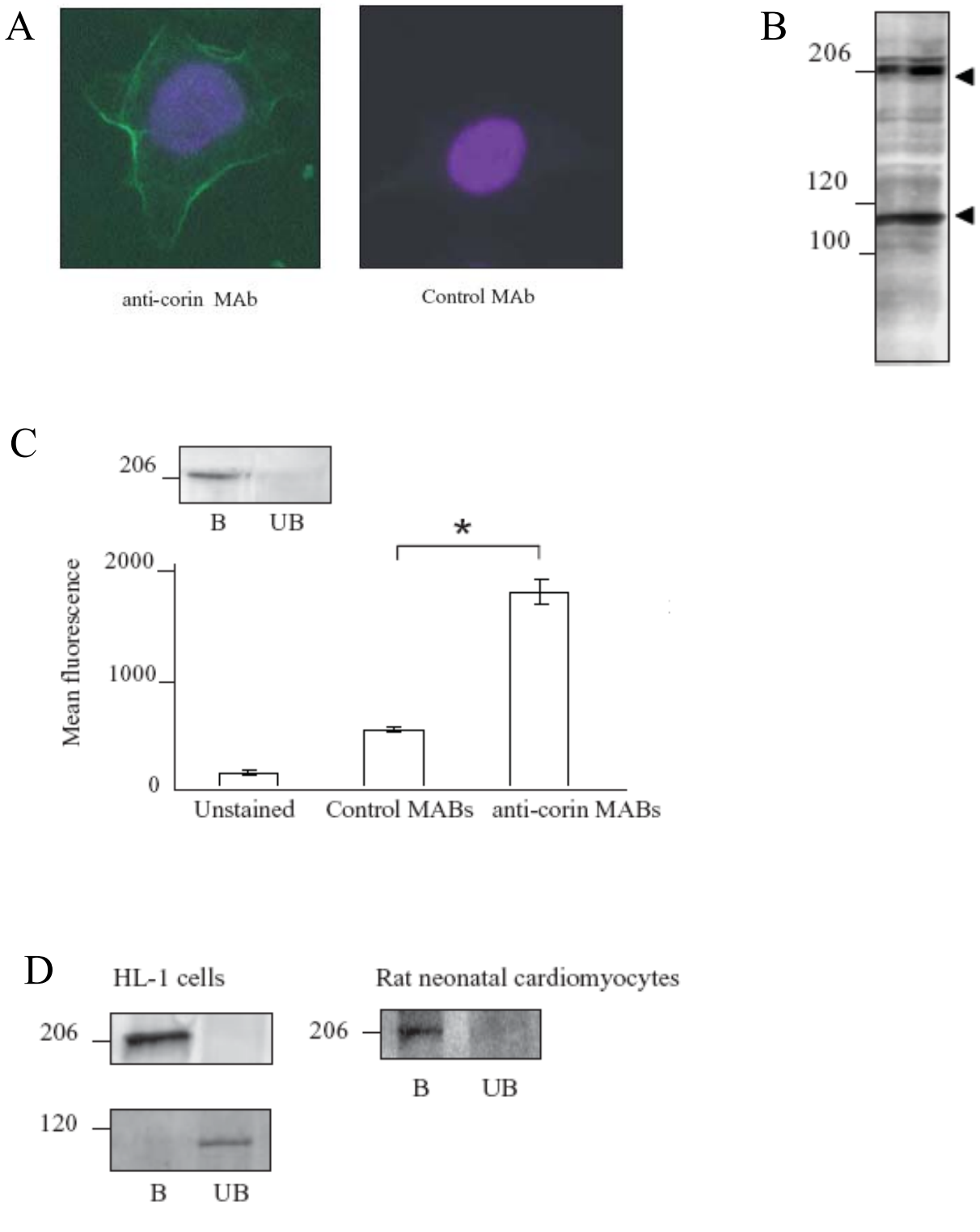

Fig. 8. Expression and localization of corin in HL-1 cells. **(A)** Non-permeabilized cells were stained with anti-corin MAb and detected with Alexa Fluor 488 secondary antibody.

Mouse monoclonal IgG was used as a negative control. Cells were visualized with a 20x lens. Merged images of membrane (green) and nuclei (blue) staining are shown. **(B)** Western blot of HL-1 cell extract under non-reducing conditions probed with pooled anti-corin MAbs (14H1/16E6/12G8). Arrows indicate corin. **(C)** Flow cytometry analysis of corin on the surface of HL-1 cells. Cells were labeled with anti-corin MAbs and detected with Alexa Fluor 488-labeled secondary antibodies. Mouse monoclonal (IgG + IgM) and unstained cells were used for controls. Data are expressed as mean fluorescence intensity. Each value represents the average of three experiments with standard deviations indicated by error bars. The difference in mean fluorescence between control and anti-corin MAbs was statistically significant (paired *t*-test, $*p < 0{,}001$). **Inset**, The lysate of biotin surface-labeled HL-1 cells was separated on Immobilized Streptavidin and bound and unbound fractions were subjected to Western blotting under conditions described in **(B)**. **(D)** HL-1 cells or rat neonatal cardiomyocytes were lysed and subjected to affinity chromatography on Concanavalin A-Sepharose followed by Western blotting under conditions above. *UB*, corin unbound, *B*, bound to affinity columns.

3. Acute hemodynamic load increases natriuretic peptide level both in healthy and diabetic dogs

A, Changes in cardiovascular parameters due to acute hemodynamic load in diabetic and metabolically healthy dogs

Mean arterial blood pressure, heart weight as well as left and right ventricle to body weight ratios were measured in diabetic and healthy dogs, with and without acute hemodynamic load in each group (Table 1). As a model for acute hemodynamic strain, arterio-venous shunts were used, and cardiovascular parameters were evaluated on the first and third days of operation. Diabetic dogs had significantly lower blood pressure as compared with healthy controls (79±2 mmHg vs. 89±6 mmHg, respectively; $p<0.05$). Acute hemodynamic strain on postoperative day 1 further decreased the blood pressure in these dogs (72±3 mmHg vs. 79±2 mmHg; $p<0.05$). However, in metabolically healthy dogs shunting did not have any effect on blood pressure. Heart weight/body weight ratio, as well as left and right ventricle to body weight ratios separately, were increased upon shunting; however, there was no difference between the metabolically healthy and the diabetic group (Table 1).

Table 1. Mean arterial blood pressure, heart weight/body weight, left and right ventricular weight/body weight ratios, and body weight data. *P<0.05 vs. the respective control group.

	Mean arterial blood pressure (mmHg)	Heart weight/ Body weight (g/kg)	Left ventricular/ Body weight (g/kg)	Right ventricular/ Body weight (g/kg)	Body weight (kg)
Healthy Sham	89±6	6.0±0.2	3.89±0.32	1.21±0.1	22.6±1.7
Diabetic Sham	79±2*	6.1±0.5	3.92±0.64	1.27±0.3	24.2±2.1
Healthy 1 day shunt (H1)	90±5	6.3±0.3	4.05±0.25	1.27±0.1	25.7±1.6
Healthy 3 days shunt (H3)	89±10	7.4±0.2 *	4.8±0.2 *	1.53±0.1*	25.8±3.4
Diabetes 1 day shunt (D1)	72±3 *	7.7±0.5	5.03±0.42*	1.47±0.1	21±0.9
Diabetes 3 days shunt (D3)	82±11	7.6±0.5	4.89±0.35	1.52±0.2*	25.2±2.4

B, Endothelin levels in healthy and diabetic dogs with normal circulation and after acute hemodynamic load

Diabetes mellitus has a series of cardiovascular complications, including endothelial dysfunction [173]. We measured endothelin-1 (ET-1) level in the plasma, coronary sinus and pericardial fluid of metabolically healthy and diabetic dogs without and with hemodynamic load. ET-1 level in the peripheral plasma was significantly higher in diabetic dogs than in the healthy controls (Fig. 9A). Shunting did not change endothelin-1 level in the healthy dogs, but significantly decreased the level in the diabetic group. Similar changes were observed in the coronary sinus (Fig. 9B). However, in the pericardial fluid, ET-1 level was higher than that of the plasma, and neither shunting nor diabetes changed it (Fig. 9C).

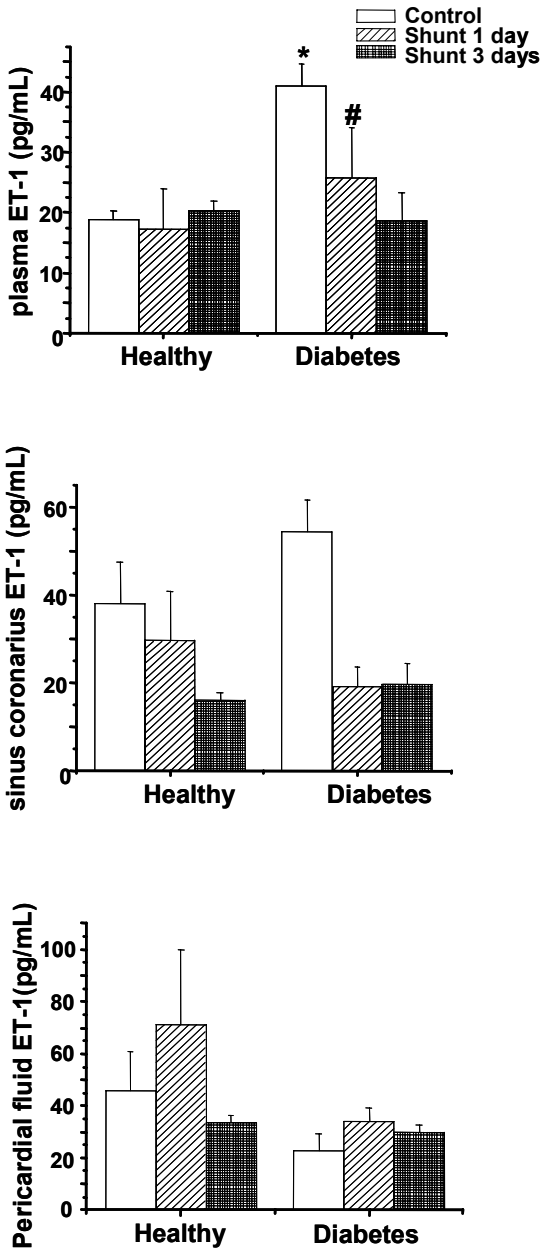

Fig. 9. Endothelin-1 (ET-1) levels in healthy and diabetic dogs with normal circulation and 1 and 3 days after shunting. * $p<0.05$, diabetes control vs. healthy; # $p<0.05$ diabetes on the 3. postoperative day vs. metabolically healthy control on the 3. postoperative day. **(A)** ET-1 Level in the peripheral plasma. **(B)** ET-1 level in the coronary sinus plasma. **(C)** Levels of ET-1 in the pericardial fluid.

C, Acute hemodynamic load increases NT-proANP levels in different compartments of diabetic and healthy dogs

Plasma NT-proANP level is a sensitive marker for hemodynamic strain [44, 81]. However, natriuretic peptide secretion is also regulated by several hormones, among those ET-1 had been shown to have a great influence [63]. To further investigate the combined effect of altered ET-1 levels and hemodynamic load on natriuretic peptide secretion, we measured NT-proANP levels in the plasma, coronary sinus and pericardial fluid of metabolically healthy and diabetic dogs without and with hemodynamic load (Fig. 10). Levels of NT-proANP showed similar patterns in all three compartments, being comparable under basal conditions but significantly increased upon shunting (Fig. 10A-C). However, elevation on NT-proANP levels due to shunting was higher in the diabetic group than in the metabolically healthy controls.

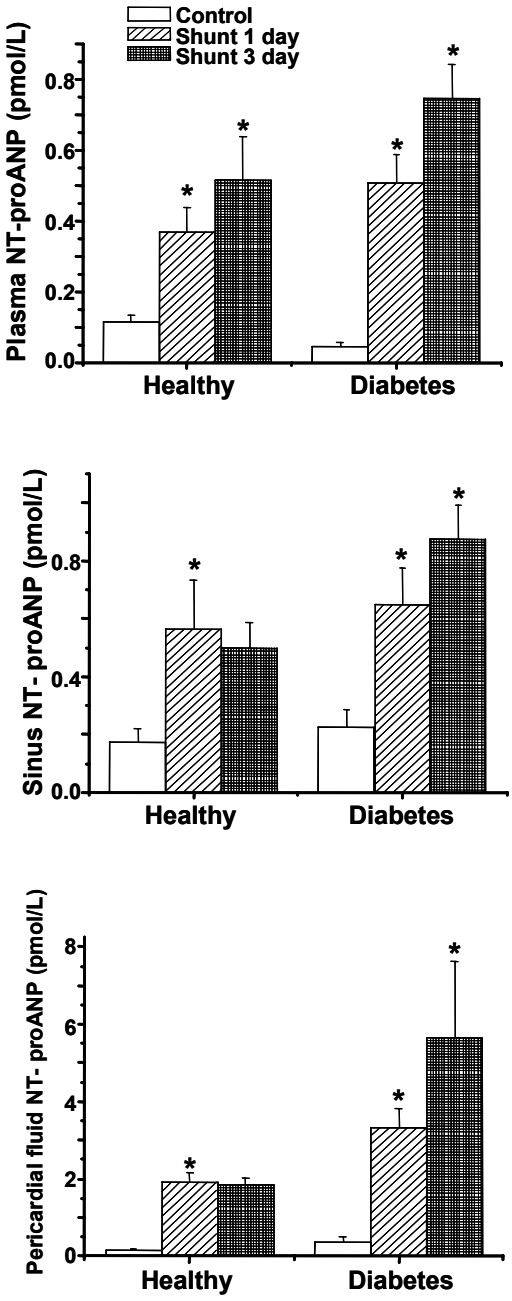

Fig. 10. N-terminal pro-atrial natriuretic peptide (NT-proANP) levels in different compartments of healthy and diabetic dogs with normal circulation and 1 and 3 days after shunting. * $p<0.05$ vs. the respective control group. **(A)** NT-proANP levels in the peripheral plasma. **(B)** Coronary sinus NT-proANP levels. **(C)** NT-proANP levels in the pericardial fluid.

4. Bradycardia can induce increased serum natriuretic peptide level

Case report

A 94 year-old woman was admitted to the hospital after 4 days of worsening weakness and dyspnea. She had no symptoms of angina. Her medical history included recurrent basal cell carcinoma, vertebrobasilar insufficiency, and hypertension. Beta-blocker (5 mg bisoprolol per day) and thiazide diuretics were administered as an antihypertensive therapy. At admission, her blood pressure was 120/80 mmHg, pulse rate was 40 bpm. There was no sign of overt heart failure. The 12-lead ECG showed sinus arrest with narrow-QRS complex junctional escape rhythm. (Fig.11.) Routine laboratory tests (including TSH and troponin-T) displayed normal values. However, NT-proBNP serum level was highly increased (22420pg/ml; normal reference value is below 270pg/ml). Echocardiographic findings displayed normal systolic ventricular function and mild degenerative mitral and aortic insufficiency.

Beta-blocker therapy was discontinued for assumed therapy-induced bradyarrhythmia. Due to the stabile hemodinamic parameters temporary pacemaker was not implanted.

The patient's bradyarrhythmia resolved in 48 hours and sinus rhythm with 90/min frequency returned. (Fig.12.) Control NT-proBNP serum level was markedly lower (2117pg/ml). Free from her actual symptoms the patient was discharged from the hospital.

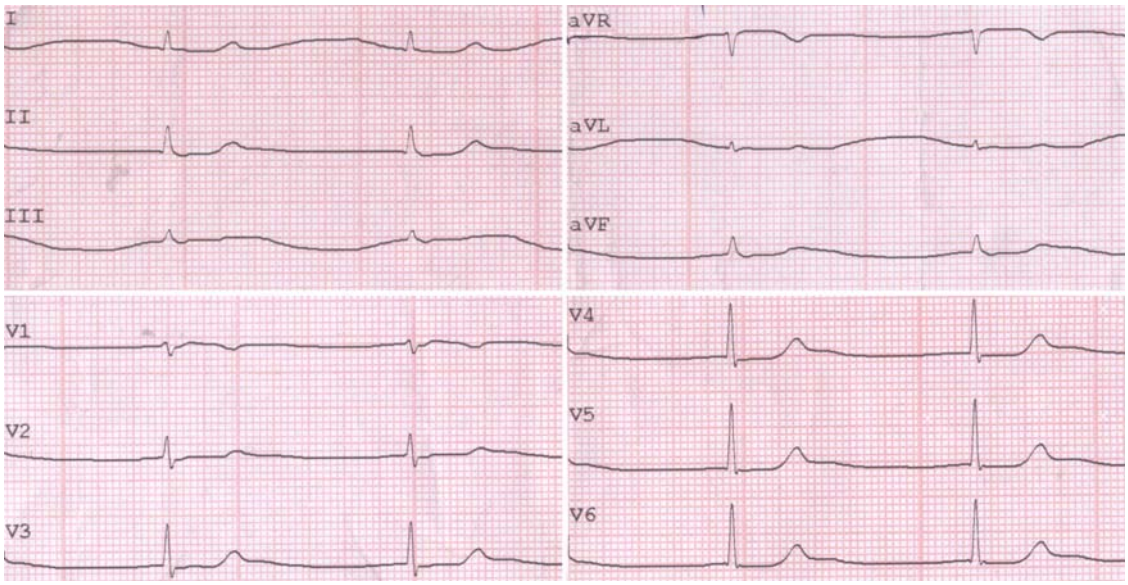

Fig.11. Electrocardiogram at admittance shows sinus arrest with junctional escape rhythm (HR: 44 bpm).

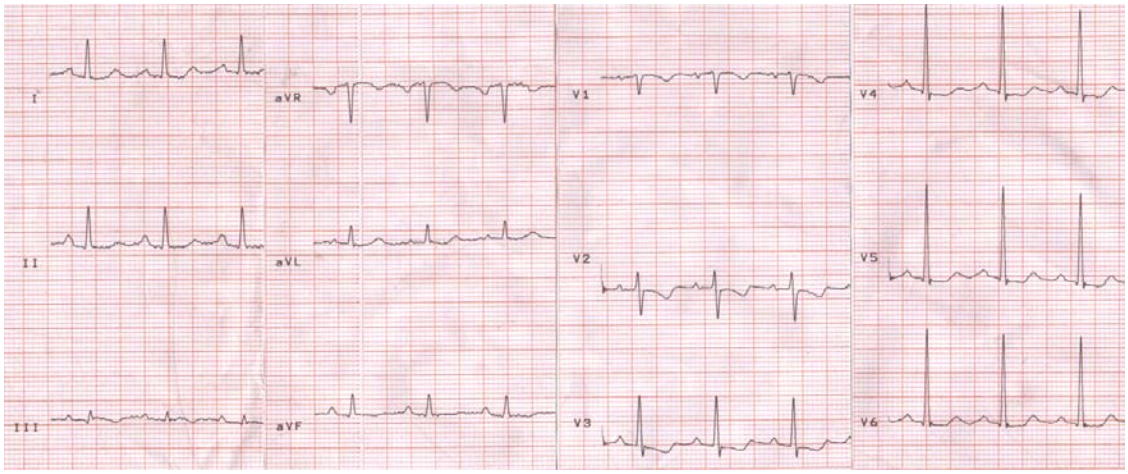

Fig.12. Electrocardiogram after cessation of the beta-blocker therapy shows restored sinus rhythm (HR: 90 bpm).

VI. Discussion

1. Mechanism of ANP secretion

A, Munc18s and SNAREs

Although there is rapidly increasing amount of knowledge in vesicle trafficking and membrane fusion, how Munc18c regulates secretion is still poorly understood [75]. The importance of Munc 18c is proved by the observation that its genetic deletion is lethal [174], and there is abundant genetic and biochemical evidence that it regulates and is required for secretion in non-neuronal cells [175-180]. Studies with inhibitory peptides, Munc18c antibodies, and overexpression as well as loss-of-function mutations have been shown that Munc18c controls exocytosis in several secretory cells, such as the insulin-dependent trafficking of GLUT4 in adipocytes [175, 177, 178, 180] and skeletal muscle cells [179]. It also plays a role in insulin secretion in pancreatic β-cells [181], dense granule secretion in platelets [182], cytokine exocytosis in macrophages [183], and amylase release in parotid acinar cells [184]. Functional studies suggested that Munc18 family of molecules exert an inhibitory effect on secretion through binding to syntaxin; whereas elimination of syntaxin binding caused Munc18 to promote secretion [76, 185, 186]. This observation was supported by early structural analyses showing that the Munc18 class of molecules binds to the closed conformation of syntaxin to inhibit the formation of a SNARE complex (with members of the SNAP-25 and VAMP families). However, more recent experiments with overexpressed proteins show that Munc18c binds both to the SNARE complex and to the monomeric syntaxin 4 [187]. The picture is further complicated by the fact that Munc18 molecules appear to interact with a number of other secretory molecules (e.g., members of the Rab [188], Rab effector [189], Doc2 [190], Mint families [191]). Still, the finding that Munc18c binds to syntaxin 4 in cardiomyocytes and that these MAbs inhibit this interaction [182], it appears plausible that Munc18c exerts its negative effect on pro-ANP secretion through syntaxin 4 in these cells.

Adult and neonatal cardiomyocytes also contain syntaxin 2. Although different splice variants of syntaxin 2 have been described, Peters et al. [165] identified only syntaxin 2C transcripts in cardiomyocytes, while an earlier study by Quinones et al. [192] detected syntaxin 2A, B and C transcripts in heart lysate as well. The finding that the syntaxin 2 variant found in cardiomyocytes is not specifically targeted to plasma membrane structures suggests that it may have a role in earlier vesicle trafficking processes than the final phase of secretion. Lower affinity interactions between syntaxin 2 and Munc18c have been detected using in vitro systems, but little if any interaction between these molecules has been detected in cells [182]. The diffuse granular distribution of syntaxin 2 throughout the cells raises the possibility that it might colocalize with ANP containing granules but we were unable to confirm that because of limitations with the antibody reagents for these molecules.

Syntaxin 6 has been recently identified by proteomics studies as the most abundant SNARE protein in atrial secretory granules [193]. However, syntaxin 6 is known to be a ubiquitous member of the trans Golgi network and endosomes in most cells [194-196]. We observed that syntaxin 6 was localized to perinuclear structures and granules containing ANP, which is consistent with expression in the Golgi, endosomes or nascent secretory granules. Syntaxin 6 is different from other syntaxins (which are classified as Qa SNAREs) because it has structural similarities to both the syntaxins and the Qbc SNAREs or the SNAP25 family (reviewed in [197]). The ability of syntaxin 6 to form SNARE-complexes is regulated by Vps45, another member of the Sec1/Munc18 gene family [195]. In accordance with these studies, we found no interaction of syntaxin 6 with Munc18.

The role of Ca^{2+} in triggering secretory granule release from cardiomyocytes has been controversial [43]. Recently, Peters et al. [165] demonstrated in cardiomyocytes that photo-triggered release of caged Ca^{2+} compounds alters membrane capacitance in a manner consistent with exocytosis or membrane fusion events. Our studies extend these results by providing strong evidence that increases in intracellular Ca^{2+} trigger pro-ANP release from cardiomyocytes. In addition, we found that priming of the cells with ATP was necessary for Ca^{2+}-triggered secretion (data not shown). This parallels results in other cells where ATP and GTP dependent priming is required for regulated secretion [72, 74].

B, Corin

Mouse corin consists of 1113 amino acid residues with a calculated molecular mass of 123 kDa [30]. However, we detected corin in the mouse heart tissue and murine HL-1 cell extracts as a 205-210 kDa protein (Fig. 5B, 7A, 8B). The glycosylation, which is common for most TTSPs [28, 30, 198-201], seems to be the most reasonable explanation for this molecular mass discrepancy. Separation on Concanavalin-Sepharose confirmed that the 205-210 kDa form of corin is a glycosylated protein (Fig. 8D). The failure to detect a glycosylated form of corin (205-210 kDa) in human heart tissue extract by Western blotting previously [202] could be explained by the inability of the anti-corin peptide antibody used in that study to recognize the extensively glycosylated protein. Even if mouse corin is glycosylated at all 16 putative *N*-glycosylation sites, its calculated molecular size still should be smaller than 205-210 kDa. Therefore, a lower than expected migration of this extensively glycosylated protein on SDS-PAGE, is likely to be explained by its complicated structure or additional post-translational modifications to the protein molecule. Among those, *O*-glycosylation could not be excluded.

Based on cDNA sequence it was proposed that corin is expressed by cardiomyocytes as a zymogen that requires proteolytic cleavage to become an active serine protease [28, 30]. A putative disulfide bond anchors the active protease domain to the stem domain, and active corin remains tethered to the cell-surface [28, 30]. Recent studies of recombinant soluble corin confirmed the zymogen nature of corin [31, 203]. However, it had been unclear whether both forms, zymogen and active, exist on the cell surface of cardiomyocytes. We addressed this question by using Western blotting of the membrane fraction of the mouse heart extract. Under the reducing conditions, anti-corin antibodies detected several protein bands corresponding to cleaved fragments of the corin molecule (Fig. 7B), indicating that mouse heart extract contains potentially active forms of corin. The existence of a zymogen form of corin was clearly confirmed by the ability of polyclonal anti-corin stem domain antibody (R34) to recognize reduced full-length corin after immuno-precipitation of the membrane fraction of mouse heart extract by anti-corin protease domain MAbs (Fig. 7A).

Halomethyl ketone inhibitors form an irreversible covalent complex with serine and cysteine proteases and are widely accepted as convenient tools for investigation of these classes of proteases *in vitro* and in animals [172]. It should be noted that to date the specific inhibitor of corin has not been identified [203]. In this study we used FITC-FPR-CMK, which repeats the sequence of the chromogenic substrate of corin, S-2302 [203], and is consistent with the corin cleavage sequence in pro-ANP, to show that an active form of corin exists on the surface of cardiac cells. Only cardiac cell-localized proteases may be tagged by FITC-FPR-CMK under our experimental conditions. Our data showed that FITC-FPR-CMK recognized active protease in murine cardiac cells *in situ* (detected by blotting of the membrane fraction of mouse heart extract, Fig. 7C, right lane). Despite the fact that corin is the only known TTSP on the extracellular plasma membrane surface of cardiac cells [25], there are a number of potential targets for FITC-FPR-CMK in cardiac cells in addition to active corin. Since FITC-FPR-CMK may be cell-permeable, it might potentially recognize also active intracellular cysteine proteases (caspases, cathepsin B, calpains) and serine proteases (tissue kallikrein and mast cell proteases) in murine cardiac cells [25, 172, 204]. However, caspases, the generally accepted executioners of apoptosis, have the absolute requirement for an Asp in the P1 position and can be excluded as potential targets on this basis [205]. Cathepsin B and calpains do not have such a strong requirement for Asp in the P1 position [172], but cathepsin B and calpains are implicated in the execution phase of apoptosis and/or necrosis [204]. A significant level of cell death is unlikely in a healthy heart tissue, therefore cysteine proteases also are not attractive candidates for interaction with FITC-FPR-CMK under our experimental conditions. Mast cell proteases are sequestered inside mast cell granules in an acidic environment, which inhibits the activity of the enzymes to avoid self-digestion within cell [25], and as a result can also be excluded as a potential target. Considering all said above, it is highly improbable that the detected FITC-tagged protease covalent complex is due to the inhibition of a protease other than corin. This was additionally confirmed by using the corin anti-protease domain antibody to identify the FITC-tagged protease. Protein bands containing the FITC tag were recognizable by anti-corin protease domain antibody by Western blotting (Fig. 7C, left), confirming that these bands correspond to corin. Detection

of these immuno-reactive fragments suggests that corin undergoes proteolysis at one or more sites to become a catalytically active protease.

Whether corin activity is regulated physiologically by endogenous inhibitors or zymogen activation remains to be determined. Human plasma had a small inhibitory effect on the pro-ANP cleavage activity of the recombinant soluble corin [203]. Although more detailed studies are necessary, the availability of corin for FITC-FPR-CMK (Figure 7C) suggests that corin may be protected from the inhibition by the physiological high molecular mass protease inhibitors. This suggestion is supported by the differential effect of soybean trypsin inhibitor on soluble and membrane forms of corin. Soluble but not membrane-bound recombinant corin was efficiently inhibited by this inhibitor [24, 31, 32, 203]. The fact that low molecular mass inhibitors were effective against corin in cell-based experiments [24, 32] indicates that steric hindrance might be responsible for the resistance of cell-bound corin to soybean trypsin inhibitor.

Corin is suggested to function as an upstream regulator of the ANP signaling pathway [24, 25, 32]. Therefore, inappropriate regulation of corin activity could compromise the entire ANP-mediated pathway. Normal human plasma contains cleaved ANP as the major circulating form while pro-ANP is absent (or present at low concentration). However the pro-ANP level is markedly raised in the plasma of patients with heart and/or kidney failure [123], resulting in a relative lack of ANP. The presence of both zymogen and catalytically active form of corin on cardiomyocyte surface might indicate that zymogen corin serves as a reserve which might be easily activated upon increased demand. However, the failure of corin activation can be responsible for decreased plasma ANP/pro-ANP ratio.

2. ANP and endothelin in response to hemodynamic strain in diabetes mellitus

Alloxan-monohydrate induced diabetes is a well-known, long-term used model of type I diabetes mellitus [206]. Diabetes mellitus has a series of cardiovascular complications

including endothelial dysfunction, tissue proliferation, atherosclerosis and altered cardiac endocrine activity [173]. The presented study examined the changes in natriuretic peptide and endothelin levels in response to acute hemodynamic load in alloxan-induced type I diabetes mellitus.

Measurement of cardiovascular parameters showed that severe unopposed hypoinsulinemia and consequent hyperglycemia caused significant drop in mean arterial blood pressure compared to the metabolically healthy controls (Table 1), which can be explained by the profound fluid and electrolyte depletion [207]. Acute hemodynamic stress further decreased the blood pressure in diabetic dogs, while there was no change in blood pressure in the control group. This observation is in accordance with earlier studies, which have shown that isolated diabetic hearts exhibit functional abnormalities when exposed to high workload [208, 209]. In a study by Ingebretsen et al. [209], where further compensating hormonal changes were excluded in the experimental settings, cardiomyopathy was suggested as a possible explanation. However, the simple fact that mechanical deterioration of the diabetic heart could be overcome by high glucose concentration and/or insulin in the perfusion media suggested that relative intracellular hypoglycemia might be responsible for the cardiac dysfunction [209, 210]. A recent study reported similar results *in vivo* [211].

Apart from the mechanical cardiac function, in vivo blood pressure is specifically controlled by the autonom nervous system and the endocrine system. Among the several hormones regulating blood pressure, endothelin and natriuretic peptides have essential, but counter regulatory effects; endothelin is a potent vasoconstrictor, while the natriuretic peptides cause vasodilation. We found that endothelin levels responded differently to acute hemodynamic load in alloxan-induced diabetic dogs and in metabolically healthy dogs. In metabolically healthy dogs, plasma endothelin level did not change upon acute hemodynamic stress modeled by shunted circulation (Fig. 9). In accordance with the literature, in diabetic dogs basal plasma endothelin level was significantly higher compared to metabolically healthy controls [212]. However, plasma endothelin level significantly decreased in response to the initiation of acute hemodynamic strain (Fig. 9). This finding might explain, at least partially, the observed lower blood pressure in diabetic dogs

exposed to hemodynamic stress (Table 1). In the coronary sinus and in the pericardial fluid, endothelin levels were higher than in the systemic plasma, corresponding to the previous reports [213], suggesting that the heart actively contributes to the maintenance of endothelin level. Changes in the coronary sinus levels of endothelin were similar to the changes in the plasma levels, whereas there was no change in the pericardial fluid endothelin levels, suggesting that the pericardial space is a functionally different compartment.

In addition of the potent vasoconstrictor property, endothelin has a series of other cardiovascular effects. The vasoconstrictor and arrhytmogenic effect [214] can be harmful in both the short term and the long term, and the induction of tissue proliferation is disadvantageous in the long term. On the other hand, endothelin is a positive inotropic agent and influences the inotropic responses by modulating the Frank-Starling mechanism [215], which can be beneficial in acute situations. We did not observe any changes in cardiac rhythm or signs of myocardial ischemia. Left ventricular dP/dt in the healthy-shunted animals was not different from the diabetic shunted animals. However, cardiac function deteriorates with progression of diabetes. Reduction of plasma endothelin levels in response to acute hemodynamic load in our diabetic model can be regarded as a potentially adaptive mechanism, since it reduces the chances of vasospasm and cardiac arrhythmias. Long-term and functional experiments are needed to prove these assumptions.

As opposed to endothelin, atrial natriuretic peptide decreases blood pressure due to its diuretic/natriuretic and vasodilator properties [112]. ANP secretion is triggered by cardiomyocyte stretch *and by endothelin* [63], and increased plasma ANP level is a well-known indicator of cardiac strain [21]. In our study, NT-proANP levels were significantly elevated upon shunting, both in metabolically healthy and in diabetic dogs, indicating that the experimental model initiated cardiac strain. However, elevation of NT-proANP levels due to shunting was somewhat higher in the diabetic group than in the metabolically healthy controls (Fig. 10). Although we did not find significant difference in the NT-proANP levels between the diabetic and the healthy controls without hemodynamic strain, clinical [216, 217] and basic [218] studies have reported increased ANP and BNP levels in type 1 diabetic patients. In addition to the increased natriuretic peptide level, decreased

natriuretic peptide receptor (NPR-A) level have been demonstrated in type 1 diabetes mellitus [218]. Furthermore, an earlier study reported impaired vasodilator response to atrial natriuretic peptide in diabetic patients [219]. Taking together, the observed increased ANP level in the diabetic animals in our experiments might be explained by the increased need of natriuretic peptides due to acute cardiac strain, and the parallel decreased natriuretic peptide receptor concentration due to the hypoinsulinemia. Since 1) diabetes is an important risk factor for cardiovascular disease; 2) the measurement of plasma natriuretic peptide is a useful tool in the diagnosis and prognosis of several cardiovascular diseases; and 3) natriuretic peptide level has been shown to be increased in patients with diabetes mellitus, even in the absence of structural heart disease [220] the correlation of natriuretic peptide level, diabetes mellitus and cardiac strain is an important question in the clinical practice as well, and requires further investigation.

3. Bradycardia and increased natriuretic peptide level

From several experimental and clinical studies we know that the most important trigger for natriuretic peptide secretion is myocardial stretch [42]. Regardless of the physiological/pathological mechanism, any process which leads to the stretch of the cardiac wall results in increased natriuretic peptide gene expression and exocytosis, and as such, elevates plasma natriuretic peptide level. However, apart from the major trigger myocardial stretch, several other factors influence the secretion of natriuretic peptides, and thus complicate the interpretation of plasma natriuretic peptide level in the clinical setting.
While tachycardia-related cardiomyopathy is a well-known and frequent cause of arrhythmia-induced elevated natriuretic peptide level [167, 168, 221], bradycardia-related cardiomyopathy is a rare phenomenon. In our case report, the beta-blocker therapy triggered bradyarrhythmia (sinus arrest with junctional escape rhythm) was followed by a parallel marked increase in the serum NT-proBNP-level, which promptly decreased after the cessation of the arrhythmia. In the present case, increased myocardial wall-stretch was

induced by bradyarrhythmia; however, the bradyarrhythmic episode was not long enough to cause left ventricular systolic dysfunction or dilation.

Our aim with this study was to point out that not only tachycardia, but bradycardia can cause cardiomyopathy, and elevated NT-proBNP level caused by increased myocardial wall-stretch might indicate even the clinically not evident cardiomyopathy [134].

Recently, Koch et al. [222] analyzed 43 children and adolescents with high-grade second degree or complete atrioventricular conduction block. They reported that the loss of atrioventricular synchrony could induce elevated plasma BNP level, similarly to our observation, where the lack of adequate atrial contraction resulted in increased BNP level. Furthermore, the authors reported that BNP level was higher in patients with VVI pacemaker than that of patients after DDD pacemaker implantation. The latter shows that the loss of atrioventricular synchrony – or the lack of adequate atrial contraction, as in our case – can as well induce elevated plasma BNP level.

These observations are further supported by the finding that in hypertonic patients the heart-rate decreasing beta blocker therapy increases plasma atrial natriuretic peptide (ANP) and BNP concentrations [223]. The most probable explanation for these characteristics is that, at lower heart rate, the stroke volume increases in order to keep up the cardiac output. Increased stroke volume results in increased wall tension, which in consequence triggers natriuretic peptide secretion. The clinical importance of this theory is that it explains why beta-blocker therapy causes atrial fibrillation more often than the angiotensin receptor blocker losartan therapy [224]. Figure 13 summarizes the mechanisms of correlated bradycardia and increased plasma BNP level.

The clinical challenge is now to predict in which patients the beta-blocker therapy causes adverse effects by triggering a cascade of events, starting from increased filling pressure leading to increased wall tension, which in turn leads to elevated ANP/BNP levels and atrial fibrillation. Our case report demonstrated such an example, when low dose of bisoprolol caused considerable bradycardia and extremely elevated plasma BNP concentration.

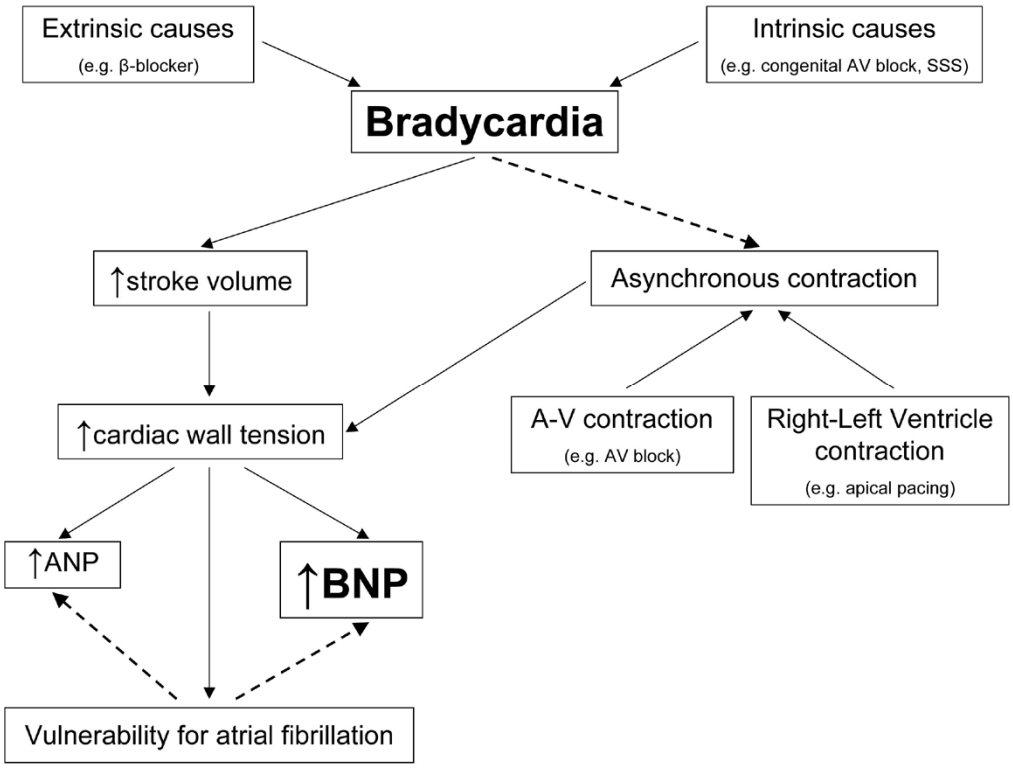

Fig.13. Flowchart depicting the mechanisms through which bradycardia can cause increased plasma BNP level. *SSS*: sick sinus syndrome; *AV block*: atrioventricular block; *ANP*: atrial natriuretic peptide; *BNP*: B-type natriuretic peptide; *A-V contraction*: atrio-ventricular contraction; ↑: increased. Dashed arrow indicates a potential consequence.

VII. Conclusion

1. Munc18c regulates ANP secretion

Although cardiac secretion of natriuretic peptides plays an important role in normal physiology and in pathological states such as heart failure, there is limited knowledge of the molecular machinery regulating the exocytosis of pro-ANP [165]. Here we demonstrated that neonatal cardiomyocytes contain the secretory molecules syntaxin 2, 4 and 6, as well as Munc18c. Syntaxin 4 and Munc18c displayed a pattern of expression consistent with localization to the plasma membrane (Fig. 1). In contrast, syntaxin 2 showed a more diffuse granular distribution throughout the cytoplasm of cardiomyocyte. Syntaxin 6 partially co-localized with pro-ANP in peri-nuclear granules, although it was not found in pro-ANP containing granules at the periphery of the cell. The plasma membrane expression pattern of syntaxin 4 and Munc18c suggested that these two molecules may interact in the heart and this interaction was confirmed by co-immunoprecipitation studies. To examine the potential role of Munc18c in pro-ANP secretion, a permeabilization system of cultured cardiomyocytes was developed for triggering pro-ANP secretion in the presence of different antibodies. In this system, streptolysin-O selectively permeabilized the plasma membrane of cardiomyocytes without affecting the leakage of pro-ANP from secretory granules. Pro-ANP secretion was reproducibly induced by increased intracellular Ca^{2+}. Introduction of a Munc18c-specific MAb into permeabilized cardiomyocytes significantly increased Ca^{2+}-triggered secretion of pro-ANP from the heart whereas a control MAb had no effect. This provided strong evidence that pro-ANP secretion from cardiomyocytes was modulated by Munc18c.

In summary, this study demonstrates that ANP secretion in cardiomyocytes is regulated by Munc18c, and ANP exocytosis in cardiomyocytes proceeds through a Ca^{2+}/ATP-dependent mechanism.

2. Corin is co-expressed with pro-ANP and localized on the cardiomyocyte surface in both zymogen and catalytically active forms

The discovery of corin [28, 30] and its establishment as the pro-ANP convertase [33] is considered a major step in our understanding of the hormonal control of blood pressure [225]. However, cellular localization, molecular forms, and post-translational modification of native corin remained unclear. A series of anti-corin polyclonal and monoclonal antibodies directed against the stem and protease domains allowed us to identify for the first time the native corin protein on the cell surface of rat neonatal cardiomyocytes and murine HL-1 cardiomyocyte-like cells by immunofluorescence staining, flow cytometry and biotin cell surface-labeling techniques (Fig. 6A, 8A, C).

Furthermore, we showed for the first time that native corin is expressed in cardiomyocytes that also express its substrate, pro-ANP (Fig. 6B), providing further evidence that corin may contribute to the regulation of the natriuretic peptide system *in vivo* by pro-ANP processing**.**

Our finding of both zymogen and active forms of corin in mouse heart suggests that regulation of corin activity might control the ratio between circulating pro-ANP and cleaved ANP. The zymogen form could be considered as repository for corin on the membrane surface of cardiomyocytes which could be easily activated during periods of increased demand such as increased hemodynamic strain, where an enzymatic cleavage of pro-ANP may play a key role.

3. Cardiac strain reduces ET-1 and increases ANP level in diabetes mellitus

Diabetes mellitus is associated with endothelial and cardiac dysfunction. Natriuretic peptides are sensitive markers of cardiac strain, widely used in the clinical practice to aid the diagnosis of several cardiac diseases, however, little is known about the hormonal

responses to acute hemodynamic burden in diabetes. We found that ET-1 plasma and sinus coronarius levels were not influenced by hemodynamic strain in metabolically healthy animals, however, diabetes caused higher basal ET-1 levels which significantly decreased upon shunting. Pericardial fluid contained higher ET-1 level in both metabolically healthy and diabetic animals, and its level did not change due to shunting neither in diabetes nor in the healthy controls. NT-proANP levels in different compartments are increased upon cardiac strain both in diabetes and in metabolically healthy controls, although NT-proANP levels tend to be higher in the diabetic animals. We conclude that 1) the pericardial space behaves as a functionally different compartment for systemic ET-1 level; 2) NT-proANP levels are increased in response to hemodynamic load both in diabetes and in metabolically healthy controls, supporting the value of natriuretic peptide measurement for the diagnosis of acute cardiac strain in diabetes mellitus 3) higher NT-proANP levels in diabetic animals support the need for different cutoff values for diagnostic purposes in diabetes mellitus.

4. Bradycardia can induce increased plasma BNP level

Tachycardia induced natriuretic peptide plasma level elevation is a well-known phenomenon, and it indicates increased cardiac wall stretch. However, not only tachycardia, but bradycardia, or asynchronous contraction might lead to cardiac wall distention, and as such, increased plasma natriuretic peptide level. In the case of an elderly woman, we observed extremely elevated natriuretic peptide-level due to bradycardia induced by sinus arrest with a junctional escape rhythm provoked by beta-blocker therapy. Cessation of the medication resulted in sinus rhythm and a rapid decrease of the natriuretic peptide-level. According to these findings, we conclude that elevated natriuretic peptide-levels might indicate subclinical cardiomyopathy induced by bradycardia.

VIII. Summary

Cardiac natriuretic peptides (atrial natriuretic peptide: ANP, B-type natriuretic peptide: BNP) are important regulators of the cardiovascular homeostasis in health and disease, and the measurement of their plasma concentration facilitates diagnosis and prognosis in the clinical practice of several disorders. This thesis reports 1) *in vitro and ex vivo* experiments for the better understanding of the physiologic mechanism of ANP secretion from cardiomyocytes *and* the cardiac localization and molecular forms of the pro-ANP convertase corin; 2) *in vivo* studies of altered natriuretic peptide and endothelin secretion in response to acute hemodynamic load in animal model of alloxan-induced type I diabetes mellitus; and finally, 3) evaluation of natriuretic peptide secretion in bradycardia in the *clinical practice*.

Cell culture studies have shown that syntaxin 4 and Munc18c, which are essential regulators of secretion in other specific secretory cells, interact with each other and are co-localized to the plasma membrane in pro-ANP containing cardiomyocytes. Functional studies have shown that cardiac secretion of ANP is triggered by increased Ca^{2+} and is specifically regulated by Munc18c. Further experiments demonstrated that corin, the enzyme responsible for pro-ANP activation to active peptide ANP, is present on the cell-surface of pro-ANP-containing cardiomyocytes. Native corin is a glycosylated protease that exists on the plasma membrane in both zymogen and catalytically active forms.

Animal studies have shown that NT-proANP plasma levels are increased in response to hemodynamic load both in diabetes and in metabolically healthy controls. Higher NT-proANP levels in diabetic animals supported the need for different cutoff values for diagnostic purposes with concomitant diabetes mellitus.

Finally, a clinical example demonstrated that bradycardia can manifest in highly elevated natriuretic peptide plasma level even in the absence of heart failure signs. Cessation of the junctional escape rhythm responsible for the bradycardia resulted in a rapid fall in the NT-proBNP serum level. This study accentuated that any pathophysiology that leads to increased myocardial wall stretch might cause clinically elevated natriuretic peptide level.

IX. References

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X. List of publications

1. Related

Kováts T and Tomcsányi J: Bradycardia and B-type natriuretic peptide. Int J Cardiol. 2008 Jul 3. [Epub ahead of print]

Gladysheva IP, Robinson BR, Houng AK, Kováts T and King SM: Corin is co-expressed with pro-ANP and localized on the cardiomyocyte surface in both zymogen and catalytically active forms. J Mol Cell Cardiol. 2008 Jan;44(1):131-42.

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Skoumal R, Seres L, Soós P, Balogh E, Kováts T, Rysa J, Ruskoaho H, Tóth M and Horkay F: Endothelin Levels in Experimental Diabetes Combined with Cardiac Hypertrophy. J Cardiovasc Pharmacol. 2004 Nov;44:S195-S197.

2. Unrelated

Pósa I, Horkay F, Seres L, Skoumal R, Kováts T, Balogh E, de Chatel R, Tóth M and Kocsis E: Effects of Experimental Diabetes on Endothelin-induced Ventricular Arrhythmias in Dogs. J Cardiovasc Pharmacol. 2004 Nov;44:S380-S382.

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