REGULATION OF ADRENOMEDULLIN GENE EXPRESSION IN THE RAT HEART

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Abstract

Adrenomedullin (AM), a novel vasorelaxant 52 amino acid peptide, was found from human pheochromocytoma in 1993. AM gene is expressed in various tissues, including heart. Although hemodynamic overload and rapid ventricular pacing are known to alter the expression of the AM gene, it has not yet been established whether wall stretch acts directly or via local paracrine and autocrine factors liberated in response to hemodynamic load. The aim of the present study was to examine AM gene expression in response to acute and chronic mechanical stress in the hearts of normal and hypertensive rats *in vivo*. The second purpose was to study the mechanism(s) involved in the induction of the AM gene expression.

The acute increased workload of the heart was produced by the infusion of vasopressin (AVP), and the involvement of angiotensin II (Ang II) and endothelin-1 (ET-1) in the early activation of AM gene expression was studied. The acute increase in left ventricular AM gene expression occured within 2 h, whereas in the left atrium the induction of AM gene expression was observed earlier, at 30 min. The induction of left atrial and ventricular AM gene expression was shown to be Ang II- and ET-1-independent by using Ang II receptor type 1 (AT_1) antagonist (losartan) and mixed ET-1 receptor types A and B antagonist (bosentan), respectively.

AT_1 receptor antagonism and angiotensin converting enzyme (ACE) inhibition for 10 weeks were used to evaluate the chronic regulation of AM gene expression in normotensive and hypertensive rats. The decreases in systolic blood pressure and cardiac hypertrophy induced by losartan and enalapril treatments had no effect on ventricular AM gene expression. The expression of AM, ANP and BNP was differently regulated both in the left ventricle and atria in response to AT_1 receptor antagonism and ACE inhibition. Moreover, C-type natriuretic peptide (CNP), a selective agonist of natriuretic peptide receptor type B (NPR_B), was infused for 2 weeks in normotensive rats to examine the effects of CNP on cardiac AM gene expression. CNP infusion increased left ventricular and plasma ir-CNP levels, and those increases were associated with significant decreases in AM and BNP mRNA levels.

In conclusion, AM was shown to react rapidly against increased cardiac workload, and cardiac wall stretch seemed to be a major stimulus for the early induction of AM gene expression in the left ventricle and atrium, mimicking the rapid induction of proto-oncogenes in response to hemodynamic stress. The acute induction of left atrial and ventricular AM gene expression was shown to be Ang II- and ET-1-independent. During chronic cardiac overload, AM synthesis was an insensitive marker of chronic changes in hemodynamic load or myocyte hypertrophy. CNP was shown to have a paracrine role in the regulation of left ventricular AM gene expression. The cardiac secretion of AM was small, at least in the acute phase of cardiac pressure load, suggesting a paracrine and/or autocrine role for AM in the regulation of cardiac function.

Keywords: pressure, cardiac overload, endothelin, angiotensin

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Oulu, November 22 Hannu Romppanen

Abbreviations

AM	adrenomedullin
ACE	angiotensin converting enzyme
ACTH	adrenocorticotropic hormone
Ang II	angiotensin II
AMI	acute myocardial infarction
ANP	atrial natriuretic peptide
AP-1	activator protein 1
AP-2	activator protein 2
AT_x	angiotensin receptor subtype
AVP	arginine^8-vasopressin
bFGF	basic fibroblast growth factor
BNP	B-type natriuretic peptide
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
CO	cardiac output
CNP	C-type natriuretic peptide
CREB/ATF	cyclic AMP response element-binding protein
DAG	1,2-diacylglycerol
Gd^{3+}	gadolinium
ERK	extracellular signal regulated kinase
ET	endothelin
ET_x	endothelin receptor subtype
FAK	focal adhesion tyrosine kinase
GAPDH	glyceraldehyde 3-phosphate-dehydrogenase
G proteins	guanine nucleotide- binding proteins
GTP	guanosine triphosphate
IGF-I	insulin-like growth factor-I
IL	interleukin
IP_3	inositol-1,4,5-triphosphate
ir	immunoreactive

JAK	janus kinase
JNK	c-Jun N terminal kinase
LPS	lipopolysaccharide
LVH	left ventricular hypertrophy
MAPK	mitogen-activated protein kinases
MAP	mean arterial pressure
MEK	MAPK/extracellular signal regulated kinase
MHC	myosin heavy chain
NEP	neutral endopeptidase
NO	nitric oxide
PAMP	proadrenomedullin N-terminal peptide
PDGF	platelet derived growth factor
PI-3 kinase	phosphatidylinositol 3-kinase
PKA	cyclic AMP-dependent protein kinase
PKC	protein kinase C
PLC	phosphoinositide phospholipase C
PLD	phosphoinositide phospholipase D
PTK	protein tyrosine kinase
RAMP	receptor-activity-modifying proteins
RAS	renin angiotensin system
RIA	radioimmunoassay
SAPK	stress-activated protein kinase
SHR	spontaneously hypertensive rat
STAT	signal transducer and activator of transcription
TCF	ternary complex factor
TGF-β	type-β transforming growth factor
TGR(mREN-2)27	(mREN-2)27 transgenic rat
TNF-α	tumor necrosis factor alfa
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
WKY	Wistar-Kyoto

α	alfa
β	beta
δ	delta
ε	epsilon
γ	gamma
η	eta
ι	iota
θ	theta
ζ	zeta
λ	lambda

List of original papers

This thesis is based on the following articles, which are referred to in the text by Roman numerals:

I Romppanen, H., Marttila, M., Magga, J., Vuolteenaho, O., Kinnunen, P., Szokodi, I. & Ruskoaho, H. (1997) Adrenomedullin gene expression in the rat heart is stimulated by acute pressure overload: blunted effect in experimental hypertension. Endocrinology. 138: 2636-2639.

II Romppanen, H., Puhakka, J., Vuolteenaho, O. & Ruskoaho, H. (1999) Endothelin-1- and angiotensin II-independent induction of cardiac adrenomedullin gene expression in response to pressure overload (submitted).

III Magga, J., Kalliovalkama, J., Romppanen, H., Vuolteenaho, O., Pörsti, I., Kähönen, M., Tolvanen, J-P. & Ruskoaho, H. (1999) Differential regulation of cardiac adrenomedullin and natriuretic peptide gene expression by AT_1 receptor antagonism and ACE inhibition in normotensive and hypertensive rats. J Hypertension 17: 1543-1552

IV Romppanen, H., Vuolteenaho, O., Pääkkö, P., Soini, Y. & Ruskoaho, H. (1999) The effect of C-type natriuretic peptide and sinorphan on adrenomedullin gene expression in rat heart (manuscript).

Contents

1. Introduction

Left ventricular hypertrophy (LVH) is seen in both physiological and pathophysiological conditions. The athlete´s heart grows in response to repetitive short-term physiological hemodynamic load, and this hypertrophy is lost rapidly after cessation of training (for reviews see Ehsani 1992, Pelliccia 1992). However, in response to long-term fixed pathological overload, LVH decreases only partially when the load is decreased (for review see Dahlof *et al.* 1992). LVH resulting from hypertension is an independent risk factor for premature cardiovascular morbidity and mortality (Kannel *et al.* 1970, Casale *et al.* 1986, Levy *et al.* 1990, Mosterd *et al*. 1999). LVH has been associated with impaired coronary hemodynamics in experimental animals (Marcus *et al.* 1979) and patients (Pichard *et al.* 1981, Opherk *et al.* 1984, Motz & Strauer 1996), leading to ventricular fibrosis, decreased coronary blood flow and endothelial dysfunction (Frochlich 1999). LVH is associated with a higher risk for cardiac arrhythmias, cardiac failure and accelerated atherosclerosis of epicardial arteries (Frohlich *et al.* 1992).

External load plays a critical role in determining heart hypertrophy, but it is not known whether the signals coupling load to growth are intrinsic to the normal adult cardiocyte, or whether activation of endocrine, paracrine, or autocrine intermediaries might be required. Load itself is a sufficient stimulus for the induction of cardiac growth at the levels of the intact heart, isolated cardiac tissue, and isolated cardiocytes (for review see Morgan *et al.* 1987). A variety of endocrine, paracrine, and autocrine factors have been shown to stimulate cardiac growth, but it has not been shown that any of these factors could serve as the primary stimulus to cardiac hypertrophy in response to hemodynamic load increases. The central problem in understanding the molecular basis of cardiac hypertrophy is finding out how mechanical input is transduced into a growth response. In this transduction, endocrine, paracrine, and autocrine factors could either themselves cause cardiocyte growth, or act as necessary intermediary factors that modify cardiocytes.

Adrenomedullin (AM) is a new potent vasorelaxing and natriuretic peptide that plays an important role in cardiorenal regulation (for reviews see Kitamura *et al.* 1993a, Samson 1999, Eto *et al.* 1999). In humans, the concentration of circulating AM is increased in diseases such as congestive heart failure (Jougasaki *et al.* 1995b, Kato *et al.* 1996, Kobayashi *et al.* 1996b), essential hypertension (Kitamura *et al.* 1994, Ishimitsu *et al.* 1994, Kohno *et al.* 1996b), chronic renal failure (Ishimitsu *et al.* 1994, Washimine *et al.* 1995b), pulmonary hypertension (Shimokubo *et al.* 1995, Nishikimi *et al.* 1997) and

myocardial infarction (Miyao *et al.* 1998, Yoshitomi *et al.* 1998). Left ventricular AM mRNA levels are increased after left coronary artery ligation in rats (Kaiser *et al.* 1998), in hypertensive Dahl salt-sensitive rats on high-salt diet (Shimokubo *et al.* 1996) and during aortic banding in Sprague-Dawley (SD) rats (Morimoto *et al.* 1999). The right ventricular AM mRNA and ir-AM levels are increased in experimental pulmonary hypertension in the rat (Shimokubo *et al.* 1995).

Although cardiac overload (Nishikimi *et al.* 1997) and rapid ventricular pacing (Jougasaki *et al.* 1997) are known to alter the expression of the AM gene, it has not yet been established whether wall stretch acts directly or via local paracrine and autocrine factors liberated in response to hemodynamic load. The aim of the present study was to examine AM gene expression in response to acute and chronic mechanical stress in the hearts of normal and hypertensive rats. The second purpose was to study the mechanism(s) involved in the induction of AM gene expression.

2. Review of the literature

2.1. The cellular and molecular response of cardiac myocytes to mechanical stretch

2.1.1. Stretch-activated cardiac gene expression

Physical forces induce profound changes in cell phenotype, shape and behavior. These changes occur in cardiac hypertrophy as a result of pressure overload, for example. At the molecular level, mechanical stimuli are converted into chemical ones and lead to modulation of gene expression of different cardiac genes whose encoded proteins help the cells to adapt to their environment. The term hypertrophy defines an increase in cellular size based on concordant increase in all of the cellular constituents. Hypertrophy proceeds transmurally from the endocardium towards the epicardium. It has been shown that in hypertrophy produced by supra-renal aortic constriction, there is a transmural gradient in the V3 myosin isoform and cell cross-sectional area, i.e. V3 myosin expression and cell cross-sectional area are increased more in the endocardium than in the epicardium (Toffolo & Ianuzzo 1994). In hypertrophy, there is a quantitative up-regulation of the vast majority of the genes encoding the full protein complement of the cardiocyte. In attempting to explain such a generalized transcriptional up-regulation it is suggested that the transcriptional program responsible for developmental cardiogenesis is reinitiated at the onset of hypertrophy. Indeed, there are reports demonstrating that hypertrophy is accompanied by a shift of the myocytes to a fetal phenotype (Schwartz *et al.* 1986, Izumo *et al.* 1988, Nadal-Ginard & Mahdavi 1989, Parker & Schneider 1991, Delcayre *et al.* 1992). Various models of mechanical stress have been used to demonstrate the early changes in cardiac gene expression leading to hypertrophy. The genes responsive to an increased workload can be divided into two classes, early responsive and late responsive load-inducible genes.

Table 1. Stretch-activated cardiac gene expression associated with cardiac hypertrophy.

Early response	Intermediate response	Late response
c-fos	ANP	α-actin
c-myc		β-MHC
c-jun		
Egr-1		
BNP		

2.1.1.1. Early response load-inducible genes

The first load-inducible genes are called early response, or immediate early genes, and their transcription is activated rapidly and transiently within minutes of stimulation (Table 1). This transcriptional induction is independent of new protein synthesis, suggesting a regulatory role in the cellular response to external stimuli. Activation of immediate early genes is therefore often considered to be the hallmark of stretch-induced hypertrophy.

The induction of immediate early genes such as *c-fos*, *c-myc* and c-Ha-ras by aortic constriction was first reported in rat hearts (Mulvagh *et al.* 1987, Izumo *et al.* 1988, Komuro *et al.* 1988). The induction of the *c-myc* gene also occurs after pressure overload in the feline ventricle (Pollack *et al.* 1994) and stretch of myocytes has been shown to stimulate the expression of *c-fos*, *c-myc*, *c-jun* and Egr-1 *in vitro* as well (for review see Yamazaki *et al.* 1995c). These results suggest that mechanical stretch can directly induce the expression of immediate early genes. Furthermore, it has recently been shown that in end-stage human cardiomyopathy, expression of junD is decreased while *c-jun* mRNA remains unchanged (Pollack *et al.* 1997). The load-response element of the *c-fos* gene promoter was first examined in order to get a clue for understanding the mechanisms of stretch-induced gene expression. It was found that a serum responsive element was required for the efficient transcription of the *c-fos* gene by myocyte stretch (Sadoshima & Izumo 1993a). Stretch induced *c-fos* induction was inhibited by both protein kinase C (PKC) inhibitors and down-regulation of PKC (Komuro *et al.* 1991). There are some data showing that immediate early gene expression in cardiac myocytes is directly associated with the induction of late response genes coding for structural proteins that are known to be upregulated during the development of cardiac hypertrophy. Activator protein 1 (AP-1), the transcriptional factor whose major constituents are Fos and Jun, activates the skeletal α-actin promoter (Bishopric *et al.* 1992), and Fos and Jun also increase the expression of atrial natriuretic peptide (ANP) in cardiac myocytes (Kovacic-Milivojevic & Gardner 1992).

2.1.1.2. Late response load-inducible genes

In the second class of cardiac load-inducible genes, gene expression is induced more slowly, over hours or days, and requires protein synthesis (Table 1). Many of these late response genes are contractile protein genes, like skeletal α-actin and β-myosin heavy chain (β-MHC). Induction of contractile protein genes includes skeletal α-actin and β-MHC that are normally expressed in embryonic development (Schwartz *et al.* 1986, Izumo *et al.* 1987, Izumo *et al.* 1988, Delcayre *et al.* 1992). In contrast to those upregulated genes, some of the cardiac muscle genes are noninducible, like Na^+ channel (Chien *et al.* 1991), and some genes get down-regulation by hemodynamic overload. The mRNA level of Ca^{2+}-ATPase of the sarcoplasmic reticulum is gradually decreased by pressure overload in the animal model (Komuro *et al.* 1989, Nagai *et al.* 1989).

2.1.1.3. Natriuretic peptides

Hypertrophy also results in induction of noncontractile protein genes, such as ANP and B-type natriuretic peptide (BNP) (for reviews see Lang *et al.* 1992a, Nakao *et al.* 1992a, Ruskoaho 1992). ANP, BNP and C-type (CNP) natriuretic peptides are the known members of the mammalian natriuretic peptide system. ANP was first purified and sequenced from atria (Flynn *et al.* 1983, Atlas *et al.* 1984), and some years later BNP and CNP were isolated from porcine brain (Sudoh *et al.* 1988, Sudoh *et al.* 1990). ANP and BNP are cardiac peptide hormones, while CNP is principally found in the central nervous system and vascular endothelial cells (for reviews see Needleman *et al.* 1989, Nakao *et al.* 1992a, Rosenzweig & Seidman 1991, Ruskoaho 1992, Yandle 1994). Natriuretic peptides share a common structural motif, consisting of a 17-amino acid loop formed by an intramolecular disulphide linkage between two cysteine residues and only five of the 17 amino acids in the ring differ among the three peptides (for review see Yandle 1994). The biologically active form of ANP is a 28-amino acid peptide, whose amino acid sequence is highly homologous between species. In contrast, the sequence variability of BNP between species is large, and the predominant circulating forms of BNP are 26, 45 and 32 amino acid peptides in pigs, rats and humans, respectively (for review see Nakao *et al.* 1992a). The mRNA of these peptides is translated to preproANP, preproBNP and preproCNP, and the first 25, 26 and 23 amino acids, respectively, seem to be cleaved as the signal peptide (for review see Nakao *et al.* 1992a). The storage form of ANP in the heart is preANP (Kangawa *et al.* 1985), and the proteolytic cleavage of proANP to N-peptide and active ANP occurs just before or during its secretion (Sugawara *et al.* 1985). The major storage form of BNP in the heart is cleaved mature peptide (Saito *et al.* 1989, Kambayashi *et al.* 1990, Ogawa *et al.* 1990, Mukoyama *et al.* 1991a).

ANP and BNP are natriuretic and diuretic hormones, and cause vasorelaxation. ANP is mainly synthesized in the atria of the normal adult heart, but ventricular hypertrophy is characterized by an augmentation of the synthesis and release of ANP from ventricles (for review see Ruskoaho 1992). BNP is expressed both in atria and ventricles, but is mainly released from ventricles (Hosoda *et al.* 1991, Ogawa *et al.* 1991). The major determinant of ANP and BNP secretion is myocyte stretch (Lang *et al.* 1992a, Ruskoaho *et al.* 1986a,

Kinnunen *et al.* 1993, Mantymaa *et al.* 1993). Raised plasma levels of ANP and BNP are observed in conditions associated with cardiac pressure and volume overload, such as chronic heart failure, acute myocardial infarction and essential hypertension, as well as in chronic renal failure (Mukoyama *et al.* 1990, Mukoyama *et al.* 1991a, Mukoyama *et al.* 1991b, Kohno *et al.* 1993, Naruse *et al.* 1994, Lang *et al.* 1992b). Ventricular levels of BNP mRNA are substantially increased in response to chronic cardiac overload in the human heart (Hosoda *et al.* 1991, Takahashi *et al.* 1992). Similarly, transcription of the BNP gene and BNP peptide levels in the ventricles are increased in experimental models of cardiac overload, including spontaneously hypertensive rats (SHR) (Ogawa *et al.* 1991, Dagnino *et al.* 1992, Kohno *et al.* 1992, Roy *et al.* 1992, Kinnunen *et al.* 1993, Yokota *et al.* 1993) and rats with myocardial infarction produced by coronary artery ligation (Hama *et al.* 1995).

In human heart atrial myocytes, ANP is localized in almost all of the secretory granules, whereas BNP is co-localized with ANP in some of the granules (Nakamura *et al.* 1991). ANP and BNP are co-localized also in ventricular myocytes, although there are very few secretory granules in ventricular myocytes (Nakamura *et al.* 1991). BNP is probably secreted from ventricular cells promptly after synthesis via a constitutive pathway, whereas it is stored in the secretory granules and released via a regulated pathway in the atrium (Lang *et al.* 1992a, Nakao *et al.* 1992a, Ruskoaho 1992, Thibault *et al.* 1992, Nakagawa *et al.* 1995). It has been shown that BNP is co-secreted with ANP from atrial cardiocytes (Iida *et al.* 1990) and that the time course of atrial stretch-induced secretion of these peptides is similar (Mantymaa *et al.* 1993).

Although cardiac overload is known to alter the expression of the ANP and BNP genes, it has not yet been established whether wall stretch acts directly or via local paracrine and autocrine factors liberated in response to hemodynamic load. The local renin-angiotensin system in particular may play an important role, since stretching of cardiac myocytes *in vitro* causes release of angiotensin II (Ang II) acutely (10-30 min), and increases the expression of the angiotensinogen gene in the long term (Sadoshima & Izumo 1993c). Another candidate is endothelin-1 (ET-1), since ET-1 is released rapidly when cultured endothelial cells are stretched (Macarthur *et al.* 1994, McClellan *et al.* 1994). Recently it has been reported that Ang II and ET-1 are not obligatorily required for stretch to trigger BNP gene expression in rat left ventricle, whereas in the left atrium BNP gene expression is ET-1 dependent (Magga *et al.* 1997).

Natriuretic peptide receptors type A (NPR_A), type B (NPR_B) and type C (NPR_C) are identified at present (Koller & Goeddel 1992, Maack 1992, Anand-Srivastava & Trachte 1993). NPR_A and NPR_B receptors are guanylyl cyclase -linked receptors, whereas the NPR_C receptor is not coupled to guanylyl cyclase (Chang *et al.* 1989, Chinkers *et al.* 1989, Schulz *et al.* 1989). ANP and BNP are primary ligands for the NPR_A receptor, and CNP binds preferentially to the NPR_B receptor (for reviews see Nakao *et al.* 1992b, Suga *et al.* 1992). The NPR_C receptor seems to act as a clearance receptor (for review see Anand-Srivastava *et al.* 1993) and the rank order in the binding affinity for the NPR_C receptor is ANP>CNP>BNP, which is consistent with the slower clearance of BNP than ANP from the circulation (Mukoyama *et al.* 1991a). Natriuretic peptide receptor binding sites have been localized to atrial and ventricular endocardium, aorta, pulmonary arteries and epicardial mesothelium (Rutherford *et al.* 1992). The progressive cardiac hypertrophy in the rat produced by aortovenocaval fistula results in increased mRNA levels for NPR_A and

NPR_B receptors in the heart (Brown & Zuo 1993). In contrast, the NPR_C receptor mRNA levels are decreased during the development of cardiac hypertrophy (Brown *et al.* 1993). Since natriuretic peptides are expressed in cardiac tissue, ANP and BNP may have paracrine/autocrine effects.

In addition to the NPR_C receptor, ANP and BNP are metabolized by neutral endopeptidase (EC 3.4.24.11; NEP), which is a zinc-containing membrane-bound enzyme widely distributed in the organism with a high activity in the brush border membranes of proximal renal tubules. NEP is a major metabolizing enzyme for ANP (Olins *et al.* 1987, Sonnenberg *et al.* 1988). Cleavage at the Cys_{105}-Phe_{106} and Ser_{123}-Phe_{124} bonds of ANP by NEP would destroy the essential ring structure and result in biological inactivation of ANP (Olins *et al.* 1987, Koehn *et al.* 1987). This membrane peptidase seems to be largely responsible for the activation of not only endogenous and exogenous ANP but also many other peptides, including BNP, CNP (Erdos & Skidgel 1989) and AM (Lisy *et al.* 1998). Neutral endopeptidase inhibitors prevent ANP from degradation, increase the half-life of ANP and promote ANP-mediated actions like diuresis, natriuresis and vasodilation (Dussaule *et al.* 1993, Lefrancois *et al.* 1990, Kahn *et al.* 1990, Lang *et al.* 1992c, Wegner *et al.* 1996). Plasma BNP concentration has been reported to increase in patients with heart failure after acute administration of an NEP inhibitor (Lang *et al.* 1992c). In aortovenocaval fistular (AVF) rat model of heart failure the neutral endopeptidase inhibitor, ecadotril, improved the reduced kidney function in AVF rats by raising natriuretic peptides in plasma, suggesting useful therapeutic possibilities for ecadotril in the treatment of heart failure (Wegner *et al.* 1996).

2.1.2. Growth factors and mechanical stress

Important mechanisms in the pathogenesis of stretch-induced organ hypertrophy may involve the presence of autocrine/paracrine growth factors (for review see Vandenburgh 1992). Usually, *c-fos* gene expression, mitogen activated protein (MAP) kinase activation, an increase in the rate of protein synthesis and induction of fetal type genes are considered to be major markers of stretch-induced hypertrophy. It has been demonstrated that in cardiac cells, mechanical stretch stimulates production and/or secretion of growth factors, which may mediate mechanical stretch-induced cell growth responses. Particularly, the local renin-angiotensin system (RAS) may play an important role in the adaptation of the heart to pressure and volume overload (for reviews see Lindpaintner & Ganten 1991, Baker *et al.* 1992). There is also evidence of the existence of a cardiac RAS in the heart (for reviews see Lindpaintner & Ganten 1991, Baker *et al.* 1992, Dostal *et al.* 1992, Dostal & Baker 1999), and angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor type 1 (AT_1 receptor) antagonists produce significant regression of left ventricular hypertrophy in patients with hypertension and in animal models of pressure or volume overload (Sen *et al.* 1980, Dunn *et al.* 1984, Baker *et al.* 1990, Ruzicka *et al.* 1993, Kojima *et al.* 1994, Ruzicka & Leenen 1995, Bruckschlegel *et al.* 1995).

2.1.2.1. Angiotensin II

The role of Ang II as a mediator of stretch-induced hypertrophy has been studied in the neonatal rat cardiac myocyte system *in vitro*. It has been shown in cultured neonatal rat heart cells that Ang II directly causes the hypertrophy of cardiac myocytes and the hyperplasia of cardiac fibroblasts, and it induces the fetal phenotype of genes in cardiac myocytes (Sadoshima & Izumo 1993b). Saralasin (an antagonist of both AT_1 and AT_2 receptors), and losartan and CV-11974 (an AT_1 receptor selective antagonist) have been shown to suppress activation of MAP kinases, and MAP kinase activators stimulated by stretching of myocytes (Kojima *et al.* 1994, Shyu *et al.* 1995, Yamazaki *et al.* 1995b). Moreover, cyclical stretch-induced expression of the mRNA angiotensinogen, the precursor of Ang II, has been reported to be suppressed by the losartan treatment (Sadoshima *et al.* 1993c). In addition, increases have been found in myocardial angiotensinogen mRNA levels, ACE activity, rate of Ang II production and AT_1 receptor mRNA levels in pressure-overload cardiac hypertrophy in rats (Baker *et al.* 1990, Schunkert *et al.* 1990, Suzuki *et al.* 1993b). Mechanical stretch of neonatal rat cardiac myocytes has been shown to induce Ang II secretion (Lin *et al.* 1995, Yamazaki *et al.* 1995a, Sadoshima *et al.* 1993c), and Ang II causes an increase in protein synthesis, protein content, immediate-early gene expression, fetal gene expression and growth factor gene expression in neonatal rat cardiac myocytes (for review see Sadoshima *et al.* 1997). Furthermore, Ang II activates many signal transduction pathways including tyrosine kinases, MAP kinases, ribosomal S6 kinases, Ras, inositol-1,4,5-triphosphate (IP_3), Ca^{2+}, 1,2-diacylglycerol (DAG), PKC, phosphatidic acid, phosphoinositide phospholipase D (PLD), arachidonic acid release and cAMP (for review see Sadoshima *et al.* 1997). These results suggest that Ang II may play a critical role in stretch-induced hypertrophy in the neonatal rat myocyte culture system. Recently oral administration of ACE inhibitor and AT_1 receptor antagonist has been shown to decrease mRNA levels for skeletal α-actin, ANP, type-β1 transforming growth factor (TGF-β1) and collagen types I, II and IV and increase α-myosin heavy chain (α-MHC) mRNA in the left ventricle of adult stroke-prone SHRs (Kim *et al.* 1996). In contrast, mechanical stetch was observed to induce hypertrophic responses, such as activation of MAPK and *c-fos* gene, in cardiac myocytes of AT_1 receptor knockout mice (Kudoh *et al.* 1998). Moreover, pressure overload was observed to induce cardiac hypertrophy in AT_1 receptor knockout mice, suggesting that AT_1-mediated signaling is not essential in pressure overload-induced cardiac hypertrophy (Harada *et al.* 1998).

2.1.2.2. Endothelin

An important mediator of heart hypertrophy could be ET-1, which is a 21-amino acid peptide with diverse cardiovascular effects, including potent vasoconstrictor properties on resistance and capacitance vessels as well as positive inotropic effects. Furthermore, ET-1 has been shown to act as a potent growth factor in several cells *in vitro*, including fibroblasts and cardiomyocytes. ET-1 has also been shown to induce several phenotypic markers of hypertrophy in isolated cardiomyocytes (Rubanyi & Polokoff 1994). It has

been demonstrated that ventricular expression of ANP mRNA due to aortic constriction was partially blocked at 1 week by subcutaneous administration of the type A endothelin receptor (ET_A receptor) antagonist BQ 123 (Ito *et al.* 1994). Another ET_A receptor antagonist, FR139317, was shown to block the hypertrophic response to the pressure increase within 21 days (Ichikawa *et al.* 1996). Pressure overload due to aortic constriction (Ito *et al.* 1994, Arai *et al.* 1995) or pulmonary hypertension (Miyauchi *et al.* 1993) and norepinehrine-induced ventricular hypertrophy (Kaddoura *et al.* 1996) have been shown to be associated with large increases in ventricular expression of ET-1 mRNA. Production of ET-1 has been shown to increase in the hypertrophied rat heart due to pressure overload as well (Yorikane *et al.* 1993), further suggesting that endogenous cardiac production of ET-1 may play a functional role in mechanical load-induced cardiac gene expression. ET-1 is synthetized in and secreted from cardiac myocytes (Ito *et al.* 1993b) and ET-1 is released rapidly when cultured endothelial cells are stretched (Macarthur *et al.* 1994, McClellan *et al.* 1994). ET-1 has potent effects on cell growth and induces hypertrophy of cultured cardiac myocytes (Shubeita *et al.* 1990, Ito *et al.* 1991a). It has also been suggested that ET-1 mediates the effects of hypertrophic stimuli such as Ang II *in vitro* (Ito *et al.* 1993b) and norepinephrine *in vivo* (Kaddoura *et al.* 1996). In addition, ET-1 has been shown to potentiate Ang II-induced activation of Raf-1 and MAP kinase through an ET_A receptor-dependent mechanism (Yamazaki *et al.* 1996). Recently it has been reported that ET-1 has an important role in cardiac overload-induced BNP gene expression in the left atrium of rat heart (Magga *et al.* 1997).

2.1.2.3. Others

Stretch causes the gene expression and release of other growth factors such as insulin-like growth factor-I (IGF-I), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) (Kudoh *et al.* 1998, Goldspink *et al.* 1995, Malek *et al.* 1993). These growth factors may also be capable of causing hypertrophy or modulating actions of other paracrine factors such as Ang II and ET-1. In support of this, TGF-β has been demonstrated to mediate the stretch-induced activation of vascular endotelial growth factor (VEGF) gene expression (Li *et al.* 1997) in perfused rat heart preparation.

2.1.3. Mechanosensors

The central problem in understanding the molecular basis for cardiac hypertrophy is to understand how mechanical input is transduced into a growth response. It is unknown which molecules are directly activated by stretch in the cardiac myocytes, and which molecules are indirectly activated by other modulators. Stretch-activated ion channels of the plasma membrane have been suggested to be the primary stretch sensors in different types of mechanotransductory processes (Fig. 1)(for review see Morris 1990). A number of stretch-activated ion channels have been found using the single-channel, patch-clamp technique, where the open probability of the channel is increased by negative pressure

applied in the suction patch electrode (Penner & Neher 1989). These channels include nonselective cation channels and K^+ channels with different ionic conductances, and a trivalent lanthanide, gadolinium (Gd^{3+}), has been shown to block these channels (Sadoshima *et al.* 1992, Sigurdson *et al.* 1992, Pollack *et al.* 1997). Gd^{3+} has been shown to block the stretch-induced increase in BNP mRNA levels in isolated rat atrium, suggesting that Gd^{3+} -sensitive stretch-activated channels may be involved in stretch-induced hypertrophic responses (Laine *et al.* 1996). However, treatment of myocytes with Gd^{3+} does not affect stretch-induced *c-fos* expression or a stretch-induced increase in the rate of protein synthesis (Sadoshima *et al.* 1992). Mechanical stress caused by whole-cell stretch or hypotonic swelling affects a wide variety of channels and currents in the heart, including K^+-ATP channel (Van Wagoner 1993), a delayed K^+ rectifier channel (Sasaki *et al.* 1992), a Cl^- channel (Hagiwara *et al.* 1992), an L type Ca^{2+} channel (Matsuda *et al.* 1996), the Na^+/K^+ pump current (Sasaki *et al.* 1994) and Na^+/H^+-exhanger (Yamazaki *et al.* 1998), which may be involved in stretch-induced hypertrophic responses. In addition, there is an immediate up-regulation of Na^+-Ca^{2+} exchanger gene expression with cardiac loading (Kent *et al.* 1993). It has also been suggested that stretch-activated channels directly interact with the cytoskeleton, and thus may intrinsically sense the cell stretch (Hamill *et al.* 1992).

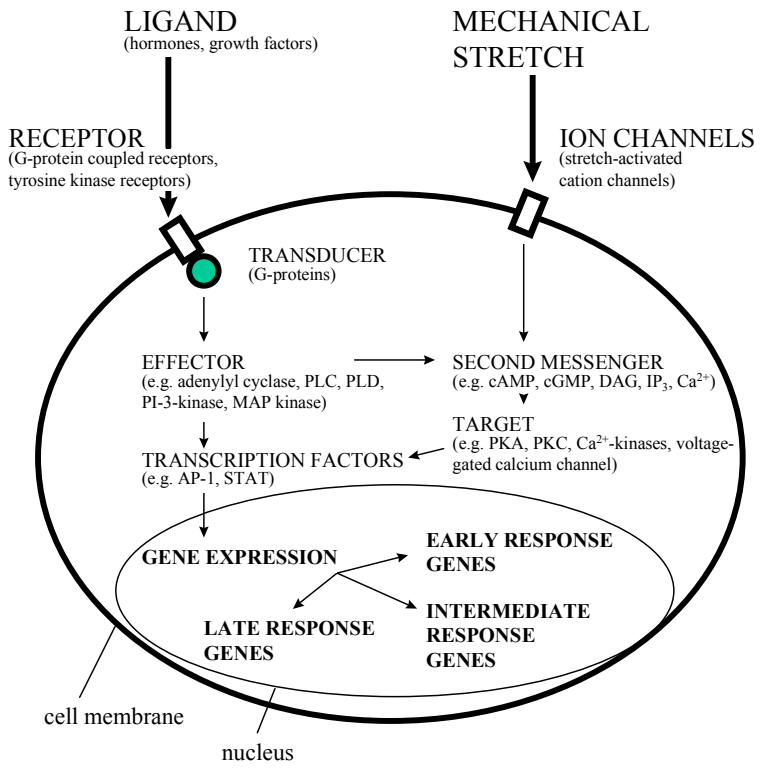

Fig. 1. A model of stretch-activated gene expression in cardiac myocyte.

2.1.4. Signal transduction pathways activated by mechanical stretch

Signal transduction explains cell behavior by elucidating the molecular events by which extracellular signals finally elicit an intracellular response. As shown in Fig. 1., hormones, growth factors and autocrine/paracrine factors act by binding to specific receptors to initiate diverse cellular events. Second messengers are transient molecules that mediate the transduction of the extracellular signals and they are usually generated from a pre-existing precursor strictly in response to a specific signal. Myocytes and the surrounding cells release various autocrine or paracrine factors, such as growth factors, hormones, neurotransmitters, and autacoids, and most of these signalling molecules do not enter the cell but affect receptors at the cell surface.

Mechanical stress-induced signal transduction is characterized by simultaneous activation of multiple second messenger systems (for reviews see Vandenburgh 1992, Sadoshima & Izumo 1993a). Since it is difficult to elucidate the signal transduction pathways in intact animal, the majority of signaling pathways have been identified in cardiomyocytes. In cultured neonatal cardiac myocytes, mechanical stretch has been shown to cause activation of phosphoinositide phospholipase C (PLC), PLD, and phosphoinositide phospholipase A_2 (PLA_2), tyrosine kinases, Ras, Raf-1 kinase, MAPK/extracellular signal regulated kinase (MEKK), MAP kinases and their activators, c-Jun N terminal kinase (JNK), ribosomal S6 kinase and PKC (Sadoshima *et al.* 1992, Sadoshima & Izumo 1993a, Yamazaki *et al.* 1993, Yamazaki *et al.* 1995b). Mechanical stretch has been shown to stimulate the activation of all components of the Raf-MEK-extracellular signal regulated kinase (ERK) signaling cascade in neonatal cardiac myocytes, mainly through the secreted Ang II (Sadoshima & Izumo 1993a, Yamazaki *et al.* 1995a). Mechanical stretch has been shown to activate the MEKK-JNKK-JNK/stress-activated protein kinase (SAPK) pathway as well (Komuro *et al.* 1996). Unlike ERKs, the activation of SAPKs by stretch was not dependent on the secreted Ang II. Furthermore, the chelation of extracellular Ca^{2+} or down-regulation of PKC did not attenuate activation of SAPKs. In addition to the JNKK-JNK/SAPK pathway, MEKK also activates ERKs through MEK (Komuro *et al.* 1996). It has been demostrated in cultured porcine aortic endothelial cells that shear stress-induced increases in ET-1 mRNA levels may be mediated by the disruption of actin fibers. Furthermore, this induction of ET-1 gene expression occurs in a PKC-independent manner (Morita *et al.* 1994).

Receptor type tyrosine kinases have transmembrane segments, and some of the nonreceptor-type tyrosine kinases, such as the Src family tyrosine kinases, are anchored to the inner surface of cell membranes (for review see Sadoshima *et al.* 1997). It is therefore possible that membrane stretch directly causes conformational changes of tyrosine kinases, thereby activating them. In support of this hypothesis, mechanical stretch of cardiac myocytes is shown to cause a rapid increase in the phosphotyrosine content of proteins such as p42, p44, p60, p70, p85, p120, and p170 within 1 min (Sadoshima & Izumo 1993a). However, the mechanism of tyrosine kinase activation by mechanical stretch is unknown at present.

Second messengers generated by different pathways can activate DNA-binding proteins (for review see Hill *et al.* 1995), which specifically regulate gene expression. The transcription factors, such as ternary complex factor (TCF), cAMP response element binding protein (CREB/ATF), AP1, signal transducer and activator of transcription

(STAT), NF-κB, GATA family and Nkx2-5 are targets for signaling pathways. These transcription factors must be located in the nucleus, bind DNA, and interact with the basal transcription apparatus to activate or repress transcription. Different signals may activate different transcription factors, but they may also converge on the same factor. A number of transcription factors contain signal-regulated transcriptional activation domains, and regulated phosphorylation may facilitate their interaction with the basal transcriptional machinery or coactivator proteins. This activation of transcription factor can occur in the nucleus, at the cell membrane, or in the cytoplasm. Both ERK and JNK/SAPK pathways can activate transcription factors in the nucleus. The JAK/STAT pathway is an example of the activation at the membrane. STAT proteins are phosphorylated by tyrosine kinase family JAK and migrate to the nucleus. NF-κB is activated in the cytoplasm by many agents, including tumor necrosis factor α and activation of PKC. Finally, the signal-regulated transcriptional activation is complicated by the fact that activation of a single transcriptional factor may require signaling via more than one signal pathway (for review see Hill *et al.* 1995).

2.2. Adrenomedullin

2.2.1. Discovery and gene cloning

AM was discovered and isolated from human pheochromocytoma extracts (Kitamura *et al.* 1993a). This 52-amino-acid peptide was observed to stimulate adenylyl cyclase activity in a platelet bioassay (Kitamura *et al.* 1993a). AM showed 27 % homology with the calcitonin gene-related peptide (CGRP) family (Fig. 3) and was found to have potent and long-lasting vasorelaxing effect (Kitamura *et al.* 1993a). The AM gene was cloned from a cDNA library from the human pheochromocytoma tissue (Kitamura *et al.* 1993b). As shown in Fig. 2., the human preproAM is 185 amino acids in length and is then processed to a 164-amino acid peptide, which is called proAM. The biologically active peptide is 52 amino acids in length and formed from proAM (Kitamura *et al.* 1993b). Suprisingly, it was found that the preproadrenomedullin contained a unique 20-residue sequence followed by Gly-Lys-Arg in the N-terminal region. This peptide was termed proadrenomedullin N-terminal 20 peptide (PAMP) (Kitamura *et al.* 1993b) and observed to have a dose-dependent hypotensive effect in rats (Kitamura *et al.* 1994b).

Fig. 2. Scheme of human adrenomedullin (AM) and human proadrenomedullin N-terminal peptide (PAMP) synthesis from preproadrenomedullin.

AM has a unique 6-residue ring structure and C-terminal amidation, which is similar to CGRP and amylin. The genomic DNA of human AM consists of 4 exons and 3 introns, and the 5´flanking region contains TATA, CAAT and GC boxes (Ishimitsu *et al.* 1994). The fourth exon codes for the mature form of AM (Ishimitsu *et al.* 1994). There are also multiple binding sites for activator protein-2 (AP-2) and cAMP regulated enhancer element (Ishimitsu *et al.* 1994). Southern blot analyses have indicated that the human AM gene is situated in a single locus on chromosome 11(Ishimitsu *et al.* 1994). Rat AM was cloned and it differs from human AM in only six positions and is 50 amino acids in length (Sakata *et al.* 1993). Human and rat PAMP are 20 amino acids in length and differ in three positions (Sakata *et al.* 1993).

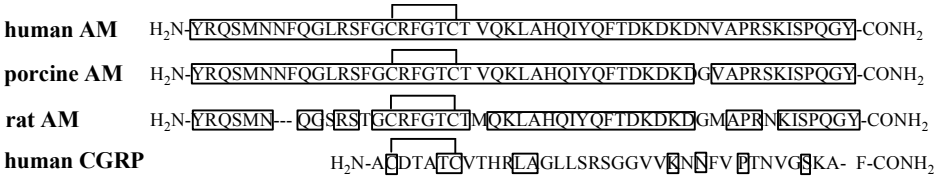

Fig. 3. Comparison of amino acid sequence of human adrenomedullin (AM), porcine AM, rat AM and human calcitonin gene-related peptide (CGRP). Identical residues between human AM and other peptides are boxed.

2.2.2. AM receptors

Initially, the pharmacological actions of AM were thought to be mediated through the CGRP receptor, because of the significant structural homology between AM and CGRP and because some of the actions of AM were blocked by CGRP antagonist. However, today there are controversial results concerning AM receptor(s). Originally, AM was shown to generate increased production of cAMP from rat vascular smooth muscle cells (VSMC) (Ishizaka *et al.* 1994) and in the same study, specific binding sites for ^{125}I-AM were competitively inhibited by human AM, but not CGRP. However, in this experiment $CGRP_{8-37}$, a CGRP antagonist, competitively inhibited cAMP production by AM. The vasorelaxant effect of AM in cerebral arterioles and mesenteric arteries was also blocked by CGRP 8-37 (Mori *et al.* 1997, Lang *et al.* 1997, Berthiaume *et al.* 1995, Nuki *et al.*

1993). On the other hand, the blood pressure-lowering effect of infused AM was not blocked by $CGRP_{8-37}$ (Nandha *et al.* 1996, Haynes *et al.* 1995, Hjelmqvist *et al.* 1997). Moreover, the effect of AM to increase renal blood flow in isolated or *in situ* kidney was not blocked by $CGRP_{8-37}$ (Haynes *et al.* 1995, Gardiner *et al.* 1995). Owji *et al.* confirmed the presence of an abundant and specific binding site for AM in rat heart, lung, spleen, liver, skeletal muscle and spinal cord, with lesser binding in thyroid, adrenal, stomach, kidney and central nervous system (Owji *et al.* 1995). The binding was highest in rat heart and lung. In lung this binding was inhibited only by AM, whereas the binding in the heart was competitively inhibited by amylin and CGRP, suggesting regional variation in AM receptor specificity. In structure and activity studies (Eguchi *et al.* 1994) performed in rat VSMC cultures, human AM induced cAMP production that was augmented by GTP. Human AMs 1-52, 13-52 and 16-52 all inhibited equally binding of radiolabeled AM and caused similar stimulation of cAMP. C-terminal deletions of human AM exhibit reduced receptor binding and reduced cAMP production. Cysteine-to-cysteine six amino acid ring structure deletions of human AM exhibit no binding. These results confirm that AM receptors are coupled to adenylate cyclase via G-protein and that both the cyclic ring structure and C-terminal amidation are essential for full binding and activity.

The cDNA for AM receptor has been cloned from rat lung tissue (Kapas *et al.* 1995). This cDNA codes a polypeptide of 395 residues which possesses seven putative, α-helical transmembrane domains. Thus the AM receptor resembles other members of the G-protein linked receptor super-family. However, the degree of interaction by AM and CGRP with one others receptor has been unclear. In rat kidney mRNAs for AM and AM-receptor were found in renal vessels, glomeruli, and inner medullary collecting ducts. Ten days of feeding a low-salt (0.02%) or a high-salt diet (4%) did not change AM mRNA or AM-receptor mRNA in any kidney zone (Jensen *et al.* 1998). Most recently, McLatchie *et al.* (McLatchie *et al.* 1998) reported that receptor-activity-modifying proteins (RAMPs) are regulating the specificity of CGRP receptor. The CGRP receptor can function as either AM or CGRP receptor depending on which members of a new family of single-transmembrane-domain proteins, RAMPs, are expressed. RAMP1 presents the receptor at the cell surface as a mature glycoprotein and a CGRP receptor. RAMP2-transported receptors are core-glycosylated and function as AM receptors. Therefore, AM receptors and tissue distribution need to be studied further.

2.2.3. Biological effects of AM

Two hallmark actions of AM, hypotension and diuresis/natriuresis, suggest that adrenomedullin plays an important role in cardiorenal regulation (for reviews see Richards *et al.* 1996, Samson 1999). AM has important effects on renal function (for review see Samson 1999). In the brain AM inhibits water intake (Murphy & Samson 1995) and, in a physiologically relevant manner, salt appetite (Samson & Murphy 1997). Recently, AM antisense oligonucleotide treatment significantly lowered AM peptide content in the hypothalamic paraventricular nucleus and exaggerated the consumption of sodium, supporting a physiological role for AM in the central regulation of sodium homeostasis (Samson *et al.* 1999). Intrarenal infusion of AM in anesthetized dogs caused significant

increases in renal blood flow (RBF), urine flow and sodium excretion in a dose-dependent manner without changes in heart rate or mean arterial pressure (MAP) (Ebara *et al.* 1994). These results indicated direct vascular and tubular effects of AM. In further studies, it was found that the effects of AM on RBF were mediated by nitric oxide (Miura *et al.* 1995). In anesthetized rats, intrarenal AM infusion had similar effects as in dogs, increases in RBF, arterial conductance, glomerular filtration rate (GFR), urine flow and sodium excretion (Haynes *et al.* 1995, Vari *et al.* 1996, Elhawary *et al.* 1995), and these effects were not blocked by a CGRP antagonist (Elhawary *et al.* 1995). Increases in RBF and urine flow were maintained also during systemic infusion of AM, which was associated with a marked decrease in MAP (Vari *et al.* 1996) and an increase in NO release in the kidney (Hirata *et al.* 1995).

Binding sites for AM and PAMP have been found in outer cortex and medulla of human adrenal gland (Nussdorfer *et al.* 1997). AM has been reported to inhibit angiotensin II- and potassium-stimulated, but not basal- or ACTH-stimulated, aldosterone release from cultured rat adrenal cells (Yamaguchi *et al.* 1996). Moreover, intravenous infusion of AM has been described to lower circulating cortisol levels and ACTH in sheep (Parkes *et al.* 1995).

Intravenous infusion of AM has been reported to cause prolonged hypotension in cat, rat, rabbit, sheep and humans (Ishiyama *et al.* 1995, Parkes *et al.* 1997, Hjelmqvist *et al.* 1997, Lainchbury *et al.* 1997, Parkes *et al.* 1995, Feng *et al.* 1994, Fukuhara *et al.* 1995, Nakamura *et al.* 1997, Parkes 1995, Shirai *et al.* 1997, Champion *et al.* 1997), largely through the generation of nitric oxide (NO) in the vasculature (Miura *et al.* 1995, Hirata *et al.* 1995, Feng *et al.* 1994). Bolus injections of AM (0.1-3.0 nmol/kg) in anesthetized SHR and Wistar-Kyoto (WKY) rats caused significant decreases in blood pressure (24-92 mmHg in SHRs vs 12-62 mmHg in WKY rats). In the same study, constant infusion of AM (0.03 nmol/min/kg) for 30 min reduced blood pressure 23 % and 20 % in SHR and WKY rats, respectively, without any effect on heart rate. In conscious rabbits, bolus injections of AM (10 and 3000 pmol/kg) dose-depently decreased MAP up to 27 % and the decrease in MAP was associated with an increase in heart rate (Fukuhara *et al.* 1995). In conscious SHR and SD rats, intravenous high dose (1670 and 5000 ng/kg) bolus injections of AM decreased blood pressure in both groups (He *et al.* 1995), while heart rate and cardiac output (CO) were increased. In the same study, using radioactive microspheres, AM infusion was shown to increase blood flow in the lungs, spleen, kidneys, adrenal glands and small intestine. The flow rates in brain and skin remained unchanged, whereas the flow rates were decreased in skeletal muscle and testes. In conscious sheep, 90 min AM infusion at 100 ng/min/kg reduced MAP by 12 mmHg, increased heart rate by 20 beats/min and CO 3 l/min. Moreover, plasma renin activity was elevated during AM infusion, whereas plasma aldosterone was not affected, and plasma norepinephrine levels fell (Charles *et al.* 1997). Recently, AM infusion at low (0.01 µg/kg/min) and high (0.05 µg/kg/min) doses was observed to exert diuresis and natriuresis without hypotension in experimental heart failure rats (Nagaya *et al.* 1999a). Moreover, in this study CO, increased suggesting a beneficial role for AM in heart failure. The increased heart rate is not secondary to the hypotension, because increased contractility was also observed in conscious sheep cardiac when pressure was maintained constant (Parkes 1995). In humans, intravenous infusion of AM (2 ng and 8 ng/min/kg) reduced blood pressure, but did not have any effect on urine volume and electrolyte excretion

(Lainchbury *et al.* 1997). Intravenous injection of DNA constructs containing the human AM cDNA fused to the cytomegalovirus promoter, induced a long-lasting reduction in blood pressure in SHR and the maximal decrease in blood pressure was 22 mmHg (Chao *et al.* 1997).

The effect of AM has been studied in isolated organs (for reviews see Richards *et al.* 1996, Samson 1999). AM was reported to have a vasodilatory effect on perfused rat mesenteric vascular bed and this vasodilatation was inhibited by $CGRP_{8-37}$ (Nuki *et al.* 1993). In this study atropine and propranolol did not have any effect on AM-induced vasodilatation, suggesting that AM induced noncholinergic and nonadrenergic vasodilatation. AM has been shown to induce a dose-dependent vasorelaxation in basilar (Baskaya *et al.* 1995), mesenteric, coronary, renal and femoral arteries isolated from the dog (Nakamura *et al.* 1995). It is noteworthy that vasorelaxing effects were slightly greater in endothelium-intact arteries than in denuded arteries (Nakamura *et al.* 1995). In the rat isolated perfused kidney, AM caused a dose-dependent vasodilator response which was blocked dy $CGRP_{8-37}$ (Haynes *et al.* 1995, Gardiner *et al.* 1995). In isolated rat hearts bolus infusion of AM caused a dose-dependent and long-lasting coronary artery vasodilation, which was markedly attenuated by CGRP (8-37) (Entzeroth *et al.* 1995). Moreover, AM induced dose-depently an increase in developed tension in isolated perfused heart preparation, indicating a positive inotropic effect for AM (Szokodi *et al.* 1998). This effect was not altered by $CGRP_{8-37}$ or cAMP-dependent protein kinase inhibitor.

Table 2. Important biological functions of adrenomedullin

Tissue or cell type	Function
Platelet	cAMP elevation
Vasculature	vasodilation and hypotension stimulation of nitric oxide synthesis inhibition of of VSMC proliferation
Heart	inotropic effect coronary artery vasodilation
Kidney	increase in RBF natriuresis and diuresis increase in nitric oxide synthesis
Adrenal gland	inhibition of aldosterone secretion inhibition of cortisol secretion
Pituitary	inhibition of ACTH secretion
CNS	inhibition of salt appetite inhibition of water drinking

Both proliferative and antiproliferative effects of AM have been observed *in vitro*. In rat VSMCs, enhancement of thymidine incorporation and cell number by fetal calf serum

is inhibited by AM and the inhibitory effect of AM was blocked by $CGRP_{8-37}$ (Kano *et al.* 1996). Furthermore, in cultured human coronary artery VSMCs, angiotensin-II stimulated cell migration was inhibited by AM (Kohno *et al.* 1997). These results suggest a role for AM in preventing pathological vascular remodeling. Moreover, AM blocked mitogen-stimulated mesangial cell proliferation in cell culture via the cAMP pathway (Chini *et al.* 1995). In contrast, in Swiss 3T3 fibroblast cell culture AM was observed to stimulate DNA synthesis and cell proliferation (Withers *et al.* 1996).

2.2.4. Gene expression of AM

AM mRNA has been found in several human tissues including adrenal medulla, cardiac ventricle, lung and kidney (Kitamura *et al.* 1993b). Using *in situ* hybridization, the expression of AM mRNA was examined in various tissues in rat and mouse. Intense expression of AM mRNA was observed in endometrium and epithelial cells lining the uterus and mouse adrenal medulla. Moderate levels of expression were detected in kidney glomerulus and cortical distal tubules, ovarian corpus luteum and follicles, epithelial cells lining the bronchioles, cardiac atrium and ventricle, posterior pituitary, stomach, small intestine, spleen and pancreas. Lower levels were observed in pulmonary alveoli, anterior pituitary and submandibular gland (Cameron & Fleming 1998). Rat and porcine vascular endothelial cells have been reported to synthetize and secrete AM (Sugo *et al.* 1994b). The most abundant transcription of AM is detected in rat endothelial cells, at an intensity 20 to 40 fold higher than that in the adrenal gland (Sugo *et al.* 1994a).

The changes in AM gene expression have been studied under different physiological and pharmacological stimuli *in vitro* and *in vivo* (for reviews see Richards *et al.* 1996, Samson 1999). By far the best-characterized activators of AM gene expression are cytokines and growth factors. In cultured VSMCs lipopolysaccharide (LPS), interleukin-1 (IL-1) and tumor necrosis factor alfa (TNF-α) stimulated AM gene expression. IL-1 and TNF-α were the most powerful, resulting in a 5 to 6-fold increase in AM gene expression at 14 hours (Sugo *et al.* 1995). This is most likely mediated via induction of the nuclear factor for interleukin-6 expression (NF-IL6) (Ishimitsu *et al.* 1998). Moreover, AM gene expression has been reported to be stimulated by IL-1 and TNF-α at 24 and 48 hours in both cultured rat cardiac myocytes and non-myocytes (Horio *et al.* 1998). In rats, LPS administration for 3 hours elevates AM gene expression 2 to 7-fold in aorta, lung, adrenal gland, skeletal and cardiac muscle, ileum/jejunum, brain and kidney (Shoji *et al.* 1995).

Adrenocortical steroids were observed to increase AM mRNA levels in cultured VSMCs (Imai *et al.* 1995, Minamino *et al.* 1995), and the increase in AM mRNA levels was enhanced in the presence of cycloheximide, whereas actinomycin D completely suppressed the AM gene expression (Minamino *et al.* 1995). These data indicated a rapid turnover of AM mRNA. Recently, Ando *et al.* showed that oxidative stress stimulates AM gene expression in cultured rat VSMCs at 24 hours (Ando *et al.* 1998). In contrast, shear-stress has been reported to down-regulate AM gene expression in cultured human aortic endothelial cells (Shinoki *et al.* 1998). In the rat model of cerebral ischemia, AM mRNA levels were reported to increase up to 20-fold (Wang *et al.* 1995). In hyperthyreoid rats,

AM gene expression is increased in the lung compared to hypothyreoid rats (Murakami *et al.* 1998).

Cardiac AM gene expression responses to various stimuli have been studied in different animal models (for reviews see Richards *et al.* 1996, Samson 1999). Jougasaki *et al.* reported that in experimental congestive heart failure produced by rapid ventricular pacing in dogs, ventricular AM is activated during the progression of heart failure (Jougasaki *et al.* 1997). Furthermore, there was a positive correlation between left ventricular mass index and left ventricular AM concentrations, suggesting that ventricular AM expression may be increased by the ventricular hypertrophy in this model of heart failure (Jougasaki *et al.* 1997). Left ventricular AM mRNA levels were also significantly increased, 40 % by left coronary artery ligation in rats at 28 days (Kaiser *et al.* 1998), 28-34 % in hypertension of Dahl salt-sensitive rats on high-salt diet at 3 weeks (Shimokubo *et al.* 1996) and 25 % during aortic banding in SD rats at 1 day (Morimoto *et al.* 1999). The right ventricular AM mRNA and ir-AM levels were increased in rat experimental pulmonary hypertension (Shimokubo *et al.* 1995).

Although cardiac overload (Nishikimi *et al.* 1997a) and rapid ventricular pacing (Jougasaki *et al.* 1997) are known to alter the expression of the AM gene, it has not yet been established whether wall stretch acts directly or via local paracrine and autocrine factors liberated in response to hemodynamic load. Little is known about the AM gene expression in cardiac atria, although AM immunostaining was observed to be more intense in rat cardiac atria than ventricles (Jougasaki *et al.* 1995a). In addition, the time-course of cardiac AM transcription in the acute phase of cardiac overload is unclear.

2.2.5. Production and release of AM

The tissue distribution of ir-AM and AM mRNA have been widely examined in rat, man, pig and mouse (for reviews see Richards *et al.* 1996, Samson 1999, Eto *et al.* 1999). Ir-AM is detectable in multiple human tissues including adrenal medulla, heart, aorta, kidney, brain, lung, gastrointestinal organs, spleen, brain and thyroid (Ichiki *et al.* 1994, Satoh *et al.* 1997, Washimine *et al.* 1995a). The highest tissue concentrations of AM are found in the adrenal. Next in ranking is cardiac atrium, having only 3-4 % of the concentration present in adrenal (Ichiki *et al.* 1994). In the rat, ir-AM is present in various tissues, and in high concentrations in the adrenal gland, lung and cardiac atrium (Sakata *et al.* 1994). The AM immunostaining in the atria of canine heart is more intense than in the ventricles (Jougasaki *et al.* 1995a).

In humans, AM is circulating in the low pg/ml range (Kitamura *et al.* 1994a, Sato *et al.* 1995). The plasma half-life of AM is estimated to be about 22 minutes in humans (Meeran *et al.* 1997). In humans, the concentration of circulating AM has been shown to be increased in patients with congestive heart failure (Jougasaki *et al.* 1995b, Kato *et al.* 1996, Kobayashi *et al.* 1996b), essential hypertension (Kitamura *et al.* 1994a, Ishimitsu *et al.* 1994, Kohno *et al.* 1996b), chronic renal failure (Ishimitsu *et al.* 1994, Washimine *et al.* 1995b), pulmonary hypertension (Shimokubo *et al.* 1995, Nishikimi *et al.* 1997b), myocardial infarction (Miyao *et al.* 1998, Yoshitomi *et al.* 1998), septic shock (Hirata *et al.* 1996, Nishio *et al.* 1997), thyreotoxicosis (Taniyama *et al.* 1996) and acute asthma

(Kohno *et al.* 1996b). In hypertension plasma AM is increased and the increase has been reported to be pronounced in hypertension with target organ damage (Ishimitsu *et al.* 1994). Furthermore, it has been reported that hypertension-induced plasma AM levels did not fall with effective treatment of hypertension (Kohno *et al.* 1996b). In chronic renal impairment plasma AM levels are 2 to 3-fold compared to normal control values depending of the severity of renal impairment (Ishimitsu *et al.* 1994). In human heart failure plasma AM levels have been reported to be elevated (Jougasaki *et al.* 1995b, Kato *et al.* 1996, Kobayashi *et al.* 1996b) and the plasma AM levels decreased with effective treatment (Kita *et al.* 1998). Moreover, Jougasaki *et al.* demonstrated that immunohistochemical staining of AM is significantly increased in the failing human ventricular myocardium compared with the normal human ventricle (Jougasaki *et al.* 1995b). Failing human ventricular myocardium has been shown to secrete AM (Jougasaki *et al.* 1996). In acute myocardial infarction (AMI), plasma levels of AM rose to a peak after 48-72 h and remained above preinfarction level for up to 3 weeks (Kobayashi *et al.* 1996a). Moreover, plasma AM on day 2 after AMI was found to be associated with long-term mortality, and AM could thus be used as a prognostic indicator (Nagaya *et al.* 1999b). In rats, circulating AM is increased in hypertension of Dahl salt-sensitive rats on a high-salt diet (Shimokubo *et al.* 1996), in experimental pulmonary hypertension (Shimokubo *et al.* 1995) and in heart failure rats (Nishikimi *et al.* 1997a).

2.3. C-type natriuretic peptide

CNP was originally isolated as a 22-amino-acid peptide from the porcine brain (Sudoh *et al.* 1990). The rat CNP precursor molecule, preproCNP, consists of 126 amino acid residues. It is a product of a gene that is distinct from the ANP or BNP genes and is highly conserved among species, with over 95 % homology among rat, human and porcine forms (for review see Nakao *et al.* 1992a). The biologically active CNP consists of 22 amino acids. The N-terminal-extended form, CNP-53, is the major form in porcine brain (Minamino *et al.* 1990). Immunoreactive CNP is found throughout the brain in the rat and humans, but outside the central nervous system little immunoreactive CNP is detected (Komatsu *et al.* 1991). However, in human breast tissue CNP immunostaining has been localized to vascular endothelial cells of small caliber arteries (Heublein *et al.* 1992). Using the RT-PCR method, transcripts of the CNP gene were detected in rat heart and kidney (Vollmar *et al.* 1993, Suzuki *et al.* 1993a), and CNP-like immunoreactivity has been found in human plasma using specific radioimmunoassay (Stingo *et al.* 1992b). CNP is synthesized and released from cultured endothelial cells (Suga *et al.* 1992). In cultured bovine endothelial cells both ANP and BNP stimulate CNP synthesis and secretion (Nazario *et al.* 1995). Other potent stimuli of CNP synthesis and secretion are cytokines and growth factors. TGF-β has been observed to induce an over 2-fold increase in CNP secretion in vascular endothelial cells (Suga *et al.* 1992). Moreover, CNP secretion is stimulated by TNF-α, IL-1, LPS, basic fibriblast growth factor (bFGF) and thrombin (Suga *et al.* 1993). Therefore, major components of the vascular wall, such as endothelial cells, platelets, and macrophages, secrete a number of factors that play a role in CNP synthesis and release. CNP acts as a specific agonist of NPR_B (Suga *et al.* 1992), which

has been found in almost all tissues studied, including brain, kidney, aorta, liver, lung (Tallerico-Melnyk *et al.* 1992), aorta (Komatsu *et al.* 1992) and heart (Nunez *et al.* 1992). Activation of this receptor stimulates production of cGMP (Koller *et al.* 1991), which probaply mediates the biological effects.

The biological effects of CNP show important differences from those of ANP and BNP. In *in vitro* studies on isolated saphenous, femoral and renal canine veins, CNP was observed to be a selective endothelium-independent venodilator compared to ANP (Wei *et al.* 1993a). In contrast to the marked actions on veins, CNP was noted to be less potent in relaxing peripheral arteries isolated from dogs. These studies demonstrated that ANP is a more selective arterial vasodilator than CNP, supporting the concept that CNP has distinct vascular actions in the control of peripheral vascular tone (Wei *et al.* 1993a). Recently, it has been reported that CNP mediates relaxation of canine femoral veins trough activation of large-conduction, calcium-activated potassium channels and activation of particulate and soluble guanylate cyclase (Banks *et al.* 1996). Although CNP may lack peripheral arterial vasorelaxation actions, it has been observed to be a potent coronary vasodilator in preconstricted canine coronary arteries (Wei *et al.* 1994). CNP has been found to act as a coronary vasodilator through activation of cGMP by activating a particulate gyanylate cyclase (Wright *et al.* 1996). Intravenous administration of CNP has been reported to lower blood pressure and to produce a fall in cardiac output in dogs (Stingo *et al.* 1992a). In humans, CNP has been reported to produce vasodilation of the forearm vascular bed and the vasodilation appeared to be significantly less potent than that elicited by ANP (Nakamura *et al.* 1994). Systemic CNP infusion in normal humans had no effect on basal or Ang II stimulated hemodynamics, aldosterone production and natriuresis (Hunt *et al.* 1994). In addition, Gargill *et al.* have reported that systemic CNP infusion in humans did not have any effect on pressor responses in systemic or pulmonary hemodynamics before and after Ang II administration (Cargill *et al.* 1995). CNP has been reported to inhibit the proliferation of vascular smooth muscle cells (Furuya *et al.* 1991). CNP is observed to be 5 to 10-fold more potent than ANP in stimulating cGMP in bovine cultured VSMCs, and low concentration of CNP inhibited up to 80 % of the [^{3}H]-thymidine incorporation induced by bFGF (Porter *et al.* 1992). Moreover, CNP infusion has been reported to inhibit the neointima formation in rat common carotid artery after experimental artery injury by balloon angioplasty (Furuya *et al.* 1993).

Immunoreactive CNP (ir-CNP) has been studied in various cardiovascular diseases including hypertension, congestive heart failure and atherosclerosis (for review see Chen & Burnett 1998). There was an association between hypertension and plasma ir-CNP in hypertensive humans, whereas a clear relationship was observed between hypertension and plasma BNP protein (Cheung & Brown 1994). To date, no difference in plasma CNP has been observed between normotensive and hypertensive humans or animals (for review see Chen & Burnett 1998). In congestive heart failure plasma levels of CNP are not elevated, although atrial and ventricular CNP content is increased (Wei *et al.* 1993b), suggesting a paracrine role of CNP. Thus, unlike ANP and BNP, CNP appears to act as a autocrine/paracrine factor within vascular endothelium rather than being a circulating hormone.

3. Aims of the research

The aim of the present study was to examine AM gene expression in response to mechanical stress. The second purpose was to study the mechanism(s) involved in the induction of AM gene expression.

The specific aims of the present study were

1. to study the effect of acute and chronic pressure overload on AM gene expression in the hearts of normal and hypertensive rats.
2. to study the time course of the acute pressure overload-induced AM gene expression and the role of ET-1 and angiotensin II in acute pressure overload-induced activation of cardiac AM gene expression in normotensive rats
3. to study the chronic effects of AT_1 receptor antagonism and ACE-inhibition on the regulation of AM and natriuretic peptides gene expression in normo- and hypertensive rats.
4. to examine the effects of chronic CNP infusion and NEP-inhibitor treatment on gene expression of AM and natriuretic peptides in the hearts of normotensive rats.

4. Materials and methods

4.1. Materials

The chemicals and supplies used in this study were: arginine^{8}-vasopressin (AVP) (Peninsula Laboratories Europe Ltd, Merseyside, U.K.), formaldehyde and guanidine isothiocyanate (Fluka Chemie AG, Buchs, Switzerland), LiCl (JT Baker Chemicals BV, Holland), CsCl (Serva Feinchemica GmbH & Co, Heidelberg, Germany), agarose NA (Pharmacia, Sweden), heparin (Leiras, Helsinki, Finland), BAS 85 nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany), synthetic rat $ANP_{99\text{-}126}$, [^{32}P]-deoxycytidine-5'-triphosphate (dCTP), radioiodine and rat [^{125}I] ANP (Amersham, Buckinghamshire, England, U.K.), and X-ray films (Eastman Kodak, Rochester, NY, USA, and Amersham). Bosentan was generously supplied by Dr. Martine Clozel (F. Hoffmann-La Roche Ltd., Basel, Switzerland), losartan and enalapril (DuPont Merck Pharmaceutical Company, Wilmington, USA), C-type natriuretic peptide (CNP-22, Neosystem Laboratoire, Strasbourg, France; Biocenter Oulu, Oulu, Finland), sinorphan (a generous gift from Dr. Stasch, Cardiovascular and Arteriosclerosis Research, Bayer AG, Wuppertal, Germany). Other chemicals were from Sigma.

4.2. Animals

Male 8- and 12-week-old Sprague-Dawley (SD) rats (260 to 400 g) from the Center for Experimental Animals at the University of Oulu, Finland, hypertensive 8-week-old homozygous TGR(mREN-2)27 rats (340-385 g) generated by Mullins (Mullins *et al.* 1990), and 7-week-old Wistar-Kyoto (WKY) rats (250 to 310 g) and 7-week-old SHRs (250-320 g) from the Center for Experimental Animals at the University of Tampere, were used. The WKY and SHR strains were originally obtained from Møllegaards Avslaboratorium, Skensved, Denmark and TGR(mREN-2)27 rats from Breeding Centre, Schönwalde, Germany. The rats were housed in plastic cages in a room with a controlled 40 % humidity and temperature of 22 °C, and a 0600 h on, 1800 off environmental light cycle was maintained. The maintenance diet of the rats contained NaCl 0,65 %. The

experimental design was approved by the animal experimentation committee of the University of Oulu. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

4.3. Experimental protocols

4.3.1. Acute cardiac load experiments (I,II)

The rats were anesthetized with fentanyl citrate 0,315 mg/ml, midazolam 5 mg/ml and aqueous solution (1:1:2) 3,3 ml/kg i.p. A PE-60 catheter was placed into the abdominal aorta through the right femoral artery for measurement of blood pressure, heart rate and for collection of blood samples, as described previously (Ruskoaho *et al.* 1989b). PE-50 catheters were inserted into the right atrium through the jugular vein for measurement of right atrial pressure and into the femoral vein for infusion of donor blood, vehicle, or drugs. All catheters were exteriorized behind the neck, filled with a heparinized (150 IU/ml) saline solution, and plugged with a stainless steel pin. After operation, the rats had free access to food and water. The day after the operation, the arterial and right atrial catheters were attached to pressure transducers (Model MP-15, Micron Instruments, Los Angeles, CA) and a Grass polygraph (Model 7DA, Grass Instruments, Quincey, MA) for recording mean arterial pressure, heart rate, and right atrial pressure. The venous catheter was connected to a syringe or an infusion pump (B. Braun Perfusor ED, Braun Melsungen AG, Melsungen, Germany). The animals were left undisturbed for 30 min to become acclimatized to the laboratory.

The experiments were started by measuring mean arterial pressure, heart rate and right atrial pressure in the conscious, freely moving animals for 30 min before 1.0 ml of blood was withdrawn from the arterial catheter for measurement of plasma ir-AM an ir-BNP (Study II). The baseline hemodynamic measurements were done 5 minutes later, when blood pressure, heart rate, and right atrial pressure were stabilized near to the control values. In study II, a new non-peptide mixed ET_A/ET_B receptor antagonist bosentan (10 mg/kg)(Clozel *et al.* 1993), AT_1 receptor antagonist losartan (10 mg/kg) or vehicle (0.9 % NaCl) was administered iv as a bolus injection. Injection volume was 0.1 ml/100 g body weight. AVP (0.05 µg/kg/min) or vehicle was infused intravenously for 2 hours (Study I) and for 15 min, 30 min, 1, 2 or 4 hours (Study II) via an infusion pump. The infusion rate was 37.5 µl/min. Arterial blood samples were taken 15 min, 30 min, 1 h, 2 h, and 4 h after the start of administration of vehicle or AVP. All blood samples were replaced by an equal volume of blood from a donor rat. Donor blood was obtained from conscious rats to which this volume was replaced by 0.9 % NaCl. Blood samples were taken into pre-cooled tubes containing 1.5 mg ethylenediamine tetra-acetic acid per 1 ml blood on ice and immediately centrifuged (2000 x g, 10 min, at +4 °C), and the plasma was stored at - 20 °C until assayed by radioimmunoassays (RIA).

The rats were immediately decapitated after the experimental period, the abdominal cavity was opened and the heart removed. The aorta and pulmonary artery were carefully excised close to the ventricular surface, and the right ventricle and the right and left

auricles and other atrial tissue were removed. To avoid possible contamination of the left ventricular-septal portion (combined right and left septa) sample by atrial tissue, the ventricles were cut into superior (about 15-20 % of total weight) and inferior parts, the latter being used for ventricular immunoreactive peptides and mRNA determinations. The left ventricle was cut into 40-µm slices on a cryostat at -20 °C, and the slices were combined to represent two equal layers (endocardium and epicardium) of the left ventricular wall as described previously (Ruskoaho *et al.* 1989b). All cardiac tissue samples were blotted dry, weighed, immersed in liquid nitrogen and stored at -70 °C until assayed (Table 2).

4.3.2. Chronic drug treatments experiments (I, III, IV)

4.3.2.1. Ang II infusion

SD rats were anesthetized with fentanyl citrate 0,315 mg/ml, midazolam 5 mg/ml and aqueous solution (1:1:2) 3,3 ml/kg i.p., then Alzet 2002 minipumps were placed subcutaneously through a neck wound. The rats received either Ang II (33 µg/h/kg) or 0.9 % NaCl infusion by using 200 µl minipumps s.c.. After 2 weeks the rats were decapitated. Blood samples were taken and heart was prepared as previouly described (see 4.3.1.).

4.3.2.2. AT_1 and ET-1 receptor antagonism treatment in normo- and hypertensive animals

The systolic blood pressures of conscious animals were measured at +28 °C by the tail-cuff method (Model 129 Blood Pressure Meter; IITC Inc., Woodland Hills, Ca., USA). At 7 weeks of age SHR and WKY rats were given an AT_1 receptor antagonist (losartan, 15 mg/kg/day) or an ACE inhibitor (enalapril, 4 mg/kg/day) in drinking water, whereas untreated SHR and normotensive WKY rats were kept on normal drinking fluid. The consumption of drinking water was measured to get the precise drug doses for rats. The doses of losartan and enalapril were adjusted so that they induced similar lowering of systolic blood pressure in SHR, but did not change blood pressure in WKY rats. Enalapril and losartan treatments and systolic blood pressure measurements were continued for 10 weeks. Thereafter, enalapril and losartan administrations were withdrawn a day before the rats were decapitated and exsanguinated. The hearts were removed and the ventricles and left and right atria were weighed and frozen in liquid nitrogen. The tissues were stored at -70 °C until the extraction of total RNA. To compare whether the regulation of AM differs from those of natriuretic peptides, cardiac mRNA and peptide levels of ANP and BNP were measured in losartan- and enalapril-treated normotensive and hypertensive rats (Table 2).

4.3.2.3. CNP infusion and sinorphan treatment in normotensive rats

SD rats were anesthetized with fentanyl citrate 0,315 mg/ml, midazolam 5 mg/ml and aqueous solution (1:1:2) 3,3 ml/kg i.p., then Alzet 2002 minipumps were placed subcutaneously through a neck wound. The rats received either CNP (1 µg/min/kg) or 0.9 % NaCl infusion by using 2 ml minipumps s.c.. In addition, the rats were treated orally by gavage either with sinorphan (30 mg/kg twice a day, administered as a suspension of polyethyleneglycol (PEG) / carboxymethyl cellulose (CMC) solution, 2 ml/kg) or only with the PEG/CMC suspension.

After 2 weeks the rats were decapitated. Blood samples were taken and the heart was prepared as previouly described (see 4.3.1.) (Table 2).

Table 3. Summary of the in vivo experimental protocols

Study	Rats	Experimental model	Duration	Drugs
I	SD TGR	pressure load (AVP)	2 h	
	SD	pressure load (Ang II)	2 weeks	for 2 weeks, angiotensin II (33 µg/kg/h)
II	SD	pressure load (AVP)	0.5 - 4 h	bolus injections, losartan (10 mg/kg) bosentan (10 mg/kg)
III	WKY SHR	drug treatment p.o.	10 weeks	for 2 weeks, enalapril (4 mg/kg/day) losartan (15 mg/kg/day)
IV	SD	CNP infusion by minipumps and sinorphan treatment by gavage	2 weeks	for 2 weeks, CNP (1 µg/kg/min) sinorphan (60 mg/kg/day)

4.4. Isolation and analysis of cytoplasmic RNA (I-IV)

RNA was isolated from left ventricle and atrium by the guanidine thiocyanate-CsCl method (Chirgwin *et al.* 1979). Northern blot hybridization, in which the size and amount of specific mRNA molecules in preparations of total RNA are determined (Alwine *et al.* 1977) and dot blot hybridization, in which a small sample of the RNA preparation is spotted onto nitrocellulose membrane (Kafatos *et al.* 1979), were done after isolation of RNA. For the RNA Northern blot and dot blot analysis, 10 µg samples of the RNA from

atria (Studies II, III) and 22 µg from ventricles (Studies I-IV) were transferred to the Schleicher & Schuell BAS 85 nitrocellulose membrane. A 390 bp fragment of rat BNP cDNA probe (Ogawa *et al.* 1991) (a generous gift from Dr. Kazuwa Nakao, Kyoto University School of Medicine, Kyoto, Japan), full-length rat ANP cDNA probe (Flynn *et al.* 1985) (a generous gift from Dr Peter L. Davies, Queen´s University, Kingston, Canada), full-length cDNA probe complementary to glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) (Fort *et al.* 1985), oligonucleotide probe complementry to rat 18 S ribosomal RNA (Lee *et al.* 1988), PCR amplified rat AM cDNA probe (nucleotides 287-736) (Study I) were labeled with [^{32}P]-dCTP with Quick Prime Kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). The membranes were hybridized overnight at +42 °C in 5 x SSC (saline sodium citrate, 1 x SSC = 0.15 mol/L NaCl, 0.015 mol/L trisodium citrate, pH 7), 0.5 % sodium dodecyl sulfate (SDS), 5 x Denhardt's solution, 50 % formamide and 100 µg/mL sheared herring sperm DNA. After hybridization, the membrane was washed in 0.1 x SSC, 0.1 % SDS three times for 20 min at +55 °C and exposed at -70 °C to X-ray film with Cronex Lighting Plus intensifying screens (DuPont, Wilmington, Delaware) or to Phosphor Screen (Molecular Dynamics, Sunnyvale, CA, USA) at room temperature. Autoradiograms generated by dot blots were scanned with a densitometer (Millipore Corp. Imaging Systems, Ann Arbor, MI). Phosphor Screens were scanned with Phosphor Imager.

4.5. Radioimmunoassay of ir-AM , ir-ANP, ir-BNP and ir-CNP (I-IV)

Tissue samples were extracted by Sep-Pak C_{18} cartridges as previously described (Kinnunen *et al.* 1993, Magga *et al.* 1994). Eluates were lyophilized and redissolved in RIA buffer. For the AM radioimmunoassay, guanidine isothiocyanate supernatans were diluted with 0.1% trifluoroacetic acid (TFA) and extracted with Sep-Pak C_{18} cartridges (Waters) as described (Magga *et al.* 1994, Kinnunen *et al.* 1993). The extracts were dried and redissolved in radioimmunoassay buffer. The AM content was examined with specific radioimmunoassay as follows: synthetic rat AM 1-50 standards (Phoenix Pharmaceuticals, Mountain Wiew, CA, USA) and the tissue extracts were incubated for 16-24 h at +4 ° C with rabbit anti-rat AM serum (Phoenix Pharmaceuticals). ^{125}I-rat AM 1-50, prepared with chloramine-T iodination and purified with Sephadex G-25 (Pharmacia, Uppsala, Sweden) gel filtration followed by Vydac C_{18} (Separations Group, Hesperia, CA, USA) reverse phase HPLC, was added and the incubation was continued for another 16-24 h. The free and bound fractions were separated by double antibody precipitation. The rat AM antiserum does not cross-react with rat AM 1-20, human AM antiserum or its fragments, human amylin or ET-1. The sensitivy of the assay was 1 fmol/tube and the intra- and interassay coefficients of variation were <10% and <15%, respectively.

For the ANP radioimmunoassay, the atrial and ventricular guanidine thiocyanate extracts were diluted 5x10^4- and 400-fold, respectively. For the BNP radioimmunoassay, the atrial and ventricular guanidine thiocyanate extracts were diluted 100- and 50-fold, respectively. The extracted samples were incubated in duplicates of 100 µL with 100 µL of the specific rabbit BNP (Kinnunen *et al.* 1993) or ANP antiserum (Vuolteenaho *et al.*

1985). Synthetic rat BNP_{51-95} (BNP-45) and synthetic rat ANP_{99-126}, ranging from 0-500 pg per tube, were incubated as standards. The BNP and ANP tracers were prepared by chloramine-T iodination of synthetic rat [Tyr_0]-BNP_{51-95} and rat ANP_{99-126} followed by reverse phase HPLC purification. After incubation for 48 hours at +4 °C the immunocomplexes were precipitated with sheep antiserum directed against rabbit gammaglobulin in the presence of 500 µL of 8 % Polyethylene Glycol 6000, pH 7, followed by centrifugation at 3000 g for 30 min. The sensitivities of the BNP and ANP assays were 2 fmol/tube and 1 fmol/tube, respectively. Fifty percent displacements of the respective standard curve occurred at 16 fmol/tube for ANP and at 25 fmol/tube for BNP. The intra- and inter-assay variations were less than 10 % and 15 %, respectively. Serial dilutions of tissue extracts showed parallelism with the standards. The ANP antiserum recognized ANP and proANP with equal avidity but did not cross-react with BNP or CNP (<.01 %). The BNP antiserum did not recognize ANP or CNP (<.01 %). Tissue BNP and ANP are expressed as a concentration per mg wet weight.

For the CNP radioimmunoassay, the atrial and ventricular guanidine isothiocyanate extracts were concentrated with SepPak C18 extraction as described previously (Magga *et al.* 1994). The extracts were dissolved in RIA buffer. The assay utilized a specific CNP antiserum purchased from Peninsula Laboratories Europe (Merseyside, UK, cat. No. RAS9030). The antiserum cross-reacts 100 % with CNP-53, but does not recognize CNP-53 1-29, ANP, BNP or ET-1. Tyr0-CNP-22 (Peninsula) was radioiodinated and purified as described above for ANP and BNP. The RIA procedure was identical to that of the ANP assay. The least detectable dose was 1 fmol/tube and the 50 % displacement was at 10 fmol/tube. The intra- and interassay coefficients of variation were < 10 % and 15 %, respectively, for each assay.

4.6. Statistical Analysis

The results are expressed as mean±SEM. For the comparison of statistical significance between two groups, Student's *t*-test was used (Studies I-IV). The hemodynamic variables (Studies I-III) and plasma ir-AM and ir-BNP levels (Study II) were analyzed with one-way ANOVA followed by Student-Newman-Keuls´s post hoc test. Repeated measures ANOVA was used for multivariate analysis. Differences at the 95 % level were considered statistically significant.

5. Results

5.1. Acute cardiac overload in SD and TGR rats (I)

5.1.1. Hemodynamic variables

Baseline mean arterial pressure, measured directly in chronically cannulated conscious animals, was markedly higher in twelve-week-old male homozygous TGR(mREN-2)27 rats than in the normotensive Sprague-Dawley rats. Also the baseline heart rate was higher in the TGR rats than in the SD rats, whereas their right atrial pressure did not differ significantly. The average body weight was similar for the controls and transgenic rats. Left ventricular weight was significantly greater in the TGR rats than in the SD rats. Consequently, the ratios of left ventricular weight to body weights were also significantly greater in the TGR rats than those of age-matched normotensive SD rats.

In the normotensive SD rats, acute pressure overload produced by infusion of arginine^{8}-vasopressin (AVP, 0.05 μg/kg/min iv) for 2 hours increased mean arterial pressure maximally by 30 % ($p<0.05$). During AVP infusion, mean arterial pressure reached maximum values within 30 min, and it remained elevated throughout the experimental period. In the TGR rats changes in mean arterial pressure in response to AVP infusion were similar to those in controls; the mean arterial pressure increased by 28 % ($p<0.05$) during the first 30 minutes and remained elevated during the whole experimental period. Heart rate decreased both in the normotensive SD and TGR rats during AVP, but the change was statistically significant only for the normotensive rats (35 %, $p<0.05$). Right atrial pressure did not change significantly in response to AVP infusion in SD nor TGR rats.

5.1.2. Left ventricular AM gene expression and immunoreactive AM

RNA Northern blot analysis with AM cDNA probe identified a single 1.4 kb mRNA species in both the epicardium and the endocardium of left ventricle. Despite the marked

hypertension and left ventricular hypertrophy in the TGR(mREN-2)27 rats, the baseline AM mRNA levels in the epicardium and endocardium of left ventricle did not differ significantly between SD and TGR(mREN-2)27 rats. In contrast, the basal concentrations of ir-AM in the left ventricular epicardium and endocardium were significantly higher in the TGR(mREN-2)27 rats than in the SD rats.

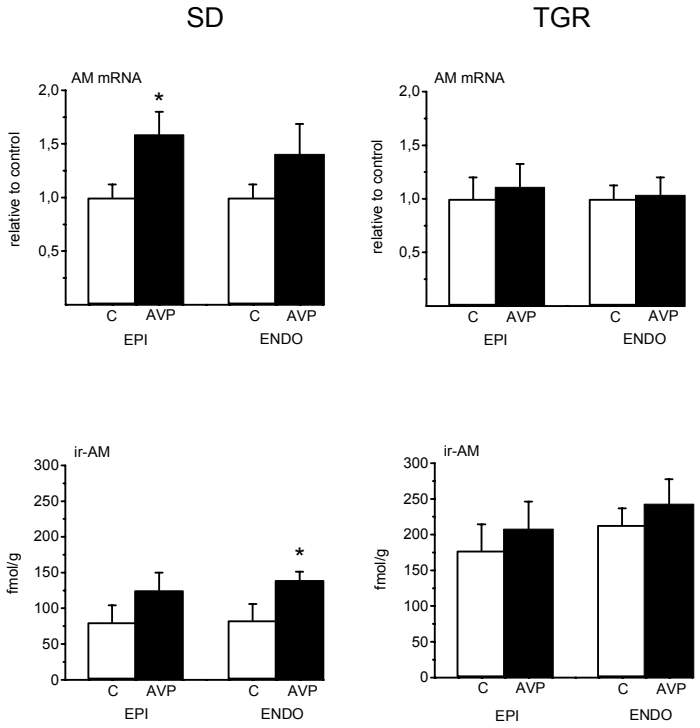

Fig. 4. Bar graphs showing the effects of AVP infusion on adrenomedullin (AM) mRNA levels and immunoreactive AM (ir-AM) levels in epicardium (EPI) and endocardium (ENDO) of left ventricle in normotensive SD rats (n=8) and hypertensive TGR rats (n=8). C, vehicle; AVP, AVP infusion for 2 h. Results are expressed as mean±SEM. *, p<0.05 (AVP vs control, Student´s t-test).

Arginine^{8}-vasopressin infusion for 2 hours induced a stimulation of AM gene expression in normotensive SD rats: a 1.6-fold increase ($p<0.05$, Fig. 4) in AM mRNA levels in the epicardium and a 1.8-fold increase in the ir-AM levels in the endocardium of left ventricle ($p<0.05$, Fig. 4) was observed. There was also tendency for AM mRNA levels in the endocardium and ir-AM levels in the epicardium to increase, but these changes were not statistically significant (Fig. 4). In transgenic rats the infusion of AVP for 2 hours did not have a significant effect on AM mRNA synthesis (Fig. 4). In addition, in transgenic rats the AVP infusion did not have a significant effect on ir-AM peptide levels (Fig. 4). Similarly in old SHR rats, on the basis of Northern blot analysis, we found

that vasopressin-induced acute pressure overload had no effect on left ventricular AM mRNA levels.

AVP infusion stimulated BNP mRNA synthesis in SD rats resulting in 1.7-fold ($p<0.01$) and 2.0-fold ($p<0.01$) increases in the endocardium and in the epicardium of left ventricle, but this activation of BNP synthesis was attenuated in transgenic animals. Baseline ANP mRNA levels in transgenic rats were 32 times higher in the endocardium and 40 times higher in the epicardium than those in SD rats. Further, AVP infusion had no effect on ANP mRNA levels in either transgenic or SD rats.

5.2. Role of endothelin-1 and angiotensin II in AM gene expression (II)

5.2.1. Characterization of pressure overload model

Since the AVP infusion induced left ventricular AM gene expression at 2 hours in study I, the acute pressure overload model produced by AVP infusion was chosen to study the regulatory mechanisms for induction of cardiac AM gene expression. To clarify the precise time-course of the early induction of AM gene expression, AVP was infused for 15 min, 30 min, 1 h, 2 h and 4 h. To validate the model, the expression of endogenous BNP mRNA levels was examined in the heart. AVP infusion caused marked elevation in blood pressure. The mean arterial pressure raised rapidly and reached maximum value in 15 minutes, associated with a significant decrease in heart rate when compared to vehicle group (Fig. 5). There were no changes in mean arterial pressure or heart rate in the vehicle group (Fig. 5), and right atrial pressure did not differ between vehicle- and AVP groups. AVP infusion caused significant increases in BNP mRNA levels both in the endocardial and the epicardial layers of left ventricle from 1 hour onwards, followed by significant increases in ir-BNP levels at 2 and 4 hours. Left atrial BNP mRNA levels increased within 30 minutes and the increase was greatest at 4 hours. The increase in left atrial BNP mRNA was associated with significant decreases in ir-BNP levels at 2 and 4 hours.

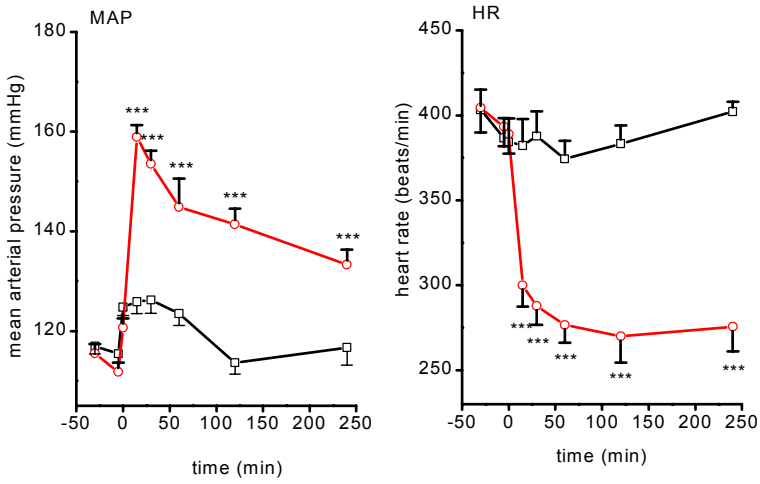

Fig. 5. Mean arterial pressure (MAP) and heart rate (HR) in SD rats during AVP infusion for 4 hours. Open circle, AVP infusion; open squere, vehicle infusion. Data are expressed as mean±SEM. ***, p<0.001 vs vehicle. (Student´s t-test)

5.2.2. Left ventricular AM gene expression

The AVP infusion caused significant increases in AM mRNA levels both in the endocardium and the epicardium of left ventricle (Fig. 6AB). A significant increase in AM mRNA levels was observed at 2 hours both in the endocardium and in the epicardium of the left ventricle (1.7- and 1.9-fold, respectively) (Fig. 6AB). The maximal response was seen after 4 hours of AVP infusion, AM mRNA levels increased 2-fold both in the endocardium and in the epicardium (Fig. 6AB). Despite the induction of AM gene expression, there were no differences in ir-AM levels in the left ventricle during AVP infusion (Fig. 6AB).

5.2.3. Left atrial AM gene expression

The AVP infusion caused significant induction of AM mRNA levels in the left atrium. A 1.3-fold increase in AM mRNA levels was already seen after 30-min AVP infusion (Fig. 6C). A maximal increase, 3.5-fold, was observed after 4 hours of infusion (Fig. 6C). Similarly like left atrial ir-BNP peptide levels, ir-AM peptide levels were decreased in the left atrium by 46 % (from 267±38 to 144±18 fmol/g, $p<0.05$) and by 40 % (from 284±21 to 173±29 fmol/g, $p<0.01$) after 2 and 4 hours infusion of AVP, respectively (Fig. 6C). In the vehicle group, left atrial concentration of ir-AM (243±15 fmol/g) was higher than in the left ventricle (endocardium: 62±3 fmol/g, epicardium: 80±2 fmol/g).

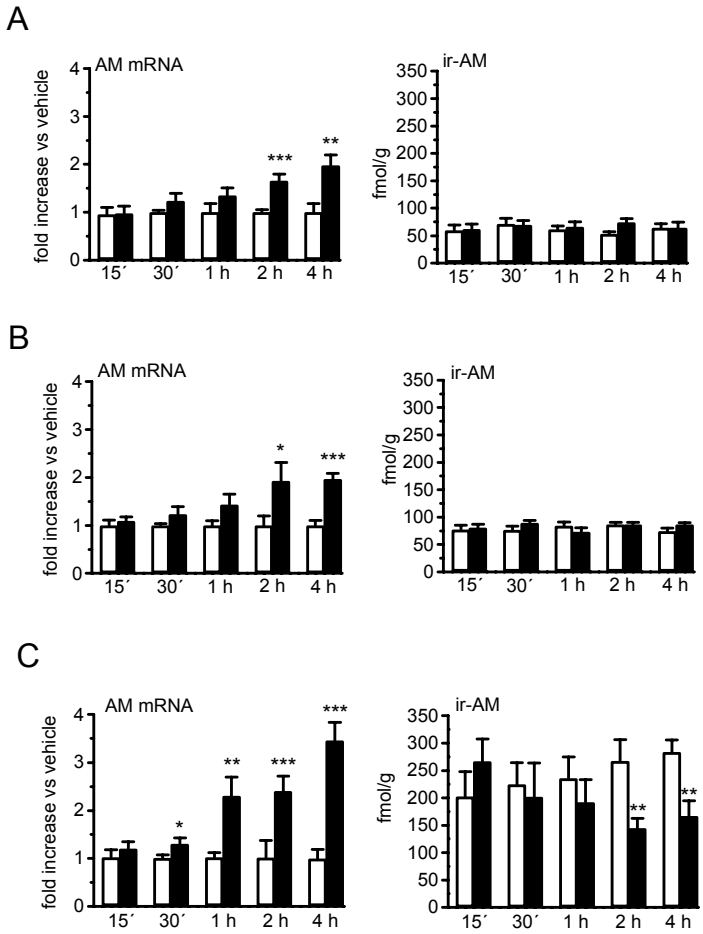

Fig. 6. Bar graphs showing the effect of AVP infusions on left ventricular adrenomedullin (AM) mRNA and immunoreactive AM (ir-AM) levels in SD rats. Panel A: the endocardium of the left ventricle. Panel B: the epicardium of the left ventricle. Panel C: the left atrium. White bar, vehicle; black bar, AVP infusion. *, p<0.05; **, p<0.01; ***p<0.001; AVP infusion vs vehicle.

5.2.4. Effect of bosentan and losartan on hemodynamic variables

Because different stimuli themselves invoke a complex array of hemodynamic, neural and hormonal responses in conscious animals, the changes in degree of cardiac load were carefully measured between vehicle- and drug-infused animals. The doses of bosentan and losartan, used in this study, have been previously validated to be effective (Leskinen *et al.* 1997). Since the significant increase in AM mRNA levels in response to AVP infusion

was observed at 2 hours, this time point was chosen for examining the effects of ET-1 and Ang II receptor antagonism on cardiac AM. The bolus injections of losartan and bosentan alone decreased significantly mean arterial pressure in vehicle-treated animals at 2 hours time point (Fig. 7AB.). During the losartan and bosentan treatment alone, the response to AVP infusion was similar as without the drug treatments; the mean arterial pressure increased and the heart rate decreased significantly (Fig. 7AB.). The combination treatment of losartan and bosentan decreased significantly mean blood pressure already at 30 min and remained decreased up to 2 h (Fig. 7C.). The combination treatment had no effect on AVP responses to mean arterial pressure and heart rate (Fig. 7C.). The results show that injections of losartan or bosentan did not alter the hemodynamic response evoked by AVP infusion, thus allowing the examination of the direct action of load versus a requirement for Ang II and ET-1 to mediate load-induced initiation of early cardiocyte response associated with the onset of cardiac hypertrophy.

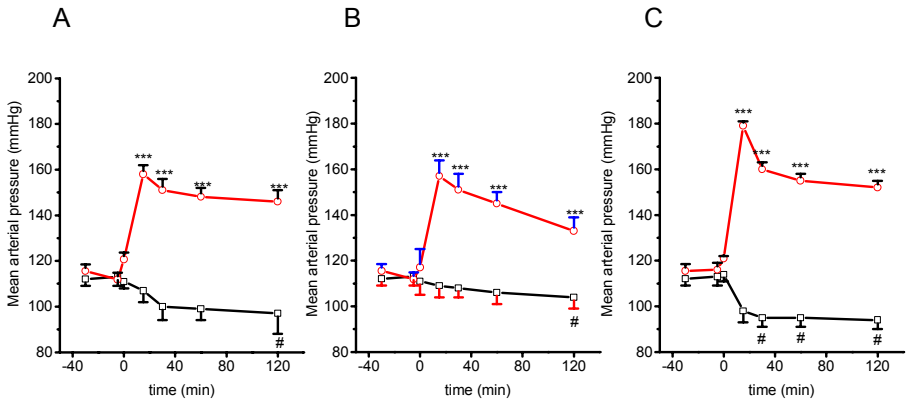

Fig. 7. Mean arterial pressure (MAP) in SD rats during AVP infusion for 2 hours. Treatment with losartan (A), bosentan (B), and the combination of losartan and bosentan(C). Open circle, AVP infusion; open square, vehicle infusion. Data are expressed as mean±SEM. ***, p<0.001 vs vehicle; #, p<0.05 vs time point 0 min in the vehicle group. (Student´s t-test).

5.2.5. Effect of bosentan and losartan on AM gene expression

The bolus injections of losartan, bosentan and their combination did not have any effect on the activation of left ventricular AM gene expression in response to 2 h AVP infusion. In the endocardial layer of left ventricle, AVP infusion caused significant increases in AM mRNA levels in animals treated with bosentan (1.6-fold), losartan (1.5-fold) and their combination (1.8-fold)(Fig. 8A). In the epicardium, the increases in AM mRNA levels in response to AVP infusion were 1.55-, 1.9- and 2.0-fold in animals treated with bosentan-, losartan- and their combination, respectively (Fig. 8B). In addition, injections of losartan, bosentan and their combination had no significant effect on baseline AM mRNA levels in the endocardium and epicardium of the left ventricle (Fig. 8AB). There were no

differences in left ventricular ir- AM levels between vehicle- and AVP-infused animals during these drug treatments.

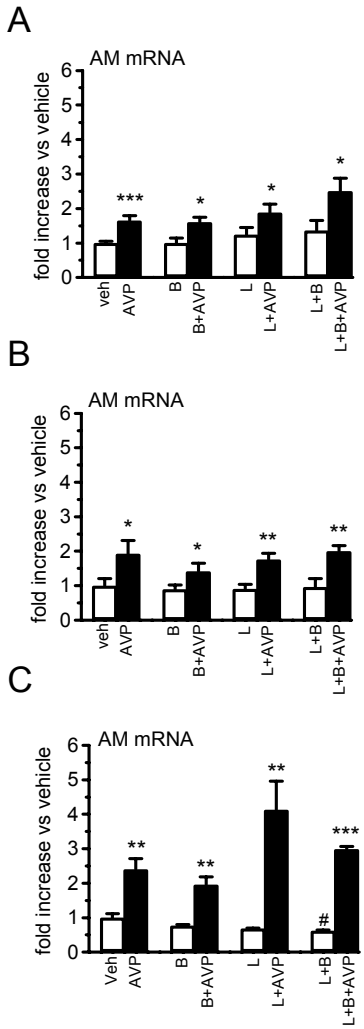

Fig. 8. Bar graphs showing the effects of drug treatments on left ventricular adrenomedullin (AM) mRNA and immunoreactive AM (ir-AM) levels in SD rats during the vehicle- and vasopressin infusion for 2 hours. Panel A: the endocardium of the left ventricle. Panel B: the epicardium of the left ventricle. Panel C: the left atrium. White bar, vehicle; black bar, AVP infusion. Veh = vehicle, AVP = vasopressin, B = bosentan, L = losartan. ***p<0.001; AVP infusion vs vehicle. *, p<0.05; **, p<0.01; B or L or L+B vs B+AVP or L+AVP or L+B+AVP.

In the left atrium, losartan, bosentan and their combination had no effect on the induction of AM mRNA levels during AVP infusion (Fig. 8C). In bosentan treated animals, AVP infusion caused 1.9-fold increase in AM mRNA levels (Fig. 8C). Similarly

in animals treated with losartan and combination of bosentan and losartan, AVP infusion induced 4.1- and 3.0-fold increases in AM mRNA levels, respectively (Fig. 8C). Furthermore, the combination of losartan and bosentan decreased baseline left atrial AM mRNA levels (Fig. 8C). After 2 hours` infusion of AVP, a 47 % decrease (from 367±40 to 195±34 fmol/g, $p<0.01$) was noted in the combination treated animals.

5.2.6. Immunoreactive AM in plasma

There was a significant difference in plasma ir-AM levels after 4 hours` infusion between vehicle- and AVP-infused SD rats (36.9±5.2 vs. 49.7±7.7 pmol/l, $p<0.05$). Otherwise, the plasma ir-AM levels were similar between vehicle- and AVP-infused animals. The treatment with losartan or bosentan and with their combination did not have any effect on the plasma ir-AM levels in the vehicle- and AVP-infused animals at 2 hours.

5.3. Effect of short-term Ang II infusion on AM gene expression (I)

To examine the effect of short-term cardiac overload on AM gene expression, Ang II was infused for 2 weeks in normotensive 8-week-old SD rats. Ang II infusion increased significantly by 10 % left ventricular weight to body weight ratio compared to vehicle-infused group. This Ang II-induced increase in LVH was associated with a 50 % ($p<0.05$) increase in the left ventricular AM gene expression.

5.4. Effect of ACE inhibitor and AT_1 receptor antagonist on cardiac AM (III)

5.4.1. Effect of losartan and enalapril treatments on blood pressure and cardiac hypertrophy

The systolic blood pressure of 7-week-old SHR was already higher than in age-matched WKY rats, the difference being 34 mm Hg ($P<0.001$). There were no significant differences in systolic blood pressure between the various treatment groups of SHR, nor between the various treatment groups of WKY prior to the beginning of the treatments. Systolic blood pressure increased by 101±14 mm Hg ($F=194$, $P<0.001$) in vehicle-treated SHR and by 44 mm Hg ($F=61$, $P<0.001$) in vehicle-treated WKY rats within 10 weeks. Administration of both losartan and enalapril in drinking water completely inhibited the increase of systolic blood pressure occurring with aging in SHR, while in WKY rats the difference in systolic blood pressure between vehicle- and drug-treated groups at the end

of experimental period was not statistically significant. The blood pressure effects of losartan and enalapril were similar throughout the treatments in both strains.

After 10 weeks` treatments, the ratio of heart weight to body weight was significantly greater in vehicle-treated SHR than in age-matched normotensive vehicle-treated WKY rats. Also the heart weight of 17-week-old vehicle-treated SHR was higher than that of WKY rats. Administration of losartan and enalapril effectively reduced cardiac hypertrophy in SHR. Losartan and enalapril both also produced significant decreases in the heart weight to body weight ratio in WKY rats ($P<0.05$). The decrease in heart hypertrophy was greater in the losartan-treated SHR than in the WKY rats. Body weights of SHR and WKY rats were similar at the end of the experimental period. The mesenteric arteries of these rats were used for vascular studies and it was observed that losartan and enalapril treatments enhanced vasodialtion in the mesenteric artery of SHR (Kahonen *et al.* 1999).

5.4.2. Cardiac gene expression of AM in losartan- and enalapril-treated SHR and WKY rats

After the experiments, the concentrations of AM mRNA and IR-AM in the left ventricle and atrium were similar in vehicle-treated SHR and WKY rats (Fig. 9). Losartan or enalapril treatments had no statistically significant effect on AM mRNA and IR-AM levels in the left ventricle in either strain. However, in SHR both drugs (losartan, 63 %, $P<0.001$: enalapril, 39 %, $P<0.01$) increased left atrial concentration of IR-AM. Furthermore, a 31 % increase ($P<0.05$) in losartan-treated WKY rats and a 25 % increase ($P<0.05$) in enalapril-treated SHR in left atrial AM mRNA levels was noted when compared to their respective vehicle-treated groups (Fig. 9). Losartan or enalapril treatments had no effect on the right atrial AM mRNA and IR-AM levels.

5.4.3. Cardiac gene expression of ANP and BNP in losartan- and enalapril-treated SHR and WKY rats

After 10 weeks` experimental time, the left ventricular concentrations of ANP mRNA and ir-ANP were similar in vehicle-treated SHR and WKY rats. In WKY rats, losartan treatment reduced left ventricular levels of ANP mRNA and ir-ANP by 36 % ($P<0.01$) and 55 % ($P<0.001$), respectively. In enalapril-treated WKY rats, similar decreases in levels of ANP mRNA (35 %, $P<0.001$) and ir-ANP (58 %, $P<0.001$) were noted. In SHR, losartan and enalapril treatments reduced levels of ANP mRNA by 81 % ($P<0.001$) and 65 % ($P>0.001$), respectively. This reduction in left ventricular ANP mRNA levels was greater in the losartan-treated SHR than in the WKY rats. Furthermore, left ventricular ir-ANP levels at the end of the treatment period were 67 % ($P<0.001$) and 69 % ($P<0.001$) lower in losartan- and enalapril-treated SHR than in the vehicle-treated SHR.

In the left atrium, the concentration of IR-ANP was 20 % lower in vehicle-treated SHR than in age-matched vehicle-treated WKY rats. In SHR, losartan treatment increased left

atrial ir-ANP concentration by 36 % (P<0.05), and similar increase in left atrial ir-ANP concentration was noted in enalapril-treated SHR, but this change was not statistically significant. In contrast, left atrial IR-ANP concentrations remained unchanged in WKY rats in response to losartan and enalapril treatments. Furthermore, the left atrial levels of ANP mRNA were similar in vehicle-treated SHR and WKY rats, and drug-treatments had no effects on left atrial ANP mRNA levels in either strain.

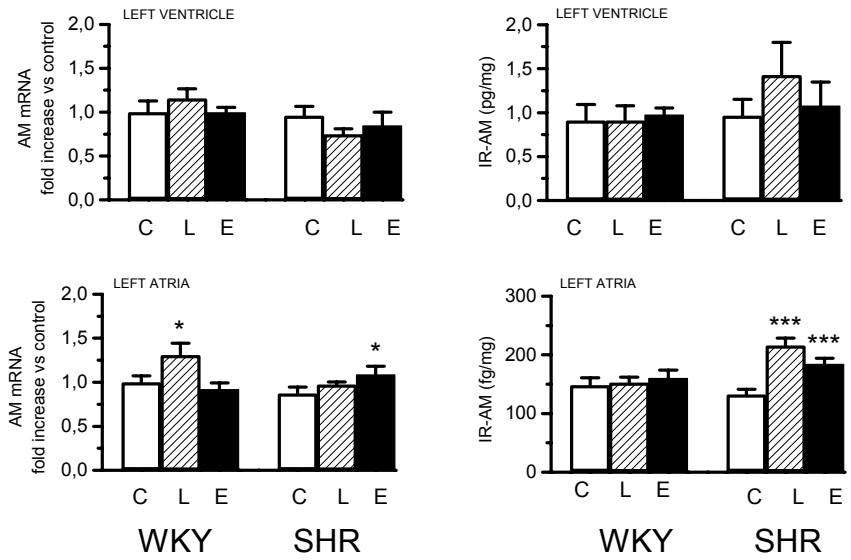

Fig. 9. Effects of administration of losartan or enalapril on left ventricular and atrial AM mRNA and IR-AM levels in SHR and WKY rats after 10 weeks treatment. C, control (SHR, n=14, WKY, n=15); L, losartan (SHR, n=10, WKY, n=10); E, enalapril (SHR, n=10, WKY, n=10). Data represent mean±SEM. ***, p<0.001 vs control (student´s t-test).

The left ventricular BNP mRNA level was higher in the vehicle-treated SHR than in the age-matched vehicle-treated WKY rats, whereas the ir-BNP concentration in the left ventricle was 28 % lower in SHR than in WKY rats. Losartan and enalapril treatments decreased significantly the BNP mRNA concentration in SHR, but had no effect on left ventricular BNP mRNA levels in WKY rats. Furthermore, this decrease in left ventricular BNP mRNA levels was more pronounced in losartan-treated SHR (68 %) than enalapril-treated SHR (35 %, P<0.001) rats. Overall, the changes in left ventricular concentrations of ir-BNP during drug treatments were small when compared to those induced by losartan and enalapril in left ir-ANP ventricular concentrations, particularly in SHR. A small but statistically significant decrease in the concentration of IR-BNP in the left ventricles was noted in drug-treated (losartan, 25 %; enalapril, 16 %) WKY rats, while the left ventricular

ir-BNP concentration was significantly higher (28 %) in enalapril-treated SHR than in the vehicle-treated SHR.

The left atrial ir-BNP concentration was higher in vehicle-treated SHR than in vehicle-treated WKY rats, whereas there was no difference in left atrial BNP mRNA levels between the strains. Losartan and enalapril decreased consistently left atrial ir-BNP levels in both strains, but had no effect on left atrial BNP mRNA levels. In WKY rats, losartan treatment for 10 weeks reduced left atrial ir-BNP concentration by 32 % ($P<0.05$) and enalapril treatment by 34 % ($P<0.05$) when compared to vehicle-treated group. Similar decreases in levels of ir-BNP during losartan treatment (22 %, $P<0.05$) and enalapril treatment (36 %, $P<0.01$) were noted in SHR.

5.5. Effects of CNP infusion and sinorphan on cardiac AM gene expression in normotensive SD rats (IV)

5.5.1. The effects of CNP infusion

CNP infusion (1 μg/min/kg) for 2 weeks did not have any effect on left ventricular weight (LW) to body weight ratio (BW). However, LW and BW were significantly lower in CNP-infused rats than in vehicle-infused rats. CNP infusion increased plasma immunoreactive CNP (ir-CNP) levels markedly compared to vehicle group. Moreover, the left ventricular ir-CNP concentration increased in response to CNP infusion. CNP infusion significantly decreased left ventricular AM mRNA levels (by 54 %), whereas the ir-AM levels remained unchanged compared to vehicle-infused rats (Fig. 10). The left ventricular BNP mRNA levels decreased by 72 % in response to CNP infusion, and were associated with a significant decrease in ir-BNP levels compared to vehicle-infused rats (from 100±4 to 50±10 pmol/g, $p<0.001$). In contrast, CNP infusion had no significant effects on left ventricular ANP mRNA or ir-ANP levels. There was a trend for plasma ir-ANP and ir-BNP levels to rise, but these changes were not statistically significant.

5.5.2. The effects of sinorphan treatment

Sinorphan administration for 2 weeks did not have an effect on LW to BW ratio, but it significantly decreased LW and BW compared to vehicle-infused rats. Sinorphan treatment decreased AM mRNA levels in the left ventricle by 40 %, but there were no changes in ir-AM levels in the left ventricle (Fig. 10) or in plasma. In contrast to AM, sinorphan treatment had no effect on left ventricular BNP mRNA or ir-BNP levels. Furthermore, the ANP mRNA and ir-ANP levels in the left ventricle and in plasma natriuretic peptide and AM levels remained unchanged during the sinorphan treatment.

5.5.3. The effects of combined treatment with CNP infusion and sinorphan

The combined treatment with CNP infusion and sinorphan did not have effect on LW to BW ratio, although LW and BW were lower than in vehicle-treated rats. The combination treatment increased plasma ir-CNP concentration and left ventricular ir-CNP levels compared to vehicle infusion, similarly to CNP infusion alone. During the combined treatment AM gene expression decreased by 50 % in the left ventricle, and this decrease in AM mRNA levels was associated with a significant decrease in ir-AM levels (from 107±7 to 83±7 fmol/g) (Fig. 10). Similarly, BNP and ANP mRNA levels decreased during the combined treatment by 43 % and 46 %, respectively. These decreases in BNP and ANP mRNA levels in CNP and sinorphan-treated rats were followed by decreases in left ventricular ir-BNP and ir-ANP levels. (BNP: from 100±4 to 70±6 pmol/g; ANP: from 287±74 to 80±24 pmol/g). In addition, plasma ir-AM levels decreased and ir-ANP levels increased during the combined treatment.

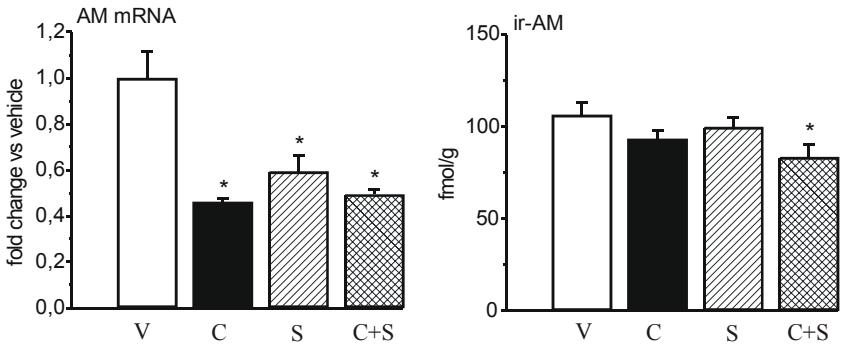

Fig. 10. The effects of CNP infudion and sinorphan treatment for 2 weeks on left ventricular AM mRNA and ir-AM levels. V, vehicle; C, CNP infusion; S, sinorphan treatment; C+S, CNP infusion + sinorphan treatment. Data expressed as mean±SEM. *, $p<0.05$ vs vehicle (Student´s t-test).

Table 4. Summary of the main results.

	Left ventricle		Left atrium	
Study	AM mRNA	ir-AM	AM mRNA	ir-AM
Acute AVP infusion	↑	↔	↑	↓
AT_1 receptor antagonism during acute AVP infusion	↑	↔	↑	↓
ET-1 receptor antagonism during acute AVP infusion	↑	↔	↑	↓
Chronic AT_1 receptor antagonism in WKY rats	↔	↔	↑	↔
Chronic ACE-inhibition in WKY rats	↔	↔	↔	↔
Chronic AT_1 receptor antagonism in SHR rats	↔	↔	↔	↑
Chronic ACE-inhibition in SHR rats	↔	↔	↑	↑
Chronic CNP infusion	↓	↔		
Chronic sinorphan treatment	↓	↔		
Chronic CNP infusion + sinorphan treatment	↓	↓		

6. Discussion

6.1. Effect of acute pressure overload on cardiac AM gene expression

Cardiac pressure overload induces rapid activation of BNP gene expression in the heart of normal and hypertensive rats (Magga *et al.* 1994), mimicking the rapid induction of proto-oncogenes to hemodynamic stress (Chien *et al.* 1991, Morgan *et al.* 1987). In this study, a similar time-course for AM and BNP gene expression was observed in response to acute cardiac pressure load. In the left ventricle, the significant induction of AM gene expression was noted at 2 hours, and the induction was maximal at 4 hours. In contrast to AVP-induced induction of AM gene expression in normotensive SD rats, the transgenic TGR rats showed a blunted increase in AM gene expression in response to acute pressure overload.

The pressure load significantly increased the AM gene expression also in the left atrium of rat heart. In the left atrium, the AM gene expression increased earlier than in the left ventricle, already at 30 min, and was maximal at 4 hours. Thus, left atrial AM gene expression reacted to cardiac load more sensitively than left ventricular AM gene expression. Similarities between AM and BNP gene expression were observed also in the left atrium, both AM and BNP gene expression increased as a result of cardiac pressure load, except for the fact that BNP gene expression was more pronounced than AM gene expression. Thus, BNP gene expression seems to be a slightly more sensitive marker for acute increased cardiac pressure load than AM.

Moreover, the increased left atrial AM and BNP mRNA levels, in response to cardiac pressure load, were followed by a decrease in ir-AM and ir-BNP levels at 2 and 4 hours. Thus, the decreases in left atrial ir-AM and ir-BNP levels associated with increases in plasma peptide levels suggest an important role for the left atrium in the secretion of AM and BNP proteins, which may in combination be important in lowering the blood pressure and inducing both natriuresis and diuresis. On the other hand, there was a difference between ir-AM and ir-BNP responses in the left ventricle. BNP protein increased in the ventricular myocytes within 4 hours of pressure overload, as previously reported (Magga *et al.* 1994), but there was no pressure overload response in ventricular AM protein levels. In the left ventricle AM may act as a paracrine/autocrine factor causing positive inotropic (Szokodi *et al.* 1996) and antihypertrophic (Tsuruda *et al.* 1998) effects. The present results are consistent with the hypothesis that AM, as a natriuretic and vasodilating peptide (Kitamura *et al.* 1993a), plays an important compensatory role in the maintenance of

optimal intravascular volume and cardiac filling pressures during increased cardiac workload, similarly to ANP and BNP (for review see Ruskoaho 1992).

6.2. Endothelin-1 and angiotensin II in acute pressure load-induced AM gene expression

Mechanical stretch may directly induce cardiac AM gene expression, or autocrine/paracrine mechanisms such as Ang II are activated by pressure overload, which then evokes rapid induction of AM gene expression. In support of this, stretching of cardiac myocytes *in vitro* causes release of Ang II in the short term (10 to 30 minutes), and increases the expression of the angiotensinogen gene in the long term (Sadoshima *et al.* 1993c). Neonatal rat cardiocytes subjected to passive stretch increased protein synthesis within 24 hours and the expression of the immediate early gene *c-fos* within 30 minutes. Both of these responses were blocked by AT_1 receptor antagonist (Sadoshima & Izumo 1993b). Because Ang II also stimulates cardiac protein synthesis (Sadoshima & Izumo 1993b, Schunkert *et al.* 1995), these results have led to the conclusion that mechanical strech-induced myocyte hypertrophy is dependent on Ang II acting through the AT_1 receptor. The present results in intact normotensive animals disagree with these reports in cultured cardiocytes. It was noted that the pressure overload-induced increase in AM mRNA levels in the left ventricle and atrium was not altered by the selective AT_1 receptor antagonist losartan. Thus Ang II is not needed in early events of transducing mechanical load signal into AM gene expression in the left ventricle or atrium. However, these results do not exclude the possibility of involvement of AT_2 receptor in the induction of cardiac AM gene expression.

Autocrine/paracrine mechanisms other than Ang II are also activated by pressure overload and could evoke rapid induction of AM gene expression. An important candidate is ET-1, because ET-1 is released rapidly when cultured endothelial cells are stretched (Macarthur *et al.* 1994, McClellan *et al.* 1994). The stretch of neonatal cardiac myocytes stimulates secretion and production of ET-1 (Yamazaki *et al.* 1996). Production of ET-1 has also been shown to increase in the hypertrophied rat heart as a result of pressure overload (Yorikane *et al.* 1993). Pressure overload due to aortic constriction (Ito *et al.* 1994, Arai *et al.* 1995) or pulmonary hypertension (Miyauchi *et al.* 1993) and norepinephirine-induced ventricular hypertrophy (Kaddoura *et al.* 1996) have been shown to be associated with large increases in ventricular expression of ET-1 mRNA, further suggesting that endogenous cardiac production of ET-1 may play a functional role in mechanical load-induced cardiac gene expression. In this study, the mixed ET_A /ET_B receptor antagonist bosentan was tested in pressure overload-induced early activation of AM gene expression. The results show that the production of ET-1 does not play a causal role in the induction of ventricular and atrial AM gene expression.

The changes in cardiac AM gene expression under basal conditions (without pressure overload) were also analyzed, and it was found that the combination of losartan and bosentan significantly decreased basal AM mRNA levels in the left atrium at 2 hours, whereas losartan or bosentan alone caused slight, but not significant decreases. Moreover, the drug treatments had no effect on basal left ventricular AM mRNA levels. MAP

decreased during the drug treatments, and the decrease was greatest with the combination treatment of losartan and bosentan, which could have been responsible for the decrease in left atrial AM mRNA levels. Thus, it seems that left atrial AM gene expression is more sensitive than left ventricle for changes in pressure load.

6.3. The effect of acute pressure overload on cardiac AM secretion

In humans, the concentration of circulating AM has been shown to be increased in patients with congestive heart failure (Jougasaki *et al.* 1995b), hypertension (Kitamura *et al.* 1994a) and chronic renal failure (Ishimitsu *et al.* 1994). In rats, circulating AM is increased in hypertension of Dahl salt-sensitive rats on a high-salt diet (Shimokubo *et al.* 1996) and in experimental pulmonary hypertension (Shimokubo *et al.* 1995). Failing human ventricular myocardium has been shown to secrete AM (Jougasaki *et al.* 1996). To date, the major source of circulating AM is unclear. In this study, the plasma ir-AM levels were similar, except for the significant difference between vehicle- and AVP-infused rats after 4 hours` infusion. It seems that during the early phase of cardiac pressure load, the heart is not the main source of circulating AM. Similar findings have been reported previously in humans with chronic ischemic heart disease (Nishikimi *et al.* 1994). Moreover, AVP infusion induced high AM mRNA levels and decreased ir-AM levels in the left atrium, suggesting an important role for the left atrium in the secretion of AM protein. Left ventricle might also contribute to AM secretion despite the similar AM protein levels between vehicle- and AVP-infused animals. However, the cardiac secretion of AM is small at least in the acute phase of cardiac pressure load, suggesting a paracrine and/or autocrine role for AM in regulation of cardiac function, rather than a classical endocrine one. AM secretion seems to be constitutive and in support of this no AM granules have been found in the heart (Jougasaki *et al.* 1995a). Furthermore, plasma ir-AM response to acute pressure load was not altered in ET_A/ET_B and AT_1 receptor antagonist-treated animals, suggesting that endogenous ET-1 and Ang II do not play a role in cardiac AM release.

6.4. Effect of ACE inhibitor and AT_1 receptor antagonist on cardiac gene expression

AM has been considered an additional tissue marker for left ventricular hypertrophy, because a positive correlation has been observed between left ventricular mass index and left ventricular AM peptide concentration (Jougasaki *et al.* 1997, Morimoto *et al.* 1999). In this study, the possible regulatory effects of 10 weeks` treatment with AT_1 receptor antagonism or ACE inhibition on cardiac AM gene expression were examined in normotensive WKY and hypertensive SHR rats.

In the present study, the doses of losartan and enalapril were adjusted so that they induced similar lowering of blood pressure of SHR throughout the experiments, whereas neither of the compounds influenced systolic blood pressure in the WKY rats. Similar

differential effects of antihypertensive drugs on blood pressure have been reported previously in SHR and WKY rats (Trippodo & Frochlich 1981). In SHR, the achieved decrease in blood pressure produced both by losartan and enalapril treatments was associated with complete normalization of cardiac hypertrophy, the results being consistent with previous investigations using these or other inhibitors of Ang II actions (Sen *et al.* 1980, Kojima *et al.* 1994). In addition, AT_1 receptor antagonism and ACE inhibition decreased cardiac mass in the normotensive WKY rats, suggesting an important role for Ang II in the regulation of normal cardiac growth occurring with aging. Previously, ACE inhibitors have been shown to decrease left ventricular mass in normotensive animals (Pfeffer *et al.* 1982, Azizi *et al.* 1989), and recently long term treatment of AT_1 receptor antagonism was observed to decrease cardiac mass in normotensive animals (Kahonen *et al.* 1999).

6.4.1. AM gene expression and release

In contrast to the findings of natriuretic peptides, ventricular AM synthesis is an insensitive marker of changes in blood pressure and ventricular hypertrophy in response to chronic ACE inhibitor and AT_1 receptor antagonist treatments in rats. In support of this, there was no correlation between cardiac mass reduction and AM or ir-AM levels, whereas a significant correlation between a decrease in cardiac hypertrophy and both ANP mRNA and IR-ANP levels was noted. Furthermore, there was no difference in ventricular AM gene expression between SHR and WKY rats. This finding is consistent with the lack of change in ventricular AM mRNA levels in other experimental models of pressure-induced hypertrophy including aortic banding (Kaiser *et al.* 1998) or hypertensive transgenic TGR(mREN-2)27 rats (Study I). These results differ from those described recently in experimental models of heart failure. Jougasaki *et al.* reported that in experimental congestive heart failure produced by rapid ventricular pacing in dogs, ventricular AM is activated in the progression of heart failure (Jougasaki *et al.* 1997). Furthermore, there was a positive correlation between left ventricular mass index and left ventricular AM concentrations, suggesting that ventricular AM expression may be increased by the ventricular hypertrophy in this model of heart failure (Jougasaki *et al.* 1997). More recently, a positive correlation between left ventricular mass index and left ventricular AM concentrations was observed during aortic banding for 28 days in rats, whereas in the same study left ventricular AM gene expression was induced at 1 day and after that decreased to the baseline level (Morimoto *et al.* 1999). Left ventricular AM mRNA levels are also increased by left coronary artery ligation in rats (Kaiser *et al.* 1998) and in hypertension of Dahl salt-sensitive rats on a high-salt diet (Shimokubo *et al.* 1996), and right ventricular AM mRNA and ir-AM levels are increased in experimental pulmonary hypertension (Shimokubo *et al.* 1995). Taken together, these studies suggest that ventricular AM gene expression is activated in well-described experimental models of heart failure, but not during chronic phase of pressure-induced cardiac hypertrophy in rats.

As described above for natriuretic peptides, the regulation of AM synthesis is also chamber-specific. Although losartan and enalapril treatments did not have any effect on left ventricular AM mRNA and ir-AM levels, both drugs increased left atrial irAM levels

in SHR. Thus, reduction of atrial AM peptide levels appears to reflect changes in systolic blood pressure. However, small but statistically significant increases in losartan-treated WKY rats and in enalapril-treated SHR in left atrial AM mRNA levels were also noted, suggesting that Ang II may also directly contribute to the long-term regulation of atrial AM gene expression. Thus, although further studies are required, both changes in hemodynamic load and autocrine/paracrine factors may play a role in the long-term regulation of atrial AM synthesis. Furthermore, the effects of chronic treatments with AT_1 receptor antagonist and ACE inhibitor on atrial AM differed from those of ANP and BNP, showing a distinct regulation pattern of synthesis for these three vasoactive peptides.

The mechanisms responsible for tissue-specific AM expression as well as the differential pattern of regulation of AM expression compared with those of ANP and BNP remains to be identified. The increased AM expression may be a response to other hormones/factors than Ang II that are activated in hemodynamic overload in failing heart. On the other hand, the lack of change in ventricular AM expression in the setting of significant alterations in cardiac mass suggests that AM is not involved in the long-term cardiac adaptive response to pressure overload, at least in rats. Moreover, the absence of increased AM expression in response to chronic pressure overload suggests that increased wall stress is not directly involved. However, acute cardiac pressure overload, produced by vasopressin infusion for 2 h, stimulated significantly AM gene expression in the left ventricle of heart in normotensive rats suggesting that hemodynamic overload may indeed regulate AM gene expression (Study I). Also cardiac overload produced by Ang II infusion for two weeks stimulated ventricular AM gene expression (study I). Therefore, the induction of AM expression may be a part of a selective change in cardiac gene expression in response to acute but not chronic hemodynamic load, and may play an important role in early cardiac failure and left ventricular hypertrophy. Taking into account the potent positive inotropic effect of AM (Szokodi *et al.* 1998) and increased ventricular AM production, AM may locally play a role in the compensatory mechanisms against acute deterioration of cardiac performance by enhancing myocardial contractility. Systematically, the natriuretic and vasorelaxing actions of AM secreted from the heart (Jougasaki *et al.* 1996) could play a compensatory role in order to decrease intravascular volume and peripheral resistance in congestive heart failure. Nevertheless, the functional consequences of cardiac AM requires further investigation, since in addition to AM levels potential changes in receptor number, binding and signaling mechanisms may modulate the actions of AM in the hypertrophic heart.

6.4.2. ANP and BNP gene expression and release

The changes in cardiac mass produced by losartan and enalapril treatments were accompanied by markedly reduced left ventricular ANP mRNA and immunoreactive ANP levels. This reduction in ANP synthesis may reflect a direct effect of Ang II on ventricular ANP gene expression, since the decrease in left ventricular ANP mRNA and immunoreactive ANP levels was seen in both SHR and WKY rats. The results agree with a previous study in which ACE inhibitors and AT_1 receptor antagonists were shown to decrease ANP gene expression in hypertrophied myocardium independent of blood

pressure levels (Kim *et al.* 1996). It was further found that the behavior of atrial ANP synthesis differs from that of ventricular ANP. The finding of decreased left atrial ANP storages in SHR as well as increased ANP concentration in the left atrium of SHR on losartan treatment can be explained by changes in ANP release from the atria secondary to alterations in left atrial pressure, because atrial ANP mRNA levels did not change in drug-treated SHR, and the treatments did not influence atrial ANP synthesis in normotensive rats. These results on ventricular and atrial ANP gene expression are in line with earlier results obtained with several other models of experimental hypertension (for review see Ruskoaho 1992). The finding that left atrial IR-ANP levels in SHR increased less in response to ACE inhibitor treatment than during AT_1 receptor blocker treatment may be related to differential actions of these drugs on tissue levels of bradykinins, prostaglandins and nitric oxide.

BNP gene expression in both atria and ventricles reacts more rapidly to hemodynamic overload than ANP, mimicking the rapid induction of proto-oncogenes in response to hemodynamic stress (Magga *et al.* 1994). In the present study, the effect of chronic administration of ACE inhibitors and AT_1 receptor antagonists on ventricular BNP gene expression was different between the strains. In SHR, both losartan and enalapril decreased significantly left ventricular AM gene expression, whereas drug treatments had no effect on AM gene expression in WKY rats. This suggests, as was previously reported (Ogawa *et al.* 1996), that the reduction in ventricular BNP gene expression could be more closely related to changes in systolic blood pressure than those in ventricular ANP gene expression. However, Ang II may directly contribute to the long-term regulation of atrial BNP synthesis, since left atrium levels of IR-BNP were decreased in both rat strains in response to drug treatments. This potential differential role of Ang II in regulating atrial and ventricular BNP gene expression is consistent with a recent study, in which endothelin-1 was reported to be an important paracrine regulator of BNP gene expression in the atria, but not in the left ventricle (Magga *et al.* 1997). Taken together, autocrine/paracrine factors may play greater role in the regulation of ventricular ANP and atrial BNP gene expression than atrial ANP and ventricular BNP gene expression.

6.5. Effect of CNP infusion and NEP inhibitor on cardiac AM gene expression

Since natriuretic peptides are expressed in cardiac tissue, ANP, BNP and CNP may have paracrine/autocrine effects in the heart. NPR_A receptor knockout in mice caused hypertension and cardiac hypertrophy associated with lethal events (Oliver *et al.* 1997). However, the effects of NPR_B receptor stimulation on the heart are not known. CNP, a selective agonist of NPR_B-receptors, was infused for 2 weeks in normotensive rats to examine the effects of CNP on cardiac AM gene expression and to compare whether the regulation of left ventricular AM gene expression differs from that of ANP and BNP. Since neutral endopeptidase (EC 3.4.24.11; NEP) is involved in metabolism of ANP (Olins *et al.* 1987), BNP, CNP (Erdos & Skidgel 1989) and AM (Lisy *et al.* 1998), sinorphan alone and in combination with CNP was administered to determine whether neutral endopeptidase inhibition potentiates the cardiac effects of CNP.

There was a trend for plasma ir-ANP and ir-BNP levels to rise in response to CNP infusion, at a dose that markedly increased ir-CNP levels in plasma. Interestingly, CNP infusion decreased left ventricular AM and BNP gene expression, whereas ANP gene expression remained unchanged. Furthermore, the decrease in BNP mRNA levels was associated with a significant decrease in ir-BNP levels. Thus, BNP and especially ANP seemed to respond more sensitively than AM to CNP infusion. As these effects were associated with an increased concentration of left ventricular ir-CNP, CNP may be an important paracrine factor in the regulation of left ventricular AM and BNP gene expression.

Cleavage at the Cys_{105}-Phe_{106} and Ser_{123}-Phe_{124} bonds of ANP by NEP will destroy the essential ring structure and result in biological inactivation of ANP (Olins *et al.* 1987). This membrane peptidase seems to be largely responsible for the activation not only of endogenous and exogenous ANP but also many other peptides, including BNP, CNP (Erdos & Skidgel 1989) and AM (Lisy *et al.* 1998). *In vivo*, neutral endopeptidase inhibitors prevent ANP and BNP from degrading, increase the half-life of ANP and BNP, and promote ANP- and BNP-mediated actions like diuresis, natriuresis and vasodilation (Lang *et al.* 1992). In this study, sinorphan treatment decreased left ventricular AM gene expression, but it did not have any effect on cardiac ANP and BNP gene expression or plasma ir-AM, ir-ANP and ir-BNP levels. Yet, NEP inhibitor sinorphan enhanced the cardiac effects of CNP by decreasing AM, BNP and ANP gene expression, and those decreases were associated with significant decreases in ir-AM, ir-BNP and ir-ANP levels. In addition, plasma ir-AM decreased and plasma ir-ANP increased. Since the NPR_C receptor has been thought to act as clearance receptor (Anand-Srivastava & Trachte 1993) and the rank order in the binding affinity for the NPR_C receptor is ANP>CNP>BNP, increased plasma ir-ANP may be due to decreased elimination of ANP by blocking of NPR_C receptors and inhibition of neutral endopeptidase.

In conclusion, intact animals were used to evaluate the role of CNP in the regulation of cardiac AM, ANP and BNP gene expression in the hearts of normotensive rats. It was also tested whether sinorphan potentiates the effects of CNP. The results show that CNP infusion increased left ventricular and plasma ir-CNP followed with significant decreases in AM and BNP mRNA levels, suggesting a paracrine role for CNP in the regulation of left ventricular AM and BNP gene expression. Sinorphan treatment alone decreased AM gene expression, but when combined with CNP infusion, sinorphan potentiated the effects of CNP infusion on the regulation of ANP gene expression and peptide secretion, possibly by decreasing the elimination of ANP.

6.6. General overview of AM gene expression in the rat heart

Pressure overload rapidly stimulated AM gene expression in the heart of normotensive rats, well before the development of left ventricular hypertrophy. The acute increase in left ventricular AM gene expression occured within 2 h, whereas in the left atrium the induction of AM gene expression was observed earlier, at 30 min. The induction of AM gene expression mimics the rapid induction of proto-oncogenes in response to hemodynamic stress. Thus, locally generated AM in the heart muscle may play a

significant role in cardiac adaptation to acute changes in mechanical load. The signal transduction pathways regulating AM gene expression are not known. On the basis of this study, Ang II and ET-1 are not obligatorily required for wall stretch to trigger the increased AM gene expression in left atrial and ventricular myocytes in response to acute cardiac overload.

Chronic increased workload of the heart produced by angiotensin II infusion for 2 weeks resulted in increased left ventricular AM gene expression. CNP infusion and neutral endopeptidase inhibitor (sinorphan) downregulated AM gene expression in normotensive rats at 2 weeks. Furthermore, 10 weeks` treatment with AT_1 receptor antagonist or ACE inhibitor did not regulate left ventricular AM gene expression in SHR and WKY rats, whereas left atrial AM gene expression increased in losartan-treated WKY rats and in enalapril-treated SHR when compared to their respective vehicles. Long-term regulation of cardiac AM gene expression differs from that of natriuretic peptides. It seemed that, in addition to pressure overload, Ang II acting via the AT_1 receptor may contribute to the long-term regulation of ventricular ANP gene expression, while changes in ventricular BNP gene expression are more related to changes in hemodynamic load. Interestingly, systolic blood pressure, cardiac hypertrophy and Ang II inhibition did not influence ventricular AM gene expression in long term. The regulation of all three peptides appeared to be tissue-specific, both pressure and Ang II probably contributing to the atrial AM synthesis. These and previous findings suggested a distinct role for AM in cardiovascular homeostasis and pathophysiology, probably in early adaptation of pressure overload as well as in the failing heart.

7. Summary and conclusions

1. The results of the present study showed that cardiac wall stretch was a major stimulus for the early induction of AM gene expression in the left ventricle and atrium, mimicking the rapid induction of proto-oncogenes in response to hemodynamic stress. The acute increase in left ventricular AM gene expression occured within 2 h, whereas in the left atrium the induction of AM gene expression was observed earlier, at 30 min, showing that AM reacted rapidly against increased cardiac workload.

2. The left atrium seemed to have a role in contributing at least partly to the increased circulating AM. However, the cardiac secretion of AM was small at least during the acute phase of cardiac pressure load, suggesting a paracrine and/or autocrine role for AM in the regulation of cardiac function.

3. Mechanical stretch may directly induce cardiac AM gene expression, or autocrine/paracrine mechanisms such as Ang II or ET-1 are activated by pressure overload, which then evokes rapid induction of AM gene expression. Thus intact animals were used to map the role of two paracrine/autocrine factors, ET-1 and Ang II, in the acute induction of AM gene expression in response to cardiac overload. The induction of left atrial and ventricular AM gene expression was shown to be Ang II- and ET-1-independent.

4. AT_1 receptor antagonism and ACE inhibition for 10 weeks resulted in the regression of cardiac hypertrophy associated with a decrease in left ventricular ANP gene expression in normotensive and hypertensive rats, although systolic blood pressure did not change significantly in WKY rats. These changes in systolic blood pressure and cardiac hypertrophy induced by losartan and enalapril treatments had no effect on ventricular AM gene expression, suggesting that ventricular AM synthesis is an insensitive marker of chronic changes in hemodynamic load or myocyte hypertrophy. The expression of AM, ANP and BNP was differently regulated both in the left ventricle and atria in response to AT_1 receptor antagonism and ACE inhibition.

5. CNP infusion-induced increases in left ventricular and plasma ir-CNP levels were associated with significant decreases in AM and BNP mRNA levels, suggesting a paracrine role for CNP in the regulation of left ventricular AM and BNP gene expression. Sinorphan treatment alone decreased only AM gene expression, but combined with CNP infusion, sinorphan potentiated the effects of CNP infusion on the

regulation of ANP gene expression and peptide secretion, possibly due to decreased elimination of ANP.

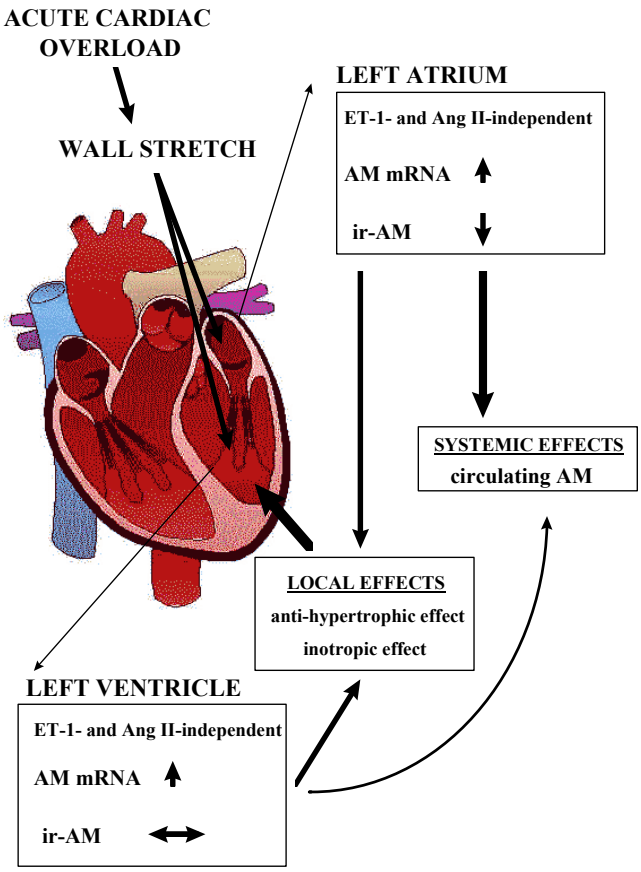

Fig. 11. A model of acute cardiac-overload induced AM gene expression in the left atrium and ventricle of rat heart.

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