THE ROLE AND MECHANISMS OF ANGIOTENSIN II IN REGULATING THE NATRIURETIC PEPTIDE GENE EXPRESSION IN RESPONSE TO CARDIAC OVERLOAD

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**Abstract**

Heart responds to pathological hemodynamic stress by increasing cardiac myocyte size, reprogramming gene expression and enhancing contractile protein synthesis. Neurohumoral factors mediate hypertrophic adaptation either directly via specific receptors or indirectly by increasing blood pressure and cardiac load. The aim of this study was to evaluate the role of angiotensin II (Ang II) in the atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) gene expression during cardiac overload. Furthermore, the mechanisms of action of Ang II in regulating cardiac gene expression were studied.

Hemodynamic stress was produced by Ang II or nitric oxide (NO) synthase inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) administration in conscious rats. Despite hypertension and increased left ventricular ANP and BNP mRNA levels, L-NAME administration for 8 weeks did not induce left ventricular hypertrophy. Ang II type 1 receptor (AT\textsubscript{1}) antagonism decreased significantly L-NAME-induced hypertension and ventricular ANP gene expression. Ang II-induced cardiac overload produced significant increase in ventricular ANP and BNP mRNA levels at 12 and 72 h, respectively. To study whether the factors synthesized by adrenals modulate the response of Ang II, the effects of adrenalectomy were studied. In Ang II-treated rats, adrenalectomy either abolished or blunted the early activation of ANP and BNP gene expression, respectively.

Ang II infusion for 2 weeks increased cardiac mass and blood pressure measured by telemetry, and produced changes in diastolic function detected by echocardiography. By using direct plasmid DNA injections into the rat myocardium, BNP promoter activity was observed to increase at 2 h and remain up-regulated up to 2 weeks of Ang II infusion, except at 12 h. BNP mRNA levels increased at 2 h but decreased to basal levels after 72 h. Mutation of GATA elements of the BNP promoter and DNA binding assays revealed that GATA4 mediates the Ang II-responsiveness of the BNP gene.

These results indicate that Ang II plays an important role in regulating natriuretic peptide gene expression during cardiac overload. ANP and BNP gene expression in the rat heart is modulated by the adrenal factors during Ang II-stimulated hemodynamic stress and the AT\textsubscript{1} receptor antagonism in NO-deficient hypertension. Moreover, ventricular BNP gene expression in Ang II-induced hypertension *in vivo* is controlled by posttranscriptional mechanisms and GATA elements.

**Keywords:** natriuretic peptides, angiotensin II, transcription factors, hypertrophy
To my family
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Oulu, April 2002

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Abbreviations

ACE angiotensin converting enzyme
ADX adrenalectomy
AM adrenomedullin
AMI acute myocardial infarction
Ang angiotensin
ANOVA analysis of variance
ANP atrial natriuretic peptide
AP-1 activator protein-1
AR adrenoceptor
AT1 angiotensin receptor subtype
ATF activating transcription factor
AVP arginine8-vasopressin
BNP B-type natriuretic peptide
[Ca2+]i intracellular calcium concentration
CaM calmodulin
CaMK calcium-calmodulin-dependent kinase
cAMP cyclic adenosine monophosphate
cGMP cyclic guanosine monophosphate
CHF congestive heart failure
CNP C-type natriuretic peptide
CRE cAMP response element
CT-1 cardiotrophin-1
DAG diacylglycerol
DNP dendroaspis natriuretic peptide
DOCA deoxycorticosterone acetate
E/A early rapid diastolic filling wave (E) to late diastolic filling wave (A)
ECM extracellular matrix
EDHF endothelium-derived hyperpolarizing factor
EF ejection fraction
EPI epinephrine
ERK extracellular signal regulated kinase
ET  endothelin
ETα  endothelin receptor subtype
FGF  fibroblast growth factor
FOG  friend of GATA
GDP  guanosine diphosphate
GPCR  G-protein-coupled receptor
GTP  guanosine triphosphate
HPLC  high-pressure liquid chromatography
IGF  insulin-like growth factor
IL  interleukin
i.p.  intraperitoneally
IP3  inositol-1,4,5-triphosphate
ir  immunoreactive
ISO  isoproterenol
i.v.  intravenously
JAK  janus kinase
JNK  c-Jun N-terminal kinase
L-NAME  Nω-nitro-L-arginine methyl ester
luciferase  luc
LVEDD  left ventricular end-diastolic dimension
LVESD  left ventricular end-systolic dimension
LVFS  left ventricular fractional shortening
L VH  left ventricular hypertrophy
LVW/BW  left ventricular weight to body weight
MAP  mean arterial pressure
MAPK  mitogen-activated protein kinase
MEF2  myocyte enhancer factor-2
MHC  myosin heavy chain
MKK  mitogen-activated protein kinase kinase
MKKK  mitogen-activated protein kinase kinase kinase
MKP-1  mitogen-activated protein kinase phosphatase-1
MLC-2  myosin light chain-2
NADPH  nicotinamide adenine dinucleotide phosphate
NE  norepinephrine
NEP  neutral endopeptidase
NF-AT  nuclear factor of activated T-cells
NF-κB  nuclear factor κB
NKE  Nkx-2.5 response element
NO  nitric oxide
NOS  nitric oxide synthase
NPR  natriuretic peptide receptor
NRSE  neuron-restrictive silencer element
NT-proANP  amino-terminal proANP
Oct-1  octamer-1
PDGF  platelet-derived growth factor
PE  phenylephrine
<table>
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<tr>
<th>Acronym</th>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>PTPase</td>
<td>protein tyrosine phosphatase</td>
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<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
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<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>SAC</td>
<td>stretch-activated ion channel</td>
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<tr>
<td>SAPK</td>
<td>stress-activated protein kinase</td>
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<tr>
<td>s.c.</td>
<td>subcutaneously</td>
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<td>sCP</td>
<td>salmon cardiac peptide</td>
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<tr>
<td>SD</td>
<td>Sprague-Dawley</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>Ser/Thr</td>
<td>serine/threonine</td>
</tr>
<tr>
<td>SH-PTP1</td>
<td>rc homology domain-containing protein tyrosine phosphatase 1</td>
</tr>
<tr>
<td>SHR</td>
<td>spontaneously hypertensive rat</td>
</tr>
<tr>
<td>SHRSP</td>
<td>spontaneously hypertensive rat-stroke prone</td>
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<tr>
<td>SRE</td>
<td>serum response element</td>
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<tr>
<td>SRF</td>
<td>serum response factor</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SV</td>
<td>simian virus</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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<td>WKY</td>
<td>Wistar-Kyoto</td>
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<td>zeta</td>
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<tr>
<td>lambda</td>
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List of original papers

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1 Introduction

Myocardial hypertrophy is an adaptive process in response to cardiac overload resulting from the interaction between mechanical forces and neuroendocrine factors. Hypertrophic response of cardiac myocyte consists of several qualitative and quantitative changes including altered gene expression, increased protein synthesis and enhanced sarcomeric organization. In the long term, compensated hypertrophy may progress to maladaptive decompensated hypertrophy and heart failure (for review, see Sugden & Clerk 1998). One of the most important hypertrophic factors is circulating and locally produced angiotensin II (Ang II), a biologically active component of the renin-angiotensin system (RAS). The mechanisms of Ang II in regulating cell growth may involve hemodynamic changes, augmented secretion of other neurohumoral/paracrine factors or direct hypertrophic effects (Sadoshima & Izumo 1993, for review, see Kim & Iwao 2000). The biological effects of Ang II have been shown to be mediated mainly via Ang II type 1 (AT₁) receptor, whereas the importance of Ang II type 2 (AT₂) receptor remains unclear. Ang II-induced complex signaling pathways transducing the hypertrophic stimulus from the cellular membrane to the nucleus, and transcription factors interacting with cis regulatory elements of target genes are widely studied (Zhang & Pratt 1996, Kudoh et al. 1997, Izumi et al. 2000). However, exact mechanisms leading to increased DNA binding of nuclear transcription factors and subsequent induction of gene expression remain unclear (for review, see Swynghedauw 1999).

The heart responds to cardiac stress by synthesizing polypeptides including atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), adrenomedullin (AM) and bradykinin. These peptides play a compensatory role in the maintenance of intravascular volume and cardiac filling pressures during increased myocardial load (for reviews, see Nakao et al. 1992a, Matsubara 1998, Samson 1999). ANP and BNP are cardiac-specific genes, since they are not expressed in the skeletal muscle cells. Mechanical wall stretch increases BNP mRNA levels within an hour, while ANP gene expression is activated in response to more sustained stimulus (for review, see Ruskoaho 1992, Mäntymaa et al. 1993). The aim of this work was to study the role of Ang II in the gene expression of cardiac natriuretic peptides in response to hemodynamic overload. Moreover, the mechanisms of actions of Ang II in regulating the cardiac gene expression, particularly BNP, were evaluated.
2 Review of the literature

2.1 The response of cardiac myocytes to hypertrophic stimulus

Adult cardiac myocytes are terminally differentiated cells, which have lost their ability to divide. The enlargement of heart muscle in response to hypertrophic stimuli is thus achieved by an increase in myocyte size rather than number (for review, see Hefti et al. 1997). Cardiac myocyte hypertrophy also involves specific qualitative alterations in cardiac gene expression and cell phenotype (for review, see Chien et al. 1991). In an attempt to normalize excessive forces and work performed per contractile unit, myocardial hypertrophy unloads the heart by accumulation of sarcomeres in order to distribute tension across a greater mass (Grossman et al. 1975). Furthermore, molecular and biochemical modifications known to occur during pressure overload-induced hypertrophy include changes in contractile protein isoforms, increased dependence on glycolysis for energy metabolism, and changes in intracellular calcium concentration ([Ca^{2+}]_{i}) regulation and excitation-contraction coupling (for reviews, see Sugden & Clerk 1998, Bers 2000). Thus, cardiac hypertrophy represents one of the most important adaptive responses to increase the ability of the heart to function properly in response to augmented workload. Interestingly, a recent work has shown that transplanted heart contains undifferentiated cells of the recipient origin, and these cells undergo active proliferation and acquisition of the mature phenotype of cardiac myocytes, suggesting that the heart may regenerate under certain circumstances (Quaini et al. 2002).

In addition to increased cell size and contractility, hypertrophic changes in cardiac myocytes concern either upregulation or downregulation of the gene expression. The expression of immediate-early proto-oncogenes, including c-fos, c-jun, Egr-1 and c-myc, increases rapidly and transiently within an hour in response to hypertrophic stimuli, independent of new protein synthesis (for review, see Yamazaki et al. 1995). The activation of the c-fos mRNA levels have been shown to increase within 15 minutes, peak at 30 minutes and decline to undetectable levels after 4 h of cardiac myocyte stretching in vitro (Komuro et al. 1990). Similarly, cardiac load produced by aortic coarctation activates the gene expression of c-fos and c-myc within an hour in vivo (Izumo et al. 1988). The c-fos mRNA levels are proportional to the magnitude of left ventricular
systolic wall stress in an isolated perfused rat heart (Schunkert et al. 1991). Furthermore, Ang II rapidly activates the expression of immediate-early genes in vitro (Sadoshima & Izumo 1993, Kudoh et al. 1997). Translated protein products of the c-fos and c-jun genes are suggested to play an important role as transcription factors of cardiac genes (Hai & Curran 1991). In addition to the early induction of proto-oncogenes, the cardiac peptides BNP and AM are also activated rapidly in response to hemodynamic stress (Magga et al. 1994, Romppanen et al. 1997).

In rodents, two isoforms of myosin heavy chain (MHC) genes, α- and β-MHCs, are expressed in the cardiac ventricle, β-MHC predominantly in fetal hearts and α-MHC mainly in healthy adult hearts (for review, see Yamazaki et al. 1995). Similarly, skeletal α-actin with greater contractility is predominantly activated in the fetal and neonatal mouse heart and cardiac α-actin in the healthy adult mouse heart (Hewett et al. 1994). Since also ANP is highly expressed during fetal development, β-MHC, skeletal α-actin and ANP are considered to be fetal-type cardiac genes (for review, see Sugden & Clerk 1998). The expression of β-MHC, skeletal α-actin and ANP genes is reactivated in response to hypertrophic stimulus following the expression of immediate-early genes (Kim et al. 1995, Yokota et al. 1995). Pressure overload in vivo by aortic coarctation induces the expression of skeletal α-actin within 2 days (Izumo et al. 1988), while Ang II infusion increases left ventricular skeletal α-actin, β-MHC and ANP mRNA levels without blood pressure elevation in conscious rats within 24 h (Kim et al. 1995). Furthermore, constitutively expressed contractile protein genes, myosin light chain-2 (MLC-2) and cardiac α-actin, are upregulated in the longer term (for review, see Sugden 1999). The activation of the fetal and late response gene expression has been shown to require new protein synthesis (for review, see Sadoshima & Izumo 1997).

2.2 Neurohumoral and paracrine/autocrine factors

2.2.1 Renin-angiotensin system

2.2.1.1 Synthesis, location and regulation

Several neurohumoral and paracrine/autocrine factors and systems are involved in mediating the cardiac overload response (for review, see Hefti et al. 1997). One of these is the RAS, which regulates blood pressure, and electrolyte and volume homeostasis (for review, see Kim & Iwao 2000). The first precursor of biologically active Ang II is angiotensinogen, which is cleaved by aspartyl protease renin to form an inactive decapeptide Ang I. Ang I is subsequently processed mainly by the zinc metallopeptidase angiotensin converting enzyme (ACE) to synthesize the octapeptide Ang II (Fig. 1). In some tissues, Ang II may also be generated by alternative enzymes, such as chymase, whose participation in the formation of Ang II remains unclear (Urata et al. 1990, Kokkonen et al. 1997). Ang II is further degraded into a heptapeptide, Ang III, and a hexapeptide, Ang IV, by the aminopeptidases and into a heptapeptide, Ang-(1-7), by neutral endopeptidases (NEPs). While angiotensinogen is mainly produced in the liver,
the main site for renin and ACE synthesis are the kidney and lung, respectively (for review, see Blume et al. 1999).

Fig. 1. The renin-angiotensin system and the connection of this vasoconstrictive system to the bradykinin-stimulated vasodilating effectors (for review, see Lindpaintner & Ganten 1990). The numbers in parentheses indicate the numbers of the amino acids of the respective peptides. ACE, angiotensin converting enzyme; EDHF, endothelium-derived hyperpolarizing factor; NEP, neutral endopeptidase; NO, nitric oxide.

Originally, the RAS was considered to be solely an endocrine system, in which the circulating Ang II functions as a hormonal effector (for review, see Dostal et al. 1997). However, the RAS has been shown to reside within several organs and cells, such as heart, kidney, lung, brain, cardiac myocytes, fibroblasts, endothelial cells and vascular smooth muscle cells (VSMCs), where it is believed to act as a paracrine/autocrine factor (for review, see Dostal & Baker 1999). Indeed, all enzymes and substrates of the RAS and Ang II receptors are expressed in the myocardium (Pieruzzi et al. 1995, van Kats et al. 1998). Small amounts of the intracellular Ang II in the neonatal rat ventricular cardiac myocytes have been reported to exist in the secretory vesicles (Sadoshima et al. 1993). Cardiac RAS is suggested to be regulated by local mechanical stress in vivo (Lee et al. 1996). In addition to local production in the heart, all components of the RAS are synthesized in the brain in low amounts (for review, see Unger et al. 1988). In vascular endothelial cells, ACE and renin have been demonstrated to be synthesized locally (Lincoln et al. 1990, Okamura et al. 1992). However, renin is also suggested to be taken
up from the circulation by the endothelium for Ang I formation and not to be generated in the vascular endothelial cells (Hilgers et al. 2001).

Several studies have reported that the RAS is activated in the hypertrophied heart (Kojima et al. 1994, Takemoto et al. 1997a). In response to volume overload, expression of renin and ACE but not angiotensinogen and AT$_1$ receptor genes have been reported to increase in left ventricular tissue (Boer et al. 1994). Pressure overload increases left ventricular angiotensinogen and AT$_1$ receptor gene expression (Wang et al. 1997). In human and rat myocardial infarction, renin, angiotensinogen and ACE mRNA levels are elevated (for review, see Dostal & Baker 1999, Silvestre et al. 1999). The administration of glucocorticoids, estrogen, thyroid hormone in vivo and β-adrenergic agonists in vitro stimulates angiotensinogen mRNA levels (Campbell & Habener 1986, for review, see Dostal & Baker 1999). Mechanical stretch increases the expression of angiotensinogen, renin, ACE and AT$_{1A}$ genes (Malhotra et al. 1999). In addition, stretching of cardiac myocytes in vitro induces the release of Ang II from cardiac myocytes acutely within 10 minutes and the expression of angiotensinogen gene after 6 hours of the induction (Sadoshima et al. 1993). Ang II downregulates the gene expression of renin and angiotensinogen in cardiac fibroblasts, but upregulates the mRNA levels of these genes in cardiac myocytes (Sadoshima & Izumo 1993, Malhotra et al. 1999, for review, see Dostal 2000). Moreover, AT$_1$ and AT$_2$ receptors are upregulated in many pathological conditions including aortic banding, myocardial infarction and cardiomyopathy (for review, see Matsubara 1998). Thus, these distinct responses of the components of RAS to hypertrophic stimuli show that the RAS is an important modulator of the cardiac function.

2.2.1.2 Receptors and effects of angiotensin II

Two of the four known Ang II receptor subtypes, AT$_1$ and AT$_2$, have been cloned and characterized as G-protein-coupled receptors (GPCR) consisting of seven transmembrane domains (Chiu et al. 1989, Sasaki et al. 1991, Mukoyama et al. 1993). In rodents, AT$_1$ receptors are further divided into two receptor subgroups of AT$_{1A}$ and AT$_{1B}$, which are the products of different genes (MacTaggart et al. 1997). AT$_1$ receptors are expressed in the heart, blood vessels, kidney, adrenal gland, liver, brain and lung in adult tissues (for review, see Allen et al. 2000). The well-known physiological effects of Ang II in the cardiovascular system, such as elevated blood pressure, vasoconstriction, aldosterone and catecholamine release, and renal sodium and water absorption, are mediated by AT$_1$ receptors (for review, see Timmermans et al. 1993). Ang II stimulates upstream activators of glucocorticoids, corticotropin-releasing hormone and adrenocorticotropin hormone, via AT$_1$ receptors in response to stress (Sumitomo et al. 1991, Armando et al. 2001). Furthermore, AT$_1$ receptors induce positive inotropic and chronotropic actions in cardiac tissue both directly by increasing Ca$^{2+}$ influx during the plateau phase of action potential and indirectly by facilitating adrenergic neurotransmission in the heart (for review, see Kim & Iwao 2000). AT$_1$ receptors mediate the Ang II-stimulated collagen synthesis and enhance cardiac remodeling referred to as perivascular and myocardial fibrosis, as well as vascular medial thickening (Tharaux et al. 2000, Lim et al. 2001). Several studies
imply that Ang II can stimulate, via activation of AT₁ receptors, growth of the cardiac muscle (Dostal & Baker 1992, Sadoshima & Izumo 1993) and interstitium (for review, see Weber & Brilla 1991) in vitro and in vivo independent of mechanical stress.

The role and mechanisms of Ang II in myocardial hypertrophy have been targets of great interest. The expression of many immediate-early genes (c-fos, c-jun, junB, Egr-1 and c-myc) and fetal marker genes of cardiac hypertrophy (ANP, skeletal α-actin) is stimulated by AT₁ receptor activation (Sadoshima & Izumo 1993). Indeed, Ang II has been shown to increase left ventricular ANP mRNA levels independent of hemodynamic load (Baltatu et al. 2000). Furthermore, targeted deletion of the angiotensinogen gene produces decrease in blood pressure and complete loss of plasma Ang I in mice (Tanimoto et al. 1994), whereas overexpression of the angiotensinogen gene in cardiac myocytes results in myocardial hypertrophy in the absence of hypertension in transgenic mice (Mazzolai et al. 2000). In order to study whether Ang II can induce cardiac hypertrophy directly via AT₁ receptor, mice overexpressing the human AT₁ receptor were generated. These transgenic mice develop cardiac hypertrophy and remodeling despite the lack of increase in blood pressure, suggesting a direct effect of Ang II on myocardial hypertrophy (Paradis et al. 2000). Moreover, several studies have shown that ACE inhibitors and AT₁ receptor antagonists have antihypertrophic effects on cardiac myocytes in vitro and in vivo (Sadoshima & Izumo 1993, Kojima et al. 1994, Kim et al. 1996). Also, increased left ventricular mass produced by aortic coarctation can be completely prevented by the ACE inhibitor without any change in afterload (Baker et al. 1990). AT₁<sub>α</sub> gene knockout mice do not express any identifiable abnormal phenotype (Chen et al. 1997), whereas double mutant mice homozygous for both AT₁<sub>α</sub> and AT₁<sub>β</sub> null mutation are characterized by marked hypotension and abnormalities in morphology of several organs (Tsuchida et al. 1998). Interestingly, AT₁<sub>α</sub> gene knockout mice have been reported to develop ventricular hypertrophy in response to pressure overload, suggesting that AT₁ receptors are not required for the cardiac hypertrophy (Hamawaki et al. 1998, Harada et al. 1998).

AT₂ receptors are found in developing fetal tissues, but the gene expression of these receptors decreases rapidly after birth. In adults, AT₂ receptors are expressed mainly in the uterus, ovary, brain, heart and adrenal medulla (for review, see Kim & Iwao 2000). Blood pressure has been shown to increase in AT₂ receptor gene knockout mice, suggesting that AT₂ receptors are involved in the regulation of systemic blood pressure by counteracting the effects of AT₁ receptors (Hein et al. 1995, Ichiki et al. 1995). Mice overexpressing AT₂ receptor resist Ang II-stimulated vasoconstrictive and blood pressure actions, and this vasodilative effect of AT₂ receptor is blocked by inhibitors of bradykinin type 2 receptor and nitric oxide synthase (NOS) (Tsutsumi et al. 1999). Moreover, several other studies have demonstrated that Ang II induces vasodilation and cardioprotection via kinin/nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) system by activating AT₂ receptors (for review, see Matsubara 1998). In addition to hypotensive effects, AT₂ receptor stimulation has been shown to induce programmed cell death (Yamada et al. 1996) and inhibit AT₁ receptor-mediated DNA synthesis and cell growth in cardiac myocytes and fibroblasts (Booz & Baker 1996, Ohkubo et al. 1997). However, apoptosis is not increased in cardiac myocytes of transgenic mice overexpressing the AT₂ receptor in response to Ang II infusion (Sugino et al. 2001). Furthermore, the AT₂ receptor antagonist is not able to attenuate the Ang II-induced
cardiac myocyte apoptosis, whereas AT₁ receptor antagonist inhibits the programmed ventricular myocyte cell death (Kajstura et al. 1997). These studies show that the role of Ang II receptors in regulating programmed cell death remains obscure. Interestingly, the effects of AT₁ receptor may vary depending on the tissue, since the AT₁ receptor gene knockout mice develop myocardial hypertrophy but not coronary arterial thickening or perivascular fibrosis in response to aortic banding (Akishita et al. 2000). Although AT₁ receptors have been suggested to mediate the hypertrophic actions of Ang II, deletion of mouse AT₁ receptor gene has been able to prevent the left ventricular growth in transgenic mice in response to pressure overload in vivo (Senbonmatsu et al. 2000). Similarly, AT₂ receptor gene knockout mice do not develop myocardial hypertrophy or cardiac fibrosis in contrast to wild-type animals in response to pressure overload, suggesting that AT₂ receptors are involved in mediating the myocardial hypertrophic response (Ichihara et al. 2001). Thus, the precise function of this receptor subtype in regulating myocyte hypertrophy remains to be clarified.

2.2.1.3 Aldosterone

Aldosterone, a corticosteroid hormone, is produced mainly in the zona glomerulosa cells of the adrenal cortex (Ratajska et al. 1994). However, aldosterone is also synthesized in other tissues such as blood vessels, heart and brain (Mellon & Deschepper 1993, Takeda et al. 1995, Takeda et al. 2000a). Cardiac aldosterone production is controlled by the RAS, since cardiac aldosterone synthesis increases in the Ang II-infused rats and decreases in the rats treated with ACE inhibitor (Takeda et al. 2000a). The specificity of the mineralocorticoid action in aldosterone target tissues is based on the 11β-hydroxysteroid dehydrogenase (Young et al. 1994). A nuclear mineralocorticoid receptor, mediating the responses of aldosterone, and 11β-hydroxysteroid dehydrogenase enzyme have been detected in the vascular endothelial cells, VSMCs and cardiac myocytes (for review, see Farman & Rafestin-Oblin 2001). Aldosterone regulates myocardial norepinephrine (NE) uptake, electrolyte balance and cardiac fibrosis (Brilla & Weber 1992, Barr et al. 1995). In rats, peripheral infusion of aldosterone induces cardiac hypertrophy and fibrosis without increasing blood pressure (Young et al. 1995). Myocardial aldosterone production is enhanced in a genetically hypertensive rat strain, spontaneously hypertensive rats-stroke prone (SHRSP), and in rats with myocardial infarction and humans with left ventricular dysfunction (Silvestre et al. 1999, Takeda et al. 2000a, Mizuno et al. 2001a). Treatment with spironolactone, a competitive mineralocorticoid receptor antagonist, reduces myocardial fibrosis, hypertrophy and inflammation independent of blood pressure in Ang II-infused rats (Fiebeler et al. 2001). In addition to spironolactone, AT₁ receptor antagonist may prevent aldosterone–induced cardiac fibrosis (Robert et al. 1999).
2.2.2 Endothelin-1

Endothelins (ETs) constitute a family of vasoactive peptides originally found in vascular endothelial cells (Yanagisawa et al. 1988). Three 21-amino acid isoforms have been identified and named ET-1, ET-2 and ET-3 (Inoue et al. 1989). ET-1 is the main isoform of the ETs in the vascular tissue and is produced also in the heart (for review, see Ito 1997). The effects of ETs are mediated by specific GPCRs, ETₐ and ETₐ, predominantly via phospholipase C (PLC), inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) – mediated signaling pathway. The ETₐ receptor is expressed in cardiac myocytes and smooth muscle cells, but not in endothelial cells (Hosoda et al. 1991), and it is mainly responsible for the vasoconstrictor and cardiac actions of ET-1 (for review, see Ito 1997). ETₐ receptors are highly expressed in endothelial cells (Ogawa et al. 1991b), and this receptor subtype induces NO and prostacyclin-mediated vasodilation (Suzuki et al. 1991). The overall physiological effect of ET-1 seems to be an increase in blood pressure, as suggested by the hypotensive effects of mixed ETₐ/ETₐ receptor antagonists in healthy humans (Haynes et al. 1996).

Mechanical stretching of cardiac myocytes in vitro and cardiac overload in vivo effectively increase ET-1 mRNA levels and peptide concentrations (Ito et al. 1994, Yamazaki et al. 1996). Furthermore, Ang II administration in cultured rat cardiac myocytes and RAS overexpression in transgenic mice have been shown to induce myocardial hypertrophic responses by endogenous synthesis and release of ET-1 (Ito et al. 1993, Kudoh et al. 1997, Maki et al. 1998). In addition, Ang II induces the paracrine release of ET-1 from cardiac fibroblasts in neonatal rat cell culture to promote cardiac myocyte hypertrophy (Gray et al. 1998). ET-1 and Ang II receptors share common signaling pathways, and ETₐ receptor blockade largely attenuates the hypertrophic responses induced by Ang II, mechanical load or ET-1 (Ito et al. 1993, Yamazaki et al. 1996). ET-1 has been shown to dose-dependently inhibit renin synthesis and directly stimulate aldosterone production from the adrenals (for review, see Rossi et al. 1999).

In the cardiovascular system, ET-1 enhances positive inotropic and chronotropic effects, sympathetic nervous system activity and mitogenesis, and modulates salt and water balance (for review, see Giannessi et al. 2001). Although ET-1 has long been considered the most powerful vasoconstrictor substance (for review, see Rubanyi & Polokoff 1994), urotensin II may be even more potent mammalian vasoconstrictor, in the order of ten-times more potent than ET-1 (Ames et al. 1999). ET-1 is a potent stimulus for ANP and BNP transcription and release in vivo and in vitro (Fyhrquist et al. 1993, Nakagawa et al. 1995, Magga et al. 1997a, Kovacic et al. 1998, He & LaPointe 2001a). In response to hypertrophic stimulus in neonatal rat cardiac myocytes, ET-1 stimulates the gene expression of contractile proteins including MLC-2, skeletal α-actin and troponin I (Ito et al. 1991, Nakagawa et al. 1995). In animals and humans with heart failure, cardiac levels of ET-1 are elevated according to the severity of the disease, and ET receptor antagonists have been shown to have a beneficial effect on both cardiac hypertrophy and symptoms of congestive heart failure (CHF) (for review, see Spieker et al. 2001). Taken together, ET-1 is an important vasoactive peptide, which may provide a useful target molecule for the treatment of heart failure.
2.2.3 Catecholamines

The sympathetic nervous system is activated and plasma levels of catecholamines, epinephrine (EPI) and NE, are elevated in response to cardiac overload and CHF (for review, see Esler et al. 1997). Catecholamines, released from the adrenal medulla and sympathetic nerve endings, have positive chronotropic and inotropic effects in the heart (Wong et al. 1990, for review, see Scheuer 1999). Specific GPCRs, α- and β-adrenoceptors (α- or β-AR), mediate the catecholamine-induced myocardial gene expression and growth of cardiac myocytes (for review, see Brodde & Michel 1999). The activation of α-AR is transduced mainly via Gq-protein connected with the PLC/IP3/DAG pathway, whereas the stimulation of β-AR is mediated predominantly through cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) pathways via Gs-protein (for review, see Scheuer 1999). In contrast, parasympathetic stimulation via muscarinic acetylcholine GPCRs induces negative chronotropic and inotropic effects (for review, see Brodde & Michel 1999).

In rats, α-AR stimulation by NE and β-AR stimulation by isoproterenol (ISO) induce myocardial hypertrophy, which is prevented by specific receptor antagonists (for review, see Zimmer 1997). In vivo pressure overload-induced cardiac hypertrophy and the activation of intracellular hypertrophic signaling pathways are inhibited in mice lacking endogenous NE and EPI (Rapacciuolo et al. 2001). Stimulation of cultured cardiac myocytes with α- or β-adrenergic agonists increases protein synthesis and cardiac myocyte growth, suggesting a direct effect of catecholamines independent of mechanical stress (for review, see Simpson et al. 1991). As a sign of myocardial hypertrophy, NE-stimulation has been shown to induce early increase in the expression of proto-oncogenes, c-fos and c-myc, in isolated perfused rat heart (for review, see Zimmer 1997). Furthermore, α- and β-adrenergic agonists activate transcription of the BNP and ANP genes, and increased half-life of BNP mRNA may be due to α-AR-mediated posttranscriptional stabilization by α1-adrenergic receptor agonist phenylephrine (PE) (Hanford et al. 1994, Hanford & Glembotski 1996, He et al. 2000a).

The cardiac sympathetic nervous system is activated by Ang II, since the stimulation of AT1 receptor facilitates the release of NE from cardiac sympathetic nerve terminals (for review, see Kim & Iwao 2000). In Ang II-administered rats, surgical cardiac sympathectomy or treatment with β-AR antagonist prevents cardiac myocyte necrosis, implicating interactions between the sympathetic nervous system and RAS in regulating cardiac damage (Henegar et al. 1998). The sympathetic nervous system is a potent stimulatory factor for the control of renin secretion and renin mRNA levels in response to hemodynamic stress (Churchill et al. 1983, Rapacciuolo et al. 2001). Furthermore, Ang II selectively downregulates α1-AR mRNA in cardiac myocytes in a time- and dose-dependent manner (Li et al. 1997). In the adrenal medulla, AT1 receptor mediates a major part of the catecholamine secretion by Ang II (Wong et al. 1990). On the basis of these studies, Ang II and catecholamines act in concert to regulate functional responses of the cardiovascular system.
2.2.4 Nitric oxide

In 1980, vascular relaxation by acetylcholine was demonstrated to require endothelium to release a factor to induce vasodilation (Furchgott & Zawadzki 1980). This factor was first named endothelium-derived relaxing factor, and later, based on the similarities in the pharmacological, physiological and chemical behavior, the endothelium-derived relaxing factor was reported to be NO (Palmer et al. 1987). NO is a soluble gas and an important cell signaling molecule with diverse actions on cardiovascular, renal and immune cell function (for reviews, see Moncada et al. 1991, Kone 2001).

2.2.4.1 Synthesis

Normal vascular homeostasis depends on the balance between neurally and humorally mediated vasoconstriction and local endothelium-dependent vasodilation. The pulsatile flow, shear stress and neurohumoral mediators stimulate the generation of NO (for review, see Moncada et al. 1991). NO is synthesized by three principal isoforms of NOS that catalyze the conversion of the amino acid L-arginine to NO and citrulline in the presence of oxygen (O₂) and nicotinamide adenine dinucleotide phosphate (NADPH) (for review, see Kelly et al. 1996). Constitutively active NOS-1 (nNOS) and NOS-3 (eNOS) were originally identified in neuronal tissue and endothelial cells, respectively. Inducible NOS-2 (iNOS) is active in several cells and tissues (for review, see Alderton et al. 2001). The eNOS is present in the endothelium, platelets, neurons and cardiac myocytes, iNOS in the epithelium, platelets and macrophages, and nNOS is expressed in the skeletal muscle, epithelium and neurons (for review, see Kone 2001). Each isoform is present in the myocardium of patients with heart failure (Satoh et al. 1997, Khadour et al. 1998, Stein et al. 1998). Mice lacking the NOS-2 gene express improved left ventricular contractile function and decreased apoptosis during the late phase of myocardial infarction (Sam et al. 2001). NOS-3 gene knockout mice are hypertensive and more vulnerable to myocardial ischemia than wild-type mice (Huang et al. 1995, Lee et al. 2000a). Thus, constitutive expression of NO is suggested to be protective, whereas inducible production of NO may be involved in the pathogenesis of myocardial dysfunction (Huang et al. 1995, Arstall et al. 1999).

2.2.4.2 Effects

NO-induced biological effects are mediated by activating soluble guanylate cyclase, and subsequently forming intracellular cGMP and decreasing [Ca²⁺]. Major target proteins for cGMP in myocardium are cGMP-dependent protein kinases and cGMP-regulated cyclic nucleotide phosphodiesterases. The former alter function via phosphorylation, while the latter modulate intracellular levels of cGMP (for review, see Shah 1996). Also, the effects of NO are suggested to be mediated via cGMP-independent pathways (Tanner et al. 2000).
The actions of NO on the heart can be divided into indirect effects on the myocardial function and direct effects on the cardiac myocytes (for reviews, see Moncada et al. 1991, Shah 1996). The regulation of cardiac muscle function by coronary vascular and endocardial NO has been reported in isolated heart muscle preparations (Smith et al. 1991). NO induces positive and negative inotropic effects, inhibits β-adrenergic-responsiveness, and modulates sarcolemmal Ca\(^{2+}\) influx and Ca\(^{2+}\) release from the sarcoplasmic reticulum (for reviews, see Kelly et al. 1996, Shah 1996). High levels of NO induce negative contractile response by cGMP, whereas low levels of NO increase positive inotropic effect by cAMP (Vila-Petroff et al. 1999). NO dilates blood vessels, and inhibits proliferation of VSMCs and aggregation of platelets (Palmer et al. 1987, Radomski et al. 1987, Tanner et al. 2000). By regulating venous return and capacitance, and thus filling volume, NO modulates cardiac output via Frank-Starling mechanism (for review, see Shah 1996). *In vivo*, endothelium-derived NO has been reported to be an anti-inflammatory and antiarteriosclerotic molecule (Moroi et al. 1998).

NO attenuates Ang II-induced proliferation of rat cardiac fibroblasts (Takizawa et al. 1997) and induces antihypertrophic effect on cultured cardiac myocytes (Calderone et al. 1998). Effects of ACE inhibition and AT\(_1\) receptor antagonism on cardiac function and left ventricular mass are attenuated in bradykinin receptor gene knockout mice during heart failure (Yang et al. 2001), suggesting a role for bradykinin-NO pathway in mediating beneficial cardiac actions of ACE inhibition or AT\(_1\) receptor antagonism. However, an *in vivo* study has suggested that NO and prostaglandins, but not kinins, are involved in the antihypertensive effect of AT\(_1\) receptor antagonists and ACE inhibitors in rats (Cachofeiro et al. 1995).

In the hearts of spontaneously hypertensive rats (SHR), the expression of constitutive NOS and synthesis of NO are upregulated (Kelm et al. 1995, Nava et al. 1995), and long-term ACE inhibition increases further the activity of NOS and concentrations of NO in SHRs (Wiemer et al. 1997). In addition, chronic L-arginine administration attenuates cardiac growth independent of changes in blood pressure and increases myocardial levels of cGMP in SHRs (Matsuoka et al. 1996), suggesting that abnormalities of the NO pathway may be involved in the pathogenesis of cardiac hypertrophy. Furthermore, pharmacological inhibition of NO synthesis has been shown to produce hypertension (Arnal et al. 1993, Sander et al. 1997). Chronic administration of the potent competitive inhibitor of NOS, N\(^{\text{G}}\)-nitro-L-arginine methyl ester (L-NAME), induces coronary microvascular remodeling, myocardial fibrosis and structural changes in resistance arteries (Li & Schiffrin 1994, Numaguchi et al. 1995, Takemoto et al. 1997a). Interestingly, L-NAME-stimulated pressure overload is associated with preserved myocardial contractility and increased ventricular compliance despite the lack of myocardial hypertrophy in several experimental animal models (Matsubara et al. 1998, Bartunek et al. 2000).

Ang II is also suggested to modulate L-NAME-induced hypertension by increasing the concentrations of ET-1 (D’Amours et al. 1999). In addition, the sympathetic nervous system may play an important role in NO-deficient hypertension, since guanethidine-induced sympathectomy is involved in the pathogenesis of L-NAME-stimulated hypertension in conscious rats in the chronic phase (Sander et al. 1997), and long-term L-NAME treatment increases catecholamine levels in rats (Zanchi et al. 1995). Furthermore, NO is involved in the central regulation of sympathetic outflow, suggesting
that both neuronal and endothelial NO synthesis may contribute to the regulation of vasomotor tone in vivo (Owlya et al. 1997). Both ANP and NO have been reported to induce antiadrenergic actions by inhibiting the growth-promoting effects of NE in cardiac myocytes and fibroblasts (Calderone et al. 1998). Furthermore, acute L-NAME treatment has been reported to revert the ANP-induced hypotensive effect, suggesting a common vasodilating pathway for NO and ANP in rats (Costa et al. 2000). In isolated rat heart, both arginine-vasopressin (AVP) and acetylcholine administration inhibit ANP secretion indirectly by stimulating NO (Melo & Sonnenberg 1996). However, blockade of cardiac NO enhances the inotropic response to β-adrenergic stimulation, but natriuretic peptide receptor antagonist HS-142-1 has no effect on β-adrenergic-responsiveness in dogs with severe CHF (Hart et al. 2001). Taken together, NO acts in concert with other biological effectors in regulating cardiovascular function.

### 2.2.5 Adrenomedullin

AM is a vasoactive peptide first found in human pheochromocytoma (Kitamura et al. 1993a). Preproadrenomedullin is cleaved from its precursor into proadrenomedullin N-terminal peptide and AM, which consists of 52 amino acids in humans and a disulfide bond that forms a six-membered ring structure (Kitamura et al. 1993b). Rat AM is 50 amino acids in length and differs from human AM at only six positions (Sakata et al. 1993). AM belongs to the calcitonin gene-related peptide superfamily, and is highly expressed in the heart, adrenal gland, kidney, and lung of both rats and humans (Kitamura et al. 1993b, Sakata et al. 1993). The receptors mediating the pharmacological actions of AM appear to depend on the species and the model studied (for review, see Jougasaki & Burnett 2000). Although many actions of AM may be mediated by intracellular cAMP, also ion channels and enzymes connected with GPCRs are suggested to transduce the effects of AM. Furthermore, the NO-dependent mechanism may be involved in mediating vasodilative responses of AM (for review, see Samson 1999).

The gene transcription and release of AM are increased in the heart by hormonal, physical and genetic factors (for review, see Jougasaki & Burnett 2000). In cardiac myocytes, mechanical stretch-induced increase in the synthesis and secretion of AM has been reported to decrease by AT₁ receptor antagonism (Tsuruda et al. 2000), suggesting a regulatory role for Ang II. Left ventricular AM mRNA and peptide concentrations increase rapidly in the rat heart in vivo, although transcriptional regulation of the AM gene may differ in response to pressure and volume overload (Romppanen et al. 1997, Yoshihara et al. 2000). However, left ventricular AM mRNA levels have also been shown to increase only in the advanced stage of heart failure in volume overloaded rats (Willenbrock et al. 1999). In pressure overloaded rat hearts, the ventricular AM mRNA concentrations have been reported to increase with the development of myocardial hypertrophy (Morimoto et al. 1999). Circulating levels and tissue production of AM increase in patients with hypertension, obstructive cardiomyopathy, myocardial infarction and CHF (for review, see Jougasaki & Burnett 2000). Thus, AM may have prognostic utility in left ventricular dysfunction (Richards et al. 2001).
Although AM appears to stimulate local paracrine and autocrine actions within tissues, it also circulates in plasma. In humans and experimental animal models, AM induces diuresis, natriuresis and vasodilation, and decreases blood pressure, peripheral resistance and left atrial pressure (Dobrzynski et al. 2000, Nagaya et al. 2000). AM increases cardiac output and left ventricular contractility in vivo (Parkes & May 1997), and exerts direct inotropic effects in vitro (Szokodi et al. 1998). In contrast, positive inotropic effect of AM has not been demonstrated in a canine model in vivo (Lainchbury et al. 2000). Ang II-stimulated aldosterone secretion from rat adrenal cells (Yamaguchi et al. 1996) and plasma aldosterone levels in humans with CHF are reduced by AM (Nagaya et al. 2000). AM inhibits Ang II-stimulated protein synthesis and downregulates ANP gene expression and secretion in cultured cardiac myocytes (Sato et al. 1997, Tsuruda et al. 1998), although AM has been reported to increase Ang II infusion-stimulated plasma ANP levels in healthy humans (Petrie et al. 2001). Furthermore, BNP-generated increase in plasma cGMP levels has been reported to decrease during AM infusion in patients with CHF (Lainchbury et al. 1999), suggesting interactions between AM and natriuretic peptides.

2.2.6 Other factors

Several other biologically active mediators of the myocardial functions, including proinflammatory cytokines and peptide growth factors, are involved in the regulation of cardiac hypertrophic and fibrotic responses (for review, see Baumgarten et al. 2000). Cytokines are signaling molecules, which act as local transducers in communication between cells (for review, see Hefti et al. 1997). Circulating and local cardiac levels of several cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-2, IL-6 and interferon-γ are increased in humans with heart failure, and especially TNF and IL-6 are considered to be stress-activated cytokines. Furthermore, increased circulating levels of TNF, IL-6 and cytokine receptors have been reported to independently predict mortality in patients with CHF (for review, see Baumgarten et al. 2000, Deswal et al. 2001).

In cardiac myocytes, TNF-α stimulation induces cell growth and synthesis of sarcomeric proteins (Yokoyama et al. 1997). Furthermore, a recent in vitro study has shown that IL-1β rapidly stimulates cardiac myocyte hypertrophy and ANP gene expression via mitogen-activated protein kinases (MAPKs) (Ng et al. 2001). IL-1β also regulates human BNP promoter activity through p38 MAPK and Ca²⁺-dependent pathways (He & LaPointe 1999, He & LaPointe 2000b). Moreover, the stimulative effect of IL-1β on the secretion of ANP and BNP appears to require factors derived from nonmyocytes (Harada et al. 1999). Cardiotrophin-1 (CT-1), a member of the IL-6 family, has been reported to be a local regulator of cardiac hypertrophy, since the secretion of ANP and BNP and cardiac myocyte protein synthesis are inhibited by anti-CT-1 antibodies in vitro (Kuwahara et al. 1999). In addition, CT-1 increases angiotensinogen mRNA levels, whereas AT₁ receptor antagonism attenuates CT-1-induced cardiac myocyte hypertrophy (Fukuzawa et al. 2000), suggesting interactions between RAS and cytokines on cardiac function.
Peptide growth factors, including fibroblast growth factor (FGF), transforming growth factor beta (TGF-β), insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF), have been shown to induce hypertrophic responses in cardiac myocytes in vitro (for review, see Hefti et al. 1997). Ang II upregulates the gene expression of TGF-β1, PDGF, basic FGF and IGF in the heart (for review, see Sadoshima & Izumo 1997). Moreover, the hypertrophic actions of Ang II may be partly mediated by TGF-β1 released from cardiac fibroblasts (Gray et al. 1998). The secretion of ANP and BNP from cardiac myocytes is enhanced by TGF-β1, and the nonmyocytes, possibly via ET-1, are involved in this process (Harada et al. 1997). The receptors of most growth factors, with the exception of TGF-β1 and IGF-II, are transmembrane tyrosine kinases. Binding of PDGF and FGF leads to receptor dimerization, which enables two cytoplasmic domains to phosphorylate each other on multiple tyrosine residues. The effects of TGF-β are transduced by the heteromeric complex of type-I and type-II receptors, which are serine/threonine (Ser/Thr) kinases (for review, see Hefti et al. 1997).

**2.3 Signal transduction and transcription factors in cardiac myocyte hypertrophy**

A number of intracellular signaling pathways have been implicated as important downstream transducers of neuroendocrine factors and hemodynamic stress in cardiac myocytes. Hypertrophic stimulators activate MAPKs, protein kinase C (PKC), calcineurin and many other cytoplasmic signaling cascades via GPCRs, ion channels and integrins to alter cardiac gene expression (for review, see Molkentin & Dorn 2001). Ang II is known to induce a wide spectrum of signaling pathways (Fig. 2), but the contribution of these cascades on the gene expression and transcriptional regulation at the nuclear level requires further study.

Transcription factors are proteins which regulate transcription of mRNA from the respective complementary DNA. Transcription factors bind to DNA, possibly with cofactors, and interact with RNA polymerase II. Tissue-specific gene expression results from the complex interactions between nuclear trans-acting factors and DNA cis regulatory elements (for review, see Martin 1991). A number of transcription factors, such as activator protein-1 (AP-1), GATA4, serum response factor (SRF), Elk-1, nuclear factor of activated T-cells 3 (NF-AT3), nuclear factor κB (NF-κB) and myocyte enhancer factor-2 (MEF2), have been reported to be involved in cardiac myocyte hypertrophic responses (Paradis et al. 1996, Hasegawa et al. 1997, Molkentin et al. 1998, Takemoto et al. 1999, Baba et al. 2000, Passier et al. 2000).
Fig. 2. Putative model of signal mechanisms induced by angiotensin II (Ang II) in cardiac myocytes (modified from Molkentin et al. 1998, Yano et al. 1998, Kim & Iwao 2000, Purcell et al. 2001). AP-1, activator protein-1; AT₁, angiotensin II receptor subtype; CaM, calmodulin; DAG, diacylglycerol; IP₃, inositol-1,4,5-triphosphate; JAK, janus kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MKKK, MAPK kinase kinase; MKP-1, MAPK phosphatase-1; NF-AT3, nuclear factor of activated T-cells 3; NF-κB, nuclear factor κB; NO, nitric oxide; PKC, protein kinase C; PLC, phospholipase C; PP2A, protein phosphatase 2A; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; SH-PTP1, Src homology domain-containing protein tyrosine phosphatase 1; STAT, signal transducer and activator of transcription.
2.3.1 Mechanotransduction

Cardiac load activates mechanosensitive molecules, which are suggested to locate in the plasma membrane and sense extracellular tension. Although the exact molecular mechanisms of the mechanical strain responses at the cellular level remain unclear, ion channels, integrins and tyrosine kinases have been suggested to conduct hypertrophy in cardiac myocytes (for review, see Sadoshima & Izumo 1997).

Stretch-activated ion channels (SACs) allow passage of ions such as \( \text{Na}^+ \) and \( \text{Ca}^{2+} \) (for review, see Tavi et al. 2001). In neonatal rat atrial cells, at least two types of SACs are found, one of them is sensitive to stretch and the other to swelling (Kim 1993, Kim & Fu 1993). In the rat atria, the inhibition of \( \text{Ca}^{2+} \)-permeable SACs by gadolinium prevents the stretch-induced release of ir-ANP and the pressure overload-stimulated synthesis of BNP mRNA (Laine et al. 1996). However, treatment of cardiac myocytes with gadolinium does not inhibit the stretch-induced expression of immediate-early genes and protein synthesis (Sadoshima et al. 1992a).

The components of the extracellular matrix (ECM) accumulate in the failing heart and induce changes in cardiac myocyte gene expression (Ogawa et al. 2000). Integrins are heterodimeric transmembrane proteins, composed of \( \alpha \) and \( \beta \) subunits, which act as receptors for ECM proteins (for review, see Giancotti & Ruoslahti 1999). Several members of the integrin family are expressed in the heart, and integrin-mediated signaling is suggested to be involved in cardiac hypertrophy (Ross et al. 1998). Integrins transduce signals by transmembrane growth factor receptors, non-receptor protein tyrosine kinases, small GTPases and MAPKs (for review, see Giancotti & Ruoslahti 1999, Laser et al. 2000, Liang et al. 2000a). The cell-matrix interactions may play an important role in regulating the ventricular BNP gene expression, since inhibition of integrin signaling decreases the mechanical stretch-induced human BNP promoter activity (Liang et al. 2000a). Furthermore, overexpression of \( \beta_1 \) integrin in the cardiac myocytes has been shown to increase ANP gene expression and protein synthesis (Ross et al. 1998).

2.3.2 G-protein-coupled receptors

In the cardiovascular system, three functional classes of GPCRs are important transducers of stimulatory and inhibitory signals in response to hypertrophic stimuli (for review, see McKinsey & Olson 1999). Ang II (AT\(_1\) receptor), \( \alpha \)-adrenergic and ET-1 receptors couple primarily to \( \text{Ga}_q \), \( \beta \)-ARs to \( \text{Go}_s \) and cholinergic receptors to \( \text{Go}_i \). Heterotrimeric G-proteins consist of separate \( \text{Ga} \) and \( \text{G}_\beta\gamma \) subunits. Agonist binding to receptor catalyzes the associated guanosine diphosphate (GDP) to guanosine triphosphate (GTP) exchange on the \( \text{Ga} \) subunit and subsequent dissociation of \( \text{Ga} \) from \( \text{G}_\beta\gamma \).

Ang II stimulates PLC through \( \text{Ga}_q \) coupled to AT\(_1\) receptor and subsequently the formation of DAG and IP\(_3\), that activate PKC and release of \( \text{Ca}^{2+} \) from the sarcoplasmic reticulum (Baker & Singer 1988, Booz et al. 1994, Schunkert et al. 1995). The importance of \( \text{Go}_i \) signaling in cardiac myocyte growth and ANP gene expression \textit{in vitro} and pressure overload-induced hypertrophy \textit{in vivo} has been reported (LaMorte et al. 1998).
The expression of ANP, β-MHC and skeletal α-actin genes is also increased in Gaq overexpressing transgenic mice (D’Angelo et al. 1997). Although the Gaq signaling pathway is involved in initiating the hypertrophic response, it also mediates the responses of cardiac hypertrophy leading to CHF (Mende et al. 1998, Sakata et al. 1998).

AT1 receptor is coupled to Gaq (Zhang & Pratt 1996) and subsequently to several phosphatases including tyrosine and Ser/Thr phosphatases (Huang et al. 1996a, Tsuzuki et al. 1996). Increased expression of Gaq has been demonstrated in humans and experimental animal models during heart failure (Hershberger et al. 1991, Vatner et al. 1996). The mRNA levels of Gaq- and Gαs-proteins in the failing heart after myocardial infarction are suggested to be chamber-specific and dependent upon the stage of CHF (Sethi et al. 1998).

### 2.3.3 Intracellular Ca²⁺

Intracellular Ca²⁺ is a central regulator of cardiac contractility, and it is shown to be a primary stimulus for the hypertrophic response induced by neurohumoral agonists including Ang II, ET-1, PE and ISO in cardiac myocytes in vitro and in vivo (for reviews, see Sadoshima & Izumo 1997, Sugden & Clerk 1998). Increased [Ca²⁺]i results from the influx through ion channels and exchangers, which further trigger the release of Ca²⁺ from the sarcoplasmic reticulum, leading to activation of contractile proteins (for review, see Bers 2000). In cardiac myocytes, diastole occurs by removing Ca²⁺ from the cytosol mainly by sarcoplasmic reticulum Ca²⁺-ATPase and sarcolemmal Na⁺-Ca²⁺exchanger (Bassani et al. 1994). The gene expression of sarcoplasmic reticulum Ca²⁺-ATPase is decreased and the gene expression of Na⁺-Ca²⁺exchanger is increased in cardiac hypertrophy and CHF (Takizawa et al. 1999, de Boer et al. 2001).

Intracellular Ca²⁺ binding protein, calmodulin (CaM), appears to be an important mediator of cardiac myocyte growth in transgenic mice (Gruver et al. 1993). The Ca²⁺-CaM-dependent kinase (CaMK) is a downstream target of CaM, and myocardial hypertrophy has been reported to develop in the mouse heart overexpressing CaMK (Passier et al. 2000). In addition, ANP gene expression is induced by the CaMKII in vitro and in vivo, while the mechanisms of transcriptional regulation appear to differ (Ramirez et al. 1997a, Colomer & Means 2000).

Calcineurin is a Ca²⁺/CaM-dependent Ser/Thr phosphatase, which is suggested to regulate hypertrophic growth and gene expression in cultured cardiac myocytes and rat heart (Molkentin et al. 1998, Lim et al. 2000). The Ca²⁺/CaM complex binds to the catalytic subunit of calcineurin during increased [Ca²⁺], resulting in the activation of calcineurin phosphatase (for review, see Molkentin & Dorn 2001). Instead of CaMKII and MAPKs, which are regulated by transient Ca²⁺ spikes, calcineurin is associated with prolonged increases in basal [Ca²⁺] (Dolmetsch et al. 1997). In response to hypertrophic stimuli, such as Ang II and PE, calcineurin dephosphorylates NF-AT3, which is thereafter translocated from the cytoplasm to the nucleus (Molkentin et al. 1998). The NF-AT3, a member of the NF-AT family, is expressed in various tissues including the myocardium (de la Pompa et al. 1998). The role of calcineurin during cardiac hypertrophy is unclear,
since myocyte growth may be either prevented or remain unaffected by specific calcineurin inhibitors cyclosporin A and FK 506 in vitro and in vivo (Molkentin et al. 1998, Zhang et al. 1999, Sakata et al. 2000).

2.3.4 Protein kinase C

PKC and its various isoforms are phospholipid-dependent Ser/Thr kinases with pleiotropic effects and they act as intracellular signaling molecules mediating the actions of cardiotoxic factors such as GPCR agonists, phorbol esters and mechanical stimuli (Komuro et al. 1991, for review, see Sugden & Clerk 1998). The PKCs are divided according to their structure and substrate requirements into three groups: conventional PKCs (cPKCs), activated by Ca\(^{2+}\), DAG and phosphatidylserine, novel PKCs (nPKCs), activated by DAG and phosphatidylserine, and atypical PKCs (aPKCs), which are sensitive to phosphatidylserine. When the PKCs are activated, they translocate to distinct subcellular sites (for review, see Naruse & King 2000).

In the heart, changes in the gene expression of certain PKC isozymes occur in response to myocardial growth, CHF and ischemic preconditioning (D'Angelo et al. 1997, Qiu et al. 1998, Bowling et al. 1999). The hypertrophic responses of cardiac myocytes are prevented by the pharmacological inhibitors of PKC and substances manipulating the interaction of the PKC isozymes with their specific anchoring proteins in vitro and in vivo (Komuro et al. 1991, Chen et al. 2001). Furthermore, the overexpression of constitutively active PKC\(\varepsilon\) induces cardiac hypertrophy in transgenic animals (Takeishi et al. 2000). In transgenic mice with an increased number of L-type Ca\(^{2+}\) channels, the activation of PKC\(\alpha\) precedes the development of cardiac hypertrophy, suggesting a regulatory role for PKC signaling in cardiac myocyte growth (Muth et al. 2001). PKC also mediates the neurohumoral and mechanical stretch-induced ANP and BNP gene transcription and secretion (Suzuki et al. 1992, LaPointe & Sitkina 1993, Magga et al. 1997b), and increases the promoter activities of ANP, BNP and MLC-2 in cardiac myocytes in response to \(\alpha\)-adrenergic hypertrophic stimulus (Shubeita et al. 1992, Hanford & Glembotski 1996). Furthermore, partly via MAPK-dependent pathway, PKC may be involved in the regulation of the BNP gene by posttranscriptional stabilization (LaPointe & Sitkina 1993, Nakagawa et al. 1995, Hanford & Glembotski 1996).

A recent study has shown that translocation of transcription factor NF-\(\kappa\)B into the nucleus of cardiac myocyte and NF-\(\kappa\)B DNA binding activity in response to Ang II stimulation are prevented by inhibitor of PKC, suggesting a role for PKCs in Ang II-induced gene expression in vitro (Rouet-Benzineb et al. 2000). However, the role of PKC in regulating NF-\(\kappa\)B may be cell specific, since PKC inhibitor has no effect on the Ang II-induced increase in NF-\(\kappa\)B DNA binding activity in VSMCs (Ruiz-Ortega et al. 2000).
2.3.5 Mitogen-activated protein kinases

MAPKs are a family of protein Ser/Thr kinases, divided into three subfamilies, which are defined by the terminal kinase in the pathway: extracellular signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPKs (for review, see Seger & Krebs 1995). The upstream regulators of ERKs are mitogen-activated protein kinase kinases (MKKs) MKK1 and MKK2, whereas JNKs are activated by MKK4 and MKK7, and p38 MAPKs by MKK3 and MKK6 (for review, see Garrington & Johnson 1999). MAPKs are stimulated by GPCR agonists, growth factors, cytokines and various types of stress (Clerk et al. 1998a, Clerk et al. 1998b, Ng et al. 2001). Small GTPases including Ras, Raf and Rho are associated with the MAPK signaling responses in the heart (Ramirez et al. 1997b, Thorburn et al. 1997, Shih et al. 2001). Furthermore, MAPKs regulate multiple transcription factors such as AP-1, MEF2, Elk-1, NF-AT3, GATA4 and NF-κB (for review, see Garrington & Johnson 1999, Liang & Gardner 1999, Fischer et al. 2001, Liang et al. 2001a). In addition to growth factor-induced cardiac myocyte hypertrophy via MAPKs in vitro (Zechner et al. 1997), pressure overload and myocardial infarction in rats and CHF in humans have been reported to induce MAPK signaling pathways (Cook et al. 1999, Fischer et al. 2001, Yoshiyama et al. 2001).

Inhibition of the ERK pathway has been shown to decrease ET-1-induced and strain-dependent increase in BNP promoter activity (Liang et al. 2000b), suggesting that the BNP gene is regulated at least in part by ERKs. Furthermore, ERK activation mediates other hypertrophic responses including the gene expression of ANP (Glennon et al. 1996, Clerk et al. 1998a). However, some studies have suggested a minimal role for ERKs in transducing the hypertrophic stimuli or activating inducible ANP gene expression (Post et al. 1996, Ramirez et al. 1997b). JNKs and p38 MAPKs are known as stress-activated protein kinases (SAPKs) because of their potent activation by several stress stimuli and inflammatory cytokines (Clerk et al. 1998b). Hypertrophic agonists and mechanical load increase the activities of p38 MAPKs and JNKs in vitro and in vivo, followed by hypertrophic changes, including enhanced myocyte growth and increased transcription of cardiac genes such as ANP, BNP and skeletal α-actin (Ramirez et al. 1997b, Zechner et al. 1997, Clerk et al. 1998a, Fischer et al. 2001). Indeed, several studies have reported an important role for p38 MAPKs and to a lesser extent JNKs in mediating mechanical strain-activated BNP gene expression (Liang et al. 1997, Liang & Gardner 1999, Liang et al. 2000b). JNKs and p38 MAPKs may be involved in the early development of left ventricular hypertrophy (LVH), since their activities increase in hypertensive Dahl salt-sensitive rat hearts in response to Ang II in the subacute LVH stage, but decrease later in the chronic LVH and CHF stages (Hayashida et al. 2001).

In cultured cardiac myocytes, ERKs are suggested to mediate the effects of Ang II, since a catalytically inactive mutant of ERK inhibits the Ang II-induced β-MHC promoter activity (Shih et al. 2001). Moreover, Ang II has been reported to induce hypertrophic responses by stimulating the ERK cascade followed by an increase in ANP gene expression and synthesis (Aoki et al. 2000) and activating JNKs and subsequently transcription factor AP-1 in vitro and in vivo (Kudoh et al. 1997, Yano et al. 1998, Izumi et al. 2000). Furthermore, cardiac AP-1 DNA binding is activated and DNA-protein interaction is prevented by AT, receptor antagonist in transgenic rats harboring both renin
and angiotensinogen genes (Fiebeler et al. 2001). Therefore, MAPK-regulated AP-1 activation appears to be an important pathway for Ang II-mediated hypertrophic responses.

2.3.6 GATA family

The family of GATA transcription factors plays an important role during embryogenesis and myocardial hypertrophy (for review, see Molkentin 2000). Two subfamilies, GATA1, -2 and -3, and GATA4, -5, and -6 have been described in vertebrates (Laverriere et al. 1994), and the expression of GATA genes is found in several cells and tissues (Table 1). The structure of these nuclear proteins is conserved, since the GATA genes from different species resemble each other more than the other members of the subfamily within the same species (Leonard et al. 1993, Jiang & Evans 1996). Drosophila GATA factor, pannier, is a homolog for GATA-binding proteins identified in invertebrates (Gajewski et al. 1999). GATA factors contain two conserved zinc fingers Cys-X$_{2}$-Cys-X$_{17}$-Cys-X$_{2}$-Cys, which recognize and bind to consensus DNA sequence (A/T)GATA(A/G), known as WGATAR, or closely related sequences (Arceci et al. 1993, Merika & Orkin 1993).

The C-terminal zinc finger and the adjacent basic domain modulate both DNA-binding and nuclear localization activities of GATA4 (Morrisey et al. 1997a). The N-terminus of GATA4 encodes two independent transcriptional activation domains. These activation domains are conserved across species and within the GATA4, -5 and -6 subfamily (Morrisey et al. 1997a). Moreover, deletion analysis shows that the N-terminal portion of GATA4 is required for full activity of the molecule in transactivation (Arceci et al. 1993). Similarities in DNA-binding activities among GATA proteins have been demonstrated, and specificity of the DNA-binding may concern slight differences in the GATA DNA sequences, local chromatin structure or interactions with other transcription factors (Merika & Orkin 1993).
Table 1. The expression of GATA genes in adult cells and tissues

<table>
<thead>
<tr>
<th>GATA1</th>
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<th>GATA3</th>
<th>GATA4</th>
<th>GATA5</th>
<th>GATA6</th>
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<td>progenitor cells</td>
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<td></td>
<td>Liver</td>
<td>Intestine</td>
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<td>Erythrocytes</td>
<td>Erythrocytes</td>
<td>Intestine</td>
<td>Stomach</td>
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<tr>
<td>Megakaryocytes</td>
<td>Endothelial cells</td>
<td>Stomach</td>
<td>Heart</td>
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<tr>
<td>Mast cells</td>
<td>Megakaryocytes</td>
<td>Gonads</td>
<td>Lung</td>
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<td>Eosinophils</td>
<td>Mast cells</td>
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<td>Fibroblasts</td>
<td>Pancreas</td>
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<td>Liver</td>
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<td>Spleen</td>
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<td>Kidney</td>
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GATA1, -2 and -3 are important regulators of the hematopoietic-specific gene expression (Leonard et al. 1993). Targeted mutation of the GATA1 gene in transgenic mice results in the abnormal maturation of erythroid and megakaryocytic cells, and embryonic lethality in males, since the GATA1 gene is located in the X-chromosome (Shimizu et al. 2001). Disruption of the GATA2 gene leads to embryonic lethality because of the deficient proliferation of the hematopoietic stem cells (Tsai et al. 1994). The GATA3 gene is expressed in the embryonic central nervous system and placenta, and the deficiency of this gene also results in embryonic lethality (Pandolfi et al. 1995). The GATA4 gene has been cloned and characterized in the mouse (Arceci et al. 1993), frog (Kelley et al. 1993), rat (Tamura et al. 1993), chicken (Laverriere et al. 1994) and man (Huang et al. 1996b). Transcription of the GATA4 gene is mostly restricted to the cardiac myocytes and it is undetectable in skeletal muscle cells (Grépin et al. 1994).

2.3.6.1 Role of GATA4, -5 and -6 during embryogenesis

The GATA4, -5 and -6 subfamily directs tissue-specific gene expression within the heart during embryonic development (Morrisey et al. 1996, Kuo et al. 1997, Morrisey et al. 1997b). GATA4 is one of the earliest transcription factors expressed in embryonic cardiac cells, and the expression of the GATA4 gene in the myocardium continues from the developmental stage to adulthood (Arceci et al. 1993, Grépin et al. 1994). During mouse embryogenesis, GATA4 appears first in visceral endoderm and precardiac mesoderm, while at the later phase the GATA4 gene is expressed in other cell and tissue types including mature cardiac myocytes, gut epithelium, liver and gonads (Arceci et al. 1993, Laverriere et al. 1994). Between embryonic days 7 and 10, GATA4-deficient mice express severe lethal abnormalities in the heart tube formation and ventral morphogenesis, they lack foregut and have abnormalities in the amnionic cavity and yolk.
sac formation. However, the cardiac tissue is normally differentiated in these GATA4 mutant embryos (Kuo et al. 1997, Molkentin et al. 1997). In humans, deficiency of the GATA4 gene due to deletion of chromosome region 8p23.1 has been reported to be associated with congenital heart disease (Pehlivan et al. 1999).

The mouse GATA6 gene is expressed in the primitive gut, VSMCs, urogenital system and lung (Morrisey et al. 1996), whereas the mouse GATA5 gene is expressed in smooth muscle cells of the lung and urinary bladder, and in a temporally and spatially restricted manner in the embryonic heart (Morrisey et al. 1997b). In contrast to GATA4 and -6, targeted disruption of the mouse GATA5 gene is not lethal, but results in defective development of the genitourinary tract (Morrisey et al. 1997b, Molkentin et al. 2000). Enhanced transcription of the GATA6 gene in the GATA4-deficient embryos has been suggested to compensate the actions of GATA4 in vivo (Kuo et al. 1997, Molkentin et al. 1997). In this regard, GATA4 and -6 are highly conserved within their DNA-binding domains (Jiang & Evans 1996, Morrisey et al. 1997a). Furthermore, the GATA4 and -6 proteins colocalize in the nucleus and form stable dimeric complexes (Charron et al. 1999). Indeed, the GATA6 gene, but not GATA5, is expressed at comparable levels to the GATA4 gene in the embryogenic, postnatal and adult heart, suggesting GATA6 as a potential regulator of the basal and inducible gene expression (Morrisey et al. 1996).

2.3.6.2 GATA4 in cardiac hypertrophy

In cardiac myocytes, GATA4 regulates expression of a number of cardiac structural genes including α-MHC, β-MHC, cardiac troponin C, cardiac troponin I, ANP, BNP, Na⁺-Ca²⁺ exchanger, cardiac-restricted ankyrin repeat protein, A1 adenosine receptor, m2 muscarinic receptor and MLC 1/3 (for review, see Molkentin 2000). Overexpression of GATA4 can transactivate many of these promoters in nonmuscle cells (Grépin et al. 1994, Ip et al. 1994, Molkentin et al. 1994), whereas inhibition of the GATA4 gene expression by vectors expressing GATA4 antisense sequence prevents the expression of cardiac-specific genes and the development of cardiac myocytes from the pluripotent cells (Grépin et al. 1995). In addition, adenovirus-mediated delivery of antisense gene regions directed toward GATA4 and GATA6 in cultured cardiac myocytes downregulates the expression of several cardiac genes, such as α- and β-MHC, cardiac troponin I, ANP and BNP (Charron et al. 1999). Moreover, overexpression of both GATA4 and GATA6 has been shown to be sufficient to induce cardiac myocyte hypertrophy (Liang et al. 2001b).

GATA4 is suggested to be an important transcriptional regulator in response to aortic coarctation-induced pressure overload in rats (Hasegawa et al. 1997, Herzog et al. 1997). GATA4 binding activity to the respective motifs of the ATₐ promoter increases in response to aortic coarctation for 2 days. Moreover, the mutation of the GATA site in the ATₐ promoter abolishes the pressure overload response, while the basal gene expression remains constant. In response to a longer period of cardiac loading, aortic coarctation for 12 days has been shown to increase GATA4 binding to the β-MHC promoter. Mutation of the GATA site markedly attenuates the β-MHC promoter activity in response to hemodynamic stress without affecting the basal transcriptional activity, suggesting that
the GATA site is a load-activated response element of the β-MHC promoter in this experimental animal model (Hasegawa et al. 1997). In contrast, the GATA element has been shown to be required for the basal, but not inducible β-MHC promoter activity in response to abdominal aortic coarctation for 12 days in rats (Wright et al. 2001).

Acute pressure overload induction by intravenous AVP infusion induces the BNP GATA4 binding activity rapidly and transiently in rats. ET-1 receptor antagonist inhibits this response, whereas AT1 receptor antagonist has no effect on it, suggesting a role for ET-1 in mediating the left ventricular BNP GATA4 binding activity in response to early pressure overload stimulation (Hautala et al. 2001). However, both ET-1 and Ang II are suggested to be required for the GATA4 DNA binding activity in isolated perfused rat hearts, since ET-1 and AT1 receptor antagonists inhibit the left ventricular BNP GATA4 binding activity in response to direct left ventricular wall stretch (Hautala et al. 2002). Furthermore, cardiac myocyte growth and expression of cardiac structural genes are prevented in transgenic mice overexpressing dominant-negative GATA4-engrailed repressor fusion-encoding adenovirus (Liang et al. 2001b), suggesting that GATA factors are important regulators of the hypertrophic response in vivo.

Prolonged ISO and PE infusion in vivo and electrical pacing in cultured cardiac myocytes have been reported to augment GATA4 mRNA levels in concert with hypertrophic changes in cardiac myocytes (Saadane et al. 1999, Xia et al. 2000). Despite increased DNA-binding activities, cardiac GATA4 mRNA levels did not increase significantly in response to acute hemodynamic overload induced by AVP in vivo and direct wall stretch in perfused rat hearts (Hautala et al. 2001, Hautala et al. 2002). Furthermore, PE stimulation for 24 h has not been reported to alter GATA4 and GATA6 protein levels in cardiac myocytes in vitro (Liang et al. 2001b). On the basis of these studies, GATA DNA-binding activities are suggested to be regulated by additional mechanisms than increased mRNA and protein synthesis.

Regulation of the GATA4 DNA-binding activity may involve posttranslational modifications, since GATA4 contains putative phosphorylation sites (Arceci et al. 1993). Increased GATA4 binding to the ET-1 promoter in response to α-adrenergic receptor agonism by PE is suggested to involve serine phosphorylation of GATA4 via ERK1/2 associated with Rho signaling pathway in rat cardiac myocytes (Morimoto et al. 2000, Yanazume et al. 2001). Also, ET-1 has been shown to phosphorylate GATA4 via ERK cascade (Kitta et al. 2001). Site-directed mutagenesis and phospho-specific GATA4 antiserum have revealed that serine 105, locating in the N-terminal transactivation domain, may be the primary site involved in agonist-stimulated phosphorylation of GATA4 by the ERK pathway (Liang et al. 2001a). Furthermore, RhoA has also been demonstrated to activate GATA4 by phosphorylating serine 105 activity through the p38 MAPK pathway (Charron et al. 2001). In contrast, glycogen synthase kinase 3β may attenuate GATA-activated transcription by decreasing nuclear expression of GATA4 by phosphorylation and promoting nuclear export of GATA4 (Morisco et al. 2001). Moreover, GATA4 DNA binding activity is suggested to be regulated by interactions with other nuclear transcription proteins (see below 2.3.8).
2.3.7 AP-1

Transcription factor AP-1 belongs to the family of basic leucine zipper factors, which consists of hetero- and homodimers of Jun (v-Jun, c-Jun, JunB, JunD), Fos (v-Fos, c-Fos, FosB, Fra1, Fra2) or activating transcription factor (ATF2, ATF3/LRF1, B-ATF) (for reviews, see Vogt & Bos 1990, Angel & Karin 1991). Complexity of the AP-1 transcription protein family is further described by several new members of basic leucine zipper proteins (for review, see Karin et al. 1997). Binding by Jun homodimers or Fos-Jun heterodimers produces distinct DNA bending that may result in highly specific protein-protein interactions between AP-1 factors (Kerppola & Curran 1991). AP-1 mediates transcription by binding preferentially to the DNA sequence of TGA(C/G)TCA termed AP-1 binding site (Hai & Curran 1991, Thuerauf et al. 1994). In AP-1-luciferase transgenic mice, AP-1 activity is detected at high levels in the skin, kidney, spleen, lung, brain, liver and heart (Zhong et al. 2001). A number of different stimulators, including growth factors, cytokines, neurotransmitters and UV irradiation, increase the activity of AP-1 (for review, see Karin 1995). The consensus sequences of AP-1 are present in numerous genes associated with cardiac hypertrophy and production of ECM proteins (for review, see Karin 1995). AP-1 is involved in regulating the promoter activity of the hypertrophic response genes, including ANP, BNP, skeletal α-actin, ATₐ receptor and ET-1 (Paradis et al. 1996, Herzig et al. 1997, Kudoh et al. 1997, von Harsdorf et al. 1997, Marttila et al. 2001). Moreover, overexpression of the dominant negative mutant of c-Jun has been shown to inhibit ET-1- and PE-induced cardiomyocyte hypertrophic changes including transcriptional activity of AP-1, myocyte growth, protein synthesis, and ANP and BNP gene expression (Omura et al. 2002), suggesting an important role for AP-1 in regulating cardiac hypertrophy. Interestingly, the components of AP-1, c-Fos and c-Jun, also act as negative regulators of the cardiac gene expression (McBride et al. 1993, Paradis et al. 1996).

Mutation introduced into the AP-1 consensus site abolishes the aortic coarctation-induced pressure overload response of the ATₐ gene, but has no effect on the basal activity of the promoter. Furthermore, the AP-1 DNA binding activity increases in loaded hearts, suggesting an important role for Fos-JunB-JunD complex in mediating the hypertrophic response (Herzig et al. 1997). Similarly, mutation of the AP-1-like site inhibits the inducible gene expression of ANP in response to pressure overload by aortic coarctation (von Harsdorf et al. 1997). AP-1 is not involved in the regulation of BNP inducibility in pressure overloaded hearts, although it regulates the basal gene expression of BNP (Marttila et al. 2001).

Phosphorylation of the components of AP-1 by MAPKs is suggested to account for the increased activity of AP-1 (for review, see Karin 1995). In adult rabbit cardiomyocytes, activation of PKCε increases AP-1 DNA binding activity, which is completely abolished by inhibition of the ERK and JNK pathways (Li et al. 2000). Previously, JNK has been reported to increase c-Jun transactivation by phosphorylating the N-terminal serine residues of c-Jun and to induce c-fos gene expression (Derijard et al. 1994, Cavigelli et al. 1995). In aortic constricted rat hearts after 30 minutes of the pressure overload induction, increased activities of JNK and p38 MAPK are associated with the enhanced phosphorylation of c-Jun and ATF2 and increased ventricular AP-1
DNA binding activity (Fischer et al. 2001). Ang II infusion, via AT₁ receptors, induces activation of JNK, followed by the induction of left ventricular AP-1 DNA binding activity at 6 h reaching the peak value at 24 h in conscious rats (Yano et al. 1998). Similarly, the increase in left ventricular AP-1 DNA binding activity and the increased activity of JNK in SHRSPs compared with WKYs are prevented by AT₁ receptor antagonist (Izumi et al. 2000). In hypertensive double transgenic rats for the human renin and angiotensinogen genes, cardiac AP-1 DNA binding activity is increased, but decreased in response to spironolactone and combined ETₐ/ET₆ receptor antagonism by bosentan for 3 weeks independent of blood pressure-related effects (Muller et al. 2000, Fiebeler et al. 2001), suggesting that aldosterone and ET-1 are involved in Ang II-induced AP-1 activation. In addition, bosentan and AT, receptor antagonist inhibit the wall stretch-induced increase in left ventricular BNP AP-1 binding activity in perfused rat hearts (Hautala et al. 2002). Taken together, MAPK pathways appear to mediate the AP-1 DNA binding activity in response to hypertrophic stimulus.

Myocardial ischemia induces MAPK activities followed by the increase in cardiac AP-1 DNA binding activity, whereas coronary reperfusion decreases the binding activities (Shimizu et al. 1998, Omura et al. 1999). Furthermore, in response to coronary ligation in rats, AP-1 DNA binding activity increases in the infarcted heart at 3 days and remains elevated until the end of the 2-week experiment. Ang II may be related to the increased AP-1 DNA binding activity in response to myocardial infarction, since ACE inhibitor and AT, receptor antagonist prevent the DNA binding of AP-1 (Yoshiyama et al. 2001). After balloon injury in rat carotid arteries, AP-1 DNA binding activity increases rapidly after the injury, preceded by the activation of ERK and JNK (Hu et al. 1997, Kim et al. 1998), and these activities are mediated at least in part via AT₁ receptor (Kim et al. 1998). Furthermore, Ang II-stimulated VSMC growth is associated with the increased AP-1 DNA binding activity following stimulation of ERK1/2 (Touyz et al. 2001).

2.3.8 Cofactors of GATA4 and AP-1

In the heart, GATA proteins have been suggested to modulate the gene expression by interactions with other cardiac tissue-restricted transcription factors. A recently identified multi-zinc-finger protein, friend of GATA-2 (FOG-2) is expressed in the heart, brain, testis and liver (Lu et al. 1999). The interaction of N-terminal zinc finger of GATA4 and C-terminal region of FOG-2 results in either synergistic activation (α-MHC) or repression (ANP and BNP) of GATA-dependent promoters (Lu et al. 1999).

SRF, a member of the MADS box family of transcription factors, interacts physically and functionally with GATA4 to drive the expression of the cardiac α-actin promoter (Belaguli et al. 2000). In cultured cardiac myocytes, activation of the ANP promoter in response to ET-1 administration requires transcriptional cooperativity between GATA4 and SRF, and both GATA and SRF binding abilities and respective DNA elements are needed (Morin et al. 2001). However, GATA4 transcriptional synergy with another MADS box protein, MEF2, occurs via GATA-dependent recruitment of MEF2 to target promoter and does not require the DNA-binding ability of MEF2 (Morin et al. 2000). Thus, via interaction with GATA4, MEF2 can activate ANP promoter, which does not

A recent study has reported an interaction between GATA4 and NF-AT3, and treatment with cyclosporin inhibits the hypertrophic responses synergistically mediated by GATA4 and NF-AT3 (Molkentin et al. 1998). Furthermore, overexpression of GATA4 and NF-AT3 cooperatively stimulate ET-1 promoter activity (Morimoto et al. 2001). Several studies have described interactions between GATA4 and homeodomain-containing transcription factor, Csx/Nkx-2.5, in regulating transcription of cardiac-restricted genes (Durocher et al. 1997, Lee et al. 1998a, Sepulveda et al. 1998, Shiojima et al. 1999). Depending on the target promoter and the other synergistically acting proteins, GATA4 interacts with Csx/Nkx-2.5 by utilizing different activation domains (Lee et al. 1998a, Sepulveda et al. 1998). YY1, a multifunctional repressor or activator protein essential for embryonic development, binds to YY1 element within a proximal cardiac BNP promoter cooperating with GATA4 to synergistically activate BNP gene transcription (Bhalla et al. 2001).

Synergistic interaction between AP-1 and GATA2 in regulating the transcriptional activity of the ET-1 promoter has been observed in endothelial cells (Kawana et al. 1995). Indeed, AP-1 potentiates the actions of GATA2 on the ET-1 promoter lacking a functional AP-1 element and vice versa. This synergistic regulation of AP-1 may also involve other members of the GATA family (Kawana et al. 1995). In vivo, functional interaction between GATA4 and AP-1 in activating AT1A gene has been reported in response to aortic coarctation (Herzig et al. 1997).

NF-κB is activated by phosphorylation of inhibitory protein κB-α, and this activation leads to dissociation of inhibitory κB-α from NF-κB and subsequent translocation of NF-κB to the nucleus (Baeuerle & Baltimore 1996). NF-κB dimerizes with AP-1 and has synergistic effects on gene transcription, and heterodimers of both NF-κB and AP-1 complexes bind to their respective binding sites (Yasumoto et al. 1992, Stein et al. 1993). Furthermore, AP-1 is known to be inhibited by nuclear receptors (for review, see Karin et al. 1997). Direct interaction between AP-1 and nuclear glucocorticoid receptor induces mutual transcriptional repression (Yang-Yen et al. 1990). Steroid receptor coactivator-1, a transcriptional coactivator connecting transcription factors, has been shown to stimulate AP-1-mediated transactiavtions by specifically binding to the subunits of AP-1, c-Jun and c-Fos, and relieving the transrepression between nuclear receptors and AP-1 (Lee et al. 1998b).

2.4 Natriuretic peptides

In mammals, the natriuretic peptide family consists of highly homologous polypeptide cardiac hormones: ANP, BNP and C-type natriuretic peptide (CNP) (for review, see Ruskoaho 1992). ANP and BNP play an important role in cardiovascular homeostasis, functioning as counterregulators of the actions of Ang II (for reviews, see Fyhrquist & Tikkanen 1988, Nakao et al. 1992a, de Bold et al. 1996) (Table 2). Dendroaspis natriuretic peptide (DNP) (Schweitz et al. 1992) and salmon cardiac peptide (SCP) (Tervonen et al. 1998) are new members of the natriuretic peptide family sharing
structural, biological and distributional similarities to other natriuretic peptides. Complete
disruption of ANP gene results in marked cardiac hypertrophy in mice with a modest
increase in blood pressure (John et al. 1995), whereas transgenic mice overexpressing
ANP have low heart weight (Klinger et al. 1994). Although BNP gene knockout mice
exhibit normal sized hearts, blood pressure and circulating levels of ANP, they develop
ventricular fibrosis, suggesting a cardiac myocyte-derived antifibrotic role for BNP
(Tamura et al. 2000).

Three distinct receptors exert the effects of natriuretic peptides in mammalian tissues
(for review, see Nakao et al. 1992b). Natriuretic peptide receptors-A and -B (NPR-A and
NPR-B) are transmembrane single-chain guanylate cyclase-linked polypeptide receptors
that stimulate the intracellular cGMP (Chang et al. 1989, Schulz et al. 1989). NPR-C,
also known as a clearance receptor, binds natriuretic peptides, whereafter the ligand-
receptor complex is internalized and enzymatically degraded (Maack et al. 1987). NPR-
A, -B and -C genes are expressed in several tissues, including heart, brain, kidney and
adrenal gland (for review, see Yandle 1994). Mice lacking NPR-A develop marked
cardiac hypertrophy and chamber dilatation (Knowles et al. 2001).

Cardiac natriuretic peptide gene expression and secretion are activated in response to
hypertrophic stimuli (for review, see de Bold et al. 1996). In agreement with these
findings, plasma ANP and BNP concentrations are useful cardiac-specific markers
(Dagnino et al. 1991). Administration of synthetic BNP (nesiritide), ANP (carpeditide)
and DNP as well as vasopeptidase inhibitors (omapatrilat), which are novel molecules
inhibiting the activity of NEP and ACE, have beneficial effects on hemodynamic,
neurohumoral and renal functions in CHF (Abraham et al. 1998, Troughton et al. 2000b,

<table>
<thead>
<tr>
<th>Biological effects</th>
<th>Natriuretic peptides</th>
<th>Angiotensin II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasoconstriction</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Myocyte hypertrophy</td>
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<td>↑</td>
</tr>
<tr>
<td>Renal sodium secretion</td>
<td>↑</td>
<td>↓</td>
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<tr>
<td>Diuresis</td>
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<td>↓</td>
</tr>
<tr>
<td>Sympathetic nerve activity</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Parasympathetic nerve activity</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Aldosterone concentration</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Renin secretion</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

### 2.4.1 Atrial natriuretic peptide

In 1981, de Bold et al. described the heart as an endocrine organ, since injection of an
extract of atrial muscle into rats induced vigorous natriuresis and a fall in arterial
pressure, and thereafter ANP was isolated (de Bold et al. 1981, de Bold 1985). ANP is
present as a single-copy gene and is organized into three exons separated by two introns
The ANP gene exhibits a significant homology of the nucleotide sequence among different species (for review, see Rosenzweig & Seidman 1991). The precursor of ANP, preproANP, is converted to proANP, which is a predominant storage form of ANP in specific atrial granules (Vuolteenaho et al. 1985). Next, proANP is cleaved into amino-terminal ANP and biologically active 28-amino acid ANP in mammals (for review, see Nakao et al. 1992a). In humans, transmembrane serine protease, corin, has been shown to convert proANP to ANP (Yan et al. 2000). ANP and other natriuretic peptides share a common structure of a 17-amino acid loop formed by an intrachain disulfide bond between cysteine residues (Misono et al. 1984, for review, see Rosenzweig & Seidman 1991) (Fig. 3).

In the heart, cardiac myocytes are the predominant cells for ANP production (Argentin et al. 1994), although ANP may also be synthesized by fibroblasts (Cameron et al. 2000). Atrium is the most important site for the synthesis of ANP, since the ANP mRNA levels in the atria are 100-fold higher than in the ventricles (for review, see Nakao et al. 1992a). Atrial ANP is secreted in a constitutive and regulatory manner (Ogawa et al. 1999), whereas ventricular ANP secretion occurs predominantly in a constitutive pathway (Ruskoaho et al. 1989). Cardiac myocyte stretch is suggested to be a primary stimulus for the release of ANP (Ruskoaho et al. 1986). Also, many neurohumoral secretagogues induce the release of ANP (for review, see de Bold et al. 1996). The major degradation pathways of ANP are receptor-mediated endocytosis and NEP-mediated degradation (for review, see Ruskoaho 1992).

![Fig. 3. Structure of the gene and the biosynthetic pathway of human ANP (modified from Nakao et al. 1992a). Shaded areas of the peptide indicate identical amino acids between ANP, BNP and CNP.](image-url)
2.4.1.1 ANP gene expression in response to cardiac overload

In experimental animal models, ventricular ANP gene expression and ir-ANP concentrations increase in response to cardiac overload and myocardial hypertrophy (Lee et al. 1988, Marttila et al. 1996). However, whether wall stretch acts directly or via paracrine factors liberated in response to wall distension remains to be clarified (for review, see Ruskoaho et al. 1997). Since the ANP mRNA levels increase 10- to 15-fold in response to hypertrophic stress in the adult ventricle and the basal ANP mRNA levels are relatively low, ANP may be one of the best available molecular markers for cardiac myocyte hypertrophy (Lattion et al. 1986, Kinnunen et al. 1991). Increased plasma and cardiac ANP levels have been measured in patients with CHF related to the severity of cardiac disease (Tikkanen et al. 1985, Yasue et al. 1994, de Boer et al. 2001).

It is probable that neural and endocrine factors are directly involved in stimulating ANP secretion, since ANP release declines to baseline levels despite maintained or repetitive atrial muscle stretch within few minutes both in vivo and in vitro (for review, see de Bold et al. 1996). Indeed, cardiac ANP gene expression and release are stimulated by α-adrenergic agonists, ET-1, glucocorticoids, prostaglandins, thyroid hormone, NO inhibition and Ang II (for reviews, see Ruskoaho 1992, Ruskoaho et al. 1997). The release of ANP is also modulated by β-adrenergic agonists (Agnoletti et al. 1992), acetylcholine (Antunes-Rodrigues et al. 1993), Na+/K+-ATPase inhibitors (Morise et al. 1991) and AVP (Marttila et al. 1996, Magga et al. 1997a).

Stretch does not increase atrial ANP mRNA levels for over a 2-hour period of stimulation in perfused rat heart (Mäntymaa et al. 1993), whereas stretch-induced changes in ANP gene expression occur after 24 h in cultured cardiac myocytes (Gardner et al. 1992, Sadoshima et al. 1992b). Therefore, it seems that more time is required for stretch to induce changes in ANP than BNP gene expression, suggesting that ANP gene expression has characteristics of secondary response genes (Hanford & Glembotski 1996). However, rapid induction of ANP gene expression within five hours by mechanical stretch has been observed in papillary muscle strips (Jarygin et al. 1994).

In rats, both cardiac ANP gene expression and cardiac and plasma ir-ANP concentrations have been shown to increase in response to chronic volume overload induced by deoxycorticosterone acetate (DOCA)–salt treatment and aortocaval shunt, and pressure and volume overload stimulated by binephrectomy (Lattion et al. 1986, Lear & Boer 1995, Yokota et al. 1995). In fact, aortocaval shunt increases left ventricular ANP mRNA levels before the onset of myocardial hypertrophy in rats (Su et al. 1999). In genetically hypertensive animals, SHRs and transgenic rats carrying the mouse Ren-2 renin gene, baseline cardiac ANP synthesis and release are markedly higher than in normotensive controls (Ruskoaho et al. 1989, Marttila et al. 1996). Also, pressure overload induced by aortic coarctation (Rockman et al. 1994, Ogawa et al. 1996) and dogs subjected to rapid ventricular pacing (Perrella et al. 1992) exhibit increased cardiac ANP gene expression in vivo. In response to myocardial infarction-induced cardiac hypertrophy, ANP mRNA levels have been shown to increase both in experimental animal models and in humans (Hama et al. 1995, Omland et al. 1996, Gidh-Jain et al. 1998).
2.4.1.2 *Transcriptional regulation of ANP gene*

During embryonic and fetal development, ANP gene is expressed both in the atrial and ventricular but not in the skeletal muscle cells. In the later stage of fetal development, the ANP gene appears to be switched off in the ventricular cells whereas its expression remains high in the atria (Argentin et al. 1994). However, when the ventricles are subjected to hemodynamic overload, ventricular ANP gene expression is reactivated (Lee et al. 1988).

High degree of homology exists between the rat and human ANP genes, suggesting the presence of putative well-conserved regulatory elements (Argentin et al. 1985). By using gene transfer, sequences of 400 bp upstream from the transcriptional initiation site of the human ANP promoter have been reported to be sufficient for the cardiac myocyte-specific gene expression *in vitro* (Wu et al. 1989) and atrial-specific gene expression in transgenic mice (Field 1988). Furthermore, a 700-bp sequence of the proximal rat ANP promoter is suggested to be sufficient for the stage-specific gene expression during heart differentiation and cardiac muscle-specific gene expression in differentiated atrial and ventricular myocytes (Argentin et al. 1994).

Nkx-2.5 response element (NKE) appears to contribute to the cardiac ANP gene transcription in a chamber- and stage-specific manner in collaboration with other regulatory elements of the rat ANP promoter (Durocher et al. 1996). NKE2, another Nkx-2.5 response element located more distally, is suggested to be required for the high-level activation of the ANP promoter (Shiojima et al. 1999). NKE2 may also be involved in the induction of cardiac ANP gene expression during pathological conditions (Takimoto et al. 2000). Transcription factors Nkx-2.5 and GATA4 have been reported to cooperatively activate ANP and physically interact *in vitro* and *in vivo* (Durocher et al. 1997, Lee et al. 1998a). GATA proteins are suggested to play a marked role in the ANP gene expression, since several GATA consensus sites are located between −117 and −3003 bp of the rat ANP promoter (Thuerauf et al. 1994). In this regard, two GATA motifs, residing at −290 and −122 bp of the rat and human ANP promoters, may be important elements for the basal cardiac-specific gene expression of ANP (Grépin et al. 1994). In addition, ANP mRNA levels are elevated chronically in transgenic mice overexpressing GATA4 (Liang et al. 2001b), and GATA4 and SRF synergistically increase the activity of ANP promoter in response to ET-1 administration *in vitro* (Morin et al. 2001).

The AP-1 binding site in the proximal ANP promoter is reported to be essential for the pressure overload-responsiveness in wall stress-stimulated rat hearts *in vitro* (Cornelius et al. 1997). In addition to AP-1 site, cAMP response element (CRE) is suggested to mediate the wall stretch-induced increase in the rat ANP promoter activity, since mutations of both AP-1 element and CRE are required to confer complete loss of the inducibility in beating rat hearts *in vitro* (Cornelius et al. 1997). Furthermore, AP-1-like site is suggested to be an important cis regulatory element in mediating pressure overload-responsiveness of the rat ANP gene *in vivo* (von Harsdorf et al. 1997). In contrast, there are also studies that cannot define the pressure overload responsive element to locate in the abovementioned region of the rat ANP promoter (Knowlton et al. 1995, Hasegawa et al. 1997). Moreover, components of AP-1 may also inhibit the cardiac...

Proximal phenylephrine response element and serum response element (SRE) are suggested to mediate both basal and $\alpha_1$-adrenergic agonist-induced transcriptional activation of the rat ANP gene (Ardati & Nemer 1993, Sprenkle et al. 1995, Morissette et al. 2000). Also, A/T-rich element located distally of the ANP promoter appears to mediate $\alpha_1$-adrenergic inducibility in rat ventricular myocytes (Harris et al. 1997). The ISO-stimulated transcription of the ANP gene is suppressed by dominant negative GATA4 (Morisco et al. 2001), suggesting that GATA4 plays an important role in $\beta$-adrenergic stimulation of ANP gene expression in vitro. Furthermore, ET-1-stimulated human ANP promoter activity has been demonstrated to require SRE element (Kovacic et al. 1998). The neuron-restrictive silencer element (NRSE), which is located in the 3′ untranslated region of the ANP gene, is involved in the ET-1-induced activation of ANP gene expression. However, NRSE also mediates repression of the ANP gene expression in ventricular myocytes (Kuwahara et al. 2001).

2.4.2 B-type natriuretic peptide

In 1988, Sudoh et al. discovered BNP in porcine brain with biological properties and structural homology similar to ANP (Sudoh et al. 1988, Dagnino et al. 1991). Subsequently, BNP was found to be more abundant in cardiac atria and ventricles than in the central nervous system (Ogawa et al. 1991a, Dagnino et al. 1992). The BNP gene is composed of three exons and two introns (Seilhamer et al. 1989) (Fig. 4). In contrast to ANP, BNP has considerable inter-species diversity of amino acid composition. The posttranslational processing of BNP precursors seems to be different from that of ANP, and the processing sites are not conserved between species, resulting in various lengths of BNP (for review, see Yandle 1994). 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 conversion of BNP precursor to BNP-45 in rats has been reported to demand an endoprotease furin (Sawada et al. 1997).
Fig. 4. Structure of the gene and the biosynthetic pathway of human BNP (modified from Nakao et al. 1992a). Shaded areas of the peptide indicate identical amino acids between BNP, ANP and CNP.

The major storage form of BNP in the heart is the cleaved mature peptide, although in atrial tissue also prohormones may be stored (for review, see Yandle 1994). BNP is suggested to be released constitutively after synthesis (Wei et al. 1993), although storage granules containing both ANP and BNP have been described, demonstrating also a regulatory pathway for BNP secretion (Nakamura et al. 1991, Ogawa et al. 1999). Baseline plasma BNP concentration is approximately 1 fmol/ml in humans, which is one-sixth of the plasma ANP concentration determined simultaneously (Mukoyama et al. 1991). The major stimulus controlling the release of BNP from the atria and ventricles appears to be myocyte stretch (for review, see Ruskoaho et al. 1997). The metabolism of BNP still remains quite an unknown subject, and it is likely that both ANP and BNP share similar metabolic pathways. However, although NEP cleaves ANP mainly at one site, porcine BNP appears to be cleaved at several different sites (Vogt-Schaden et al. 1989). In humans, lower binding activity of BNP than ANP to clearance receptors is suggested to be one reason for the longer plasma half-life of BNP (approximately 22 minutes) compared with ANP (Mukoyama et al. 1991).

2.4.2.1 BNP gene expression in response to cardiac overload

Compared with ANP, plasma concentration of BNP has been shown to be a better marker for impaired left ventricular function and diagnosis of CHF (Kohno et al. 1995,
In addition, plasma BNP concentrations provide prognostic information in CHF (Omland et al. 1996, Tsutamoto et al. 1997) and guide the treatment of patients with CHF (Troughton et al. 2000a). Plasma BNP concentrations and cardiac BNP mRNA levels have been shown to increase in humans with moderate and severe heart failure and not at early stage of ventricular dysfunction (Wei et al. 1993, de Boer et al. 2001). Also in volume overloaded rats, cardiac BNP gene expression is induced specifically in overt heart failure (Yokota et al. 1995, Langenickel et al. 2000), suggesting a possible role for BNP as a marker of the transition from compensated to severe cardiac dysfunction. In addition, CHF stimulated by rapid ventricular pacing in dogs demonstrates that early left ventricular dysfunction is characterized by selective increase in the atrial BNP gene expression, and overt CHF is provided with additional ventricular BNP gene expression (Luchner et al. 1998).

Cardiac volume overload induced by aortocaval shunt, DOCA-salt treatment and bilateral nephrectomy, which also produces pressure load, increases plasma BNP concentrations and left ventricular BNP mRNA and ir-BNP levels (Lear & Boer 1995, Yokota et al. 1995, Langenickel et al. 2000, Marttila et al. 2001) (Table 3). Also, pressure overload generated by aortic banding in rats stimulates BNP synthesis (Ogawa et al. 1996). In addition to cardiac overload, neural and endocrine factors such as ET-1, Ang II, adrenergic agonists and AVP activate cardiac BNP gene expression and secretion (Bruneau & de Bold 1994, Magga et al. 1994, Bruneau et al. 1996, Liang & Gardner 1998, Magga et al. 1999, Ogawa et al. 1999). In SHRs, ventricular BNP mRNA levels are elevated at the onset of the hypertensive stage and the BNP gene expression correlates with the progression of hypertension (Dagnino et al. 1992). Transgenic mice containing the proximal human BNP promoter exhibit increased promoter activities after two days of acute myocardial infarction in vivo lasting until the end of the four-week experiment. Myocardial infarction also induces endogenous mice BNP mRNA levels in the left ventricles within 48 h (He et al. 2001b). In order to study the BNP gene expression in the absence of ANP, ANP gene knockout mice were produced. Ventricular BNP gene expression increases significantly, but the plasma BNP concentrations remain unaltered in ANP gene disrupted mice (Tse et al. 2001), suggesting the inability of BNP to completely compensate the lack of ANP.

In addition to the long-term stimulation of BNP during hemodynamic stress, BNP gene expression increases as quickly as the expression of proto-oncogenes in response to hypertrophic stimuli in vitro (Mäntymaa et al. 1993, Nakagawa et al. 1995) and in vivo well before the development of LVH (Magga et al. 1994). Moreover, BNP mRNA levels are elevated rapidly in response to PE and phorbol ester stimulation, and transcript stabilization by these agents further increases the half-life of BNP mRNA (LaPointe & Sitkins 1993, Hanford et al. 1994, Hanford & Glombotski 1996). However, although mechanical strain increases BNP gene expression at the transcriptional level, it may not stabilize the BNP transcripts (Liang et al. 1997).
Table 3. Left ventricular BNP synthesis in experimental animal models

<table>
<thead>
<tr>
<th>Animals</th>
<th>Stimulus</th>
<th>LV H</th>
<th>∆RR</th>
<th>Time/ Age</th>
<th>LV BNP mRNA</th>
<th>LV ir-BNP</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1A KO mice</td>
<td>CoA</td>
<td>↔</td>
<td>↑</td>
<td>30 min</td>
<td>↑</td>
<td>↑</td>
<td>Harada et al. 1998</td>
</tr>
<tr>
<td>WKY rats</td>
<td>AVP/PE</td>
<td>↔</td>
<td>↑</td>
<td>1 h</td>
<td>↑</td>
<td>↑</td>
<td>Magga et al. 1994</td>
</tr>
<tr>
<td>SHRs</td>
<td>AVP/PE</td>
<td>↑</td>
<td>↑</td>
<td>1 h</td>
<td>↑</td>
<td>↑</td>
<td>Magga et al. 1994</td>
</tr>
<tr>
<td>TGR(mREN-2)</td>
<td>AVP</td>
<td>↑</td>
<td>↑</td>
<td>2 h</td>
<td>↔</td>
<td>↔</td>
<td>Marttila et al. 1996</td>
</tr>
<tr>
<td>Wistar rats</td>
<td>AMI</td>
<td>–</td>
<td>↓</td>
<td>4 h</td>
<td>↑</td>
<td>↑</td>
<td>Hama et al. 1995</td>
</tr>
<tr>
<td>SD rats</td>
<td>Nephr.</td>
<td>↔</td>
<td>↑</td>
<td>1 d</td>
<td>↑</td>
<td>↑</td>
<td>Marttila et al. 2001</td>
</tr>
<tr>
<td>Wistar rats</td>
<td>Ao-shunt</td>
<td>↑</td>
<td>↓</td>
<td>3 d</td>
<td>↔</td>
<td>–</td>
<td>Langenickel et al. 2000</td>
</tr>
<tr>
<td>SD rats</td>
<td>DOCA</td>
<td>↑</td>
<td>↔</td>
<td>1 w</td>
<td>↔</td>
<td>↔</td>
<td>Yokota et al. 1995</td>
</tr>
<tr>
<td>SHRs</td>
<td>DOCA</td>
<td>↑</td>
<td>↑</td>
<td>5 w</td>
<td>↑</td>
<td>↑</td>
<td>Yokota et al. 1995</td>
</tr>
<tr>
<td>SD rats</td>
<td>CoA</td>
<td>↑</td>
<td>↑</td>
<td>5 w</td>
<td>↑</td>
<td>↑</td>
<td>Dagnino et al. 1992</td>
</tr>
<tr>
<td>SHRSPs</td>
<td>-</td>
<td>↔</td>
<td>↑</td>
<td>5 w</td>
<td>↑</td>
<td>↑</td>
<td>Ogawa et al. 1996</td>
</tr>
<tr>
<td>TGR(mREN-2)</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>12 w</td>
<td>↔</td>
<td>↑</td>
<td>Marttila et al. 1996</td>
</tr>
<tr>
<td>ANP KO mice</td>
<td>-</td>
<td>↑</td>
<td>–</td>
<td>12 w</td>
<td>↔</td>
<td>↑</td>
<td>Tse et al. 2001</td>
</tr>
<tr>
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<td>-</td>
<td>↑</td>
<td>↑</td>
<td>10-22 m</td>
<td>↑</td>
<td>↑</td>
<td>Magga et al. 1994</td>
</tr>
</tbody>
</table>

Ao-shunt, aortocaval shunt; AMI, acute myocardial infarction; ANP-/- mouse, ANP gene knockout mouse; AT1, angiotensin II type 1A receptor; AVP, arginine-vasopressin; CoA, aortic coarctation; DOCA, deoxycorticosterone acetate; ir, immunoreactive; KO, knockout; LV, left ventricular; LVH, left ventricular hypertrophy; Nephr., bilateral nephrectomy; PE, phenylephrine; ∆RR, change in blood pressure; SHRSP, spontaneously hypertensive rat-stroke prone; TGR(mREN-2), transgenic rats carrying the mouse Ren-2 renin gene; WKY, Wistar-Kyoto.

### 2.4.2.2 Transcriptional regulation of BNP gene

The rat BNP promoter possesses multiple potential GATA and AP-1 binding sites in the 5' flanking sequence, which are suggested to be important elements for BNP regulation (Grépin et al. 1994, Thuerauf et al. 1994). The sequences and positions of the GATA and AP-1 motifs are highly conserved in the rat, dog and human BNP genes (Grépin et al. 1994) (Fig. 5). In rat and human BNP gene, GATA site at -30 bp likely serves as a binding sequence for TATA-protein (Seilhamer et al. 1989, Thuerauf et al. 1994). By using gene transfection into the rat cardiac myocytes in vivo, the proximal -114 bp containing GATA and AP-1-like elements have been shown to be sufficient for basal ventricular-specific expression of the rat BNP gene (Marttila et al. 2001). Also in vitro, the determinant for cardiac specificity has been suggested to locate within the proximal -114 bp, since the deletion of sequences between -2200 and -114 bp does not affect the high-level activity of the rat BNP promoter (Grépin et al. 1994). By transfecting the rat BNP-luciferase vectors into the cardiac myocyte cultures, deletion of the proximal AP-1-like motif decreases BNP promoter activity fourfold and deletion of two GATA motifs at -90 bp causes another fourfold reduction of BNP promoter activity (Grépin et al. 1994).
Moreover, co-transfection of the rat -116 bp BNP-luciferase reporter with GATA4 or GATA6 results in 4-fold activation of the BNP promoter, and the response is potentiated by PE in cardiac myocytes (Liang et al. 2001b). Furthermore, when cardiac myocytes are transfected with rat BNP-luciferase construct, mutation of two GATA sites at -90 bp decreases GATA-mediated induction by 50% in response to GATA4 overexpression in vitro (Thuerauf et al. 1994). Similarly, dominant-negative GATA4-engrailed fusion construct inhibits GATA4 or GATA6-induced BNP gene transactivation, suggesting the involvement of GATA factors in regulating BNP promoter activity (Liang et al. 2001b). Moreover, BNP mRNA levels are elevated in transgenic mice overexpressing GATA4 (Liang et al. 2001b). In rats, mutation of the AP-1-like element has no significant effect, whereas mutation of two GATA elements at -90 bp decreases left ventricular BNP promoter activity to 57% of the intact -114 BNP construct activity in response to one-day nephrectomy in vivo (Marttila et al. 2001).

Fig. 5. The proximal rat BNP promoter and the cis elements referred to in the text (modified from Thuerauf et al. 1994 and Tokola et al. 2001).

The rat BNP gene is 65% homologous with the human BNP gene (LaPointe et al. 1996). Human BNP promoter consisting of sequences between -1818 and +100 bp has been shown to be more active in cardiac myocytes than in fibroblasts in vitro. This sequence consists of positive and negative regulatory elements that contribute to the BNP gene expression in cardiac myocytes (LaPointe et al. 1996). The analysis of the proximal human BNP promoter has revealed that rather than a single element, several tandemly arranged cis elements are responsible for the myocyte-specific promoter activity (LaPointe et al. 1996). In vivo, the human BNP promoter containing sequences from −408 to +100 bp has been shown to confer the cardiac-specific BNP gene expression (LaPointe et al. 1996, He et al. 2001b).

In contrast to rat BNP promoter, the gene construct containing human BNP proximal promoter (-111 to -40 bp) is inactive when transfected into cardiac myocytes, suggesting that GATA and AP-1 elements may not be critical determinants of human BNP gene activity (LaPointe et al. 1996). However, the GATA element at −85 bp of the human BNP promoter and unidentified more distal cis elements have been shown to be targets for ET-1 (He & LaPointe 2001a). Furthermore, three NF-AT binding sites have been demonstrated in the human BNP promoter, and NF-AT3 and GATA4 transactivate human BNP promoter synergistically in neonatal rat cardiac myocytes (Molkentin et al. 1998). M-CAT binding factors, also known as transcription enhancer factor-1-like proteins, contribute to basal human BNP promoter activity by binding to M-CAT-like elements.
The proximal M-CAT element mediates the human BNP promoter activation stimulated by ISO and cAMP (He et al. 2000a). In rat BNP promoter, M-CAT may also mediate transcriptional stimulation in response to α₁-adrenergic treatment (Thuerauf & Glembotski 1997). Furthermore, mechanical strain and ET-1 have been shown to enhance human BNP promoter activity through p38 MAPK, which operates via the NF-κB element with three shear stress response element-like structures of the human BNP gene in neonatal rat ventricular myocytes (Liang & Gardner 1999, Liang et al. 2000b).
3 Aims of the research

The aim of this study was to elucidate the role of Ang II in regulating the gene expression of ANP and BNP in response to pressure overload. The second purpose was to study the mechanisms of Ang II in the regulation of cardiac genes during pressure overload.

Specific aims of this study were:

1. to examine the role of Ang II in cardiac ANP and BNP gene expression, blood pressure and vascular changes in response to cardiac overload stimulated by NOS inhibitor L-NAME.
2. to investigate whether the factors synthesized by adrenal glands modulate the Ang II-induced left ventricular ANP, BNP and AM gene expression, hemodynamics and cardiac hypertrophy.
3. to elucidate the effect of Ang II-induced pressure overload on BNP gene expression and transcription.
4. to evaluate the specific cis elements of the BNP gene mediating the Ang II-induced pressure overload-responsiveness.
4 Materials and methods

4.1 Materials

The chemicals and supplies used in this study were: formaldehyde and guanidine isothiocyanate (Fluka Chemie AG, Buchs, Switzerland), CsCl (Serva Feinchemica GmbH & Co, Heidelberg, Germany), agarose NA and T7 Quick Prime Kit (Amersham Pharmacia Biotech, Uppsala, Sweden), [32P]-deoxycytidine-5’-triphosphate (dCTP) and radioiodine (Amersham Pharmacia Biotech, Buckinghamshire, England, UK), X-ray films (Eastman Kodak, Rochester, NY, USA, and Amersham Pharmacia Biotech), angiotensin II and N\textsuperscript{G}-nitro-L-arginine methyl ester hydrochloride (Sigma Chemical Co., St Louis, MO, USA), losartan (Merck Pharmaceutical Co., Wilmington, DE, USA), spironolactone (ICN Biomedicals Inc., Ohio, USA), fentanyl citrate and fluanisone (Janssen-Cilag, Saunderton, UK), midazolam (Roche, Espoo, Finland), atipamezole and medetomidine hydrochloride (Orion Pharmaceutical Inc., Espoo, Finland) and ketamine hydrochloride (Parke-Davis, Warner Lambert Nordic AB, Solna, Sweden). Other chemicals were obtained from Sigma.

4.2 Animals

Male 2-month-old Sprague-Dawley (SD) rats (260 to 350 g) were obtained from the Center for Experimental Animals at the University of Oulu, Finland and male 11-week-old Wistar rats (400 to 480 g) were obtained from the Center for Experimental Animals at the University of Tampere, Finland. The rats were housed in plastic cages in a room with a controlled humidity of 40 % and temperature of +22 °C. A controlled environmental 12 h light-dark cycle was maintained. The experimental design was approved by the Animal Use and Care Committee of the University of Oulu and the Animal Experimentation Committee of the University of Tampere. 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

Table 4. Summary of the experimental protocols

<table>
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<td>2 h 6 h 12 h 72 h 1 week 2 weeks</td>
</tr>
<tr>
<td>IV</td>
<td>SD</td>
<td>Pressure load (Ang II s.c.)</td>
<td>Hemodynamics Cloning Direct gene transfer Reporter gene assays Gel shift RNA isolation RIA</td>
<td>6 h 2 weeks</td>
</tr>
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</table>

Ang II, angiotensin II; i.v., intravenously; L-NAME, N\(^\text{G}\)-nitro-L-arginine methyl ester; p.o., perorally; RIA, radioimmunoassay; s.c., subcutaneously; SD, Sprague-Dawley

4.3.1 Chronic cannulation and measurement of hemodynamics (I-IV)

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 intraperitoneally (i.p.). A PE-60 catheter was placed into the abdominal aorta through the right femoral artery for collection of blood samples and measurement of hemodynamics as described previously (Ruskoaho et al. 1989). A PE-50 catheter was inserted into the right atrium through the jugular vein for measurement of right atrial pressure and into the left femoral vein for infusion of donor blood, 0.9 % NaCl or Ang II. All catheters were exteriorized behind the neck, filled with
heparinized (150 IU/ml) 0.9 % NaCl solution, and plugged with a stainless pin. A day after the operation, the arterial and right atrial catheters were attached to pressure transducers (model MP-15, Micron Instruments, Los Angeles, CA, USA) and a Grass polygraph (model 7DA, Grass Instruments, Quincy, MA, USA) for recording mean arterial pressure (MAP), heart rate and right atrial pressure. The venous catheter was connected to an infusion pump (B. Braun Perfusor ED, Braun Melsungen AG, Melsungen, Germany) for infusions. During measurement, the animals were conscious and freely moving. The measurement of hemodynamics was started for 30 minutes before a 1.0 ml blood sample (for plasma peptide determination) was withdrawn from the arterial catheter. The baseline MAP and heart rate measurements were done 5 minutes later. The blood sample was replaced by an equal volume of 1.0 ml blood from a donor rat 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 (EDTA) per 1.0 ml blood on ice and immediately centrifuged (2000 x g, 10 min, at +4 °C). After the 2-hour infusions (see below 4.3.2) another arterial blood sample was collected. The rats were decapitated immediately after the experimental period. The plasma was stored at -20 °C until assayed by radioimmunoassay (RIA).

The systolic blood pressure of the conscious rats restrained in plastic holders was measured indirectly by the tail-cuff method at +28 °C (I). All the measurements were performed with an IITC Inc. Model 129 Blood Pressure Meter (Woodland Hills, CA, USA) equipped with a photoelectric pulse detector. The blood pressure of each rat was obtained by averaging three reliable recordings.

For telemetric monitoring of hemodynamics for 3 days (II) or 2 weeks (III, IV), the rats were anesthetized (as described above) and instrumented with a catheter in the descending aorta coupled with a sensor and transmitter (TA11PA-C40, Data Sciences, St. Paul, MN, USA). MAP and heart rate were measured every 10 minutes and averaged every hour. Ang II or 0.9 % NaCl infusion was started (see below 4.3.2) and adrenalectomy (ADX) or sham operation was performed (see below 4.3.4) 7 days after surgery. After the experiment, the rats were decapitated, the hearts were detached and the ventricles, auricles and other atrial tissue were removed. Ventricular and atrial tissue samples were blotted dry, weighed, immersed in liquid nitrogen and stored at –70 °C until assayed.

4.3.2 Induction of hemodynamic overload by angiotensin II (II-IV)

Ang II or 0.9 % NaCl was administered chronically by using Alzet osmotic minipumps (B & K Universal AB, Sollentuna, Sweden) model 1003D (pumping rate 1 µl/h, volume 90 µl, for 6, 12 and 72 h infusions), model 2001 (pumping rate 1 µl/h, volume 220 µl, for 1 week infusion) and model 2002 (pumping rate 0.5 µl/h, volume 230 µl, for 2 weeks 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. and the minipumps were placed subcutaneously (s.c.) into the back of the SD rats for either Ang II (33.3 µg/kg/h) or 0.9 % NaCl infusion. To the rats which were exposed to direct DNA injection into the left ventricle, minipumps for 1-week and 2-week experiments were installed at the same time
with the injection of the plasmids. For the 6, 12 and 72 h experiments, the plasmid DNA injections were made a week before the decapitation and minipumps for Ang II and 0.9 % NaCl infusion were installed under anesthesia 6, 12 and 72 h before the decapitation. For 2-hour infusions, a PE-50 venous catheter was placed in the left femoral vein of the SD rat under anesthesia on the 6\textsuperscript{th} day, and 2-hour Ang II (30 \(\mu\)g/kg/h) or 0.9 % NaCl infusion intravenously (i.v.) was started on the 7\textsuperscript{th} day of the DNA injection.

### 4.3.3 Chronic drug treatment with L-NAME and losartan (I)

Male Wistar rats were given L-NAME, losartan and their combination, which were added to the drinking fluid as follows: control (tap water), losartan (20 mg/kg/day), L-NAME (20 mg/kg/day) and losartan plus L-NAME (doses of both losartan and L-NAME 20 mg/kg/day). Drug solutions were prepared daily and given in light-proof bottles. To obtain the desired daily drug dose, the concentration in drinking water was adjusted according to 24 h fluid consumption measurements. Drug administrations were continued for 8 weeks.

### 4.3.4 Adrenalectomy (II)

SD rats were subjected to sham operation, bilateral ADX, Ang II infusion and bilateral ADX plus Ang II infusion under anesthesia (see above 4.3.1). Bilateral ADX was performed by using dorsolumbar approach and separate incisions were made on each side. The efficacy of ADX was verified by postmortem examination of the suprarenal region. In sham-operated rats for ADX, skin incision and further muscle incisions were made at both sides of the vertebral column without removing the adrenal glands. The rats treated with Ang II s.c. were subjected either to the sham operation or to ADX.

### 4.3.5 Spironolactone treatment (II)

SD rats were subjected to vehicle and spironolactone treatment, Ang II infusion and Ang II infusion plus spironolactone treatment. Spironolactone was diluted in ethanol (25 mg/ml). Spironolactone (100 mg/kg) was injected s.c. at the nape of the neck 24 h before and at the time of starting vehicle- and Ang II infusion s.c. by osmotic minipumps for 12 h. The same volume of ethanol that was injected to the spironolactone-treated rats was given to the vehicle-treated and Ang II-administered rats.
**4.3.6 Direct gene transfer into rat heart in vivo (III, IV)**

Plasmid DNA of 10 µg or 50 µg containing reporter construct driven by fragments of the rat BNP promoter were injected with 100 µg of simian virus β-gal plasmid (pSVβ-gal). SD rats were anesthetized with 250 µg/kg medetomidine hydrochloride and 50 mg/kg ketamine hydrochloride i.p. First, left lateral thoracotomy was made and the chest muscles were then blunt-dissected to the level of the ribs to expose the heart. The heart was gently exteriorized and 100 µl of circular DNA plasmid in 0.9 % NaCl was injected into the beating left ventricular free wall close to the apex by using 100 µl Hamilton precision syringe as described previously (for review, see Kass-Eisler & Leinwand 1997). After injection, the heart was placed back into the chest cavity. The muscle and skin were closed in layers, and the chest was evacuated. To antagonize the sedative effect of medetomidine, the rats received 3.3 mg/kg atipamezole i.p.

**4.3.7 Echocardiography (III)**

Transthoracic echocardiograms were performed using a commercially available Acuson Ultrasound System (Sequoia™ 512) and a 15-MHz linear transducer (15L8) (Acuson, MountainView, CA, USA). Before examination, rats were sedated with ketamine 50 mg/kg and xylazine 10 mg/kg i.p. and the chest was shaved. The rats were placed in the supine position and normal body temperature was maintained during the examination by a warming pad and lamp.

Using two-dimensional imaging, a short-axis view of the left ventricle at the level of the papillary muscles was obtained for the M-mode recording. Left ventricular end-systolic (LVESD) and end-diastolic (LVEDD) dimensions as well as interventricular septum and posterior wall thickness were measured from the M-mode tracings.

Left ventricular fractional shortening (LVFS), a measure of left ventricular systolic function, was calculated from the M-mode left ventricular dimensions using the following equation: $\text{LVFS} (%) = \frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}} \times 100$. Ejection fraction (EF) was also calculated from the M-mode left ventricular dimensions using the equation: $\text{EF} (%) = \left(\frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}}\right)^2 \times 100$. For evaluation of left ventricular diastolic function, mitral flow was recorded from an apical four-chamber view. Measurements of peak flow velocity of the early rapid diastolic filling wave (E) and peak flow velocity of the late diastolic filling wave (A) were made, and E/A ratio was calculated.

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

Total RNA was isolated from ventricular and atrial tissues by the guanidine isothiocyanate-CsCl method (Chirgwin et al. 1979). After isolation of RNA, the size and amount of specific mRNA molecules in preparations of total RNA were determined by Northern blot hybridization (Alwine et al. 1977). For the RNA Northern blot analysis, 3
µg samples of the RNA from atria (I) and 20 µg from ventricles (I-IV) were transferred to the Hybond N+ nylon membrane (Amersham Life Science, Buckinghamshire, UK). A full-length rat ANP cDNA probe (Flynn et al. 1985) (a generous gift from Dr Peter L. Davies, Queen’s University, Kingston, Canada), a 390-bp fragment of rat BNP cDNA probe (Ogawa et al. 1991a) (a generous gift from Dr Kazuwa Nakao, Kyoto University School of Medicine, Kyoto, Japan), a 1417-bp probe for rat GATA4, a 1175-bp probe for rat GATA6, a 1050-bp probe for rat c-fos, and rat AM cDNA probe (nucleotides 287-736) (Romppanen et al. 1997), and a 482-bp cDNA probe for 18S ribosomal RNA (Torczynski et al. 1983) were labeled with [\(^{32}\)P]-dCTP with \(^{32}\)Quick Prime Kit. The hybridization signals of AM, ANP, BNP, GATA4, GATA6 and c-fos mRNAs were normalized to that of 18S ribosomal RNA for each sample to correct for potential differences in loading and/or transfer. The membranes were hybridized overnight at +42 °C in 5 x SSC (saline sodium citrate, 1 x SSC = 0.15 M NaCl, 0.015 M 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 membranes were washed in 0.1 x SSC, 0.1 % SDS three times for 20 min at +60 °C (AM, ANP and BNP), +63 °C (GATA4, GATA6 and 18S) and +50 °C (c-fos), and exposed to X-ray films with Cronex Lighting Plus intensifying screens (DuPont, Wilmington, DL, USA) at -70 °C or to Phosphor Screens (Molecular Dynamics, Sunnyvale, CA, USA) at room temperature. Autoradiograms generated by Northern blots were scanned with a densitometer (Millipore Corp. Imaging Systems, Ann Arbor, MI, USA) and Phosphor Screens with Phosphor Imager (Molecular Dynamics).

4.5 Hormone measurements

4.5.1 Radioimmunoassay of peptides in plasma and cardiac tissue (I-IV)

The ir-BNP (I-IV), ir-ANP (I, II), ir-AM (II) and ir- amino-terminal proANP (ir-NT-proANP) (I, II) levels were measured by RIA (Vuolteenaho et al. 1985, Ruskoaho et al. 1989, Vuolteenaho et al. 1992). The plasma samples for the ir-ANP, ir-BNP and ir-AM assays were extracted with SepPak C\(_{18}\) cartridges before measurement. ir-NT-proANP was assayed from unextracted plasma. The cardiac tissue peptide levels were measured from aliquots of the guanidine isothiocyanate extracts prepared for mRNA determination (see above 4.4). ir-ANP and ir-NT-proANP were measured directly from the extracts diluted with the RIA buffer. No interference from the strongly chaotropic extraction buffer was detected with volumes of 1 µl or less of the extract per RIA tube. Tissue ir-BNP and ir-AM levels were determined after SepPak C\(_{18}\) extraction of the guanidine isothiocyanate supernatants.

\(^{125}\)I-ANP, \(^{125}\)I-BNP and \(^{125}\)I-NT-proANP tracers were prepared by chloramine-T iodination of synthetic rat ANP\(_{99-126}\), BNP\(_{11-35}\), and human [Tyr\(_{78}\)]-proANP\(_{79-98}\) followed by desalting in a Sephadex G-25 column and final purification in a Vydac C\(_{18}\) reverse-phase high-pressure liquid chromatography (HPLC) column. Synthetic rat ANP\(_{99-126}\) ranging
from 0 to 500 pg per tube, synthetic rat BNP\textsubscript{41-95} (BNP-45) ranging from 0 to 125 fmol per tube, and synthetic human proANP\textsubscript{79-98} ranging from 0 to 75 fmol per tube were used as standards. For the ANP and BNP RIAs, the standards and samples were incubated in duplicates of 100 µl with an equal volume of the specific rabbit ANP antiserum (final dilution 1:30 000) (Vuolteenaho \textit{et al.} 1985) or BNP antiserum (final dilution 1:50 000) (Ogawa \textit{et al.} 1991a) 24 h at +4 °C. 100 µl of \textsuperscript{125}I-ANP\textsubscript{99-126} or rat \textsuperscript{125}I-proBNP\textsubscript{51-95} was added and the incubation was continued for another 24 h at +4 °C. For the NT-proANP assay, the standard and samples (25 µl) were incubated in duplicate with 200 µl of human [Tyr\textsubscript{78}]-proANP\textsubscript{79-98} and 200 µl rabbit NT-proANP antiserum (final dilution 1:35 000) (Vuolteenaho \textit{et al.} 1992). The bound and free fractions were separated by double antibody precipitation in the presence of 8 % polyethylene glycol, followed by centrifugation at 3000 g for 20 min. The sensitivities of the ANP, BNP and NT-proANP were 2 pg/tube, 0.5 fmol/tube and 0.75 fmol/tube, respectively. The intra- and interassay coefficients of variation in the assays were <10 % and 15 %, respectively. Serial dilutions of the samples showed parallelism with the standards. The ANP antiserum recognized ANP and proANP with equal avidity but did not cross-react with BNP, CNP, Ang II, ET-1 or AVP (<0.1 %). The BNP antiserum did not recognize ANP, CNP, Ang II, ET-1 or AVP (<0.1 %). The BNP antiserum recognized rat proBNP as judged from HPLC analyses of rat heart extracts. The exact level of crossreaction, however, is not known because of lack of pure rat proBNP standard preparation. The NT-proANP antiserum cross-reacted fully with proANP but did not recognize ANP, BNP, CNP, Ang II, ET-1 or AVP (<0.1 %).

Rat ir-AM was determined with reagents purchased from Phoenix Pharmaceuticals (Mountain View, CA, USA) (Romppanen \textit{et al.} 1997). The synthetic rat AM\textsubscript{1-50} standards and the samples were incubated for 16-24 h at +4 °C with rabbit rat AM antiserum. After adding \textsuperscript{125}I-rat AM\textsubscript{1-50}, the incubation was continued for another 16-24 h. The free and bound fractions were separated by double antibody precipitation. The rat AM antiserum did not cross-react with rat AM\textsubscript{1-20}, human AM or its fragments. The sensitivity of the assay was 1 fmol/tube and the intra- and interassay coefficients of variation were <10 % and 15 %, respectively.

\textbf{4.5.2 Adrenal hormone measurements (II)}

Plasma catecholamines were purified by Al\textsubscript{2}O\textsubscript{3} extraction and NE and EPI were analyzed by HPLC with an electrochemical detector (Pullinen \textit{et al.} 2000). Corticosterone was determined from unextracted plasma (diluted 1:200) using a commercial RIA kit (DRG Instruments GmbH, Marburg, Germany). Aldosterone was first extracted from 0.3-ml plasma samples with 3 ml ethyl acetate-hexane (3:2 dilution), and aldosterone was determined using a commercial RIA kit (DRG Instruments GmbH).
4.6 Morphological measurements (I)

Third order mesenteric arterial branches were used. A 2-mm segment of the artery was isolated under dissection microscope, and transferred to a myograph chamber (Living Systems Instrumentation Inc., Burlington, VT, USA) containing 8 ml of PSS (pH 7.4) of the following composition (mM): NaCl 119.0, NaHCO₃ 25.0, glucose 11.1, CaCl₂ 2.5, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, Na₂EDTA 30. The proximal end of the vessel was cannulated and flushed before the cannulation of the distal end. The artery was superfused at 10 ml/min and aerated with 95 % O₂ and 5 % CO₂, and the intraluminal pressure was slowly raised to 100 mm Hg. The pressure was monitored by a transducer and servo perfusion system. After equilibration of 40 minutes at 100 mm Hg the arterial wall thickness and lumen diameter were recorded by a video monitoring system (Video dimension analyzer, Living Systems Instrumentation Inc., Burlington, VT, USA).

4.7 Plasmid DNA preparation and reporter gene assays (III, IV)

Rat BNP-luciferase (luc) plasmids containing various rat BNP promoter fragments were obtained by subcloning appropriate 5′ deletions of the BNP promoter (generated by restriction or by polymerase chain reaction) in the pXP-2 vector (III) or pGL3-Basic vector (Promega Co., San Diego, CA, USA) (IV). The reporter plasmid p-2200BNPluc was produced by cloning a HindIII-StI fragment encompassing nucleotides -2200 to +75 and plasmid p-114BNPluc was generated by subcloning a StI-BamH1 fragment containing the nucleotides -114 to +75 relative to the transcriptional start site of rat BNP gene into pXP-2 vector as described previously (Grépin et al. 1994) (III, Fig. 6A). The plasmid contained the firefly luciferase coding region, SV 40 splicing and polyadenylation signals. The plasmid p-5000BNPluc was produced by cloning a KpnI- BamH1 fragment encompassing nucleotides -5000 to +4 relative to the transcriptional start site of rat BNP gene into pGL3-Basic vector (IV, Fig. 6B) as described previously (Pikkarainen et al. 2002). Plasmid p-5000BNPluc was used to generate the p-534BNPluc containing the nucleotides -534 to +4 relative to the transcriptional initiation site by Double Stranded Nested Deletion Kit (Amersham Pharmacia Biotech) (Fig. 6B). To generate the p-534GATAmut and p-534AP-1mut constructs (Fig. 6B), site-directed mutations were introduced into two adjacent (-91 and -80 bp) GATA sites and the AP-1 site at -372 bp in the p-534BNPluc by QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), as previously described (Pikkarainen et al. 2002). The pSVβ-gal (Promega Co.), which contains β-galactosidase enzyme encoding the lacZ gene under the control of SV40 early promoter and enhancer unit was coinjected with all p-BNPluc constructs to correct for variation in transfection efficiency. For each construct different doses from 10 µg to 100 µg were tested to find the linear range of plasmid DNA content, and the minimum amount of plasmid that could easily be detected was chosen. Plasmid DNA consisted of 10 µg (III) and 50 µg (IV) of the BNPluc reporter constructs and 100 µg of the pSVβ-gal vector to correct for variation in transfection efficiency.

Our unpublished results (Marttila, Suo, Tóth, Nemer & Ruskoaho) indicate that the expression of directly injected genes is maximal at three days and measurable until 14
days following injection. The rats were sacrificed either one week or two weeks after plasmid DNA injections by decapitation. The heart was removed and washed in ice-cold 0.9 % NaCl. The left ventricle was divided into three pieces, including the middle one-third, the apical one-third and the basal one-third, and each part was weighed. The basal one-third of the left ventricle was homogenized in 1 ml homogenization buffer (20 mM Tris-acetate, pH 7.5, 5 mM EDTA, 20 mM KCl, 2 mM Mg-acetate and 0.5 mM dithiothreitol [DTT]) with an Ultra-Turrax T25-tissue homogenizer (Janke and Kunkel, Stauffen, Germany). Homogenates were centrifuged at 6000 g for 10 minutes at +4 °C. Luciferase and β-galactosidase activities were measured in 20 µl aliquots of the supernatants with a luminometer (model RS, Labsystems Luminoskan, Helsinki, Finland). For the luciferase and β-galactosidase activities, commercially available Luciferase Assay System (Promega Co., San Diego, CA, USA) and Luminescent β-galactosidase Detection Kit II (Clontech Laboratories, Inc., Palo Alto, CA, USA) were used, respectively. Samples in which the β-galactosidase activities were less than 1 LU (detection limit 0.5 LU) were not selected.

Fig. 6. Rat BNP-luciferase constructs and their names as referred to in the text. A. The BNP constructs used in Study III. The sequences and position of the highly conserved motifs of GATA and AP-1-like are shown. B. The BNP-luciferase constructs used in Study IV. Site-directed mutations of the two GATA motifs and AP-1 elements were introduced into the –534BNPluc construct.
4.8 Nuclear protein extraction and gel mobility shift assay (III, IV)

For gel mobility shift assays, nuclear extracts were prepared from the ventricular tissue
by using a modified procedure described by Deryckere and Gannon (Deryckere &
Gannon 1994). Ventricles were broken with a hammer and reduced to powder in a mortar
in liquid nitrogen. The thawed powder was homogenized with an Ultra-Turrax T25-tissue
homogenizer (Janke and Kunkel) in a low salt solution (0.6 % Nonidet P-40 [NP-40],
150 mM NaCl, 10 mM HEPES pH 7.9, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl
fluoride [PMSF]), and centrifuged for 30 seconds at 2000 rpm. The supernatant was
incubated for 5 minutes on ice and then centrifuged for 5 minutes at 5000 rpm. The
pelleted nuclei were resuspended in a high salt solution (25 % glycerol, 20 mM HEPES
pH 7.9, 420 mM NaCl, 1.2 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2
mM benzamidine, and 5 µg/ml of each aprotinin, leupeptin and pepstatin), and incubated
on ice for 20 minutes. Insoluble cellular debris was pelleted by a 15-s centrifugation,
and the supernatant was aliquoted, frozen in liquid nitrogen and stored at -80 °C until
assayed. Protein concentrations were determined by Bio-Rad Laboratories Protein Assay
(Bio-Rad Laboratories Inc., Hercules, CA, USA).

Double-stranded synthetic oligonucleotides containing AP-1 (5'-
GGAAGTGTTTTTGATGAGTCA
CCCACC-3') or GATA motifs (5'-
TGTTGTCTGATAATCAGAGTACCCACC-3') of the rat BNP promoter were
labeled with [α-³²P]-dCTP. Binding reactions contained 30 µg of crude nuclear extract
and 2 µg of poly-(dI-dC)-(dI-dC) in a buffer containing 10 mM HEPES pH 7.9, 1 mM
MgCl₂, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 10 % glycerol, 0.025 % NP-40, 0.25
mM PMSF and 1 µM of each aprotinin, leupeptin and pepstatin, and when appropriate,
various molar excesses of unlabeled double-stranded oligonucleotides. Reactions were
made at room temperature for 20 minutes and protein-DNA complexes were separated by
electrophoresis on 5 % polyacrylamide gel in 0.5 x Tris-borate-EDTA buffer (TBE) at +4
°C. Nonlabeled double-stranded oligonucleotides corresponding to AP-1 or GATA
binding sites of the BNP promoter and a GATA consensus sequence (Santa Cruz
Biotechnology, Santa Cruz, CA, USA) were used as specific competitor DNAs.
Nonspecific competitor DNAs included a double-stranded oligonucleotide carrying the
mutated binding site for GATA4 (5'-TGTTGTCTGATAAATCAGAGTACCCACC-
3') and Octamer-1 (Oct-1) as non-related DNA. For supershift experiments, 1-2 µg of
group polyclonal GATA4, GATA5, GATA6, c-Fos (4)-G, c-Fos (K-25)-G, c-Jun/AP-1 (N)-
G, JunB (N-17)-G or JunD (329)-G affinity purified IgG (Santa Cruz Biotechnology)
was used. After the reactions were loaded on a gel and ran at 220 V at +4 °C, the gel was
dried and exposed to the Phosphor Screens (Molecular Dynamics) and analyzed by
Image Quant.

4.9 Statistical analysis

The results are expressed as mean ± SEM. Student’s t-test was used to compare statistical
significance between two groups (III, IV). Differences between four study groups were
analyzed with Mann-Whitney test or one-way analysis of variance (ANOVA) followed
by Bonferroni post hoc test (I, II). The hemodynamic variables were evaluated with two-way repeated-measures ANOVA. The relationships between LVH and mRNA and peptide levels were determined using linear regression analysis. Echocardiographic measurements were analyzed using normalized values with one-way ANOVA followed by Student-Newman-Keuls post hoc test. A $P$ value <0.05 was considered statistically significant.
5 Results

5.1 Effect of losartan on ANP and BNP gene expression in response to L-NAME administration (I)

5.1.1 Blood pressure and ventricular weights

To examine the role of Ang II during NO deficiency induced by L-NAME in vivo, the effects of AT₁ receptor antagonist losartan on hemodynamics, natriuretic peptide gene expression and secretion, and morphology of mesenteric arteries were studied. Rats were divided into four groups: control (tap water), losartan (20 mg/kg/day), L-NAME (20 mg/kg/day) and losartan+L-NAME (both 20 mg/kg/day); in each group n=10. There were no significant differences in the baseline systolic blood pressures between the study groups measured by the tail-cuff method. Long-term administration of L-NAME elevated systolic blood pressure, which increased from 151 ± 4 mm Hg to 200 ± 4 mm Hg within 4 weeks, whereafter the blood pressure remained sustained over the rest of the 8-week treatment period. Losartan therapy significantly attenuated the increase in systolic arterial pressure induced by L-NAME. The ventricular weight to body weight ratios were similar in the control and L-NAME groups. Losartan administration reduced ventricular weight to body weight ratios slightly but significantly in the group not receiving L-NAME.

5.1.2 Morphological changes of mesenteric arteries

To identify the effect of losartan on the structure of mesenteric arteries in response to NO inhibition, the arteries were cannulated and the intraluminal pressure was raised to 100 mm Hg. The wall thickness, wall to lumen ratio, and wall cross-sectional area of the mesenteric artery increased significantly in both L-NAME and losartan+L-NAME groups when compared with the control group. There were no significant differences in the lumen- or external diameter between the groups. Thus, losartan treatment did not prevent the hypertrophic remodeling of the mesenteric resistance artery induced by L-NAME.
5.1.3 Effect of losartan on ANP gene expression

To evaluate the effect of losartan during L-NAME treatment on ANP gene expression, ventricular and atrial ANP mRNA levels and ir-ANP concentrations, and plasma ir-NT-proANP levels were measured. Left ventricular and atrial mRNA levels were expressed as the ratio of ANP mRNA to 18S ribosomal RNA. In the ventricles, a 4.5-fold increase in ANP mRNA was noted following L-NAME administration compared with the control rats (Fig. 7). Losartan therapy significantly attenuated the increase in ANP gene expression (by 69%) induced by L-NAME. L-NAME administration did not induce a significant increase in ventricular ir-ANP concentrations. While losartan did not decrease the ir-ANP levels of the L-NAME group in the ventricles (Fig. 7), losartan treatment in control rats decreased ventricular ir-ANP concentration by 30% (Fig. 7). In the left atria, L-NAME decreased ir-ANP levels by 43% when compared with the control group (control, 183.3 ± 24.5 vs. L-NAME, 105.2 ± 9.8 nmol/g; P<0.05). This change in left atrial ir-ANP concentrations was prevented by concomitant losartan therapy (losartan+L-NAME, 197.3 ± 15.7 nmol/g vs. L-NAME; P<0.05). L-NAME, losartan, or their combination had no significant effect on right or left atrial ANP mRNA levels. Losartan treatment decreased plasma ir-NT-proANP concentrations by 30% in the presence of L-NAME (L-NAME, 1.0 ± 0.1 vs. losartan+L-NAME, 0.7 ± 0.1 pmol/ml, P<0.05).

Fig. 7. Effects of administration of losartan, L-NAME and their combination on ventricular ANP mRNA and ir-ANP levels in rats. Results are mean ± SEM. *P<0.05 compared with the control group, †P<0.05 compared with the L-NAME group, and ‡P<0.05 compared with the losartan group (Mann-Whitney test).

5.1.4 Effect of losartan on BNP gene expression

To characterize the role of Ang II in BNP gene expression in response to L-NAME-induced hypertension, ventricular and atrial BNP mRNA levels, and cardiac and plasma
ir-BNP concentrations were measured. Left ventricular mRNA levels were expressed as the ratio of BNP mRNA to 18S. In the ventricles, L-NAME treatment increased BNP mRNA by 64% (Fig. 8), and ir-BNP by 61% when compared with the control group (Fig. 8). In the losartan plus L-NAME group, ventricular BNP mRNA levels did not differ from the control. Losartan therapy did not have any effect on the increase of ventricular ir-BNP levels induced by L-NAME (Fig. 8). In the rats treated with losartan alone, ventricular ir-BNP decreased by 31% when compared with the control group (Fig. 8). There were no significant changes in the right and left atrial BNP mRNA levels. In the left atria, L-NAME treatment decreased ir-BNP concentrations by 38% when compared with the control group (control, 138.8 ± 26.9 vs. L-NAME, 85.9 ± 6.5 pmol/g; \( P<0.05 \)). In the L-NAME group, plasma ir-BNP concentrations were increased by 54% when compared with the control group (control, 5.6 ± 0.4 vs. L-NAME, 8.6 ± 0.7 fmol/ml; \( P<0.001 \)), the increase of which was prevented by concomitant losartan therapy (losartan+L-NAME, 6.3 ± 0.5 fmol/ml vs. L-NAME; \( P<0.05 \)).

![Fig. 8. Effects of administration of losartan, L-NAME and their combination on ventricular BNP mRNA and ir-BNP levels in rats. Results are mean ± SEM. \(* P<0.05, \*** P<0.001\) compared with the control group, and \(\ddagger P<0.05, \ddagger\ddagger P<0.001\) compared with the losartan group (Mann-Whitney test).](image)

### 5.2 Role of adrenal factors in angiotensin II-induced activation of ANP, BNP and AM gene expression (II)

#### 5.2.1 Hemodynamic variables and left ventricular weights

To study whether Ang II-induced early gene expression of ANP, BNP and AM is modulated by adrenal factors, rats were subjected to sham operation, bilateral ADX, Ang II infusion or bilateral ADX plus Ang II infusion for 12 and 72 h. Hemodynamic
variables were measured in freely moving rats by telemetry to avoid stress. MAP and heart rate were similar in all groups before the onset of treatment. Ang II alone significantly increased MAP within 3 h, which persisted throughout the 72 h of infusion (F=8.7, \(P<0.05\)). MAP of the adrenalectomized rats during Ang II infusion did not differ from that of sham-operated rats for up to 45 h, but thereafter it tended to be lower (at 72 h: Ang II plus ADX, 130 ± 4 mm Hg, \(n=8\), vs. Ang II, 167 ± 11 mm Hg, \(n=6\)). Heart rate decreased significantly (F=25.5, \(P<0.001\)) within 5 to 10 h after the beginning of Ang II infusion in sham-operated rats, and this decrease in the heart rate was smaller in Ang II-infused adrenalectomized rats (F=39.6, \(P<0.001\)). ADX alone had no statistically significant effect on MAP, however, it did increase the heart rate (F=22.4, \(P<0.001, n=6\)) compared to sham-operated rats (\(n=6\)). The index of LVH, left ventricular weight to body weight (LVW/BW) ratio was significantly higher in Ang II-infused sham-operated rats (2.57 ± 0.02) compared to sham-operated controls (2.25 ± 0.02) and Ang II-treated adrenalectomized rats (2.52 ± 0.05) compared to the adrenalectomized rats (2.26 ± 0.03) at 72 h. ADX did not significantly change the LVW/BW ratio compared to sham-operated animals. Thus, hemodynamic parameters for up to 45 h and the degree of LVH in adrenalectomized rats during Ang II infusion were comparable to those of sham-operated rats.

5.2.2 Characterization of adrenalectomy model

To validate the ADX model, the levels of adrenal hormones were measured in sham-operated and adrenalectomized rats both with and without Ang II administration. Plasma aldosterone, corticosterone and EPI levels were undetectable in adrenalectomized rats. Ang II significantly increased plasma aldosterone levels at 12 and 72 h and EPI at 12 h, while circulating corticosterone and NE levels were similar in control- and Ang II-infused rats. ADX alone resulted in a 2-fold increase in NE levels at 72 h in both vehicle- and Ang II-infused rats.

5.2.3 Ventricular and plasma ANP levels

Ang II infusion in sham-operated rats resulted in a 3.6-fold and 20.4-fold increase in left ventricular ANP mRNA levels at 12 and 72 h, respectively (Fig. 9). Ventricular ir-ANP levels of Ang II-infused rats were 3-fold higher compared with the control group at 72 h (control, 20.6 ± 2.8 vs. Ang II, 68.3 ± 11.5 pg/mg, \(P<0.01\)). ANP mRNA levels showed a significant correlation with the LVW/BW ratio at 72 h \((r^2=0.69, n=19, P<0.001)\) but not at 12 h \((r^2=0.02, n=19\). LVH at 72 h also correlated with left ventricular ir-ANP levels \((r^2=0.32, n=21, P<0.01\).

The baseline levels of ANP mRNA (Fig. 9) and ir-ANP in the left ventricle remained unchanged in adrenalectomized rats at 12 and 72 h. However, ADX almost completely abolished (92 \%) the Ang II-induced increase in left ventricular ANP mRNA levels at 12 h and also significantly attenuated (49 \%) the increase at 72 h (Fig. 9). In
adrenalectomized rats, ANP mRNA and ir-ANP levels did not correlate with the LVW/BW ratio (ANP mRNA at 72 h: \( r^2 = 0.1, n = 31, P = 0.3 \)). The plasma ir-NT-proANP concentrations in Ang II-infused adrenalectomized rats tended to be lower compared to sham-operated Ang II-infused rats, but these changes were not statistically significant. ADX alone had no effect on baseline plasma ir-NT-proANP levels. Furthermore, to study the role of aldosterone in ventricular ANP gene expression in response to Ang II infusion, aldosterone receptor antagonist spironolactone was injected s.c. into rats (in each group \( n = 6 \)). The increase in ANP mRNA levels in response to Ang II at 12 h was similar in rats infused with Ang II plus spironolactone, suggesting that aldosterone is not responsible for the stimulation of ANP gene expression during Ang II-induced pressure overload.

5.2.4 Ventricular and plasma BNP levels

Ang II infusion for 12 h markedly increased (5.2-fold) left ventricular BNP mRNA levels (Fig. 9). A significant, but smaller increase (1.8-fold) in BNP mRNA levels in the left ventricles was observed at 72 h of Ang II infusion (Fig. 9). Ventricular ir-BNP levels also increased significantly in Ang II-infused rats compared to those of control animals, and this increase was greater at 12 h (control, 1.7 ± 0.1 vs. Ang II, 5.3 ± 0.5 pg/mg, \( P < 0.001 \)) than at 72 h (control, 1.8 ± 0.1 vs. Ang II, 3.5 ± 0.6 pg/mg, \( P < 0.01 \)). Furthermore, plasma ir-BNP concentrations were significantly increased in Ang II-infused animals at 12 h (control, 3.7 ± 0.2 vs. Ang II, 11 ± 1.0 fmol/ml, \( P < 0.001 \)) and at 72 h (control, 3.2 ± 0.1 vs. Ang II, 4.9 ± 0.7 fmol/ml, \( P < 0.05 \)).

ADX significantly attenuated the increase in BNP mRNA levels in response to Ang II infusion at 12 h (Fig. 9). Similarly, ADX significantly decreased, but did not abolish, the Ang II-induced increase in left ventricular ir-BNP and plasma ir-BNP levels at 12 h. ADX alone had no significant effect on baseline left ventricular BNP mRNA (Fig. 9) and peptide levels, but it slightly decreased plasma ir-BNP levels at 12 h. A significant correlation between left ventricular BNP mRNA levels and LVW/BW ratio was observed in sham-operated and Ang II-administered rats (at 72 h, \( r^2 = 0.36, n = 19, P < 0.01 \)), and this correlation remained significant in their adrenalectomized counterparts (\( r^2 = 0.21, n = 31, P < 0.01 \)). Similar relationships were observed between LVH and left ventricular and plasma ir-BNP levels. In agreement with results observed with ANP, spironolactone treatment did not significantly change Ang II-induced increase in BNP mRNA levels at 12 h.
5.2.5 Ventricular and plasma AM levels

Ang II induced a transient 1.4-fold increase in AM mRNA ($P<0.05$) and a 1.5-fold increase (control, $0.17 \pm 0.01$ vs. Ang II, $0.26 \pm 0.01$ pg/mg, $P<0.01$) in ir-AM levels in
the left ventricles at 12 h. While plasma ir-AM levels were increased by Ang II at 12 h, the concentrations returned to control levels at 72 h. No significant correlation was found between left ventricular AM mRNA levels and LVW/BW ratio ($r^2=0.04$, $n=38$, $P=0.6$). ADX completely abolished the Ang II-induced increases in left ventricular AM mRNA levels ($P<0.001$) at 12 h and attenuated the increase in ir-AM concentrations in the left ventricles produced by Ang II at 12 h. Interestingly, although the adrenal glands were originally suggested to be a source of circulating AM (for review, see Samson 1999), plasma ir-AM levels in adrenalectomized animals were higher than those in sham-operated animals.

5.3 Regulation of BNP gene expression in response to angiotensin II-induced pressure overload (III)

5.3.1 Hemodynamic variables, myocardial hypertrophy and cardiac function

To examine the BNP gene expression during Ang II infusion, Ang II or 0.9 % NaCl was administered to SD rats by i.v. infusion for 2 h or by minipumps s.c. for 6 h, 12 h, 3 days, 1 week and 2 weeks. In conscious rats, Ang II infusion i.v. for 2 h raised MAP from 123 ± 10 mm Hg to 163 ± 9 mm Hg ($n=18$, $P<0.001$) associated with a significant decrease in heart rate (from 384 ± 33 beats/min to 293 ± 15 beats/min, $P<0.001$). Both MAP (119 ± 13 vs. 117 ± 12 mm Hg, $n=14$) and heart rate (358 ± 11 beats/min vs. 356 ± 8 beats/min) remained unchanged in the vehicle-infused rats. When Ang II was administered s.c. by minipumps for 2 weeks, MAP increased significantly from 108 ± 11 mm Hg to 127 ± 11 mm Hg within 3 h ($n=10$). The increase in blood pressure persisted throughout the 2-week infusion (181 ± 5 mm Hg at the end of the experiment). After 6 days, the heart rate in the Ang II group started to increase significantly compared with the vehicle group and remained increased until the end of the experiment. Both MAP (104 ± 3 vs. 107 ± 8 mm Hg, $n=10$) and heart rate (371 ± 15 beats/min vs. 368 ± 4 beats/min) remained unchanged in the vehicle-infused rats. The ventricular weight to body weight ratio was significantly increased at 12 h and onwards in Ang II-treated rats. Measured by the echocardiography method, the thickness of interventricular septum ($F=26.0$, $P<0.001$) and left ventricular posterior wall ($F=22.0$, $P<0.002$) was increased during Ang II infusion at 1 week and 2 weeks. EF and LVFS were not different in vehicle- and Ang II-infused rats at any time point. However, the E/A ratio was significantly lower in Ang II-infused rats compared with control rats ($F=28.5$, $P<0.001$), suggesting changes in diastolic function.
5.3.2 Angiotensin II-induced BNP gene expression

In Ang II-treated rats, left ventricular BNP mRNA levels increased 2.2-fold by 2 h and peaked at 12 h (5.2-fold, Fig. 10A). Thereafter, BNP mRNA levels decreased (at 3 days 1.8-fold) and returned to control levels at 1 week (Fig. 10A). Left ventricular ir-BNP peptide levels increased 1.5-fold at 2 h of Ang II infusion (vehicle, 6.5 ± 0.6 fmol/mg vs. Ang II, 10.1 ± 1.3 fmol/mg; P<0.01) and peaked at 6 h (3.8-fold; vehicle, 3.7 ± 0.2 vs. Ang II, 14.3 ± 2.4 fmol/mg; P<0.001). The ventricular ir-BNP concentrations were gradually reduced to the control levels (at 2 weeks; vehicle, 4.2 ± 0.3 vs. Ang II, 4.6 ± 0.7 fmol/mg). Plasma ir-BNP concentrations increased 3.3-fold at 12 h in response to Ang II infusion (vehicle, 3.3 ± 0.1 vs. Ang II, 11.0 ± 1.0 fmol/ml; P<0.001). After 12 h, plasma ir-BNP levels decreased but remained significantly elevated until the end of the experiment (1.4-fold increase at 3 days, 1 week and 2 weeks).

5.3.3 BNP promoter activation

To study the transcriptional activity of the BNP gene in vivo in response to Ang II-induced pressure overload, direct plasmid DNA injection into the beating rat myocardium was used. To generate the p-BNPluc constructs, genomic BNP fragments containing sequences between –2200 to +75 bp were fused to the luciferase reporter gene and injected into the ventricular wall close to the apex. The pSVβ-galactosidase vector was co-injected as an internal control for transfection efficiency and the BNP promoter activity was expressed as the relative ratios of luciferase to β-galactosidase values. The expression of the luciferase reporter gene driven by 2200 bp of the rat BNP 5’ flanking sequences was evaluated in the ventricles of both sham-operated and Ang II-infused rats. The expression level of pSVβ-galactosidase was similar in the left ventricles of controls and Ang II-infused rats, indicating that increases in the expression of p-2200BNPluc are due to augmentation of the transcriptional activity of the BNP promoter sequences. The –2200 BNP promoter activity increased 3.9-fold and 2.0-fold at 2 and 6 h, respectively, in response to Ang II infusion (Fig. 10B). At 12 h after starting the infusions, the BNP promoter activity did not differ between the vehicle- and Ang II-treated groups. Thereafter, Ang II infusion significantly augmented the BNP promoter activity by 1.7-, 2.4- and 2.8-fold, at 3 days, 1 week and 2 weeks, respectively (Fig. 10B). The effect was sequence-specific as illustrated by the absence of Ang II-stimulated increases in transcription directed by the proximal 114 bp fragment of the BNP promoter.
5.3.4 Left ventricular AP-1 binding activities

In order to identify the possible mechanisms of the distinct activation of left ventricular BNP promoter activity and BNP mRNA levels during Ang II-stimulated pressure overload, gel mobility shift assays were used to analyze the DNA-binding activities of the transcription proteins. In Ang II-infused rats, BNP AP-1 DNA binding activity was increased 2.7- and 1.9-fold at 2 and 6 h, respectively. Later (at 12 h, 3 days, 1 week and 2 weeks) the BNP AP-1 binding activities were not statistically significantly different between the vehicle- and Ang II-treated rats. The specificity of the ventricular AP-1 binding activity was confirmed by competition analysis, which indicated that AP-1 binding activity was inhibited by unlabeled AP-1 oligonucleotide, whereas non-related DNA Oct-1 or mutated AP-1 site had no effect on DNA binding. Nuclear extracts were incubated with 1 µg of c-Fos (4)-G, c-Fos (K-25)-G and c-Jun/AP-1, JunB/N17 or JunD/329 in supershift experiments to confirm that the complex bound to the BNP AP-1 site contains AP-1 proteins. The presence of JunB and JunD was observed in the complex formed between BNP AP-1 probe and nuclear extracts from Ang II-treated left ventricles.
5.4 Transcription factors in angiotensin II-induced BNP gene expression (IV)

5.4.1 Transactivation of BNP promoter by angiotensin II

To analyze the mechanism of Ang II on the BNP gene expression, the reporter plasmid p-5000BNPluc containing rat BNP sequences from -5000 to +4 bp and p-534BNPluc containing sequences from -534 to +4 bp relative to the transcriptional initiation site were generated and injected into the rat hearts. Ang II or 0.9 % NaCl was administered s.c. to rats to produce pressure overload. MAP increased significantly within 3 h of the Ang II administration and persisted elevated until the end of the 2-week experiment. The BNP promoter activities and index of myocardial hypertrophy were measured at 6 h and 2 weeks of infusions. The ventricular weight to body weight ratio remained at the control level at 6 h but increased after 2 weeks of Ang II infusion. Deletion of an upstream region of BNP promoter from -5000 to -534 bp did not affect basal promoter activity. Ang II-induced pressure overload increased -5000 BNP promoter activity 2.9-fold at 2 weeks and -534 BNP promoter activity 1.8-fold at 2 weeks (Fig. 11). The promoter activities of both -5000 BNP and -534 BNP constructs remained at the control level at 6 h of Ang II infusion (Fig. 11).

To identify the potential regulatory elements required for the induction of left ventricular BNP gene expression in response to Ang II-induced pressure overload, site-specific mutations were generated and tested in vivo by DNA injection at 6 h and 2 weeks of Ang II infusion. Since AP-1 and GA T A sites are involved in the induction of natriuretic peptide gene expression in response to hemodynamic overload in vivo (von Harsdorf et al. 1997, Marttila et al. 2001), the importance of AP-1 and GA T A elements was studied. Point mutations of two GA T A motifs at -90 bp (-534GA T Amut) or AP-1 element at -372 bp (-534AP-1mut) were generated in the -534BNPluc construct to study the role of these cis-acting elements in regulating BNP promoter activation in rat hearts. The increase in -534GA T Amut construct activity was almost completely abrogated, whereas the activity of the -534AP-1mut construct did not differ significantly from the intact -534BNPluc in response to Ang II treatment for 2 weeks (Fig. 11).

5.4.2 Left ventricular GATA4 binding activities

To study GATA DNA-binding activities, gel mobility shift assays were performed. By using the left ventricular nuclear extracts obtained from the rats infused with 0.9 % NaCl and Ang II for 6 h and 2 weeks, BNP GATA binding activities were analyzed. When the left ventricular nuclear extracts of the vehicles and Ang II-administered rats were incubated with the oligonucleotide probe containing -90 bp GATA motifs, a specific complex was formed. Ang II infusion increased significantly BNP GATA4 binding activity 1.4-fold (P<0.05) at 6 h. However, BNP GATA4 binding activity did not differ from the control at 2 weeks of Ang II infusion. Competition gel mobility shift analysis was performed to determine the specificity of GATA binding activity. The formation of
complexes with the rat BNP GATA probe was effectively inhibited by the unlabeled probe and GATA consensus DNA. The binding was unaffected by an excess of oligonucleotides corresponding to the non-related competitor DNA Oct-1 or the mutated BNP GATA site. To further confirm that the complex bound to the BNP GATA site contains GATA proteins, supershift assays were made by using GATA4, GATA5 and GATA6 antibodies. Left ventricular extracts from Ang II-infused rats clearly showed antibody-induced supershift of GATA4 but not GATA5 or GATA6 complexes.

![Graph](https://via.placeholder.com/150)

**Fig. 11.** –5000, –534, GATAmut and AP-1mut BNP promoter activities expressed as the relative ratios of luciferase (luc) to β-galactosidase. Number of experiments, -5000BNP (n=7-18), -534BNP (n=13-23), GATAmut (n=20-22), AP-1mut (n=26-29), in each group. Open bars indicate vehicle and solid bars Ang II. Results are the mean ± SEM. *P<0.05 and **P<0.01 Ang II vs. vehicle.

### 5.4.3 Angiotensin II-induced cardiac gene expression

The left ventricular gene expression of BNP, GATA4, GATA6 and the component of AP-1, c-fos, were measured at 6 h and 2 weeks. The results were expressed as the ratio of BNP, GATA4, GATA6 and c-fos mRNA to 18S. The left ventricular BNP mRNA levels peaked at 6 h reaching 4.3-fold increase (P<0.001), and the gene expression remained elevated until the end of the experiment (1.8-fold, P<0.05, n=10). The left ventricular ir-BNP concentrations increased 3.5-fold (vehicle, 2.1 ± 0.2 fmol/mg vs. Ang II, 7.3 ± 0.7 fmol/mg; P<0.001) at 6 h and 3.4-fold (vehicle, 1.7 ± 0.1 fmol/mg vs. Ang II, 5.8 ± 0.6 fmol/mg; P<0.001) at 2 weeks. The levels of GATA4 and GATA6 mRNA in the left ventricle remained unchanged during the Ang II infusion, whereas left ventricular c-fos mRNA levels increased 1.3-fold at 6 h (P<0.001) but remained at the control level at 2 weeks.
6 Discussion

6.1 Role of angiotensin II in natriuretic peptide gene expression in response to hemodynamic overload by nitric oxide deficiency

NO is suggested to buffer the effects of the RAS and preserve the perfusion of tissues (Hernandez et al. 1999). Thus, NO appears to be an endogenous inhibitor of the actions of Ang II, and interaction of NO and Ang II is important in the maintenance of vascular homeostasis (for review, see Gibbons & Dzau 1994). In isolated rat atrial tissue, Ang II-induced ANP secretion is enhanced by L-NAME, suggesting that NO modulates the effect of Ang II on ANP release (Soualmia et al. 2001). Furthermore, the actions of both natriuretic peptides and NO are mediated by intracellular second messenger cGMP in cardiac myocytes (Calderone et al. 1998).

In this study, NO-deficient hypertension generated by long-term treatment with NOS inhibitor L-NAME for 8 weeks in rats produced sustained elevation in systolic blood pressure, which agrees with previous reports (Hu et al. 1994, Katoh et al. 1998, Bartunek et al. 2000). However, in contrast to several other experimental models of long-term hemodynamic overload (Dostal & Baker 1992, Yamamoto et al. 2000), myocardial hypertrophy was not developed in response to chronic L-NAME administration. Although left ventricular remodeling and changes in functional cardiac properties have been observed following L-NAME treatment in rats, there are usually no signs of myocardial hypertrophy (Banting et al. 1997, Matsubara et al. 1998, Bartunek et al. 2000). Yet, increased cardiac weight in response to chronic inhibition of NOS has also been demonstrated (Numaguchi et al. 1995, Takemoto et al. 1997a). In contrast to myocardium, administration of L-NAME induced clear hypertrophic remodeling in the mesenteric resistance arteries, as previous studies have described (Deng et al. 1993, Morton et al. 1993).

Ang II is suggested to be a mediator of NO deficiency-induced hypertension, since L-NAME administration increases plasma renin activity (Ribeiro et al. 1992, Hu et al. 1994), elevates vascular and myocardial ACE levels (Takemoto et al. 1997a) and upregulates cardiac Ang II receptors in rats (Katoh et al. 1998). Furthermore, the AT1 receptor antagonist losartan can largely prevent the development of L-NAME-
hypertension in rats (Arnal et al. 1993, Jover et al. 1993). Other vasoconstrictor mechanisms such as the sympathetic nervous system and ET-1 may also contribute to the increase in blood pressure and cardiac changes in NO deficiency-induced hypertension (Qiu et al. 1994, D’Amours et al. 1999). Thus, L-NAME-stimulated responses may be mediated by complex interactions of different regulatory mechanisms. This study indicated that losartan opposed the L-NAME-elicited increase in blood pressure, and although blood pressure was not completely normalized, Ang II seems to be a significant mediator of the L-NAME-induced hypertension. As reported previously in normotensive rats (Magga et al. 1999), losartan therapy decreased LVH significantly in the control group, which may result from the observed small but non-significant decrease in blood pressure. However, the decrease in left ventricular mass by losartan was not significant in response to NOS inhibition by L-NAME, implicating an Ang II-independent effect. Previously, both ACE inhibitor and AT1 receptor antagonist treatments have been suggested to prevent the development of vascular hypertrophy in L-NAME hypertensive rats (Takemoto et al. 1997a, Takemoto et al. 1997b). In this study, however, losartan had no effect on the mesenteric arterial hypertrophy in the L-NAME rats, even though blood pressure was reduced, suggesting that the changes in arterial structure in this model of L-NAME-induced hypertension are independent of hemodynamics and stimulation of the AT1 receptor.

Although ANP and BNP genes are activated rapidly in myocardial hypertrophy, the levels of these peptides are also elevated chronically in response to sustained cardiac overload (Lang et al. 1986, Dagnino et al. 1992, Yasue et al. 1994). In eNOS-deficient mice, cardiac ANP expression is markedly augmented and the increased synthesis either directly resulted from eNOS gene disruption or the hemodynamic changes (Gyurko et al. 2000). The present data showed that the expression of both ANP and BNP genes is dissociated from the development of ventricular hypertrophy in NO deficiency-induced hypertension, since despite the absence of compensatory myocardial hypertrophy, both ventricular ANP and BNP mRNA and peptide levels were significantly increased. Thus, the upregulation of ventricular natriuretic peptide synthesis is not a marker of myocyte hypertrophy in this experimental model of hypertension. Previously, NOS inhibition neither induced cardiac hypertrophy nor increased left ventricular ANP mRNA levels in rats (Hou et al. 1995). Furthermore, although acute intravenous administration of L-NAME has been reported to increase plasma ANP concentrations in rats (Leskinen et al. 1995), these results showed only a slight increase in circulating ir-NT-proANP levels in response to long-term L-NAME treatment. Losartan administration decreased both baseline and the NO deficiency-induced activation of ventricular ANP and BNP expression, which may reflect a direct effect of cardiac AT1 receptor antagonism or a decrease in systolic blood pressure. In addition, AT1 receptor antagonism may sensitize Ang II to act via Ang II type 2 receptors, which have been reported to activate kinins and the NO system and thus induce a cardioprotective effect (Liu et al. 1997, Matsubara et al. 1998). Previously, it has been demonstrated using both normotensive and hypertensive rats that Ang II may directly contribute to the long-term regulation of ventricular ANP, while ventricular BNP gene expression seems to be more related to hemodynamic changes (Magga et al. 1999). Furthermore, when aortic-banded rats were treated with the ACE inhibitor ramipril, the reduction in ventricular BNP gene expression was more closely related to the changes in systolic blood pressure.
than that of ventricular ANP gene expression (Ogawa et al. 1996). Thus, although further studies are required, Ang II seems to contribute also directly to the long-term regulation of ventricular natriuretic peptide synthesis.

The regulation of natriuretic peptides following L-NAME treatment appears to be tissue-specific. In the present study, NOS inhibition had no effect on the gene expression of atrial ANP and BNP. These results disagree with a study demonstrating an increase in atrial ANP mRNA levels following L-NAME treatment (Lee et al. 2000b), the duration of L-NAME treatment being in the present study 8 weeks compared to 4 weeks in the previous work. Moreover, present results showed differences between ventricular natriuretic peptide mRNA and peptide levels, since losartan treatment decreased ventricular ANP and BNP mRNA levels, while peptide levels remained unaffected in L-NAME-treated rats. The reason for the dissociation between mRNA and peptide responses is not clear, but a similar discrepancy between BNP mRNA and BNP peptide levels has previously been reported in response to acute and chronic hemodynamic overload in rats (Marttila et al. 1996, Magga et al. 1999). The increase in mRNA levels could result from transcriptional and posttranscriptional mechanisms or both, whereas the rate of release also influences tissue peptide levels (Magga et al. 1997b). Nevertheless, these results showed that the expression of ANP and BNP is differently regulated in the left ventricle and atria in response to NO-deficient hypertension. Furthermore, Ang II may play a greater role in the regulation of ventricular than atrial gene expression, since atrial levels of ANP and BNP mRNA remained unchanged in losartan-treated rats. The majority of ANP is stored in the atrial granules at high concentrations, and it is rapidly secreted to the systemic circulation in response to cardiac overload (for review, see Ruskoaho 1992). Therefore, reduced atrial ANP peptide levels in the L-NAME group, and normalized atrial ANP peptide concentrations in the losartan plus L-NAME group with unchanged ANP mRNA levels could be explained by alterations of ANP secretion into the systemic circulation secondary to changes in systolic blood and atrial pressures. Since a much smaller concentration of ANP is stored in the ventricles, the unchanged (L-NAME treatment) or slightly increased (L-NAME plus losartan treatment) ventricular ir-ANP levels may reflect increased ANP gene expression during hemodynamic overload (for review, see Ruskoaho 1992).

6.2 Effect of adrenal factors in angiotensin II-induced cardiac gene expression

Infusion of Ang II induces hypertension and expression of various cardiac genes including natriuretic peptides (for review, see Kim & Iwao 2000). Furthermore, Ang II has been shown to increase dose-dependently the secretion of adrenocortical steroids and adrenomedullary catecholamines (Breidert et al. 1996). Adrenal-derived hormones are suggested to independently induce myocardial hypertrophy and natriuretic peptide gene expression (for reviews, see Simpson et al. 1991, de Bold et al. 1996). By using the experimental model of bilateral adrenalectomy during Ang II infusion in this study, the importance of adrenal factors in Ang II-induced cardiac gene expression was examined.
The absence of adrenal glands has been reported to partially prevent the myocardial hypertrophy induced by chronic pressure overload (Beznak et al. 1952, Womble et al. 1980, Nichols et al. 1983). In order to validate the ADX model, plasma concentrations of catecholamines and corticosteroids were measured. In adrenalectomized rats, the levels of the adrenal-derived hormones aldosterone, corticosterone, and EPI were undetectable, both in basal conditions as well as after the administration of Ang II. In sham-operated rats, Ang II infusion elevated the levels of aldosterone and EPI but not those of corticosterone or NE. ANP, BNP and AM were selected to assess the effects of ADX during early induction of cardiac hypertrophy, since these genes are rapidly activated in response to hypertrophic agonists and mechanical stress in vitro (Ruskoaho et al. 1986, Bruneau & de Bold 1994, Tsuruda et al. 1998) and in vivo (for review, see Ruskoaho 1992, Magga et al. 1994, Romppanen et al. 1997).

The present results showed that ventricular ANP gene expression was upregulated even before cardiac hypertrophy developed in response to Ang II infusion. There are no previous data showing as early activation as 12 h of ANP in response to Ang II. In contrast to expression of ANP, which was further increased at 72 h, administration of Ang II resulted in transient induction of both BNP and AM gene expression, both reaching their peak values at 12 h. Previously, the induction of BNP gene expression has been shown to be one of the earliest cardiac myocyte-specific markers of hemodynamic overload, since it is activated within 1 h of pressure overload (Magga et al. 1994). Furthermore, AM mRNA and peptide levels are increased within 2 h of pressure overload produced by AVP infusion in conscious rats (Romppanen et al. 1997). In aortic-banded rats, AM gene expression has been shown to increase within 1 day of pressure overload and decrease to basal level at 3 days of cardiac hypertrophy (Morimoto et al. 1999). Thus, the pattern of reprogramming of ventricular gene expression by Ang II is similar to that induced by other mediators of hemodynamic overload.

Despite similar blood pressure (up to 45 h) and hypertrophic process as in the sham-operated controls, there was a lack of upregulation of ANP and AM gene expression in the left ventricle by Ang II-stimulated pressure overload in adrenalectomized rats. Removal of adrenal glands also abolished the correlation between ANP gene expression and left ventricular mass. These results indicate that the early phase of hypertrophic response of ANP and AM genes induced by Ang II requires factors originating from the adrenal glands. Furthermore, ADX blunted but did not abolish Ang II-induced increases in ventricular BNP gene expression, and did not modulate the correlation between BNP mRNA levels and left ventricular mass. Thus, the early upregulation of BNP gene expression by Ang II is less dependent on adrenal factors. In agreement with this, BNP production in aortic-banded hypertensive rats after six weeks was more sensitive than ANP to the load-dependent component (Ogawa et al. 1996). Interestingly, ANP gene expression was most sensitive to the inhibition by ADX at the early phase of overload (12 h), suggesting that the actions of adrenal factors, pressure load and hypertrophy on cardiac gene expression may vary during the distinct phases of Ang II-induced hypertension. Furthermore, low ventricular ANP gene expression has been shown to be genetically linked to high cardiac mass independent of blood pressure (Masciotra et al. 1999). The present results agree with that work by showing a dissociation between the hypertrophic process and increased ANP, BNP and AM gene expression.
Aldosterone could be one of the adrenal-derived factors required for Ang II-initiated genetic program of myocardial hypertrophy. Aldosterone treatment enhances Ang II binding and potentiates the Ang II-induced hypertrophic responses (Robert et al. 1999). In a chronic experimental model, mineralocorticoid receptor blockade reduced Ang II-induced cardiac damage at 7 weeks (Fiebeler et al. 2001). Aldosterone has been reported to induce cardiac hypertrophy and fibrosis both via direct and hemodynamic mechanisms (Takeda et al. 2000b). Furthermore, spironolactone treatment improves left ventricular function, decreases myocardial mass and plasma levels of ANP and BNP in patients with CHF (Tsutamoto et al. 2001). In this experimental model of ventricular hypertrophy, aldosterone appears not to be the adrenal-derived factor required for the induction of early cardiac gene expression, since spironolactone administration did not attenuate Ang II-induced increase in ANP and BNP gene expression at 12 h.

Other steroid hormones produced in the adrenal cortex, glucocorticoids, have been found to increase the synthesis and processing of proANP in rat atrial and ventricular cardiomyocytes (Shields et al. 1988). Thus, a normal plasma glucocorticoid level might be required as a permissive factor for the normal production of cardiac peptide hormones, perhaps by preserving the function of the key signal transduction pathways. Furthermore, dexamethasone administration has been shown to upregulate ANP and AM gene expression in rat ventricular myocytes, suggesting that glucocorticoids directly stimulate cardiac gene expression (Nishimori et al. 1997). In addition, catecholamines are well known stimulators of cardiac gene expression and peptide secretion (for review, see Ruskoaho 1992), and adrenergic agonists activate the transcription of BNP and ANP genes (Hanford et al. 1994, He et al. 2000a). However, these results cannot define precisely which factors are predominantly responsible for the inhibition of Ang II-induced activation of cardiac gene expression. The relative importance of the above-mentioned factors as well as identification of the role of novel factors regulating Ang II action, such as AT, receptor-associated protein (Davet et al. 1999) represents a logical target for future study.

6.3 Mechanisms of action of angiotensin II in regulating BNP gene expression

6.3.1 Posttranscriptional control of BNP gene

In order to study the mechanisms of Ang II in regulating the transient and rapid ventricular BNP gene expression observed in the present study (II) in response to cardiac overload in vivo, BNP gene constructs were generated and injected into beating rat hearts (III, IV). In response to Ang II infusion by osmotic minipumps, blood pressure increased significantly after 3 h and ventricular weight to body weight ratio after 12 h. Furthermore, Ang II induced changes in diastolic function, while systolic function of the heart remained unaffected. Despite sustained hypertension and LVH, BNP mRNA levels were only temporarily upregulated and returned to the control levels within 3 days. Left ventricular BNP peptide concentrations followed the changes in BNP mRNA levels. In
agreement with the present results, left ventricular BNP mRNA and peptide levels did not differ from those of controls in normotensive DOCA-salt treated rats (Yokota et al. 1995). Transgenic rats overexpressing human renin and angiotensinogen genes also have normal left ventricular BNP mRNA levels even in established phase of hypertension and severe myocardial hypertrophy (Marttila et al. 1999). Lack of an increase in ventricular BNP mRNA levels has also been observed in some patients with heart failure (de Boer et al. 2001, Hystad et al. 2001). The present results support the previous findings showing that left ventricular BNP mRNA levels may return to the baseline level despite cardiac overload and myocardial hypertrophy.

To determine whether alterations in the rate of transcription of the BNP gene could account for the changes observed in BNP mRNA levels in vivo, the activity of the -2200 BNP construct was measured. The 5' flanking sequences of the BNP promoter have been well characterized in in vitro studies (Grépin et al. 1994, Thuerauf et al. 1994). The sequences 2200 bp from the transcription initiation site of the BNP promoter are sufficient to direct cardiac-specific expression in vitro (Grépin et al. 1994) and in vivo in transgenic mice (Bhalla & Nemer, unpublished results, 2001). In a previous in vivo study, the -2200 BNP construct was activated within one day in response to nephrectomy-stimulated hemodynamic overload in rats (Marttila et al. 2001). Furthermore, transfection assays in cultured neonatal rat ventricular cells have shown that deletion of the sequences between -2200 and -114 bp did not affect the high-level activity of the BNP luciferase vectors (Grépin et al. 1994), suggesting that the determinant for cardiac-specificity resides within the first 114 bp. However, although the 114-bp fragment of rat BNP promoter is sufficient to confer full hemodynamic stress-responsiveness induced by nephrectomy in vivo (Marttila et al. 2001), deletion of sequences between -2200 and -114 bp abrogated the inducible activation of the BNP gene in response to Ang II infusion. Ang II administration increased -2200 BNP promoter activity within 2 h and the promoter activity remained upregulated until the end of the 2-week experiment, except at 12 h. These divergencies between the BNP promoter activity and BNP mRNA and peptide levels suggest that posttranscriptional control plays an important role in the regulation of BNP gene expression in vivo. These data are in agreement with the previous in vitro results showing that in addition to transcriptional regulation, BNP gene expression is regulated by posttranscriptional mechanisms (Hanford et al. 1994, Nakagawa et al. 1995).

In contrast to ANP, BNP mRNA contains several AU-rich elements in the 3'-untranslated region that may be involved in the translation-dependent mRNA degradation (Kojima et al. 1989, for review, see Chen & Shyu 1995). The observation that activators of PKC and MAPKs in cell culture models of myocyte hypertrophy enhance BNP transcript stability (LaPointe & Sitkins 1993, Hanford & Glembotski 1996) is consistent with a role for posttranscriptional regulation. PKC activation results in the accumulation of c-fos protein, which interacts with Jun family members to constitute AP-1 activity (for review, see Karin 1995). Since Ang II has been shown to activate PKC and MAPKs and to stimulate AP-1 activity in cardiac myocytes in vitro (for review, see Kim & Iwao 2000), BNP AP-1 binding activity in the nuclear extracts of left ventricles was analyzed. BNP AP-1 binding activity increased significantly at 2 h of Ang II treatment but decreased to control levels within 12 h. Since the normalization of AP-1 binding activity preceded the decrease in BNP mRNA levels, one possibility is that decreased stabilization
of mRNA during prolonged Ang II infusion could explain the fall of BNP mRNA to control levels. Nevertheless, further studies are needed to characterize the precise posttranscriptional mechanisms mediating the changes in BNP gene expression (Table 5).

Plasma concentration of BNP can be used as a diagnostic and prognostic marker, and as a tool for the guidance of treatment in patients with heart failure (Troughton et al. 2000a, Dao et al. 2001, Richards et al. 2001). As reported in patients with CHF, plasma BNP levels have been reported to increase according to the severity of left ventricular dysfunction (Grantham & Burnett 1997). In this study, Ang II infusion increased plasma ir-BNP levels reaching a peak value at 12 h and the circulating BNP remained elevated until the end of the experiment. Thus, increased BNP plasma levels in the absence of activation in gene expression might be explained by enhanced BNP translational efficiency or capacity. However, increased release of BNP from atria (Mäntymaa et al. 1993) as well as decreased elimination of BNP from the circulation could also result in increased plasma BNP concentrations (for reviews, see Nakao et al. 1992a, de Bold et al. 1996). These results are in line with previous data (Langenickel et al. 2000) indicating raised plasma BNP concentrations in the absence of increased ventricular BNP mRNA and peptide levels.

**Table 5. Alternative levels for posttranscriptional regulation**

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcription attenuation</td>
<td>Intracellular localization</td>
</tr>
<tr>
<td>Alternative RNA splicing</td>
<td><em>trans</em>RNA splicing and RNA editing</td>
</tr>
<tr>
<td>3' end cleavage</td>
<td>Translation start, ignorance of AUG codon</td>
</tr>
<tr>
<td>Regulation of nuclear transport</td>
<td>RNA stabilization, AU-rich sequences</td>
</tr>
<tr>
<td></td>
<td>Translational recoding, frameshifting</td>
</tr>
</tbody>
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**6.3.2 Transcription factors mediating angiotensin II-responsiveness of BNP gene**

To further analyze the mechanism of Ang II in the expression of BNP gene in response to pressure overload in vivo, the activities of the -5000 and -534 BNP constructs transfected into the rat myocardium were measured. Ang II infusion for 2 weeks increased activities of these constructs significantly, the fold induction of the -534 BNP construct activity being slightly lower than that of the -5000 BNP construct. Interestingly, the Ang II-induced activation of the -534 BNP construct did not differ from the control at 6 h, although the activity of the -2200 BNP construct (III) increased significantly at this early phase of Ang II administration. These results may be explained by additional positive cis regulatory elements located between -5000 and -534 bp (Thuerauf et al. 1994) needed for the early activation of the BNP gene in response to Ang II. Moreover, although the -534 BNP construct activity did not increase at 6 h in response to Ang II infusion, the activity of the shorter -114 BNP construct increased over 4-fold in response to hemodynamic stress by nephrectomy (Marttila et al. 2001), suggesting that a more powerful stimulus...
than Ang II-induced pressure overload may be needed for the early activation of the proximal BNP promoter. Furthermore, the -5000 BNP construct activity did not differ from the control after 6 h of Ang II infusion, which may be due to the putative negative cis regulatory elements between the sequences -5000 and -2200 bp suppressing the activation of the BNP gene (LaPointe et al. 1996, Thurauf & Glembotski 1997). As also observed in study III, left ventricular BNP mRNA levels were activated rapidly and transiently, and the left ventricular ir-BNP concentrations followed the changes in BNP gene expression. In addition to rapid activation of BNP gene expression, left ventricular c-fos mRNA levels increased at the acute phase of Ang II infusion representing a typical genetic reprogramming of the heart subjected to hemodynamic overload (for review, see Sadoshima & Izumo 1997).

In order to specify the genetic elements responsible for the Ang II-induced BNP transcription, point mutations of GATA and AP-1 elements were introduced in the -534 BNP promoter. The sequences and position of the GATA and AP-1-like motifs are highly conserved in the rat and human BNP genes (Grépin et al. 1994). Transcription factor GATA4 is a potent transactivator of several cardiac genes including BNP (Grépin et al. 1994, for review, see Molkentin 2000). Furthermore, GATA4 plays an important role in the morphogenesis of heart (Molkentin et al. 1997) and myocardial hypertrophic growth in vitro and in vivo (Liang et al. 2001a, Liang et al. 2001b). Several GATA elements have been identified within the 5' flanking sequences of the BNP promoter, and primarily the proximal GATA elements at -90 bp seem to be required for the basal and inducible gene expression of BNP in vitro (Grépin et al. 1994, Thurauf et al. 1994). Furthermore, the aortic coarctation-induced pressure overload-responsiveness of the ß-MHC and AT1A genes has been shown to be mediated by the GATA motifs in vivo (Hasegawa et al. 1997, Herzig et al. 1997). Moreover, the AP-1-like element has been reported to confer the pressure overload induction of the ANP and AT1A receptor genes in vivo (Herzig et al. 1997, von Harsdorf et al. 1997), although this result is controversial (Knowlton et al. 1995, Hasegawa et al. 1997). Site-directed mutations of two GATA elements at -90 bp in the BNP promoter decreased significantly the basal activity, and abrogated completely the induction by Ang II-stimulated hemodynamic overload. The mutation of the AP-1 motif located at -372 bp had no effect on the inducibility of the BNP promoter, but decreased markedly the basal activity of the BNP promoter. These in vivo results show that GATA elements are involved in mediating the Ang II-induced pressure overload-responsiveness of the BNP gene, whereas both GATA and AP-1 sites are needed for the basal BNP activation. These findings are in line with the previous in vivo results showing that two GATA motifs located at -90 bp are necessary and sufficient to confer transcriptional activation of the BNP gene in response to nephrectomy-induced hemodynamic stress in rats (Marttila et al. 2001). Moreover, the AP-1-like motif at -114 bp has been shown to confer the basal activation of the BNP gene expression in vivo (Marttila et al. 2001). Similarly, mutation of the proximal GATA element results in a 50% reduction of ET-1-induced human BNP promoter activity, whereas mutation of the AP-1 element has no effect on the activation of BNP promoter in response to ET-1 in cultured neonatal ventricular myocytes (He & LaPointe 2001a).

In perfused rat hearts in vitro, infusion of Ang II activates BNP GATA4 binding and direct wall stretch-induced BNP GATA4 binding activity is blocked by AT, receptor antagonist (Hautala et al. 2002), suggesting a regulatory role for Ang II. These results are
different from those obtained from in vivo AVP-induced pressure overload rat hearts, showing that the increase in BNP GATA4 binding activity is not prevented by AT1 receptor antagonism (Hautala et al. 2001). In the present study, increased left ventricular BNP GATA4 binding activity was detected at 6 h. Interestingly, BNP GATA4 binding activity did not differ from the control at 2 weeks, while the promoter activities showed that mutation of the proximal GATA motifs abolished the Ang II-stimulated pressure overload-responsiveness. Thus, additional transcription factors may act in concert with GATA4 to modulate BNP GATA4 binding activity. Previously, GATA4 has been shown to transactivate cardiac gene promoters synergistically with Nkx-2.5 (Sepulveda et al. 1998, Shiojima et al. 1999) and SRF (Belaguli et al. 2000) independently of the GATA consensus sites. Furthermore, NF-AT3 and NF-κB are suggested to be important regulators of the BNP gene expression, and these transcription factors are also targets of Ang II (Molkentin et al. 1998, Liang & Gardner 1999, Purcell et al. 2001).

ISO and PE infusion in adult mice and electrical pacing-induced cardiac hypertrophy in cultured cardiac myocytes are associated with increased GATA4 mRNA levels (Saadane et al. 1999, Xia et al. 2000), suggesting a regulatory mechanism in which the total content of GATA4 is upregulated by hypertrophic stimulus. However, in the present study the left ventricular GATA4 mRNA levels remained at the baseline level as previously described in vivo (Hautala et al. 2001). Thus, increase in BNP GATA4 binding activity appears to be regulated by additional mechanisms. Previously, GATA4 transcriptional activity and DNA binding affinity have been suggested to be regulated by protein phosphorylation of GATA4 at serine 105 by ERKs and p38 MAPKs without increasing GATA4 protein levels (Morimoto et al. 2000, Charron et al. 2001, Liang et al. 2001a, Yanazume et al. 2001). Whether Ang II-induced pressure overload alters the amount or the phosphorylation of the GATA4 protein under these experimental conditions remains to be studied.
7 Summary and conclusions

1. Ventricular expression of ANP and BNP increased in response to chronic L-NAME-induced hypertension despite the lack of myocardial hypertrophy, whereas expression of natriuretic peptide genes in the atria remained unaltered. AT₁ receptor antagonism decreased significantly left ventricular ANP, suggesting that the expression of natriuretic peptide genes in the ventricles is, at least in part, mediated by Ang II. However, the L-NAME-induced hypertrophic remodeling of mesenteric resistance arteries was Ang II-independent.

2. Ang II infusion produced early activation of left ventricular ANP, BNP and AM gene expression. These responses were either abolished (ANP and AM) or blunted (BNP) in Ang II-infused adrenalectomized rats, showing that adrenal gland-derived, load- and hypertrophy-independent factors are required for the Ang II-induced early activation of cardiac gene expression. Furthermore, the importance of adrenal factors, pressure load and hypertrophy on ANP, BNP and AM gene expression appears to vary during the distinct phases of Ang II-induced hypertension.

3. The left ventricular −2200 BNP promoter activity increased rapidly and remained upregulated in response to Ang II infusion for 2 weeks, except at 12 h. However, left ventricular BNP mRNA levels decreased after 3 days followed by a decline in left ventricular ir-BNP concentrations despite continuous Ang II infusion. These results show that posttranscriptional control plays an important role in the regulation of left ventricular BNP gene expression in vivo (Fig. 12).

4. Mutation of two proximal GATA elements abolished the increase in BNP promoter activity in response to Ang II administration. Moreover, both GATA and AP-1 elements were required for the basal activation of the BNP gene. Results from gel mobility shift assays showed increased left ventricular BNP GATA4 binding activity in Ang II-stimulated left ventricles. These results suggest an important role for GATA4 in BNP gene activation in response to Ang II-induced pressure overload (Fig. 12).
Fig. 12. A hypothetical model of the mechanisms of Ang II in regulating left ventricular BNP gene expression. Ang II, angiotensin II; AP-1; activator protein-1; AT, angiotensin receptor.
References


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