REGULATION OF CARDIAC RESPONSES TO INCREASED LOAD

Role of endothelin-1, angiotensin II and collagen XV

JARKKO PIUHOLA

Department of Pharmacology and Toxicology,
University of Oulu
Biocenter Oulu,
University of Oulu

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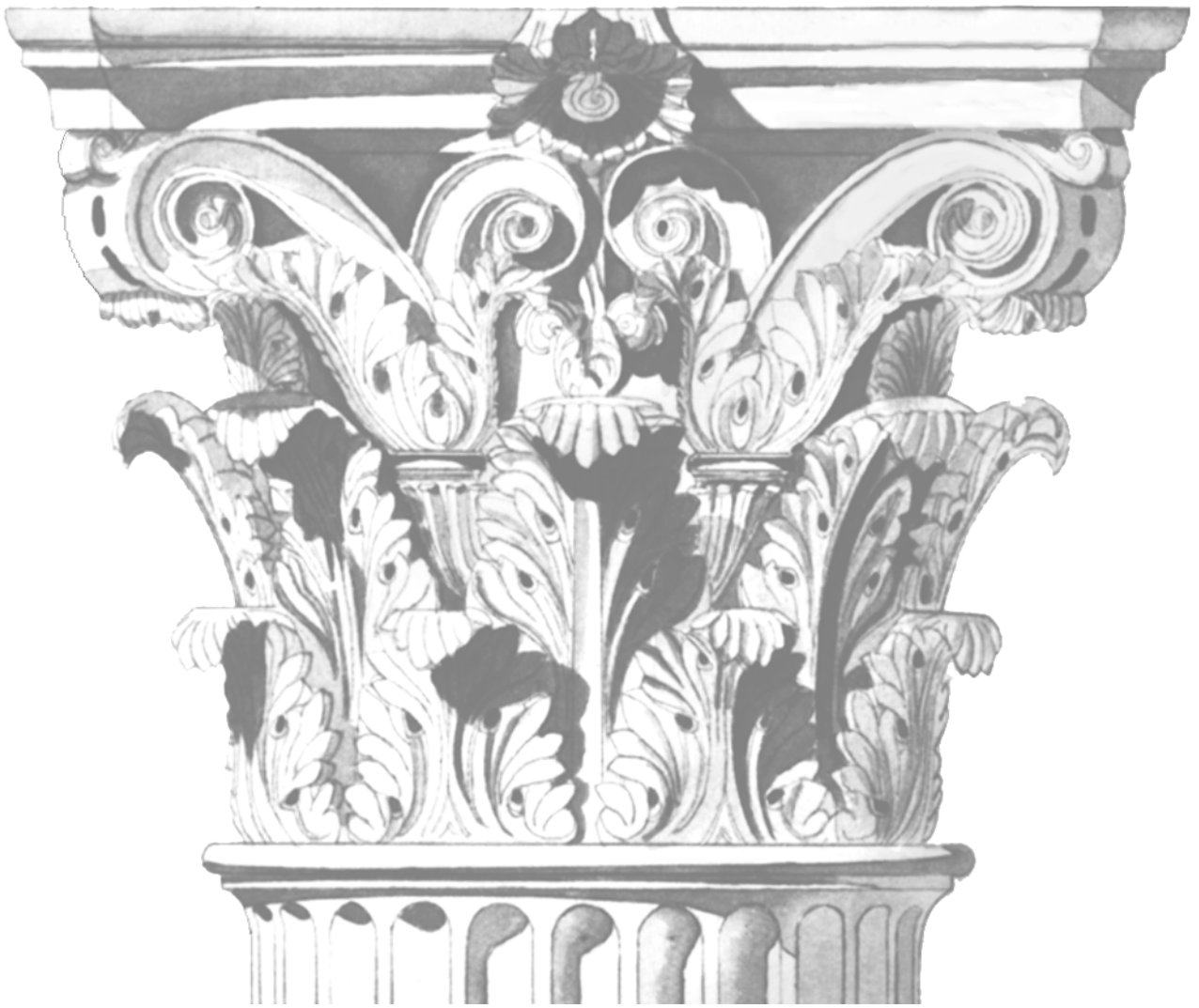

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Abstract

Chronic overload of the heart is the major cause of left ventricular hypertrophy (LVH) and eventually heart failure. It is generally accepted that autocrine/paracrine factors, such as angiotensin II (Ang II) and endothelin-1 (ET-1) contribute to the development of LVH. Cardiac hypertrophy and failure are characterized by attenuated responsiveness to β- adrenergic stimulation and accumulation of collagenous material to the left ventricular wall. The present study aimed to characterize the roles of ET-1 and Ang II in the regulation of cardiac function. The role of the plasmamembrane Ca^{2+}-ATPase (PMCA) in ET-1 induced cardiac responses and the role of type XV collagen in cardiac function were also studied.

Both ET-1 infusion and mechanical loading were able to induce positive inotropic effect and induction of early response genes in isolated perfused hearts. ET-1 also induced strong vasoconstriction. Cardiomyocyte-specific PMCA overexpression inhibited the ET-1 induced hypertrophic response, while inotropic response remained unaltered. ET-1 was found to induce release of adrenomedullin (AM), a potent vasorelaxing and inotropic peptide. Infusion of AM antagonized the vasoconstrictive effect of ET-1 independently of nitric oxide. In hypertrophied rat hearts ET-1 was found to contribute significantly to the Frank-Starling response, a fundamental mechanism regulating contractile performance of the heart. In mice hearts, ET-1 was found to play a dual role in load induced elevation of contractile strength: ET_A receptors mediated an increase, while ET_B receptors mediated an inhibitory effect on contrcatile force. Ang II was not contributing to the contractile response to load in either rat or mice hearts. Blunted response to β-adrenergic stimulus and increased vulnerability as a result of exercise was observed in mice lacking collagen XV.

In conclusion, the present results underscore the importance of the local factors, especially ET-1, in regulation of cardiac function, not only in terms of hypertrophic but also in terms of contractile response to load. The results also suggest a role for PMCA in regulation of cardiac function. Lack of type XV collagen was found to result in cardiac dysfunction with many features similar to those of early heart failure.

Keywords: angiotensin II, hypertrophy, endothelin-1, adrenomedullin, collagen XV, plasma membrane Ca^{2+}-ATPase

To Päivi

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Oulu, May, 2002 Jarkko Piuhola

Abbreviations

AC	adenylyl cyclase
ACE	angiotensin converting enzyme
AM	adrenomedullin
Ang	angiotensin
ANP	atrial natriuretic peptide
AT_x	angiotensin receptor subtype
BNP	B-type natriuretic peptide
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	3´,5´-cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cGMP	3´,5´-cyclic guanosine monophosphate
CHF	chronic heart failure
CNP	C-type natriuretic peptide
ColXV	type XV collagen
DP	developed pressure
dP/dt	derivative of intraventricular pressure
dTG	double transgenic
DAG	1,2-diacylglycerol
DP	developed pressure
DT	developed tension
EC	endothelial cell
E-C coupling	excitation-contraction coupling
EDRF	endothelium-derived relaxing factor
ES	embryonic stem cell
ET	endothelin
ET_x	endothelin receptor subtype
GAPDH	glyceraldehyde 3-phosphate-dehydrogenase
G-protein	guanine nucleotide binding protein
GPCR	G- protein coupled receptor
GTP	guanosine triphosphate
IP_3	inositol-1,4,5-triphosphate

ir	immunoreactive
L-NAME	N^{ω} –nitro-L-arginine methyl ester
LV	left ventricle
LVH	left ventricular hypertrophy
LVEDP	left ventricular end diastolic pressure
MAP	mitogen activated protein
MHC	myosin heavy chain
mRNA	messenger ribonucleic acid
NCX	Na^{+}-Ca^{2+} exchanger
NHE	Na^{+}-H^{+} exchanger
NPR_x	natriuretic peptide receptor subtype
NO	nitric oxide
NOS	nitric oxide synthase
NT-ANP	amino terminal fragment of pro atrial natriuretic peptide
NTG	non-transgenic
PKC	protein kinase C
PLC	phospholipase C
PMCA	plasma membrane calmodulin-dependent calcium ATPase
RAS	renin-angiotensin system
RIA	radioimmunoassay
RT-PCR	reverse transcriptase polymerase chain reaction
SD	Sprague-Dawley
SEM	standard error of mean
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase
SHR	spontaneously hypertensive rat
SR	sarcoplasmic reticulum
TG	transgenic
TnC	troponin C
TnI	troponin I
TnT	troponin T
TUNEL	terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling
VSMC	vascular smooth muscle cell

List of original papers

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

I Piuhola J, Hammes A, Schuh K, Neyses L, Vuolteenaho O & Ruskoaho H (2001) Overexpression of the Sarcolemmal Calcium Pump Attenuates Early Induction of Cardiac Gene Expression in Response to Endothelin-1. Am J Physiol Regul Integr Comp Physiol. 281: R699-705.

II Kinnunen P, Piuhola J, Ruskoaho H & Szokodi I (2001) AM Reverses Pressor Response to ET-1 Independently of NO in Rat Coronary Circulation. Am J Physiol Heart Circ Physiol. 281: H1178-83.

III Piuhola J, Szokodi I, Kinnunen P, Ilves M, Vuolteenaho O & Ruskoaho H (2002) Endogenous Endothelin Contributes to the Frank-Starling Response in a Rat Model of Human Renin Dependent Hypertension and Cardiac Hypertrophy. Submitted for publication.

IV Piuhola J, Mäkinen M, Szokodi I & Ruskoaho H (2002) Dual Role of Endothelin-1 via ET_A and ET_B Receptors in Regulation of Contractile Function in Mice Hearts. Submitted for publication.

V Eklund L, Piuhola J, Komulainen J, Sormunen R, Ongvarrasopone C, Fässler R, Muona A, Ilves M, Ruskoaho H, Takala T & Pihlajaniemi T (2001) Lack of Type XV Collagen Causes a Skeletal Myopathy and Cardiovascular Defects in Mice. Proc Natl Acad Sci U S A. 98: 1194-1199.

Contents

1 Introduction

Cardiovascular load leads to rapid alterations in cardiac contractile function and in the long term in cardiac structure as well. Tuning the contractile state of the myocardium is essential for the heart to adapt to the highly varying demands of the organism. Therefore, the cardiac function is under continuous regulation by various mechanisms which help the left ventricle to successfully fulfill its pump function. In addition to intrinsic mechanisms, such as the Frank-Starling law of the heart and force-frequency relationship, also extrinsic factors, such as autonomic nervous system, circulating hormones and locally acting peptide mediators, contribute to cardiovascular regulation. The development of left ventricular hypertrophy (LVH) in response to long term pressure overload may initially act as a compensatory response to decrease left ventricular wall stress. During the development of LVH, the pump function of the heart is initially improved (Strömer *et al.* 1997, Nakamura *et al.* 2001). However, in the long term LVH is accompanied by increased risk of adverse cardiovascular events and eventually by worsening of the cardiac performance (Levy *et al.* 1990). Synthesis and secretion of natriuretic peptides is also elevated, and accumulation of collagenous extracellular matrix is increased during hypertrophic process (Saito *et al.* 1989, Weber 1989). During the development of chronic heart failure (CHF), the hypertrophic compensation leads to decreased contractile performance per unit mass of myocardium (for review, see Cooper 1997), and regulation of contraction by adrenergic stimuli and force-frequency relationship are impaired (Bristow *et al.* 1982, Pieske *et al.* 1995).

During the past few decades, CHF has emerged as a major cause of mortality and morbidity in Western countries (O'Connell & Bristow 1994). In addition to the load itself the development of LVH and CHF is also affected by various autocrine/paracrine factors, such as endothelin-1 (ET-1) and angiotensin II (Ang II), which are upregulated during the process (for reviews, see e.g. Dostal & Baker 1999 and Kedzierski & Yanagisawa 2001). Therefore, these paracrine systems have been a target of intensive research in the treatment of cardiovascular disease. In addition to the hypertrophic response, ET-1 and Ang II are involved in the regulation of contractile performance of the heart (Kelly *et al.* 1990).

The aim of the present study was to evaluate the significance of locally acting peptide mediators in the regulation of cardiac contractile function and the early events of the

hypertrophic response. Using transgenic (TG) rats overexpressing plasma membrane Ca^{2+} ATPase (PMCA) (Hammes *et al.* 1998) the role of PMCA in cardiac response to ET-1 as well as to increased mechanical load were studied. The coronary vasoconstriction provoked by ET-1 was then analyzed, and interplay between ET-1 and adrenomedullin (AM), an endogenous peptide stimulated by ET-1, in the regulation of coronary vascular tone was analyzed. The roles of ET-1 and Ang II in Frank-Starling response were analyzed in both normal Sprague-Dawley rat hearts and hypertrophic double transgenic (dTG) rat hearts expressing human renin and angiotensinogen (Ganten *et al.* 1992, Bohlender *et al.* 1997). To set up a novel method for studying genetically engineered mice hearts, the effects of ET and Ang II receptor antagonists on contractility of isolated perfused mice hearts were studied. Finally, with genetically engineered collagen XV knockout mice, the role of collagen XV in cardiovascular structure and function was characterized. Both isolated perfused heart setup as well as *in vivo* loading with treadmill exercise were used for phenotype analysis of the TG mice.

2 Review of the literature

2.1 Regulation of cardiac contractile function

Contractile function of the heart is regulated by a number of intrinsic and extrinsic mechanisms. The impact of autonomic nervous system, various hormones, such as thyroid hormone, adrenocortical steroids, insulin, glucagon, and blood concentrations of O_2, CO_2 and H^+ on cardiac contractile function has been well established (See e.g. Berne & Levy 1993). Also autocrine/paracrine effectors synthesized and secreted by endothelial cells (EC), fibroblasts or cardiomyocytes themselves have been demonstrated to possess the ability to affect cardiac contractility. Examples of such regulators are ET-1 (Kelly *et al.* 1990), AM (Szokodi *et al.* 1998), natriuretic peptides (Yamamoto *et al.* 1997), nitric oxide (NO) (Prendergast *et al.* 1997b) and Ang II (Li *et al.* 1994). Intrinsic mechanisms affecting cardiac function include the Frank-Starling mechanism and the force-frequency relation. The complex interplay between all these factors is occurring continuously via both the hemodynamic state and respective feedback mechanisms, and also at the level of single cardiomyocytes. The changes in cardiac function can also be divided based on the time scale of occurrence. Acutely, within a few minutes after stimuli, changes due to posttranslational modification of proteins, such as phosphorylation, can be noted in contractile and secretory function of the heart, while the structural changes occur during a longer period as a result of altered gene expression and protein synthesis.

2.1.1 Excitation-contraction coupling

The excitation-contraction coupling (E-C coupling) includes the events which follow the wave of excitation and lead to contraction. Initially, the wave of depolarization spreads rapidly along the myocardial sarcolemma, and also into the interior of the cells via the invaginations of the sarcolemma, the T-tubules, opening the voltage dependent L-type Ca^{2+} channels and triggering a Ca^{2+} influx (Hobai & Levi 1999).

2.1.1.1 Ca^{2+} influx leading to contraction

The calcium entering the cell through the L-type Ca^{2+} channels serves as a trigger to release Ca^{2+} (Ca^{2+} induced Ca^{2+} release, CICR) from the sarcoplasmic reticulum (SR) through SR Ca^{2+} release channels known as ryanodine receptors (RyR) (Fabiato & Fabiato 1979). The RyR and L-type Ca^{2+} channels are located in close functional association, thus allowing rapid CICR to occur (Sham *et al.* 1995).

The cytosolic free Ca^{2+} is increased 10- to 100-fold during the E-C coupling process. High intracellular calcium concentration ($[Ca^{2+}]_i$) levels promote Ca^{2+} binding to specific site in the N-terminal domain of troponin C (TnC), resulting in a conformational change of the TnC molecule (Robertson *et al.* 1982, for review, see Solaro & Rarick 1998). Cardiac troponin is a heterotrimer consisting of three distinct gene products: TnC, troponin I (TnI) and troponin T (TnT). TnC acts as the Ca^{2+} receptor, TnI inhibits the actin-myosin reaction and shuttles between tight binding to actin and tight binding to Ca^{2+}-TnC and TnT binds to myosin, TnI, and TnC. As a consequence of the Ca^{2+}-signaling process and the conformational change in TnC, TnI moves from its diastolic state (tightly bound to actin) to its systolic state (tightly bound to TnC) (Tao *et al.* 1990, Solaro & Rarick 1998). The interaction between TnI and TnC is followed by moving of the tropomyosin molecule to allow the crossbridges to attach and to produce force (Opie 1995). Heads of myosins (the crossbridges or myosin) protruding from the thick filament then react with thin-filament actins in a reaction cycle that is powered by ATP (Rayment *et al.* 1993).

2.1.1.2 Factors affecting the excitation-contraction coupling

A number of factors influence the E-C coupling process. Extracellular mediators, such as ET-1, AM, Ang II, NO and catecholamines, regulate the process by activating the intracellular second messengers. Depending on the agonist, the contractile force may increase or decrease, i.e. there may be a positive or negative inotropic effect, respectively. In terms of Ca^{2+}-contractile protein interaction, in order to a positive inotropic effect to occur, either the supply of the Ca^{2+} during systole must increase, or the sensitivity of the TnC for Ca^{2+} must be elevated, which means that the response of the myofilaments at a given level of occupancy of Ca^{2+} binding sites is increased (Endoh 1998, Opie 1995). The majority of the inotropic interventions (e.g. the force-frequency relationship, β-adrenergic agonists and digitalis glycosides) alter the intracellular Ca^{2+} transient, thus acting through an upstream mechanism to increase the contractile force. The Frank-Starling mechanism, α-adrenergic agonists, ET-1 and some novel drugs, such as EMD 57033 and levosimendan, act through a downstream mechanism by increasing the sensitivity to Ca^{2+} (Krämer *et al.* 1991, Haeusler *et al.* 1997, Kentish & Wrzosek 1998) (for review, see Haikala & Linden 1995).

Intracellular signaling in response to agonist stimuli is mediated by a number of second messengers. Well characterized 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP) mediate positive and negative inotropic responses, respectively. cAMP is generated by adenylyl cyclase (AC), which is

coupled to sarcolemmal receptors, e.g. β-adrenergic receptor (β-AR) (Hajjar *et al.* 1998). cAMP then activates protein kinase A (PKA), which can phosphorylate e.g. L-type Ca^{2+} channel, phospholamban (PLB) and TnI (for review, see e.g. Walsh & Van Patten 1994, Katz & Lorell 2000). By phosphorylating TnI, PKA enhances the interaction between TnI and actin, thus decreasing the sensitivity of contractile apparatus to Ca^{2+}, but also potentially increasing the rate of relaxation (Venema & Kuo 1993). However, the potential negative inotropic effect induced by TnI phosphorylation is normally overcome by a marked increase in $[Ca^{2+}]_i$ due to stimulation of Ca^{2+} influx through L-type Ca^{2+} channels, as occurs in response to a β-receptor agonist.

Several independent signals affect cardiac function via the guanine nucleotide binding protein (G-protein) coupled receptors. The heterotrimeric G-proteins consist of separate Gα and Gβγ subunits. Agonist binding to membrane bound G-protein coupled receptors catalyzes the exchange of guanosine diphosphate to guanosine triphosphate GTP on Gα subunit and subsequent dissociation of Gα from Gβγ (for review, see Molkentin & Dorn II 2001). The Gα subunit is considered to mediate the majority of the downstream effects, but Gβγ may also have an impact on downstream signaling through mitogen activated protein (MAP) kinases (Crespo *et al.* 1994). The cardiovascular G-protein coupled receptors couple to the three major classes of G-proteins, as divided by the alpha subunit: $G\alpha_s$, $G\alpha_i$ and $G\alpha_q$. Classically, $G\alpha_s$ mediates AC activation in response to ß-AR stimulation, $G\alpha_i$ mediates cholinergic inhibition of AC and $G\alpha_q$ has been implicated in LVH development (Molkentin & Dorn II 2001). Activation of G_q for instance by ET-1 induces phosphoinositide hydrolysis by phospholipase C (PLC). The second messengers inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) induce subsequent activation of protein kinase C (PKC) and downstream effectors, such as the Na^+-H^+ exchanger (NHE) (Wang *et al.* 1993).

2.1.1.3 Removal of Ca^{2+} from cytoplasm during diastole

During the diastole, for relaxation and ventricular filling to occur, the Ca^{2+} that activated the myofilaments must be removed from the cytosol. Ca^{2+} is extruded from the cytoplasm via sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), sarcolemmal Na^+-Ca^{2+} exchanger (NCX), PMCA, and mitochondrial Ca^{2+} uniporter (for review, see Bers 2000). Quantitatively, SERCA and NCX are most important. In rat and mice ventricles, SERCA accounts for over 90% of the Ca^{2+} removal during cardiac relaxation (Hove-Madsen & Bers 1993, Li *et al.* 1998), while in human and rabbit ventricles SERCA removes ca. 70% of the Ca^{2+} from the cytosol and the NCX ca. 28%. The rest of the Ca^{2+} is removed by PMCA and mitochondrial Ca^{2+} uniporter (Pieske *et al.* 1999b, Bers 2000) (see Fig.1). Thus, most of the Ca^{2+} that activates the contractile process is released from the SR, and the SR takes up most of the released Ca^{2+} again during diastole. PLB is a 52-amino acid phosphoprotein found in the SR membranes also in cardiomyocytes. It binds to the SERCA, inhibiting the Ca^{2+} binding ability. The PLB binding to SERCA is decreased via phosphorylation in response to certain stimuli, such as β-adrenergic signaling (for review, see Kiriazis & Kranias 2000). In failing hearts, the Ca^{2+} loading of the SR may be impaired (see section 2.2.), increasing the role of extracellular Ca^{2+} in EC-coupling and

the role of NCX in Ca^{2+} transients (Pieske *et al.* 1999b). This may be partially responsible for the slowing down of the relaxation process as seen in heart failure (Kiriazis & Kranias 2000).

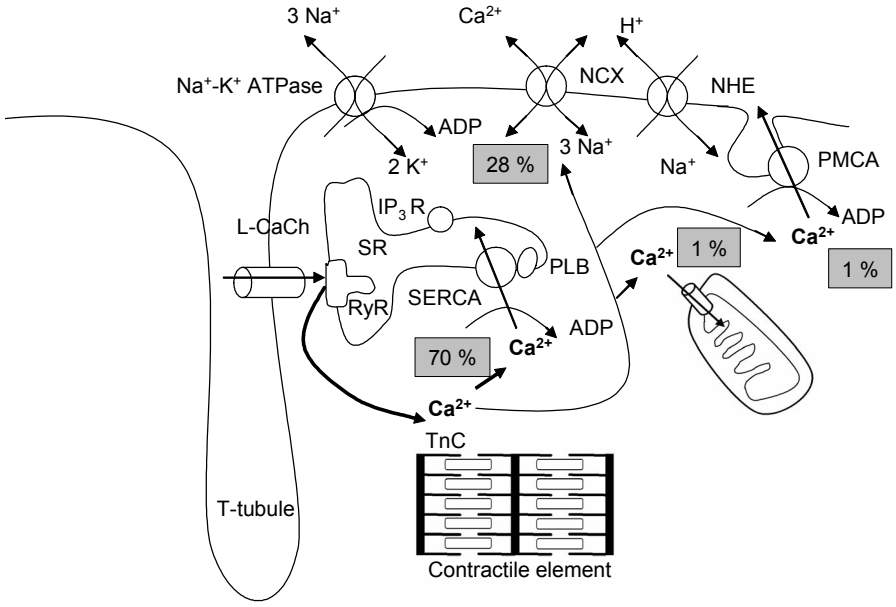

Fig. 1. Calcium fluxes during cardiac cycle. Gray boxes with per cent values indicate proportion of Ca^{2+} removal during diastole by the respective mechanism in human and rabbit hearts. IP_3R, IP_3 receptor; L-CaCh, L-type Ca^{2+} channel; NHE, Na^+-H^+ exchanger; NCX, Na^+-Ca^{2+} exchanger; PLB, phospholamban; PMCA, plasma membrane calmodulin-dependent Ca^{2+} ATPase; RyR, Ryanodine receptor; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TnC, troponin C. Modified from Bers 2000.

2.1.1.4 The role of the plasma membrane Ca^{2+}-ATPase in heart

PMCA is a ubiquitous Ca^{2+}-transporting enzyme extruding Ca^{2+} from the cell (Schatzmann 1966) (for review, see Carafoli 1992). As mentioned, in excitable cells expressing the high capacity NCX, the activity of PMCA *in vitro* is rather low compared with NCX (Bers 2000). In the myocardium, the expression of the PMCA isoforms 1, 2, and 4 has been shown (Stauffer *et al.* 1995, Hammes *et al.* 1994, for review, see Carafoli & Stauffer 1994), but the physiological significance has remained unknown. Due to the high affinity to Ca^{2+}, PMCA has been suggested to play a role in fine-tuning Ca^{2+} in the final phase of diastole in the heart (for review, see Carafoli 1994).

PMCA is known to localize in caveolae, 50- to 100-nm plasma membrane invaginations, containing receptors for ET-1 and various other ligands. Also a number of important signaling molecules, such as $G\alpha_s$, ras, PKCα, MAP kinase, AC and Src tyrosine kinase are enriched in caveolae (Fujimoto 1993, Chun *et al.* 1994, Hammes *et al.*

1998) (for reviews, see e.g. Couet *et al.* 1997, Smart *et al.* 1999). PMCA has been suggested to play a role in growth and differentiation processes in myoblasts as well as in other cell types *in vitro* (Hammes *et al.* 1996). Altered growth and differentiation responses to phenylephrine and isoproterenol were found in PMCA overexpressing neonatal cardiac myocytes *in vitro* (Hammes *et al.* 1998).

The finding that cardiac overexpression of PMCA resulted in no differences in voltage dependence, activation, and inactivation behavior of L-type Ca^{2+} current between TG cells and control adult cardiomyocytes confirmed the previous hypothesis that the significance of PMCA in Ca^{2+} extrusion is minor. Only when the SR was blocked by thapsigargin (SERCA inhibitor) and ryanodine (blocks the RyRs), a marginally different time constant of $[Ca^{2+}]_i$ decline was seen (Hammes *et al.* 1998). Thus, the role of PMCA in cardiac myocytes has remained obscure.

2.1.2 The Frank-Starling mechanism

In 1895 Frank discovered that the greater the preload, the greater the force generated by frog cardiac muscle. In 1914 Starling demonstrated the same phenomenon in canine heart-lung preparation by elevating either right atrial pressure or aortic resistance (see e.g. Berne & Levy 1993, Katz & Lorell 2000).

The Frank-Starling mechanism (heterometric autoregulation) plays a major role in intrinsic regulation of cardiac function (Sarnoff & Berglund 1954; for review, see Katz & Lorell 2000). The role of the Frank-Starling response is augmented in the elderly, who have a dimished increase in the heart rate in response to physical excercise. It is also known that this response is preserved even in hypertrophied and failing hearts (Holubarsch *et al.* 1996). In normal subjects, the Frank-Starling response contributes to cardiac output during submaximal exercise (Plotnick *et al.* 1986), and changes in posture (Drake-Holland *et al.* 1990). An increase in ventricular end-diastolic volume, produced by increased venous return or decreased aortic outflow, leads immediately to a more powerful contraction. At the molecular basis, the mechanism of this phenomenon is not well understood. The main theory of the cellular basis of the Frank-Starling law has for long been length-dependent myofilament activation (Allen & Kentish 1985). The length dependence of myofilament activation is very prominent in normal hearts, operating at sarcomere lengths less than the optimal 2.2 μm (Solaro & Rarick 1998). The length-dependent activation has been suggested to relate to increased Ca^{2+} affinity of the Ca^{2+} -binding part of the contractile element, TnC (Kentish *et al.* 1986). A possible mechanism is that the change in sarcomere length involves a change in interfilament spacing that modulates the ability of crossbridges to react with thin filaments (actin) at the same Ca^{2+} concentration, thus increasing the rate of crossbridge formation, as suggested by studies using osmotic compression of the cardiomyocytes (McDonald & Moss 1995). However, in a recent study with x-ray diffraction analysis, the osmotic compression to achieve lattice spacing typical of longer length could not produce a change in Ca^{2+} sensitivity of force (Konhilas *et al.* 2002). Other possible cellular mechanisms explaining the Frank-Starling relationship include positive cooperativity in crossbridge binding, or strain of

titin, elastic protein of the contractile element (Fitzsimons *et al.* 2001, Cazorla *et al.* 2001).

After the rapid increase in contractile force, there is a further increase in myocardial performance during the next few minutes of stretch. *In vivo* this allows the end-diastolic volume to return toward its original value (von Anrep 1912, Parmley & Chuck 1973). This slow rise in contractile strength, also known as Anrep effect or homeometric autoregulation, accounts for <10% to 25% of the overall contractile response to load in physiological temperatures (Tucci *et al.* 1984, Perez *et al.* 2001).

In isolated, blood perfused canine hearts as well as in isolated ferret papillary muscle increased intracellular Ca^{2+} and also cAMP concentrations have been shown to parallel alterations in contractile force in response to an increase in end diastolic pressure (Todaka *et al.* 1998, Calaghan *et al.* 1999). In contrast, in rat atrial preparation, stretching did not change the production of cAMP or cGMP (Tavi *et al.* 2000). Furthermore, if cAMP would mediate the slow force response, the resulting PKA activation would also lead to phosphorylation of TnI, decreasing the Ca^{2+} sensitivity of the contractile element. This hypothesis contrasts with the finding that Ca^{2+} sensitivity of the contractile elements at the beginning of the stretch is increased (Kentish & Wrzosek 1998). Alvarez *et al.* (1999) suggested that intracellular alkalinization by ET-1 and Ang II induced NHE activation accounts for the mechanism. Indeed, it seems that this mechanism might play a role in hypertrophied, failing or especially in ischemic hearts (Krämer *et al.* 1991, Perez *et al.* 1995, Tavi *et al.* 1999). However, in a further study in normal cat papillary muscle it was shown that intracellular alkalinization is not occurring in the presence of bicarbonate buffered medium (Perez *et al.* 2001). Furthermore, the NHE activation was suggested to induce a slight increase in $[Na^+]_i$, leading to activation of NCX in reverse mode (Na^+ out, Ca^{2+} in). To confirm this it was shown that intracellular Na^+ replacement by lithium or by blocking the reverse mode of NCX prevented the development of the slow force response (Perez *et al.* 2001). This mechanism would also explain the increase in $[Ca^{2+}]_i$. In a study by another group (Calaghan & White 2001), the pivotal role of the endocardial endothelium in the slow force response was confirmed, ET-1 being the key mediator, independently of Ang II.

Recent evidence suggests that the Frank-Starling mechanism is subject to paracrine regulation. Basal release of NO attenuates diastolic stiffness and thus augments the Frank-Starling response (Prendergast *et al.* 1997b). The slow phase response is regulated via stretch induced release of ET-1 and Ang II. However, at present the role of these mediators in the complete Frank-Starling response in whole organ level is unclear.

2.1.3 The force-frequency relationship

When the contractile frequency is increased, cardiac output is elevated through an increased number of beats per minute, as during exercise. In most species, including nonfailing human myocardium, increased frequency also leads to elevation of contractile force, an event also known as the Treppe phenomenon or the Bowditch effect (Miura *et al.* 1992). However, in the failing myocardium, frequency potentiation of contractile force is inverse, decreasing contractile force. The Treppe phenomenon has been

suggested to result from increased transsarcolemmal Ca^{2+} influx leading to greater filling of the SR and therefore, a higher amount of Ca^{2+} available for release during systole (Pieske *et al.* 1995). This positive inotropic effect can be further augmented with ß-AR agonist dobutamine under resting conditions, when the heart rate is modulated by pacing (Kambayashi *et al.* 1992). In failing hearts, SR Ca^{2+} uptake was significantly reduced, suggesting a possible mechanism for inverse force-frequency relationship in CHF (Pieske *et al.* 1995). Altered Ca^{2+} handling could be explained by a depressed role of SERCA combined with enhanced cytosolic Ca^{2+} extrusion via NCX (Pieske *et al.* 1999b).

2.1.4 The adrenergic system

The effectors of the sympathetic nervous system, i.e. epinephrine and norepinephrine, act on cardiac myocytes via both α- and β- adrenergic receptors. Currently, three ß-AR subtypes, designated $ß_1$-AR, $ß_2$-AR, and $ß_3$-AR, have been cloned and pharmacologically characterized. A fourth subtype ($ß_4$-AR) may also exist, but it is not well characterized (for review, see e.g. Post *et al.* 1999). All three of the cloned ß-AR subtypes belong to the large family of seven membrane-spanning GPCRs. While β_1-AR is the predominant subtype on cardiac myocytes (66% in mouse and 80% in rat), also $ß_2$-ARs are present (34% in mouse and 20% in rat cardiac myocytes) and capable of mediating positive inotropic responses (Bristow *et al.* 1986, Hilal-Dandan *et al.* 2000). Activation of $ß_1$- and $ß_2$-ARs results in an increase in intracellular cAMP after AC stimulation through G_s proteins (Post *et al.* 1999). The increase in cAMP leads to phosphorylation of PLB, calcium channels and contractile element proteins via PKA. Phosphorylation of these proteins alters their activity and leads to a functional response including positive inotropic effect. In contrast to other ß-ARs, $ß_3$-AR activation leads to negative inotropic response. Inhibitors of NO synthase successfully blocked the negative inotropism of $ß_3$-AR stimulation (Gauthier *et al.* 1998).

Also α_1-AR activation may mediate the positive inotropic responses to catecholamines or adrenergic agonists. There are three subtypes of α_1-ARs (α_{1A}-, α_{1B}-, and α_{1D}-ARs), all of which are encoded by distinct genes (for review, see e.g. Brodde & Michel 1999). All α_1-AR subtypes are GPCRs, and most commonly the intracellular second messengers are IP_3 and DAG formed by PLC activation. IP_3 mediates the Ca^{2+} release from intracellular stores, while also an increase in Ca^{2+} sensitivity of the myofilaments and NHE activation contribute to positive inotropic effect. Interestingly, in rat heart, α_{1B}-AR is the predominant subtype, while in human and also in mice hearts the α_{1A}- subtype is present in highest amounts (Brodde & Michel 1999). There are also differences in coupling of α_1-ARs to downstream effectors in mouse and rat cardiomyocytes, since no α_1-adrenergic stimulation of phosphoinositide turnover could be detected in mouse cardiomyocytes (Hilal-Dandan *et al.* 2000).

CHF is associated with a number of alterations in the activation and deactivation of beta-adrenergic receptor pathways. Continuous adrenergic stimulus results in uncoupling of β-ARs (Post *et al.* 1999). Activation of the sympathetic nervous system is considered to be one of the major pathophysiological abnormalities in patients with heart failure (Cohn *et al.* 1984). Elevated circulating norepinephrine and epinephrine have been

implicated in contributing to the ß-AR down regulation in both protein and messenger ribonucleic acid (mRNA) level and receptor uncoupling that are characteristic of end-stage heart failure, resulting in subsensitivity to ß-agonist stimulation (Bristow *et al.* 1982, Fowler *et al.* 1986) (for reviews, see e.g. Dzimiri 1999). An important mechanism for rapidly regulating ß-AR function is agonist-stimulated receptor phosphorylation by G-protein –coupled receptor kinases (GRKs), resulting in decreased sensitivity to subsequent catecholamine stimulation. ß-AR kinase (ßARK) is a member of this family of GRKs that phosphorylate and regulate a wide variety of receptors that couple to heterotrimeric-G proteins (Pitcher *et al.* 1998). Interestingly, mice that lack the ability to generate norepinephrine or epinephrine due to genetic disruption of dopamine ß-hydroxylase show increased cardiac contractility associated with a decrease in the level of ßARK1 protein and kinase activity (Cho *et al.* 1999).

β-AR antagonists were previously considered contraindicated in heart failure due to their negative inotropic effect. Thereafter, an increasing body of evidence has shown that many of the neurohumoral compensatory mechanisms that are activated during CHF are actually deleterious. During the past decade large clinical trials have provided the proof that β-AR antagonists bisoprolol, carvedilol and metoprolol are valuable drugs in treatment of CHF (CIBIS Investigators and Committees 1994, Packer *et al* 1996, MERIT-HF Study Group 1999), pointing out the importance of understanding the pathophysiological mechanisms behind complex diseases such as CHF.

2.1.5 Circulating hormones

A number of circulating hormones have an impact on the myocardial performance. The adrenomedullary hormone epinephrine exerts its effects on cardiac myocytes through α- and ß-ARs. It is likely that under normal conditions the circulating catecholamines have only minor effects on cardiac contractility compared with the influence of the sympathetic nervous system, which is usually activated in parallel with the adrenomedullary hormone release (Berne & Levy 1993). In rat, hyperthyroidism increases the cardiac contractility (Kolar *et al.* 1992), and acutely thyroid hormones exert positive inotropic effects in isolated rat heart (Segal *et al.* 1996). Interestingly, thyroid hormone seems to regulate the expression pattern of central Ca^{2+} handling proteins, SERCA and NCX, in rat heart during postnatal development inducing an increase in the SERCA mRNA, while decreasing NCX mRNA levels (Rohrer & Dillmann 1988, Arai *et al.* 1991). Hypothyroid rats have been utilized as a model of decreased contractile function and heart failure (Ng *et al.* 1991), and all three subtypes of thyroid hormone receptors in cardiomyocytes were downregulated by phenylephrine treatment in cell culture and pressure overload *in vivo*. Insulin and glucagon (Farah 1983) as well as circulating insulin-like growth factors (Cittadini *et al.* 1998) have a direct positive inotropic effect on myocardial contraction, although the physiological significance of these effects is not known. Also natriuretic peptides have direct cardiac effects (see section 2.4).

2.2 Autocrine/paracrine factors

The finding that disruption of endocardial endothelium of isolated cat papillary muscle decreases contraction force (Brutsaert *et al.* 1988) was the early evidence suggesting that endocardial cells synthesize and secrete substances affecting contractile performance of cardiac muscle. Thereafter, locally acting mediators have been described to affect not only contractile force but also the remodeling process after myocardial injury and overload and therefore pharmacological antagonists of the autocrine/paracrine factors have been intensively studied as a potential treatment of cardiac hypertrophy and failure (Kojima *et al.* 1994, Sakai *et al.* 1996a).

2.2.1 Endothelins

2.2.1.1 Structure and biosynthesis

In 1988, a 21-amino-acid vasoconstricting factor termed endothelin-1 was found from cultured porcine aortic ECs (Yanagisawa *et al.* 1988). Even before that there were pieces of evidence showing that ECs produce a vasoconstrictor substance (Hickey *et al.* 1985). Soon after the discovery of ET-1, two structurally related peptides were identified and termed endothelin-2 (ET-2) and endothelin-3 (ET-3) (Inoue *et al.* 1989). Each of these peptides is encoded by a separate gene, and the biosynthesis includes processing from prepro-form by a furin-like protease to form big ET-1, big ET-2 or big ET-3, respectively. Big ETs are further processed by endothelin converting enzymes 1 and 2 (ECE-1 and ECE-2, respectively) to form ET-1, ET-2 and ET-3 (Inoue *et al.* 1989, for review, see Kedzierski & Yanagisawa 2001) (Fig. 2). Besides the membrane-bound metalloproteases ECE-1 and ECE-2, it is likely that also other enzymes take part in conversion of big ETs to mature endothelins (Yanagisawa *et al.* 2000). Endothelial cells are the principal sites of ET-1 synthesis (Inoue *et al.* 1989), but also cardiac myocytes and fibroblasts, kidney, central nervous system and human aortic vascular smooth muscle cells (VSMC) produce ET-1 (Ito *et al.* 1993, Yamazaki *et al.* 1996, for review, see Giannessi *et al.* 2001). ET-2 is expressed by intestinal epithelial cells and at lower levels also in heart, and ET-3 is expressed by brain neurons, kidney, and intestinal epithelial cells (Matsumoto *et al.* 1989, Kedzierski & Yanagisawa 2001). In the cardiovascular system ET-2 and ET-3 are expressed at low levels, and ET-1 seems to be the predominant isopeptide in the cardiovascular system (Firth & Ratcliffe 1992).

ET-1 is secreted by ECs in a polar manner: 80% of ET-1 is secreted on the basal side of ECs (Wagner *et al.* 1992), resulting in 100-fold concentrations within the vascular wall compared to plasma levels. ET-1 concentration in the interstitial transudate of perfused hearts has been reported to be higher than the concentration in coronary effluent (Brunner 1997). Therefore, under normal physiological conditions, ET-1 is not a circulating hormone, acting rather as a paracrine factor.

The synthesis and secretion of ET-1 by ECs is increased by various growth factors, cytokines and vasoactive factors, such as Ang II, vasopressin, bradykinin, norepinephrine and ET-1 itself (Miyauchi & Masaki 1999). Low shear stress increases ET-1 mRNA, while high shear stress decreases it (Yoshizumi *et al.* 1989, Malek & Izumo 1992). The biological effects of ET-1 include strong vasoconstriction (Yanagisawa *et al.* 1988), positive inotropic and chronotropic effects (Ishikawa *et al.* 1988a, Ishikawa *et al.* 1988b, Krämer *et al.* 1991, Kinnunen *et al.* 2000), mitogenic effects on smooth muscle cells (Fujitani *et al.* 1995), influence on salt and water homeostasis, and stimulation of the renin-angiotensin-aldosterone and sympathetic nervous systems (for reviews, see e.g. Giannessi *et al.* 2001, Kedzierski & Yanagisawa 2001). ET-1 is essential for normal embryonic development (Kurihara *et al.* 1995, Yanagisawa *et al.* 2000), which has lead to difficulties in development of genetically engineered animal models with a disrupted ET system.

The clearance of ETs from plasma may occur through cleavage by neutral endopeptidase EC3.4.24.11 (Abassi *et al.* 1992), or through ET_B receptor, which especially in the lung acts as a clearance receptor (Fukuroda *et al.* 1994). Due to effective clearance, the plasma half life of infused ET-1 is only one minute (Kedzierski & Yanagisawa 2001).

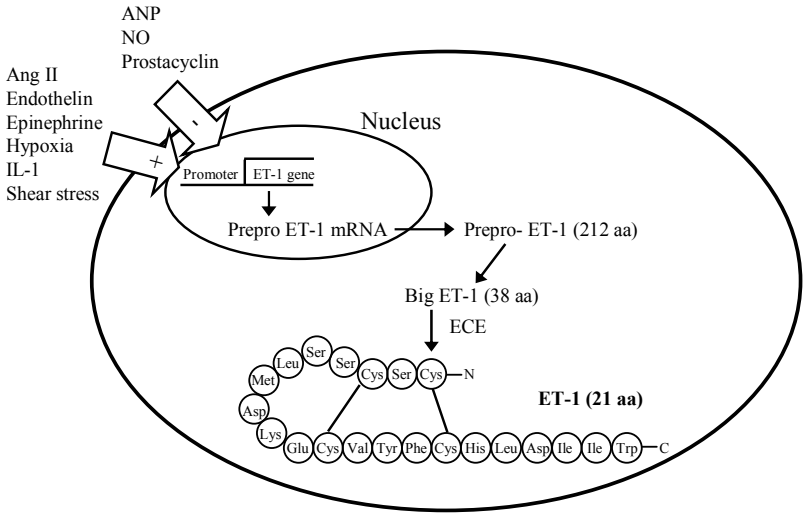

Fig. 2. Schematic presentation of biosynthesis and structure of ET-1. aa, amino acid; Ang II, angiotensin II; ANP, atrial natriuretic peptide; ECE, endothelin converting enzyme; ET-1, endothelin-1; IL-1, interleukin-1; NO, nitric oxide. Modified from Giannessi *et al.* 2001.

2.2.1.2 Receptors and intracellular signaling systems

Two endothelin receptors (ET_A and ET_B) have been identified in mammalian tissues (Arai *et al.* 1990, Sakurai *et al.* 1990) (see Table 1). ET_A receptors bind preferentially ET-1 (Hosoda *et al.* 1991), while ET_B receptors are non-isopeptide-selective (Sakurai *et al.*

1990). Both of these receptors belong to the group of GPCRs, containing seven transmembrane domains and activating an overlapping set of G-proteins (Kedzierski & Yanagisawa 2001). Initially, ET-1 signaling was shown to couple to G_q subfamily of G-proteins (Takuwa *et al.* 1990). However, there is also evidence of coupling to pertussis-toxin sensitive G_i subfamily of G-proteins, leading to inhibition of AC and phosphoinositide hydrolysis (Kelly *et al.* 1990, Hilal-Dandan *et al.* 1992, Hilal-Dandan *et al.* 1994). The study by Takagi and co-workers showed that in addition to G_q, human ET_A receptors couple to G_s and ET_B receptors to G_i, leading to subsequent induction or inhibition of cAMP formation, respectively (Takagi *et al.* 1995). Taken together, it seems that the ET_A receptor couples primarily with members of the G_q and G_s families, but coupling to other G-protein subfamilies has also been reported (Aramori & Nakanishi 1992, Mao *et al.* 1998). ET_A receptors also stimulate PLC and induce subsequent formation of IP_3 and DAG (for review, see Masaki *et al.* 1999, Clerk & Sugden 1999). Activation of the G_q mediated signaling has been suggested a major role in cellular responses to ET-1 (for review, see Clerk & Sugden 1999).

Table 1. Function of ET receptors in different cell types of cardiovascular system.

Cell type	ET_A receptors	ET_B receptors
Endothelial cells	-[1]	Vasodilation through the release of NO and prostacyclin[2] and adrenomedullin[3] ET-1 reuptake[4] Increased ET-1 gene expression[5]
Vascular smooth muscle cells	Vasoconstriction[6] Growth[7]	Vasoconstriction[8]
Cardiac fibroblasts	Growth, Fibrosis[9,10,11]	Growth, Fibrosis[12,13,14]
Cardiomyocytes	Hypertrophy[15] Positive inotropy[16] Protection from apoptosis[17]	Positive chronotropy[18,19] Hypertrophy ?

-, no expression. See text for details. [1]Hosoda *et al.* 1991, [2]De Nucci *et al.* 1988, [3]Jougasaki *et al.* 1998, [4]Lüscher & Barton 2000, [5]Saito *et al.* 1995, [6]Haynes & Webb 1994, [7]Fujitani *et al.* 1995, [8]Clozel *et al.* 1992, [9]Serneri *et al.* 2000, [10]Fujisaki *et al.* 1995, [11]Piacentini *et al.* 2000, [12]Fareh *et al.* 1996, [13]Mulder *et al.* 2000, [14]Fareh *et al.* 2000, [15]Ito *et al.* 1991, [16]Ishikawa *et al.* 1988b, [17]Araki *et al.* 2000, [18]Hori *et al.* 1992, [19]Ishikawa *et al.* 1988b. For review, see Giannessi *et al.* 2001.

2.2.1.3 Vascular effects of endothelin-1

In vasculature, ET_A receptors, located primarily on smooth muscle cells, account mostly for the vasoconstrictive action of ET-1 (Hosoda *et al.* 1991, Haynes & Webb 1994, Verhaar *et al.* 1998). ET-1 is one of the most potent vasoconstrictors in mammalian vasculature (Yanagisawa *et al.* 1988). In healthy humans, endogenous ET-1 contributes to

basal vascular tone (Haynes & Webb 1994). ET_B receptor mRNA is found in ECs, where ET_B receptors mediate vasodilation through release of endothelium-derived vasodilators, such as NO and prostacyclin (De Nucci *et al.* 1988, Ogawa *et al.* 1991a, Verhaar *et al.* 1998). However, some ET_B receptors are also located in VSMCs, mediating vasoconstriction (Clozel *et al.* 1992) (see Table 1). As a presentation of the dual action of ET-1 on vessels, infusion of exogenous ET-1 in different species results in short-term vasodilation (up to a few minutes), followed by strong, long-term vasoconstriction (Yanagisawa *et al.* 1988, Kedzierski & Yanagisawa 2001). Another regulatory interplay occurs between ET-1 and NO which can oppose each other's effect on vascular tone (Lerman *et* al. 1992). While ET-1 increases NO release via ET_B receptors (De Nucci *et al.* 1988), it also releases vasodilator adrenomedullin (AM) from ECs (Jougasaki *et al.* 1998). Administration of either ET_A selective or mixed $ET_{A/B}$ receptor antagonist bosentan in both experimental and human essential hypertension decreases blood pressure in a dose dependent manner (Krum *et al.* 1998) (for review, see Schiffrin 1999). However, since ET_B receptors in ECs mediate vasodilation, ET_A selective antagonists may be more potential as cardiovascular drugs.

2.2.1.4 Inotropic effects of ET-1

In the heart ET-1 has potent positive inotropic and chronotropic effects (Ishikawa *et al.* 1988a, Ishikawa *et al.* 1988b, Moravec *et al.* 1989, Kelly *et al.* 1990, Krämer *et al.* 1991, Kinnunen *et al.* 2000). In normal subjects, positive basal inotropic effect of ET-1 has been reported (MacCarthy *et al.* 2000). In isolated hearts, ET-1 is released into coronary circulation at a concentration high enough to exert a positive inotropic effect (McClellan *et al.* 1994). As previously mentioned, ET-1 contributes to the slowly developing part of the contractile response to increased stretch (Perez *et al.* 2001, Calaghan & White 2001). In addition to the strong body of evidence showing positive inotropic effects in isolated myocytes or isolated hearts, there are also studies with no positive inotropic effect or even negative inotropic effect in isolated hearts (Karwatowska-Prokopczuk & Wennmalm 1990, Neubauer *et al.* 1990) or *in vivo* (Beyer *et al.* 1999). This may be due to the strong vasoconstrictive effect of ET-1 limiting coronary flow and *in vivo* increasing afterload (Beyer *et al.* 1999). Both ET_A and ET_B receptors are located in fibroblasts and cardiomyocytes (Table 1) (Fareh *et al.* 1996, Kedzierski & Yanagisawa 2001), with the predominance of ET_A receptor subtype (approximately 90%) in human and rodent ventricles (Molenaar *et al.* 1993, Sakai *et al.* 1996a, Serneri *et al.* 2000). The ET_A receptor subtype has been shown to account mostly for the positive inotropic action of ET-1 (Kelso *et al.* 2000, Takeuchi *et al.* 2001).

The intracellular events leading to the positive inotropic effect of ET-1 differ clearly from that of β-adrenergic agonists, since rather a decrease than an increase is noted in intracellular cAMP concentration (Hilal-Dandan *et al.* 1992). In fact, ET-1 is able to antagonize β-AR agonist induced increase in cAMP (James *et al.* 1994, Ono *et al.* 1994). G-protein coupled pathways activated by ET-1 lead to PLC mediated phosphoinositide hydrolysis and accumulation of DAG and IP_3 (Takanashi & Endoh 1991, Hilal-Dandan *et al.* 1992, Jones *et al.* 1992, Endoh *et al.* 1998). DAG leads to inotropic response through

activation of PKC and L-type Ca^{2+} channels (Lacerda *et al.* 1988, Kelly *et al.* 1990, Wang *et al.* 1993, Pi *et al.* 1997). PKC is also able to phosphorylate and thus activate sarcolemmal NHE, inducing intracellular alkalinization (Krämer *et al.* 1991, Pi *et al.* 1997), and possibly activation of NCX in its reverse mode due to the small increase in $[Na^+]_i$ (Perez *et al.* 2001) (Fig. 3.). Also increased $[Ca^{2+}]_i$ through either reverse mode NCX or L-type Ca^{2+} channels occurs in response to ET-1 stimulus (Ballard & Schaffer 1996). An increase in both $[Ca^{2+}]_i$ and especially in Ca^{2+}-responsiveness of the contractile element has been reported with ET-1, the increase in Ca^{2+} responsiveness dominating (Wang *et al.* 1991, Yang *et al.* 1999). Thus, for a given increase in contractile strength to occur, the increase in $[Ca^{2+}]_i$ with ET-1 remains lower than with β-AR agonist isoproterenol (Endoh *et al.* 1998). Interestingly, it seems that ET-1 may modulate chemomechanical conversion efficiency, so that the rate of oxygen consumption decreases compared to contractile force (Takeuchi *et al.* 2001). ET-1 was found to raise isometric force and simultaneously decrease actomyosin Mg^{2+}-ATPase activity, indicating that force is developed with a lower rate of ATP hydrolysis and therefore with greater economy (McClellan *et al.* 1996, Takeuchi *et al.* 2001). The reduction in ATPase activity was progressively enhanced, as sarcomere length was increased. Intracellular alkalinization due to NHE activation could partially account for the increase in Ca^{2+}-responsiveness, but it does not seem to affect the efficiency of contraction (McClellan *et al.* 1996, Takeuchi *et al.* 2001). Taken together, it seems that ECs may balance the rate of work performed by the heart with the rate of energy supplied to the heart by a mechanism involving ET-1 secretion (McClellan *et al.* 1994, Winegrad 1997).

2.2.1.5 Hypertrophic effects of endothelin-1 on the heart

ET-1 is a potent hypertrophic agonist on cardiac myocytes (Ito *et al.* 1991), and it partially mediates LVH induced by aortic banding or myocardial infarct in rats (Ito *et al.* 1994a, Sakai *et al.* 1996a). However, there are also studies showing no effect with ET-1 antagonists on LVH (Oie *et al.* 1998), showing that in some conditions ET-1 is not obligatory for LVH development. Blocking ET_A and ET_B receptors could prevent induction of early genes in rat atria, but not in ventricle in response to acute pressure overload (Magga *et al.* 1997a). Exogenous ET-1 induces the expression of the early genes such as B-type natriuretic peptide (BNP) and *c-fos* in isolated rat heart (Magga *et al.* 1998a) and in cultured rat cardiomyocytes (Neyses *et al.* 1993, Liang & Gardner 1998). The hypertrophic effect *in vivo* is further augmented by the vasoconstrictive effect, which increases afterload (Yanagisawa *et al.* 1988). The activation of aldosterone synthesis and secretion via ET_B receptors may contribute to hypertrophic and especially fibrotic effects of ET-1 (Wada *et al.* 1997). Furthermore, ET-1 also plays a role in norepinephrine and Ang II induced end-organ damage in experimental *in vivo* models of LVH (Kaddoura *et al.* 1996, Müller *et al.* 2000). The signaling mechanism responsible for hypertrophic actions include G_q mediated pathway, leading to phosphoinositide hydrolysis, PKC activation and activation of MAP kinases (for review, see Sugden & Clerk 1998), eventually leading to changes in gene expression and cardiac function and structure (Shubeita *et al.* 1990, Douglas & Ohlstein 1997) (Fig. 3). Increased plasma levels of ET-1

as well as activation of cardiac ET system are seen in LVH and CHF (Stewart *et al.* 1992, McMurray *et al.* 1992, Serneri *et al.* 1999, Schunkert *et al.* 1999). Furthermore, ET-1 plasma levels correlate with the prognosis and symptoms in patients with heart failure (Wei *et al.* 1994, Pacher *et al.* 1996) and coronary artery disease (Salomone *et al.* 1996). In human dilative cardiomyopathy both ventricular ET-1 peptide concentration and ET_A receptor binding are elevated (Pieske *et al.* 1999a). Since ET-1 may initiate hypertrophic changes similar to those in human LVH and CHF, these findings support the hypothesis that ET-1 participates in the pathogenesis of these diseases.

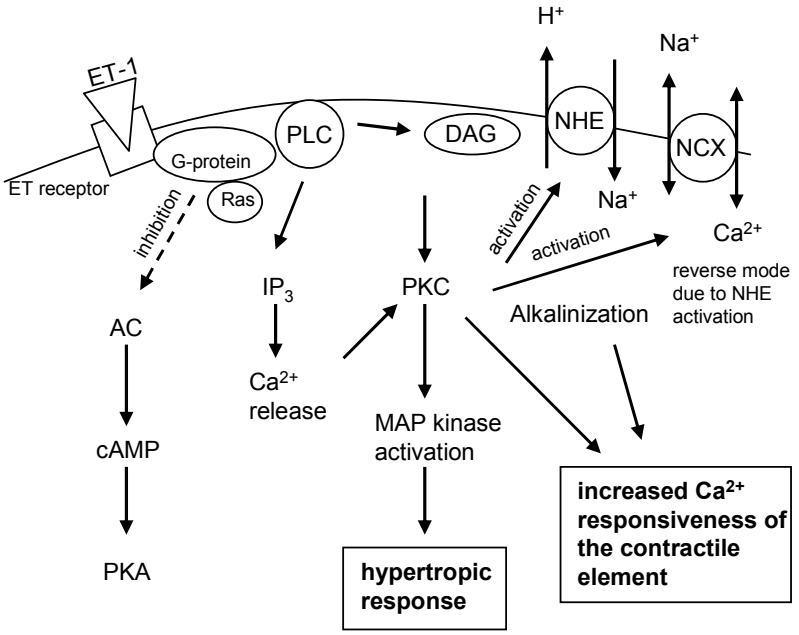

Fig. 3. An overview of cellular events leading to enhanced contractility and hypertrophic response after exposure to ET-1 in cardiomyocytes. AC, adenylyl cyclase; DAG, diacylglycerol; IP_3, inositol-1,4,5-triphosphate; MAP kinase, mitogen activated protein kinase; NCX, Na^+-Ca^{2+} exchanger; NHE, Na^+-H^+ exchanger; PKA, protein kinase A; PKC , protein kinase C; PLC, phospholipase C. Ballard & Schaffer 1996, Shigekawa & Iwamoto 2001 and Takeuchi *et al.* 2001.

2.2.1.6 The role of endothelin-1 in the pathophysiology of the cardiovascular system

The results with ET receptor antagonists in experimental CHF have been promising. Sakai et al. (1996a) reported that treatment with ET_A antagonist BQ-123 improved survival and left ventricular function and also prevented remodeling after experimental myocardial infarction in rats. Thereafter, a large number of ET receptor antagonists have been synthesized and described (for review, see Lüscher & Barton 2000). Based on a large body of evidence, it is likely that ET-1 plays a role in progression of the CHF

(Clozel *et al.* 1993, Spinale *et al.* 1997, Iwanaga *et al.* 1998). A large number of studies have found ET antagonism useful in experimental LVH and CHF (for reviews, see e.g. Yazaki & Yamazaki 1997, Kedzierski & Yanagisawa 2001). ET receptor antagonists protect from hypertensive end organ damage independently of blood pressure attenuation in spontaneously hypertensive rats (SHR) (Karam *et al.* 1996). Favorable hemodynamic responses have been described in human CHF (Cowburn *et al.* 1998), although rather similar responses have been reported with non-specific vasodilation, as in response to a warm bath or sauna (Tei *et al.* 1995). A recent study showed that cardiomyocyte-specific knockout of the ET-1 gene was sufficient to reduce hypertrophic response to aortic banding in mice with intact EC ET-1 gene (Miyauchi *et al.* 2001).

However, Sakai *et al*. (1996b) have also reported that ET-1 helps to maintain cardiac contractility after experimental myocardial infarction, since BQ-123 decreased contractile parameters such as left ventricular (LV) maximal positive derivative of intraventricular pressure ($+dP/dt_{max}$). In another study (Nguyen *et al.* 2001), increased mortality and impaired cardiac function were associated with ET receptor antagonist treatment in experimental heart failure. A recent study in humans with high-dose $ET_{A/B}$ antagonist bosentan was discontinued due to non-cardiovascular side effects (liver toxicity). Furthermore, the analysis of the results showed that initiation of the treatment with $ET_{A/B}$ antagonist bosentan is accompanied by an increased number of events leading to worsening of the clinical status (Mylona & Cleland 1999).

Taken together, it has been demonstrated that ET-1 plays a role in the pathophysiology of LVH and CHF, but there have been difficulties involved with the clinical studies. Still it seems possible that ET antagonists might contribute to the treatment of CHF, if the correct dosing and the scheduling of treatment could be found with well-tolerated compounds.

2.2.2 Angiotensin II

In 1898, Tigerstedt and Bergman made the original observation of the vasoactive substance secreted by kidney (Tigerstedt & Bergman 1898). The substance was named renin. Later the humoral renin angiotensin system (RAS) was characterized (for review, see Basso & Terragno 2001). The most important effector peptide of the RAS is Ang II. The biosynthesis occurs from angiotensinogen, which is cleaved by renin to form Ang I. Ang I is normally rapidly converted to the octapeptide Ang II, which is a much stronger vasoconstrictor, by angiotensin converting enzyme (ACE) and also probably by other enzymes, such as chymase (Balcells *et al.* 1997). Ang II induces vasoconstriction and LVH through stimulation of AT_1 receptors (Peach 1977). The rodent AT_1 receptor has 2 subtypes: AT_{1A} and AT_{1B} (Chiu *et al.* 1989). The role of AT_2 receptors is not clear, since a role opposing the actions of AT_1 was initially suggested (van Kesteren *et al.* 1997). Yet recently it has also been implicated in hypertrophic process in heart (Ichihara *et al.* 2001). The classical concept of RAS as a primarily humoral system has been revised since local RAS has been discovered in various tissues, including blood vessels, heart, adrenals and the brain (Dzau *et al.* 1987, Paul *et al.* 1993, Dostal & Baker 1999, De Mello & Danser 2000). It is though possible that the renin for the cardiac RAS may be derived from blood

(Lindpaintner *et al.* 1990, Müller *et al.* 1998). Ang II affects blood pressure and fluid and electrolyte homeostasis by various mechanisms: it increases vascular tone by contraction of vascular smooth muscle, but it also increases aldosterone biosynthesis, Na^+ reabsorbtion in kidney, water intake, and cellular growth of both VSMCs and cardiomyocytes (Peach 1977, Dostal *et al.* 1997). Ang II induces catecholamine synthesis (for review, see Paul & Ganten 1992), and adrenal glands seem to be involved in Ang II induced end organ damage (Ratajska *et al.* 1994, Földes *et al.* 2001).

The exogenous Ang II has a direct positive inotropic effect on rabbit isolated perfused heart via AT_1 receptors (Ishihata & Endoh 1993), but no inotropism was noted in dog, rat or ferret heart (Ishihata & Endoh 1995). A positive chronotropic effect has been reported as well (Allen *et al.* 1988). In cat papillary muscles and isolated myocytes, a positive inotropic effect and negative lusitropic effect has been described (Salas *et al.* 2001). Others have reported a negative inotropic effect in isolated perfused rat hearts, probably due to coronary vasoconstriction (Traquandi & Riva 1998). It has also been suggested that the effect of Ang II on inotropy may be dependent on the baseline loading of the heart (Li *et al.* 1994). The possible mechanisms of the contractile effects include acceleration of the phosphoinositide hydrolysis, with possible inter-species differences in down-stream signal-transduction (Ishihata & Endoh 1995). In cat, PKC induced increase in $[Ca^{2+}]_i$ was found to mediate positive inotropic effect (Salas *et al.* 2001). Interestingly, Alvarez *et* al. (1999) suggested that Ang II is the factor released in response to stretch, inducing ET-1 release and contributing to the slow force response in papillary muscles.

The hypertrophic effect of Ang II on cardiomyocytes and VSMCs has drawn considerably more attention than the modest inotropic effect. The role of Ang II as a mediator of LVH in response to stretch or overload has been shown in numerous experimental models (for review, see Dostal & Baker 1999), and the effect seems to be present also *in vitro*, when blood pressure changes can be excluded (Sadoshima *et al.* 1993, Schunkert *et al.* 1995, Liang & Gardner 1998). Increased Ang II is able to produce cardiac hypertrophy *in vivo*, as shown by dTG rats expressing human renin and angiotensinogen genes (Ganten *et al.* 1992, Bohlender *et al.* 1997, Luft *et al.* 1999), or AT_1 receptor overexpressing TG mice (Paradis *et al.* 2000). There is also *in vivo* evidence for blood pressure independent hypertrophic effect of Ang II in TG mice (Mazzolai *et al.* 1998) and in dTG rats (Mervaala *et al.* 2000). Additionally, administration of Ang II *in vivo* induces fibrosis and scarring, and increases ventricular wall stiffness (Ratajska *et al.* 1994). Furthermore, it seems that inflammatory processes play a role in Ang II induced end-organ damage (Mervaala *et al.* 2000). Still, it does seem that Ang II is not obligatory for development of LVH in response to hemodynamic overload, since AT_{1A} knock-out mice showed normal hypertrophic response to pressure overload (Harada *et al.* 1998). Acute ventricular activation of early response genes such as BNP and *c-fos* in response to hemodynamic load *in vivo* and by elevated systolic wall stress in isolated heart are not dependent on Ang II (Magga *et al.* 1997a, Thienelt *et al.* 1997). In various models of LVH, the total AT receptor number is elevated, while the AT_1/AT_2 ratio remains unaffected (Swynghedauw 1999).

The mechanism for the direct hypertrophic effect of Ang II has been suggested to include activation of G-protein coupled AT_1 receptors leading to activation of PLC and consequent formation of IP_3 and DAG. Also $[Ca^{2+}]_i$ is increased, and tyrosine kinase and MAP kinase are activated by phosphorylation (Thomas *et al.* 1996). Also activation of

janus kinase (JAK)/signal transducers and activators of transcription (STAT) -pathway appears to contribute to the hypertrophic response (Kodama *et al.* 1998). The growth promoting action of Ang II appears to be partially mediated by autocrine and paracrine factors, such as platelet derived growth factor, basic fibroblast growth factor and also ET-1 (Cottone *et al.* 1998). In isolated cardiac myocytes Ang II has been shown to stimulate ET-1 synthesis and secretion, and to induce a hypertrophic response through activation of ET system (Ito *et al.* 1993, Liang & Gardner 1998). ET-1 seems to contribute to Ang II induced effects also *in vivo*, since Ang II induced changes have been successfully treated with ET antagonists (Müller *et al.* 2000, Moreau *et al.* 1997). Also Ang II induced free oxygen radical production seems to play a role in hypertrophic effect of Ang II on cardiac myocytes (Nakamura *et al.* 1998). Both ACE inhibitors and AT_1 antagonists have shown their usefulness in the treatment of experimental CHF and LVH (Kojima *et al.* 1994, Richer *et al.* 1999), and both of them have also been proven useful in treatment of human CHF (Pfeffer *et al.* 1992, Pitt *et al.* 1997).

Pointing out the importance of interplay between different vasoactive peptides, combined treatment with both $ET_{A/B}$ and AT_1 antagonists inhibited deteriosation of myocyte function as well as neurohumoral activation more effectively than either of the antagonists alone in rapid pacing induced CHF in pigs (New *et al.* 2000). Similar results were also seen in Dahl salt-sensitive rats with LVH (Iwanaga *et al.* 2001).

2.2.3 Adrenomedullin

AM was originally discovered in 1993. It consists of 52 amino acids, which form one intramolecular disulfide bond (Kitamura *et al.* 1993a). AM belongs to the calcitonin gene related peptide gene superfamily. AM peptide and mRNA have been detected in various tissues since the original discovery from pheocromocytoma cells. Highest levels of immunoreactive (ir)-AM were found in human and rat adrenal medulla, but also plasma, lung, kidney, heart atrium and gastrointestinal system showed significant concentrations (Kitamura *et al.* 1993a, Ichiki *et al.* 1994, Washimine *et al.* 1995). Also AM mRNA can be found in adrenal medulla, ventricular myocardium, lung and kidney (Kitamura *et al.* 1993b). Cultured ECs, cardiac myocytes and VSMCs reportedly produce AM (Sugo *et al.* 1994a, Horio *et al.* 1998).

Physiologically, AM has been found to elicit a hypotensive effect due to reduction in vascular resistance when infused into rat (Kitamura *et al.* 1993a), sheep (Charles *et al.* 1997) or human (Lainchbury *et al.* 2000) circulation. Part of the vasorelaxing effect was lost in endothelium-denuded arteries and by treatment with N^{ω} –nitro-methyl ester (L-NAME) (Yang *et al.* 1996). AM has also diuretic and natriuretic actions (Ishiyama *et al.* 1993). Studies have also described an increase in heart rate, cardiac output and indices of contractility to associate with vasodilation during systemic administration, effects independent of sympathetic reflexes (Parkes & May 1997, Lainchbury *et al.* 2000). AM was shown to induce a potent positive inotropic effect also in isolated perfused rat heart preparation (Szokodi *et al.* 1996). The effect was independent of cAMP formation (Szokodi *et al.* 1998), although AM and its receptor were primarily shown to increase intracellular cAMP (Kitamura *et al.* 1993a, Kapas *et al.* 1995). The intracellular signaling

in response to AM is not clear. A decrease in intracellular Ca^{2+} appears to occur in VSMCs (Kureishi *et al.* 1995), in contrast to the increase in intracellular Ca^{2+} provoked by ET-1 in these cells (Hirata *et al.* 1988). In cardiomyocytes, a role for sarcoplasmic reticulum Ca^{2+} release and PKC in AM induced inotropy has been suggested (Szokodi *et al.* 1998). Taken together, these cardiovascular effects of AM suggest a possible role as a autocrine/paracrine regulator of the cardiovascular system (for review, see Samson 1999, Jougasaki & Burnett, Jr. 2000).

In the failing heart, AM secretion is known to increase (Jougasaki *et al.* 1996). AM plasma concentrations increase in certain disease states such as hypertension and CHF (Kitamura *et al.* 1994, Jougasaki *et al.* 1996). In experimental myocardial infarction, AM mRNA was found to increase by 40% at 4 weeks (Kaiser *et al.* 1998). Aortic banding increased the gene expression by 25% at 24 hours, but after that the increase could not be observed, while the increase in ir-AM persisted, showing correlation with the level of LVH development (Morimoto *et al.* 1999). In VSMCs, lipopolysaccharide, interleukin-1 and tumor necrosis factor α are powerful inducers of AM gene expression (Sugo *et al.* 1995a). In cultured neonatal rat cardiac myocytes and non-myocytes, these same factors also increase AM gene expression (Horio *et al.* 1998). Also mechanical stretch and Ang II increase AM mRNA levels in cell culture (Tsuruda *et al.* 2000), but the stretch induced increase was not blocked by CV-11974, an AT_1 receptor antagonist. In adult rat ventricular myocardium AM gene expression as well as plasma AM are increased by pressure load produced by vasopressin or Ang II infusion (Romppanen *et al.* 1997, Földes *et al.* 2001). Also ET-1 increased AM synthesis in left ventricle of isolated heart (Magga *et al.* 1998a). The increase of AM mRNA by hemodynamic overload was unaffected by AT_1 or $ET_{A/B}$ antagonists *in vivo* (Romppanen *et al.* 2001). Baseline AM gene expression was not altered in hypertensive TG rats expressing mouse renin gene, but the response to vasopressin infusion was blunted (Romppanen *et al.* 1997). In isolated, perfused rat heart, loading the hearts with increasing coronary flow based on the Gregg (See section 6.4) effect was found to increase contractility and left ventricular BNP mRNA, while AM mRNA levels decreased abruptly (Magga *et al.* 1998a).

A previous cell culture study suggested a growth inhibitory effect of AM, since it was able to inhibit Ang II -stimulated [^{14}C]-phenylalanine incorporation (Tsuruda *et al.* 1998). AM was also able to inhibit Ang II induced increases in atrial natriuretic peptide (ANP) and BNP gene expression (Luodonpää *et al.* 2001). Recently, AM was described to protect against cardiovascular damage such as perivascular fibrosis and coronary artery intimal hyperplasia induced by a high salt diet and Ang II in mice (Shimosawa *et al.* 2002).

2.2.4 Nitric oxide

In 1987 the free radical gas NO was described as possessing the characteristics of the substance that had previously been known as endothelium-derived relaxing factor (Palmer *et al.* 1987). Earlier, vasodilation in response to acetylcholine was shown to be mediated by a very labile non-prostanoid endothelium-derived relaxing factor (EDRF) or

factors, stimulating guanylate cyclase of the vascular smooth muscle, with the resulting increase in cGMP activating relaxation (Furchgott & Zawadzki 1980).

NO is produced by enzymes known as NO synthases (NOS), which catalyze the formation of NO from the amino acid L-arginine. The three NOS isoforms that have been described are each a product of a separate gene and share over 50% amino acid homology. All the isoforms are present in the heart: NOS1 (nNOS, "neuronal" NOS) has been detected in conduction tissue and intracardiac neurons; NOS2 (iNOS, "cytokine-inducible" and "Ca^{2+} insensitive" NOS) can be expressed in all cell types in the heart; NOS3 (eNOS, "endothelial-constitutive" NOS) is expressed in endothelium, endocardium and cardiomyocytes (for review, see Kelly *et al.* 1996).

NOS3 is located in caveolae, and it forms a complex with caveolin, and is thus attached to plasma membrane (Kelly *et al.* 1996). The complex is disrupted by Ca^{2+}/calmodulin, which activates NOS3 (Feron *et al.* 1998). However, also activation by phosphorylation is possible (Butt *et al.* 2000). NOS expression and activity can be regulated by various stimuli (for review, see Fleming & Busse 1999). Fluid shear stress exerted on the endothelium is the major stimulus for continuous NO production *in vivo* (Lamontagne *et al.* 1992). Basal generation of NO by NOS3 plays an important role in the regulation of basal vascular tone, blood pressure, and tissue perfusion, and in NOS3 knockout mice EDRF activity is absent and the mice are hypertensive (Huang *et al.* 1995). They also have a higher mortality rate, depressed left ventricular function and increased rate of remodeling after myocardial infarct (Scherrer-Crosbie *et al.* 2001). In humans, abnormalities in endothelial production of NO accompany atherosclerosis and hypertension (Fleming & Busse 1999).

In failing human LV, NOS2 mRNA was described to increase approximately 3-fold and NOS3 mRNA to decrease by 70% compared to normal levels, and the enhanced cardiac NO production by NOS2 was suggested to contribute to diminished β-AR responsiveness in failing hearts (Drexler *et al.* 1998). NOS2 knockout mice survived better after myocardial infarct, and cardiac function after the infarct was also better than in wild-type littermates (Sam *et al.* 2001). Very high levels of NO have been suggested to lead to peroxynitrite formation, thus impairing cardiac function (for review, see Paulus & Shah 1999). The deleterious role of increased NOS2 in LVH may relate to a NO·mediated defect in mitochondrial function in the hypertrophied heart (Dai *et al.* 2001). Contrasting with this hypothesis, a recent report showed that very high cardiac overexpression of NOS2 (260-fold) induced only a mild cardiac dysfunction in mice (Heger *et al.* 2002).

Conflicting results on the inotropic effect of NO from either endogenous or pharmacological sources have been obtained. With intracoronary NOS inhibitor, Cotton *et al*. (2001) found a small decrease (-14%) in LV $+dP/dt_{max}$ in normal subjects and no change in LV $+dP/dt_{max}$ in cardiomyopathy patients, despite myocardial expression of NOS2 in those patients. A recent study showed no effect on baseline contractility of isolated rat heart with infusion of NOS inhibitor L-NAME, but maximal inotropic response to ET-1 was augmented with the administration of L-NAME (Kinnunen *et al.* 2000). Previously, a negative inotropic effect for NO has been suggested with intracoronary infusion of the NO donor substance sodium nitroprusside, with an increased rate of relaxation and improved diastolic distensibility with intracoronary infusion in normal subjects (Paulus *et al.* 1994). The discrepancy may relate not only to

different effects of NOS inhibitors and NO donors, but also to the different actions of low and high doses of NO in the heart. Administration of low concentrations of NO was reported to induce a positive inotropic effect, while with higher concentrations a negative inotropic effect in isolated cat papillary muscle preparation was seen (Mohan *et al.* 1996, Kojda *et al.* 1996).

The effects of NO in regulation of cardiomyocyte function may be mediated either by cGMP or by other mechanisms, such as nitrosylation (Xu *et al.* 1998). The main effector molecules are protein kinase G and cGMP inhibited cAMP phosphodiesterase (Kojda & Kottenberg 1999). Calderone *et al.* (1998) reported that NO was able to antagonize growth promoting effects of norepinephrine via induction of cGMP pathway. An NO-mediated increase in Ca^{2+} transient through RyRs has been reported with myocardial stretch (Ruwhof *et al.* 2001, Petroff *et al.* 2001). Basal release of NO contributes to the Frank-Starling response in isolated ejecting guinea pig hearts by a mechanism probably involving enhancement of diastolic distensibility (Prendergast *et al.* 1997b, Paulus *et al.* 1994). NO does not seem to have much effect on force-frequency relationship in isolated muscle strips of human heart (Cotton *et al.* 2001).

2.2.5 Other paracrine mediators

In addition to ET-1 and Ang II, there are also a number of other locally acting mediators which could contribute to load induced alterations in cardiac structure and function. Arginine vasopressin (AVP), a potent antidiuretic, vasoconstricting and growth stimulating peptide, has traditionally been thought to be synthesized and secreted by hypothalamo-neurohypophysial system, and AVP infusion has been used as a model of acute pressure overload (Berne & Levy 1993, Magga *et al.* 1994). Recently, vasopressin mRNA was found in rat left ventricles, and mRNA levels were greatly elevated after 2-hour stimulation with increased wall stress in isolated, perfused hearts (Hupf *et al.* 1999). Vasopressin concentration in the coronary effluent of loaded hearts was increased, and vasopressin V_1 receptor antagonist was able to prevent the spontaneous increase in coronary perfusion pressure during experiments, further suggesting a role for local vasopressin synthesis during cardiac overload (Hupf *et al.* 1999).

The mineralocorticoid aldosterone is mainly secreted by the adrenal cortex, in response to various stimuli, e.g. Ang II, and it promotes the retention of sodium and loss of potassium, activates the sympathetic nervous system and myocardial and vascular fibrosis, and causes baroreceptor dysfunction (See e.g. Berne & Levy 1993). There is also evidence suggesting aldosterone as well as corticosteroid production from locally present substrate in isolated perfused hearts under stimulation with Ang II or adrenocorticotropin (Silvestre *et al.* 1998). The authors also demonstrated the expression of the genes of the terminal enzymes of aldosterone and corticosterone synthesis, 11β-hydroxylase- and aldosterone synthase, respectively, in adult rat hearts. Furthermore, it was recently reported that patients with left ventricular systolic or diastolic dysfunction but not control patients present increased cardiac secretion of aldosterone (Mizuno *et al.* 2001). Even before these findings, it was shown that patients with congestive heart failure gained

advantage from treatment with spironolactone, an aldosterone receptor antagonist (Pitt *et al.* 1999).

Also prostaglandin $F_{2\alpha}$ has been suggested to have a role in LVH, since it stimulates hypertrophic growth of cultured neonatal rat ventricular myocytes *in vitro* (Adams *et al.* 1996), and cardiac growth *in vivo* (Lai *et al.* 1996). Its concentration was elevated in rabbit hearts in response to acute pressure overload created by aortic stenosis (Chazov *et al.* 1979). The receptor of prostaglandin $F_{2\alpha}$ couples to phosphoinositide metabolism via G_q (Ito *et al.* 1994b), suggesting a possible mechanism for hypertrophic effect. However, it is currently not known whether cyclo-oxygenase inhibitors can affect load induced LVH (Sugden & Clerk 1998).

Among other possibly contributing factors are insulin-like growth factors (Serneri *et al.* 1999), myotrophin (Sen *et al.* 1990), basic fibroblast growth factor (Kaye *et al.* 1996), transforming growth factor-β and vascular endothelial growth factor (Li *et al.* 1997) and cardiotrophin-1, a cytokine, which has been linked to eccentric hypertrophy similar to the volume-overload induced LVH *in vivo* (Sugden & Clerk 1998).

2.3 Changes in cardiac gene expression and structure in response to increased load

When cardiac load increases, there is a rapid increase in contractile strength as previously mentioned, which is accompanied by increased secretion of various autocrine/paracrine peptides, such as Ang II and ET-1 (Sadoshima *et al.* 1993, Yamazaki *et al.* 1996) (Fig. 4). These factors then contribute to the cellular changes and adaptation to load. Studies with TG mice lacking functional G_q have further confirmed the role of G_q signaling in the development of LVH (Akhter *et al.* 1998). However, the "dominant-negative" G_q was not able to completely abolish the ventricular hypertrophy, and thus it is likely that other pathways (e.g. other G-proteins, mechanical stress itself, tyrosine kinase coupled receptors) play an important role in the development of LVH (Akhter *et al.* 1998). The rapid changes in cardiac function in response to load are mainly mediated by modification of target molecules, such as contractile element and Ca^{2+} handling proteins. In the long term mechanical load leads to changes in the gene expression pattern and structure of the heart (for reviews, see Sadoshima & Izumo 1997, Lorell & Carabello 2000, Tavi *et al.* 2001). Initially left ventricular hypertrophy may serve as an adaptative response to pressure overload, decreasing wall stress and increasing contractile force (Fig. 4) (Strömer *et al.* 1997, Nakamura *et al.* 2001). Pressure overload leads to increased deposition of sarcomeres in parallel and increased wall thickness, thus allowing the heart to adapt to the demand of greater pressure generation and decreasing wall stress. Volume load leads to different phenotype, whereby chamber volume and cardiomyocyte length is increased (for review, see Swynghedauw 1999).

Eventually, the hypertrophic compensation may lead to abnormal contractile performance per unit mass of myocardium (for review, see Cooper 1997), and thus increased load may result in cardiac failure, which has emerged as a major cause of mortality and morbidity in western countries (O'Connell & Bristow 1994). Also LVH itself has been demonstrated to be a risk factor of cardiovascular events (Levy *et al.*

1990), suggesting that normalization of wall stress by LVH may not be beneficial in the long term. In a recent study with two lines of TG mice (G_q disrupted- and dopamine ß-hydroxylase knockout) it was found that these TG mouse lines had no LVH in response to pressure overload by transverse aortic constriction. Despite the increased wall stress the cardiac function was better than in non-transgenic (NTG) littermates (Esposito *et al.* 2002). However, both of the genetic manipulations affect neurohumoral compensatory systems which have been implicated in the impairment of cardiac function during development of CHF.

The most common causes of LVH and CHF in humans are remodeling following myocardial infarction and continuously elevated blood pressure, but LVH may also be observed in certain inherited diseases (e.g. hypertrophic cardiomyopathies). It has been generally accepted that increase in cardiac size primarily occurs through cell hypertrophy instead of hyperplasia, even though cell divisions have been reported in cardiac myocytes (for review, see Swynghedauw 1999) Also migration of stem cells from other origins to the heart has been suggested to occur under some conditions (Quaini *et al.* 2002).

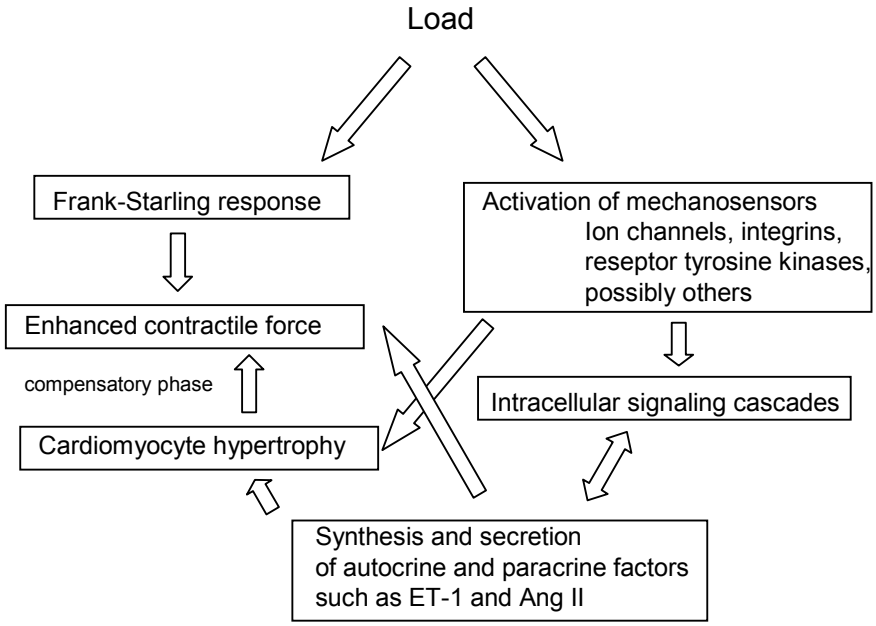

Fig. 4. A simplified summary of cardiac response to load leading to enhanced contractile force and hypertrophy. Modified from Crozatier 1996 and Perez *et al.* 2001.

2.3.1 Mechanotransduction

The process of mechanotransduction, i.e. the coupling of mechanical forces on cells to biological responses, has been studied intensively during the last few years. Many of the molecular events accompanying the increased cardiac stress have been described, but the

question of the primary mechanosensors still remains unanswered. There are a few mechanisms which have been suggested to account for the primary intracellular signal in response to load (for review, see Sadoshima & Izumo 1997, Tavi *et al.* 2001). First, a number of stretch-activated ion channels have been found using the single-channel, patch-clamp technique. Stretch-activated channels in the heart are sensitive to blockade with Gd^{3+}, but the results with Gd^{3+} have been conflicting. Gd^{3+} had no effect on stretch-induced c-*fos* expression or stretch-induced increase in the rate of protein synthesis (Sadoshima *et al.* 1992). In another study Gd^{3+} prevented stretch induced increase in atrial BNP mRNA levels (Laine *et al.* 1996).

Secondly, the cytoskeleton and the integrins are another possible mechanotransducer. Integrins are heterodimeric transmembrane receptors that couple the extracellular matrix components, mainly basement membrane, with the actin cytoskeleton (Sadoshima & Izumo 1997). Cytoplasmic domain of the ß-integrin interacts not only with actin-binding proteins, but also with the amino-terminal domain of focal adhesion tyrosine kinase (FAK), which interacts further with various signaling molecules (Parsons 1996). In a recent study, increases of diastolic pressure rapidly increased FAK tyrosine phosphorylation in isolated rat hearts (Domingos *et al.* 2002). Besides integrins, also extracellular matrix proteins ligated to the integrins, such as collagens, laminin, fibronectin and vitronectin, may play a role in the signal transduction (Ruwhof & van der Laarse 2000).

Thirdly, receptor type tyrosine kinases have transmembrane segments, and some of the nonreceptor-type tyrosine kinases are anchored to the inner surface of cell membranes through their N-terminal myristoylation site (Sadoshima & Izumo 1997). Thus it is possible that stretch directly induces conformational changes causing activation of the kinases. In support of this mechanism, stretch–induced *c-fos* gene induction was inhibited by tyrosine kinase inhibitor (Sadoshima & Izumo 1993). In isolated rat hearts, lavendustin A, an inhibitor of protein tyrosine kinases, decreased atrial wall stretch-induced ANP and BNP secretion (Taskinen *et al.* 1999). Furthermore, an increase in tyrosine phosphorylation can be observed as early as five seconds after application of mechanical stress by hypotonic fluid-induced cell swelling (Sadoshima *et al.* 1996).

Mechanotransduction is accompanied by increased activity of at least two major intracellular signaling systems affecting the gene expression pattern, known as MAP kinase pathway (Sugden & Clerk 1998) and Ca^{2+}/calmodulin activated protein kinases/phosphatases involving calcineurin (Molkentin *et al.* 1998). The level of intracellular Ca^{2+} not only defines contractile function but also acts as a second messenger and is able to control a number of cellular functions (for review, see Tavi *et al.* 2001). A recent study suggested that in cell culture systolic strain (a model of pressure overload) activated the MAP kinase pathway more effectively than diastolic strain (a model of volume overload) (Yamamoto *et al.* 2001). This has been suggested as a possible mechanism differentiating between concentric and eccentric hypertrophy.

2.3.2 Cardiac gene expression response to load

The changes in cardiac gene expression leading to structural alterations may be classified as rapid induction of proto-oncogenes (such as *c-fos*, *c-jun*, *c-myc* and Egr-1), heat-shock protein genes (hsp70) and BNP, which is followed by quantitative and qualitative changes in gene expression of other genes and changes in protein synthesis (for reviews, see Sadoshima & Izumo 1997, Magga *et al.* 1998b, Ruwhof & van der Laarse 2000). The cellular events in the development of hypertrophic response have been studied with several models, including cell culture, isolated heart models and various *in vivo* models. Ventricular myocytes in different models of persistent hypertrophy show genetic reprogramming and re-expression of several embryonic genes including ANP (Table 2) (Izumo *et al.* 1988, Ruskoaho *et al.* 1989, Marttila *et al.* 1996). In experimental conditions, the level of ANP mRNA in left ventricle starts to increase a few hours after the initiation of loading, and remains high thereafter (for review, see Ruskoaho 1992). ANP mRNA levels remain increased during transition to failure (Boluyt *et al.* 1994). BNP mRNA levels are increased rapidly upon stretch or increased wall stress in both atria and ventricles (Tokola *et al.* 2001). Also expression of several genes that encode sarcomeric proteins is changed, for instance β-myosin heavy chain (β-MHC) and skeletal α-actin mRNA levels increase, while cardiac α-actin mRNA levels decrease (Schwartz *et al.* 1986). Also NHE mRNA levels are increased during cardiac overload (Takewaki *et al.* 1995). Interestingly, a recent study showed that the NHE inhibitor cariporide was able to prevent LV remodeling induced by coronary artery ligation in rats (Kusumoto *et al.* 2001). There are also significant alterations in calcium handling proteins in LVH. The SERCA2a mRNA levels tend to decrease, while NCX mRNA is increased (Flesch *et al.* 1996). Also PLB mRNA levels decrease in failing hearts (Swynghedauw 1999). The alteration in the balance of Ca^{2+} removal from the cytoplasm towards the direction of the extracellular space instead of the SR is likely to have serious functional consequences in the long term, since the economy and velocity of NCX system is lower (Bers 2000). Additionally, also extracellular matrix undergoes alterations during the process of hypertrophy. The expression of major collagen forms I and III and also collagen XV is increased (Weber 1989, Piuhola J., Eklund L., Pihlajaniemi T., Ruskoaho H. Unpublished observation) (See also section 2.5.). In addition, several changes in isogene expression of proteins involved in energy metabolism have been described (Ruwhof & van der Laarse 2000).

As a conclusion, the regulation of cardiomyocyte growth and function is a delicate process involving a large number of factors. Therefore, it is not surprising that one-gene knockout models easily fail to prevent the structural changes induced by load. In agreement with this, antagonizing multiple neurohumoral compensatory mechanisms has proven valuable in the treatment of CHF (Hunt *et al.* 2001).

Table 2. Summary of ventricular genes induced in response to cardiac load.

Immediate early genes	Intermediate response genes	Late response genes
BNP	Angiotensinogen	Cardiac α-actin
c-fos	AM	NCX
c-jun	ANP	IP_3 receptor
c-myc	β-MHC	ACE
Egr-1	Collagen III	Collagen I
Hsp70	preproET-1	Collagen XV
	ET_A receptor	
	MLC-2	
	NHE	
	Skeletal α-actin	

Expression of a number of other genes as well is regulated during stress response. Immediate early genes show increased mRNA levels as early as 1 hour after initiation of load, while others take several hours or even days before induced expression is seen. See text for details. Modified from Lee *et al.* 1988a, Lindpaintner *et al.* 1990, Takewaki *et al.* 1995, Flesch *et al.* 1996, Crozatier 1996, Schunkert *et al.* 1999, Swynghedauw 1999 and Dostal & Baker 1999.

2.4 Natriuretic peptide system

Even though the first observations of secretory granules in atrial tissue date back to 1956 (Kisch 1956), the function of these granules and the heart as an endocrine organ only began to be understood more recently. de Bold *et al*. (1981) demonstrated that infusion of atrial (but not ventricular) tissue extracts of normal hearts into rats causes rapid natriuresis and diuresis and decrease in arterial pressure. At present, the mammalian natriuretic peptide system including ANP, BNP and C-type natriuretic peptide (CNP) has been described. The three peptides share a common 17 amino acid ring structure in which most of the amino acid residues are conserved (for reviews, see Ruskoaho 1992, Levin *et al.* 1998).

The ANP was the first member of the family to be characterized. The major site of synthesis in normal hearts is the atrium, and secretion is promptly stimulated by stretch (Flynn *et al.* 1983, Atlas *et al.* 1984, for review, see Ruskoaho 1992). In normal adult hearts ventricular tissue produces only minor amounts of ANP, but ANP is found in ventricles of fetuses and patients with left ventricular hypertrophy (Saito *et al.* 1989). The induction of LV ANP gene expression is seen in most of the clinical disorders as well as experimental models with pressure or volume overload, and the increase occurs within the first day of experimental overload (Ruskoaho 1992). The translation product of ANP gene is preproANP, from which proANP is formed by cleavage of the signal peptide. The storage form of ANP, proANP, consists of 126 amino acids in humans, and is processed during the secretory process to form $proANP_{1\text{-}98}$ (NT-ANP), and the biologically active hormone, the carboxy terminal peptide ($ANP_{99\text{-}126}$) (Vuolteenaho *et al.* 1985, Ruskoaho

1992). The plasma half-life of ANP is short, close to one minute (Ruskoaho 1992), and therefore the measurement of plasma concentrations of NT-ANP, which is co-secreted with ANP in equimolar amounts but is not subject to effective enzymatic degradation and receptor binding, has been used to characterize the secretion of the peptides (Sundsfjord *et al.* 1988). *In vivo* plasma ANP increases rapidly in response to pressure as well as volume loading (Lang *et al.* 1985, Ruskoaho 1992), and also in response to physical exercise (Vuolteenaho *et al.* 1992). In isolated perfused hearts increased atrial pressure very rapidly releases ANP to the perfusate (Ruskoaho *et al.* 1986). Besides the major cardiovascular effects, i.e. vasodilation, diuresis and natriuresis, ANP reportedly has a direct negative inotropic effect, mediated via cGMP pathway leading to decrease of intracellular pH and subsequently decreased Ca^{2+} sensitivity (Tajima *et al.* 1998).

BNP, originally termed brain natriuretic peptide, was discovered in 1988 from porcine brain (Sudoh *et al.* 1988), but it was soon discovered that the highest concentration of the peptide is found in the atria, with the total ventricular amount of BNP being even higher due to greater mass (Minamino *et al.* 1988, Ogawa *et al.* 1991b). The hemodynamic effects of BNP are largely similar to ANP. BNP gene expression in atria and ventricles is induced within 1 hour in response to overload (Mäntymaa *et al.* 1993, Magga *et al.* 1994, Nakagawa *et al.* 1995, Hama *et al.* 1995). With chronic overload, BNP mRNA levels have been suggested to remain constantly increased (de Bold *et al.* 1996). The rapid induction of BNP gene expression in response to overload has been widely used as a marker of elevated loading (for review, see Tokola *et al.* 2001). In neonatal rat ventricular cell culture, ET-1 and Ang II have been suggested to mediate 24-hour stretch-induced induction of BNP gene expression (Liang & Gardner 1998), but in adult rat the $ET_{A/B}$ antagonist bosentan and AT_1 antagonist losartan were unable to inhibit ventricular BNP mRNA elevation induced by 2-hour pressure overload (Magga *et al.* 1997a). The mRNA increase is independent of protein synthesis, and depends on the induced transcription, not the transcript stability (Magga *et al.* 1997b). Plasma BNP concentration is a sensitive and specific marker of the altered left ventricular structure and function in a patient population at risk for cardiovascular disease (Yamamoto *et al.* 1996). Plasma levels of BNP or its 1-76 amino acid N-terminal fragment have been shown to provide an objective index for guiding drug treatment in patients with stable cardiac failure (Troughton *et al.* 2000). Recombinant BNP has emerged as a potential treatment for patients with acute decompensated heart failure. It causes potent, dose-related vasodilation, improves symptoms and is well tolerated (for review, see Colucci 2001).

Two years after the discovery of BNP, the third member of the group, CNP, was found, again from porcine brain (Sudoh *et al.* 1990). It is thought to act mainly as a local regulator in brain, the major site of synthesis, and in vessels, since it causes vigorous vasorelaxation of vascular smooth muscle, but only mild diuresis and natriuresis, and its plasma concentrations are very low (Sudoh *et al.* 1990, for review, see Ruskoaho 1992).

In addition to the mammalian natriuretic peptides, two other natriuretic peptides have been discovered. A novel salmon cardiac peptide, sharing structural and biological properties with the mammalian natriuretic peptides has been discovered (Tervonen *et al.* 1998, Majalahti-Palviainen *et al.* 2000). Furthermore, dendroaspis natriuretic peptide (DNP) was discovered in the venom of green mamba by Schweitz *et al*. (1992).

In mammals, there are three distinct receptors for natriuretic peptides, termed natriuretic peptide receptors A, B and C (NPR_A, NPR_B and NPR_C, respectively) (for

reviews, see Ruskoaho 1992, Levin *et al.* 1998). NPR_A and NPR_B are linked to intracellular cGMP signaling cascade. NPR_A is responsible for mediating most of the biological effects of ANP and BNP, while the effects of CNP are mediated by NPR_B (Ruskoaho 1992, Yandle 1994). NPR_A prefers ANP binding over BNP, and no specific receptor for BNP has been found so far, but a recent study suggests that there may be an additional cGMP coupled NPR preferring BNP over ANP present in testis and adrenal gland (Goy *et al.* 2001). NPR_C is a clearance receptor (Maack *et al.* 1987), and the binding of a ligand leads to internalization of the receptor ligand complex to the cell and degradation of the ligand (Levin *et al.* 1998). Also neutral endopeptidases take part in the inactivation of natriuretic peptides, the proportion in inactivation being similar to NPR_C in sheep (Charles *et al.* 1996).

Underlining the importance of the natriuretic peptide system in regulation of cardiovascular function are several genetically engineered mouse models with alterations in the natriuretic peptide system (see Table 3). NPR_A knockout in mice results in hypertension, blood pressure-independent eccentric LVH with interstitial fibrosis and sudden death, and increased responsiveness to pressure overload by transverse aortic constriction (Lopez *et al.* 1995, Oliver *et al.* 1997, Knowles *et al.* 2001). Also BNP knockout mice have cardiac fibrosis (Tamura *et al.* 2000). ANP knockout resulted in salt-sensitive hypertension with a 8 mmHg increase in mean arterial pressure on low salt diet and 27 mmHg increase on high salt diet (John *et al.* 1995). ANP overexpression with 8- to 10-fold elevation in ANP plasma levels resulted in chronic hypotension (mean arterial pressures 75.5±0.9 mmHg vs 103.9±2.0 mmHg in control mice) with no changes in plasma and urinary electrolytes, water intake, or urine volume (Steinhelper *et al.* 1990). BNP overexpression in the livers of TG mice resulted in over 10-fold increase in plasma BNP concentration and lower blood pressure (~20 mmHg) compared to NTG littermates (Ogawa *et al.* 1994).

Table 3. Genetically engineered mice with alterations in the natriuretic peptide system

Genetic alteration	Cardiovascular phenotype	References
ANP knockout	Salt sensitive hypertension	John *et al.* 1995
ANP overexpression	Hypotension	Steinhelper *et al.* 1990
BNP knockout	Normal blood pressure, cardiac fibrosis, no LVH	Tamura *et al.* 2000
BNP overexpression	Hypotension	Ogawa *et al.* 1994
NPR_A knockout	Hypertension, LVH with cardiac fibrosis, sudden death	Lopez *et al.* 1995, Oliver *et al.* 1997
NPR_A overexpression (cardiomyocytes)	Normal blood pressure, decreased LV weight	Kishimoto *et al.* 2001
NPR_A overexpression (non tissue specific)	Hypotension, protection from high dietary salt	Oliver *et al.* 1998
NPR_C knockout	Hypotension	Matsukawa *et al.* 1999

ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; LVH, left ventricular hypertrophy; NPR_X, natriuretic peptide receptor subtype.

2.5 Cardiac extracellular matrix

The cardiac connective tissue is mainly composed of collagen, with smaller amounts of elastin, laminin and fibronectin. Approximately 85% of the total collagen in heart consists of collagen type I. In addition to type I, other fibril forming collagen types found in the heart are III and V. Type IV and VI collagens are located in basement membranes in heart as in other tissues. Collagen XIII, which has a transmembrane domain, is found also in the heart. Furthermore, type XV and XVIII collagens, two members of the heterogenous group of non-fibril-forming collagens, are found in the myocardium (Weber *et al.* 1994, Hägg *et al.* 1997a, Myllyharju & Kivirikko 2001, Sund *et al.* 2001). The obvious function of collagenous extracellular matrix is to serve as the structural network for translating the force generated by individual myocytes into organized ventricular contraction and to prevent myocyte slippage, but it also accounts for passive stiffness in diastole and prevents overstretch as well as interstitial edema (for review, see Weber *et al.* 1994). Interstitial connective tissue network may also have a role in mechanosensing process via integrins.

The alterations in cardiac collagen network occur in response to pressure or volume overload and after myocardial infarct. In hypertrophied and failing hearts, interstitial fibrosis is generally seen (for review, see Boluyt & Bing 2000). Reparative fibrosis occurs as a reaction to a loss of myocardial material after necrosis or apoptosis, due to myocardial ischemia or senescence, and it is mainly interstitial. Another type of fibrosis, reactive fibrosis, is observed in the absence of cell loss as a reaction to inflammation and is primarily perivascular. Usually during cardiac remodeling, both types of fibrosis exist (Swynghedauw 1999). Structural changes are one of the key features in cardiac failure. Type I and type III collagen mRNAs were not significantly elevated in ventricles of the non-failing SHR but were increased 4-fold in failing hearts (Boluyt *et al.* 1994). This suggests a role for collagen accumulation in transition from LVH to failure.

Initially, after myocardial injury, the remodeling process is characterized by collagen fiber degradation, edematous intermuscular spaces and increased formation of type III collagen (Weber 1989). Fibrosis may occupy as much as 30% of the myocardium. The high level of fibrosis alters the mechanical properties of myocardium significantly: stiffness increases, and impaired diastolic filling and cardiac function may result (Boluyt & Bing 2000, Lorell & Carabello 2000). However, fibrosis is not seen with all models of LVH, suggesting that it may be regulated by other factors besides load. For instance, infrarenal aortic banding induces blood pressure increase and LVH without fibrosis (Weber *et al.* 1994). LVH associated with exercise training is not associated with fibrosis. Accordingly, a major role for Ang II and also aldosterone in fibrosis and collagen I accumulation has been demonstrated (Weber *et al.* 1994). Also ETs can increase collagen synthesis and decrease collagen degradation in cultured cardiac fibroblasts (Guarda *et al.* 1993), and ET receptor blockers inhibited fibrosis in SHR independently of blood pressure changes (Karam *et al.* 1996). The effect may be partially mediated through ET_B receptor mediated aldosterone release (Wada *et al.* 1997). Natriuretic peptides and AM have been suggested to exert an antifibrotic effect in the heart (Tamura *et al.* 2000, Shimosawa *et al.* 2002).

Type I collagen in the heart is mainly synthesized by cardiac fibroblasts, and it is subject to slow metabolism with a half-life of 100 days (Swynghedauw 1999). The degradation of collagens occurs via specific collagenases (matrix metalloproteinases; MMP). The MMPs are activated by extracellular serine proteases. Tissue inhibitors of MMP form a complex with MMP in extracellular space, inhibiting collagen degradation (Weber *et al.* 1994, Swynghedauw 1999). The finding that inhibition of MMPs attenuates left ventricular dilatation in mice with experimental myocardial infarct has led to the proposal that MMP inhibitors could be used as a therapy for patients at risk for the development of heart failure after myocardial infarction (for review, see Creemers *et al.* 2001).

Type XV collagen is a homotrimer consisting of three α1(XV) chains (Rehn & Pihlajaniemi 1994). The mouse type XV collagen gene is 110 kb in size and contains 40 exons. It is characterized by a central highly interrupted triple helical domain and large N and C-terminal domains (Myers *et al.* 1992). The mRNA shows wide tissue distribution, but the highest levels in the mouse can be detected in the heart (Hägg *et al.* 1997b). The protein is localized mainly to basement membrane zones, but it is also found in the fibrillar collagen matrix near the basement membranes in certain human tissues (Myers *et al.* 1996, Hägg *et al.* 1997a). Type XV collagen has been suggested to act as a link between basement membrane and the underlying collagen matrix. The NC1 domain of the protein binds strongly to extracellular matrix proteins (Fig. 5) (Sasaki *et al.* 2000).

Interestingly, type XV and XVIII collagen share homology in the C-terminal domain, which contains the 20-kDa endostatin peptide (Sasaki *et al.* 2000). Endostatin, similar to that derived from type XVIII collagen, inhibits endothelial proliferation and potently inhibits angiogenesis and tumor growth (O'Reilly *et al.* 1997). Tumor growth and metastasis are dependent of the formation of blood vessels, and consequently the inhibition of tumor angiogenesis has been suggested as a strategy for treating cancer (Folkman 1971, Saaristo *et al.* 2000). The homology between type XV and XVIII collagen endostatin fragments is 60%. As expected, also type XV-derived endostatin has antiangiogenic functions. Proteolytically released XV-endostatins are found in mouse tissues, but the physiological function remains unclear (Sasaki *et al.* 2000). So far the significance of collagen XV for the cardiovascular structure and function has remained unclear.

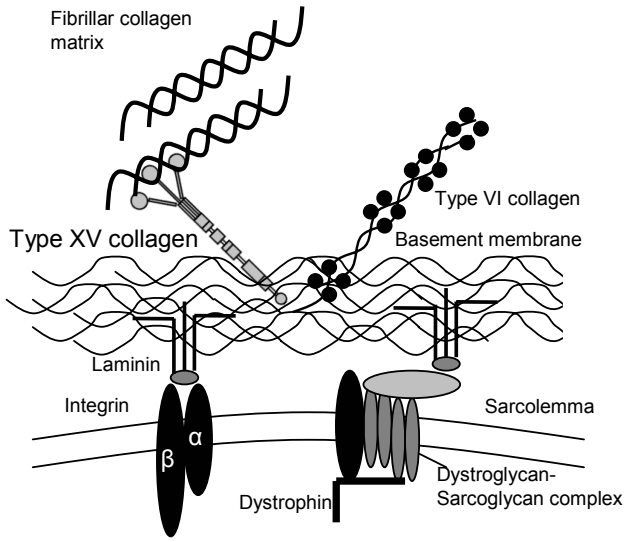

Fig. 5. A schematic presentation of basement membrane structure surrounding cardiac cells. Modified from Eklund *et al.* 2000, Sasaki *et al.* 2000 and Towbin & Bowles 2001.

The sarcoglycan subcomplex contains five subunits that are laterally associated with β-dystroglycan: α-, β-, δ–, γ– and ε-sarcoglygans. The sarcoglycan complex is involved in coupling of cells to basement membrane and to the extracellular matrix (for review, see Towbin & Bowles 2001). A common pathogenic feature for many muscular diseases could be disruption of the link between the ECM and the cytoskeleton, which may occur in the subsarcolemmal part (e.g. dystrophin), at sarcolemmal level (e.g. sarcoglycans and integrin α7) or in the ECM (e.g. laminin α2 chain and type VI collagen). Mice lacking δ-sarcoglycan and thereby disruption of the muscle cytoskeleton and the sarcoglycan-sarcospan complex in vascular smooth muscle develop cardiomyopathy (Coral-Vazquez *et al.* 1999). The primary cause of the heart phenotype is thought to be a perturbation in vascular function, and the vasodilator verapamil can save the hearts (Cohn *et al.* 2001). Dystrophin deficiency in *mdx* mice leads to myopathy associated with impaired running performance: The adult mice of this strain ran less than half of the distance achieved by wild-type mice in voluntary tread wheel tests (Carter *et al.* 1995, Wineinger *et al.* 1998).

2.6 Genetically engineered animal models in cardiovascular research

The capacity to selectively mutate genes or create excessive or deleted gene expression has given researchers the possibility to evaluate the significance of certain gene product for structure-function studies of cardiac proteins and their role in heart disease. To date, several hundred mutant mouse strains and also a few mutant rat strains have been generated (http://tbase.jax.org). The number of genetically engineered mouse lines for

cardiovascular research has been growing rapidly. Mouse is currently the model organism studied most using transgenic approach, since mice breed rapidly, the maintenance costs are lower, and the general knowledge of mouse genetics is at a high level. Germ line transmission has first been achieved in mouse embryonic stem cells. In larger mammals, such as rat, microinjection is the most widely used method (Mullins & Mullins 1996).

There are two basic approaches to mouse genomic manipulation: random chromosomal integration, which can be used for addition of an exogenous transgene, and homologous recombination of foreign DNA, which leads to targeted mutation of an endogenous gene (Williams & Wagner 2000). The first method is based on addition of DNA into fertilized oocyte, and it has been frequently used to generate "gain-of-function" mutations, in which the transgene is (over-)expressed under a desired promoter. Gene targeting via homologous recombination in embryonic stem cells is frequently used to create "loss-of-function" mutations, known as knockouts. Targeted inactivation has been in many cases performed by introducing a positive selection marker which will disrupt gene structure. The Cre/*loxP* approach, which is based on the ability of Cre recombinase to recognize a unique nucleotide sequence (*loxP* site), allows the introduction of mutations in the gene of interest, and by the controlled expression of Cre also control the expression during different time points and avoid e.g. embryonic lethality (Chien 2001).

Genetically engineered animal models offer an important method to evaluate the significance of certain proteins for cardiovascular structure *in vivo*. It has also become possible to analyze the role of the proteins of interest for the cardiac function instead of descriptive studies with gene expression rate. The availability of specific pharmacological agents activating or inhibiting the desired target molecules may also be limited. This has been the case with the cardiac membrane Ca^{2+}-handling proteins, and the research has gained great benefit from the use of genetically engineered animal models (Kiriazis and Kranias 2000). Thanks to the genetical engineering, it has also become possible to generate rodent disease models which are dependent of human regulatory system components. This is the case with the dTG rats, which present with human renin dependent hypertension and end-organ damage. There are also some obvious difficulties with TG animals. Due to the small size and rapid heart rate the physiological measurements with mice are challenging, but with miniatyrized instrumentation and development of surgical procedures many of the problems have been solved. Compensatory mechanisms may be activated during the life span of genetically engineered mice. However, it is often possible to analyze the compensatory changes and to evaluate the effects of these changes on the results. Regulation of protein synthesis of important regulatory components is often tight, resulting in an unexpectedly low increase in protein amounts independent of the high level of expression (Baker *et al.* 1998).

In many cases the benefits with transgene technology clearly outweigh the costs, and important information can be achieved through the use of genetically engineered animal models. With further advances in transgenic technology, it may be possible to control the level of expression of a specific gene product and limit cardiac compensatory changes in order to identify changes solely due to the altered gene product of interest. In addition, the ability to manipulate the particular time-point at which a gene is switched on or off in a tissue-specific manner and the introduction of specific mutations in the gene of interest will advance our understanding of regulatory processes. This may also lead to the development of novel approaches for therapeutic interventions in cardiovascular research.

3 Aims of the research

The aim of the present study was to characterize the role of autocrine/paracrine factors ET-1 and Ang II in cardiac responses to load in isolated mouse and rat heart, to describe the role of PMCA in cardiac function and to investigate the role of collagen XV in the heart.

Specifically the aims were:

1. To elucidate the effects of PMCA overexpression on ET-1- and load-induced BNP gene expression responses.

2. To study the effects of AM on ET-1 induced vasoconstriction.

3. To characterize the role of endogenous ET-1 and Ang II in Frank-Starling responses of normal and hypertrophied rat hearts.

4. To establish an isolated, Langendorff-perfused mouse heart model and to characterize the role of ET-1 and Ang II in contractile response to load in mice hearts.

5. To characterize the role of collagen XV in cardiac function.

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), LiCl (JT Baker Chemicals BV, Holland), agarose NA (Pharmacia LKB Biotechnology, Uppsala, Sweden), Hybond, N+ nylon membrane (Amersham Life Science, Buckinghamshire, UK), [^{32}P]-deoxycytidine-5´-triphosphate (dCTP, Amersham), Quick Prime Kit (Pharmacia, Sweden), X-ray films (Eastman Kodak, Rochester, NY, USA, and Amersham), ET-1 (Phoenix Pharmaceuticals Inc., CA, USA and Peninsula Laboratories, Belmont, CA, USA), rat AM-(1-50) (Phoenix Pharmaceuticals), isoproterenol, saponin and N^{ω} -nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Company, St. Louis, Missouri, USA.), BQ-123 and BQ-788 (Phoenix Pharmaceuticals). Bosentan was generously supplied by Dr. Martine Clozel (F. Hoffmann-La Roche Ltd., Basel, Switzerland and Actelion Ltd., Allschwil, Switzerland) and CV-11974 by Dr. Hajime Toguchi (Takeda Chemical Industries Ltd, Osaka, Japan). Other chemicals were from Sigma.

4.2 Experimental animals

Male 8-week-old Sprague-Dawley (SD) rats, 10-week old NMRI mice, collagen XV knockout mice and respective 129sv control mice were from the Center for Experimental Animals at the University of Oulu, Finland. PMCA overexpressing rats (Hammes *et al.* 1998) and hypertensive 7-week-old dTG rats expressing human renin and angiotensinogen (Ganten *et al.* 1992, Luft *et al.* 1999) were also used. The respective control rats for PMCA overexpressing ratline were from the same Sprague-Dawley-Hannover strain from Møllegaard Experimental Animal Center (Skensved, Denmark), and for the dTG rats from Møllegaard Breeding Centre Deutschland Gmbh., Schönwalde, Germany.

The dTG rats (Ganten *et al.* 1992) have plasma renin activity 20 to 30 times above that of control rats generated by transgenic human renin cleaving human angiotensinogen, whereas rat plasma renin is suppressed. Transgenic human renin does not interfere with rat angiotensinogen, and vice versa. As a consequence, the dTG rats develop early malignant hypertension with significant end-organ damage and die before the age of 8 weeks (Bohlender *et al.* 2000, Mervaala *et al.* 2000). The PMCA rats carry the human PMCA isoform 4CI cDNA6 under the control of the ventricle-specific rat myosin light chain-2 promoter. The total amount of PMCA protein in the adult ventricles was 1.6-fold compared with the control animals. Furthermore, a 1.8-fold increase in the Ca^{2+}-ATPase activity in rats overexpressing PMCA was noted (Hammes *et al.* 1998). The mice lacking α1(XV) collagen chains were generated by site-specific Cre/*loxP*-mediated deletion in embryonic stem (ES) cells. Briefly, the knockout targeting vector was prepared with selection marker genes (*neo*^{r} and *HSV-tk*) at the 5' end of the 120-nt second intron and *loxP* sites flanking the marker cassette and the first two exons. After homologous recombination, a Cre expression plasmid was electroporated into the targeted ES cell lines to generate Cre-mediated deletions. Two types of Cre-mediated recombination alleles were observed: *loxP*-flanked alleles identified by Southern blot analysis of EcoRI-digested genomic DNA and inactivated alleles identified by XbaI-digested genomic DNA, lacking the first two exons and the transcription start sites. No compensatory changes were observed in the level of expression of the homologous type XVIII collagen mRNA in the *Col15a1*$^{-/-}$ mice.

The animals were housed in plastic cages with free access to tap water and normal rat and mouse chow in a room with a controlled 40 % humidity and temperature of 22 ºC, and a 0600 h on, 1800 off environmental light cycle was maintained. The maintenance diet of the animals contained 0,65 % sodium chloride (NaCl). Animal Use and Care Committee of the University of Oulu approved the experimental design. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

4.3 Isolated perfused heart preparations (I-V)

The isolated, perfused rat heart preparation used in this study was a modification of that previously described (Ruskoaho *et al.* 1986, Mäntymaa *et al.* 1993). Briefly, rats were decapitated after sedation with CO_2. The abdominal cavity was immediately opened, the diaphragm transected, lateral incisions made along the both sides of the rib cage, and the heart cooled with the perfusion fluid (4-10 ºC). The aorta was cannulated above the aortic valve and retrograde perfusion was begun with a modified Krebs-Henseleit bicarbonate buffer, pH 7.4, equilibrated with 95% O_2 / 5% CO_2 at 37ºC. The composition of the buffer was (mM): NaCl 113.8, $NaHCO_3$ 22.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.1, $CaCl_2$ 2.5 and glucose 11.0. Variations in the perfusion pressure arising from changes in coronary vascular resistance were measured with a pressure transducer (model MP-15, Micron Instruments) situated on a side-arm of the aortic cannula. For measurement of the developed tension (apicobasal displacement), a small hook was attached to the apex of the heart and connected to a force-displacement transducer (model FT03, Grass

Instruments) and recorded with a Grass polygraph (model 7DA, Grass Instruments, Quincey, MA, USA). The hearts were put under a resting tension of 2.0 g. The hearts were paced (9 V, 0.5 ms) with a Grass stimulator (model S88, Grass Instruments, Quincey, MA, USA) at a rate of 300 and 400 beats per minute (rat and mice, respectively). The drugs were infused via an infusion pump (Secan PSA 55, Sky Electronics S.A.). The hearts were allowed to stabilize for 50 minutes before any interventions.

Mice hearts were prepared for perfusion as described above for the rat using miniaturized instrumentation. The mice hearts were perfused with a peristaltic pump (Minipuls 3, model 312, Gilson) at a constant flow rate of 2 (IV) or 2.5 mL/min (V), while the flow rate for rat hearts was 5 – 7 mL/min, depending on the cardiac weight. In pressure-overloaded hearts, the coronary flow rate was increased to a level of 20 mL/min for rats (I), or 4 -6 mL/min for mice hearts (IV).

For atrial stretch studies, the inferior vena cava was cannulated with a PE-50 cannula and connected to a pressure transducer (model MP-15) and the pressure was recorded with Grass polygraph. Pulmonary artery was also cannulated, and the atrial pressure was increased by elevating the tip of the pulmonary artery cannula to increase the atrial pressure to 5 mmHg.

The isometric force of contraction was measured by inserting an empty plastic balloon to the left ventricle through the mitral valve after cutting the left atrium away (III, V). The balloon was then filled with 50% ethanol to give a left ventricular end diastolic pressure (LVEDP) of 2-3 mmHg. The intraventricular pressure inside the balloon was recorded with a pressure transducer (Isotec, Hugo Sachs elektronik). The balloons were large enough so that only a negligible pressure resulted when the balloons alone were filled up to the maximum volume used. Heart rate was determined from the changes in intraventricular pressure. Analog signals were digitized at a sampling frequency of 1000 Hz. The data were analyzed and recorded on an IBM PC-compatible computer using Ponemah data acquisition software (Gould Instrument System Inc., Ohio, USA).

The left ventricular developed pressure (maximum pressure – minimum pressure) was measured, and the first derivatives of the intraventricular pressure, +dP/dT and -dP/dT, in millimeters of mercury per second, were recorded as measures of contractility and relaxation, respectively (III, V). For studying the Frank-Starling response (III), the volume of the intraventricular balloon was stepwisely increased, or in the control experiments the perfusion was continued with LVEDP = 3 mmHg. Cardiac function was assessed within one minute of each volume increment, when the heart had stabilized. The whole assessment of the Frank-Starling response was completed within 15 minutes. Comparison between dTG and NTG ventricular function was done at a similar level of LVEDP and also at the left ventricular balloon volume producing 50% of the maximal developed pressure using absolute values (Strömer *et al.* 1997). Comparison between different treatments within the same rat line was done using contractile data related to baseline level at LVEDP of 3 mmHg.

In isoproterenol-infusion studies (V), there was a 20-minute stabilization period before infusion with the vehicle was started. After 10 minutes control time, the infusion of the first dose of isoproterenol was started. Infusion was stopped when the maximal response had been observed. Then, after a 10-min equilibration period, the next dose was given. Meanwhile, vehicle was infused into the hearts to keep the flow rate constant. The dosing

of isoproterenol was begun from the dose of 0.01nM and increased 10-fold at each step. The contractile response to each dose was calculated as a ratio of the maximal level of contractility to the basal level before isoproterenol.

4.4 Exercise experiment (V)

In the exercise experiment, 11 mutant male mice aged 27-34 weeks and 11 age- and sex-matched wild-type mice were subjected to six hours of running on a motor-driven treadmill with a 6° uphill inclination at the speed of 8.5 m x min^{-1} with two 20 min pauses. 48 hours after the cessation of exercise, the animals were sacrificed together with unexercised mutant (n=7) and wild-type controls (n=7) and prepared for β-glucuronidase activity assays (Takala *et al.* 1992) and histological analysis (see section 4.10).

4.5 Experimental protocols

Table 4. Summary of the experimental protocols.

Study	Animals	Experimental model	Duration	Treatments
I	PMCA and SD rats	Isolated heart, stimulation with ET-1 or increased flow rate, contractile force measurement using apicobasal displacement	2 h	ET-1
II	SD rats	Isolated heart, perfusion pressure measurement, drug infusions	30 min	AM, L-NAME, ET-1
III	dTG and SD rats	Isolated heart, contractile force measurement, stimulation with intraventricular balloon	up to 20 minutes	Bosentan, CV-11974
IV	NMRI mice	Isolated heart, stimulation with ET-1 or increased flow rate, contractile force measurement using apicobasal displacement	30 min	Bosentan, BQ-123, BQ-788, CV-11974, ET-1, Saponin
V	Col XV $^{-/-}$ and wild-type 129sv mice	Isolated heart, isoproterenol stimulation, contractile force measurement with intraventricular balloon	< 1 h	Isoproterenol
		Exercise experiments	6 h	

AM, adrenomedullin; Col $XV^{-/-}$, type XV collagen deficient mice; dTG, double transgenic rats expressing human renin and angiotensinogen genes; ET-1, endothelin-1; L-NAME, N^{ω} -nitro-L-arginine methyl ester; PMCA, plasma membrane Ca^{2+}-ATPase overexpressing rats; SD, Sprague-Dawley rats.

4.6 Isolation and analysis of cytoplasmic RNA (I-III, V)

At the end of each experiment, both ventricles and atria were blotted dry, weighed, immersed in liquid nitrogen and stored at - 70°C until assayed. RNA was isolated from atria and ventricles by the guanidine thiocyanate-CsCl method (Chirgwin *et al.* 1979). Northern blot hybridization, in which the size and amount of specific mRNA molecules in total RNA preparations are determined, were performed after isolation of RNA. For the RNA Northern blot analyses, 20-µg samples of the RNA were transferred to Amersham Hybond N+ nylon membranes. A 390-bp fragment of rat BNP complementary deoxyribonucleic acid (cDNA) probe (Ogawa *et al.* 1991b) (a generous gift from Dr. K. Nakao, Kyoto University School of Medicine, Kyoto, Japan), a full-length mouse BNP cDNA probe (Ogawa *et al.* 1994) (a generous gift from Dr. Y. Ogawa, Kyoto University School of Medicine, Kyoto, Japan), full-length rat ANP cDNA probe (Flynn *et al.* 1985)(a generous gift from Dr. P. L. Davies, Queen's University, Kingston, Ontario, Canada), PCR amplified rat AM cDNA probe (nucleotides 287-736) (Romppanen *et al.* 1997), cDNA probe made by reverse transcriptase polymerase chain reaction (RT-PCR) for rat *c-fos* (nucleotides 231-1280), mouse type XV collagen cDNA probe encoding exons 7-10, cDNA probe corresponding to the 3' untranslated and endostatin regions of the mouse XVIII collagen mRNA, full-length cDNA probe complementary to glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) (Fort *et al.* 1985) and cDNA probe complementary to rat 18S ribosomal RNA (Lee *et al.* 1988b) were labeled with [^{32}P]dCTP with T7 Quick Prime Kit (Pharmacia LKB Biotechnology). The membranes were hybridized overnight at +42 ºC 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 +55 ºC and exposed to X-ray film with Cronex Lighting Plus intensifying screens (DuPont, Wilmington, DL, USA) at –70 ºC or to Phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA) at room temperature. Phosphor screens were scanned with Phosphor Imager (Molecular Dynamics). The hybridization signals of AM, ANP, BNP and *c-fos* mRNA were normalized to that of 18S or GAPDH mRNA for each sample to correct for potential differences in loading and/or transfer.

AT_1 receptor, preproET-1, ACE, rat angiotensinogen, PLB and NCX (III) and mouse ANP (V) mRNA levels were measured by quantitative reverse transcription-PCR analysis with an ABI 3700 Genetic Analyzer using TaqMan chemistry (Applied Biosystems, Foster City, CA) as described (Majalahti-Palviainen *et al.* 2000). Initially, the RNA was extracted as previously described (Chirgwin *et al.* 1979), a cDNA reaction was performed according to the manufacturer's protocol (Gibco BRL), after which mRNA levels were measured by quantitative RT-PCR analysis. Forward and reverse primers and probes for mRNA detection are presented in Table 5.

Table 5. Primer and probe sequences used for mRNA quantitation

Gene	Sense-primer	Antisense-primer	Probe
AT_1	GTGGCCAAAGTCACCTGCA	GTGGATGACAGCTGGCAAACT	CATCTGGCTGATGGCTGGCTTGG
ACE	GCACATTCGCAGGAACGTG	GCCCTCCAGTGCCTAGATCC	AAGGTGACTTTGACCCAGGGTCCAAGTT
mANP	GAAAAGCAAACTGAGGGCTCTG	CCTACCCCCGAAGCAGCT	TCGCTGGCCCTCGGAGCCT
rat AOGEN	CAGAGCCAACCTTTGAGCCT	CAGGGTCTTCTCATCCACGG	TGCCCATTCAGGCCAAGACCT
ET-1	ATGGACAAGGAGTGTGTCTACTTCTG	GGGACGACGCGCTCG	CACCTGGACATCATCTGGGTCAACACTC
NCX	CTCTTGTTTACCCATGTTGACCATAT	GAGCCAGTACATTCAGTGGTTTCA	TGCAGATACAGAGGCAGAAACAGGAGGAA
PLB	AAGTCTGTCGCCACCGCA	TGGTGGAGGGCCAGGTT	CCTGCACCATGCCAACGCAGC
18S	TGGTTGCAAAGCTGAAACTTAAAG	AGTCAAATTAAGCCGCAGGC	CCTGGTGGTGCCCTTCCGTCA

ACE, angiotensin converting enzyme; AOGEN, angiotensinogen; mANP, mouse ANP; NCX, Na^+-Ca^{2+} exchanger; PLB, phospholamban.

4.7 Radioimmunoassays (I- III)

For coronary effluent AM and BNP radioimmunoassay, the 5 mL perfusate sample was extracted by Sep-Pak C_{18} cartridges, lyophilized and redissolved to 500 µL of RIA buffer. For coronary effluent ANP radioimmunoassay, samples were not extracted. For tissue peptide radioimmunoassay, 150 µL of ventricular guanidine thiocyanate extract was diluted to 700 µL of RIA buffer. The tissue extracts and unextracted perfusate samples in duplicates of 100 µL were incubated with specific rabbit BNP (Ogawa *et al.* 1991b), ANP (Vuolteenaho *et al.* 1985), AM (Phoenix pharmaceuticals) or ET-1 (Peninsula Laboratories) antiserum. Synthetic rat BNP_{51-95} (BNP-45), synthetic rat ANP_{99-126}, synthetic rat AM_{1-50} and synthetic rat $ET\text{-}1_{53-73}$ were incubated as standards. The tracers were prepared by chloramine-T iodination of synthetic rat [Tyr_0]-BNP_{51-95}, rat ANP_{99-126}, rat AM_{1-50} and synthetic rat ET-1 followed by reverse phase high performance liquid chromatography purification. After incubation for 48 hours at +4ºC, the immunocomplexes were precipitated with sheep antiserum directed against rabbit gammaglobulin in the presence of 500 µL of 8% Polyethylene Glycol 6000, pH 7, followed by centrifugation at 3000 g for 30 min. The sensitivities of the BNP, ANP, AM and ET-1 assays were 2 fmol/tube, 1 fmol/tube, 1 fmol/tube and 0.3 fmol/tube,

respectively. The intra- and inter-assay variations were less than 10 % and 15 %, respectively. Serial dilutions of perfusate and tissue extracts showed parallelism with the standards. The BNP antiserum did not recognize ANP or CNP. The ANP antiserum recognized ANP and proANP with equal avidity but did not cross-react with BNP or CNP. The rat AM antiserum did not cross-react with rat AM_{1-20}, human AM antiserum or its fragment, human amylin or ET-1. The ET-1 antiserum cross-reacted 7% with human ET-2 and rat ET-3, 35% with porcine big ET-1 but not with ANP, Ang II or AVP.

4.8 Cyclic AMP measurements (V)

For the cAMP measurements, hearts from 18-month-old wild-type and collagen XV null mice were perfused as mentioned above. After the isoproterenol (0.1 nM) infusion, the left ventricles were frozen in liquid nitrogen and stored at -80°C. Cardiac samples were homogenized with 6% trichloroacetic acid at 4°C and centrifuged at 2000g for 15 minutes. The supernatants were collected and washed with 5 vol of water-saturated diethyl ether. The extracts were lyophilized and the cAMP content was measured by radioimmunoassay according to the manufacturer's protocol (Amersham).

4.9 Analysis of markers for cardiac injury (V)

After the exercise protocol the left and right ventricles were separated and the left ventricles cut into three pieces for histological, mRNA and biochemical analysis. The possibility of cardiac injury was studied by analyzing the extent of apoptosis, the activities of matrix metalloproteinase 2 (proMMP-2) and β-glucuronidase and the ANP mRNA levels.

4.9.1 TUNEL-staining

DNA fragmentation (terminal deoxyribonucleotidyl transferase -mediated dUTP nick end labeling, TUNEL assay) was detected from cryostat sections stained with the In Situ Cell Death Detection Kit (Boehringer Mannheim) according to the manufacturer's protocol. For quantitative analysis, the mean number of TUNEL-positive nuclei was counted in four sections on different depths in each sample.

4.9.2 Preparation of samples for biochemical assays

A frozen sample was placed in buffer (1:10 w/v in homogenization buffer: 0.2 M NaCl, 0.1% TritonX-100, 0.02 M Tris) and homogenized by hand using a glass probe. 50 µl of homogenate was taken for the β-glucuronidase activity assay and the rest centrifuged for 20 minutes at 13000 rpm for zymography.

4.9.3 ProMMP-2 activity

ProMMP-2 activity was measured by zymography. 7.5% running gels containing 1 mg/ml gelatin were overlaid with 4% stacking gels. The samples (cardiac homogenates mixed with a 1/1 volume of sample buffer: 0.4 M Tris, pH 6.8, 2% SDS, 20% glycerol and 0.03% bromphenol blue) were loaded into the gel and electrophoresis was carried out first at 16 mA for 1 hour and then at 24 mA until the dye front ran off the gels. The gels were incubated for 30 min in a solution containing 2.5% Tween 80 and 50 nM Tris, pH 7.5, and then at 37°C for 18 h in a solution containing 50 mM Tris, pH 7.5, 5 mM $CaCl_2$ and 10 mM $ZnCl_2$. Gelatinase activity was revealed by negative staining with Coomassie Brilliant. Purified proMMP-2 was used for identifying the enzyme activity. The degree of digestion was quantified by densitometry and area analysis.

4.9.4 β-glucuronidase activity

β-glucuronidase activity was measured in a muscle homogenate. Briefly, 450 µl of 0.1M acetate buffer (pH 4.2) was added to 50 µl cardiac homogenate. After five minutes of preincubation at 37°C, 250 µl of substrate (5 mM p-nitrophenyl-βD-glucuronidase, Sigma) was added, and incubated overnight at 37°C. The reaction was stopped by adding 1.5 ml of cold glycine buffer (0.1 M pH 10.8), followed by centrifugation at 3000 rpm for 10 minutes, after which β-glucuronidase activity was calculated based on absorbance at 420 nm.

4.10 Histology (IV, V)

4.10.1 Light microscopy

For studying the coronary endothelial structure, perfused hearts were fixed overnight in 10 % buffered formalin solution. Serial transversal sections of ventricles were embedded in paraffin. 5 µm thick sections were cut and stained for Hematoxylin and Eosin, Herovici and Verhoeff van Gieson. For immunohistochemical analysis, commercial antibodies (Dako, Klostrupp, Denmark) against von Willebrandt's factor (vWF) were used to visualize the ECs of perfused coronary arteries.

4.10.2 Electron microscopy

The samples from free wall of the left ventricles were fixed and dehydrated, and sections were viewed in a transmission electron microscope (Philips CM100). At least two samples from the heart (wild-type mice, n=6, 12-97 weeks old; *Col15a1*$^{-/-}$ mice, n=7, 12-129 weeks old), and the gastrocnemius and quadriceps femoris muscles (wild-type mice,

n=4, 12-97 weeks old; *Col15a1*$^{-/-}$ mice, n=5, 12-131 weeks old) were examined per mouse.

4.11 Statistical analysis

The results are expressed as mean±standard error of mean (SEM). For the comparison of statistical significance between two groups, Student's *t*-test was used. The non-parametric Mann-Whitney *U*-test was used to compare the means of the cardiac injury markers (V). The hemodynamic variables and peptide secretion levels (I-IV) were analyzed with one-way ANOVA followed by Student-Newman-Keul´s post hoc test. Repeated measures ANOVA was used for multivariate analysis. Differences at the 95% level were considered statistically significant.

5 Results

5.1 Cardiac overexpression of the plasma membrane Ca^{2+}-ATPase (I)

5.1.1 Effects on baseline cardiac function

The developed tension (DT) at baseline was 1.0±0.1g and 1.0±0.2 g in non-transgenic (NTG) and plasma membrane Ca^{2+}-ATPase overexpressing (PMCA) rats, respectively. Basal secretion rates of ir-AM, ir-ANP and ir-BNP were also similar: ir-AM 0.10±0.02 and 0.13±0.03 fmol/mL, ir-ANP 346±74 and 309±49 pg/mL, ir-BNP 1.7±0.2 and 1.6±0.2 pmol/mL, for NTG and PMCA rats, respectively. Baseline BNP mRNA levels were similar in left ventricles of NTG and PMCA animals (BNP/GAPDH ratio 0.79±0.08, n=4 and 0.72±0.06, n=7 densitometric units, respectively). Left ventricles weighed 532±15 and 539±15 mg in NTG and PMCA animals, respectively. The respective ventricular to body weight ratios were 1.32±0.03 and 1.34±0.03 mg/g showing that 2-month-old PMCA rats did not have left ventricular hypertrophy.

5.1.2 Effects on responses to endothelin-1

In NTG rats, BNP mRNA levels in left ventricles increased 2.0-fold in response to 2-hour infusion with 1 nmol/L ET-1 (P<0.01) (Fig. 6). In contrast, in PMCA overexpressing rat hearts ET-1 did not induce any increase in left ventricular BNP mRNA levels. Accordingly, release of ir-BNP into the perfusate in response to ET-1 infusion remained unchanged in PMCA transgenic rats while a 1.5-fold increase in BNP secretion (P<0.05) compared to baseline levels in NTG rats was noted (Fig. 7). Two hours perfusion with ET-1 resulted in 1.7- and 2.5-fold increase in AM and *c-fos* mRNA levels in left ventricles of NTG rat hearts, respectively. This early induction of AM and *c-fos* gene expression was almost completely abolished in left ventricles of PMCA rats (Fig. 6). Similarly, ET-1 induced a 2.0-fold increase in secretion of ir-AM in NTG animals but not

in PMCA rat hearts (P<0.05, ET-1 treated NTG versus PMCA rats) (Fig. 7). For number of experiments in each group, see Fig. 6A.

Perfusion with 1 nM ET-1 induced similar, significant positive inotropic responses in both strains. DT reached its maximum at 20 minutes, when 24±6 and 37±7 % increases were observed in NTG and PMCA rats, respectively (P<0.05 for both vs. baseline values). There was no significant difference in contractile force between NTG and PMCA rats in response to ET-1 infusion. When ET-1 infusion was continued, the contractile force gradually decreased, as reported previously (Baydoun *et al.* 1989).

ET-1 is a very potent vasoconstrictor, and in isolated constant flow-perfused hearts this results in an increase in perfusion pressure. In NTG and PMCA rat hearts, the perfusion with 5 mL/min gave perfusion pressures of 30±2 and 31±2 mmHg, respectively (P=NS). ET-1 induced similar increases of perfusion pressure in both strains. After 2-hour perfusion with 1 nM ET-1 the perfusion pressures were 153±13 and 153±23 mmHg for NTG and PMCA rat hearts, respectively (P<0.001 for both vs. baseline values).

The overexpression of PMCA was driven under ventricle-specific rat myosin light chain-2 promoter (Hammes *et al.* 1998). Therefore the atrial response to ET-1 was studied. In the left atria, 2-hour perfusion with 1 nM ET-1 resulted in 1.6- and 1.7-fold increases in BNP mRNA levels in NTG and PMCA rat hearts, respectively (P<0.005 vs. vehicle). In terms of absolute values, the BNP/18S ratio increased from 0.56±0.06 to 0.87±0.04 and from 0.55±0.04 to 0.92±0.09 densitometric units in NTG and PMCA left atria, respectively.

5.1.3 Effects on responses to mechanical load

To examine whether the attenuated hypertrophic responses are selective to ET-1, both NTG and PMCA rat hearts were loaded by increasing coronary flow from 5 ml/min to 20 ml/min for 2 hours, a method previously described to induce typical alterations in ventricular gene expression similar to those seen in response to loading (Magga *et al.* 1998a). The elevation of coronary flow rate increased coronary perfusion pressure from the average levels of 30±2 and 31±2 mmHg to 153±13 and 154±14 in PMCA and NTG rat hearts, respectively. This was sufficient to cause 1.6- and 1.5-fold increases (P<0.01) in left ventricular BNP mRNA levels in both strains (P<0.05 for both) (Fig. 6A).

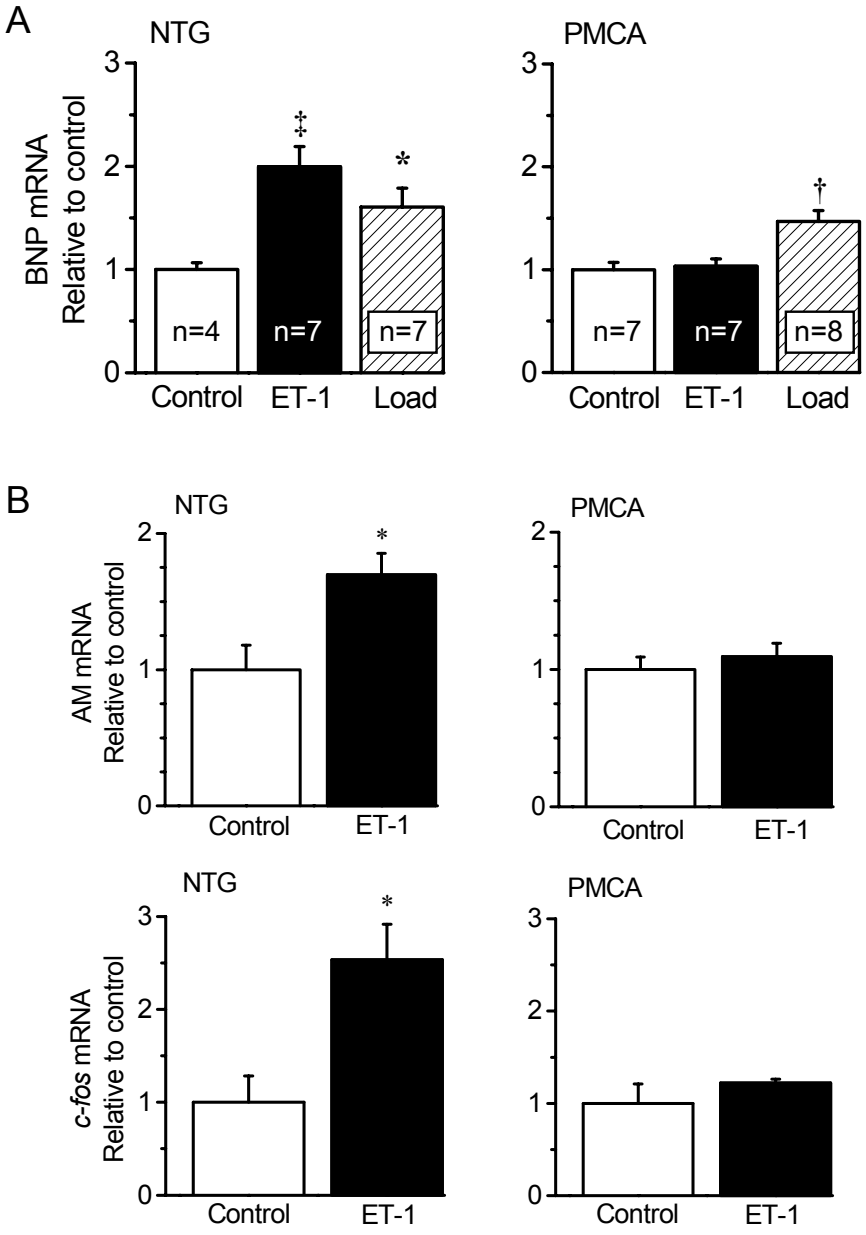

Fig. 6. Panel A. Changes in BNP gene expression in response to 2-hour stimulation with ET-1 or mechanical load (elevated coronary flow) in NTG and PMCA rat hearts. Panel B Changes in AM and *c-fos* gene expression in response to 2-hour stimulation with ET-1 in NTG and PMCA rat hearts. Results are means ± SEM. *$P<0.05$, †$P<0.01$, and ‡$P<0.001$ vs. control (Student's t-test)

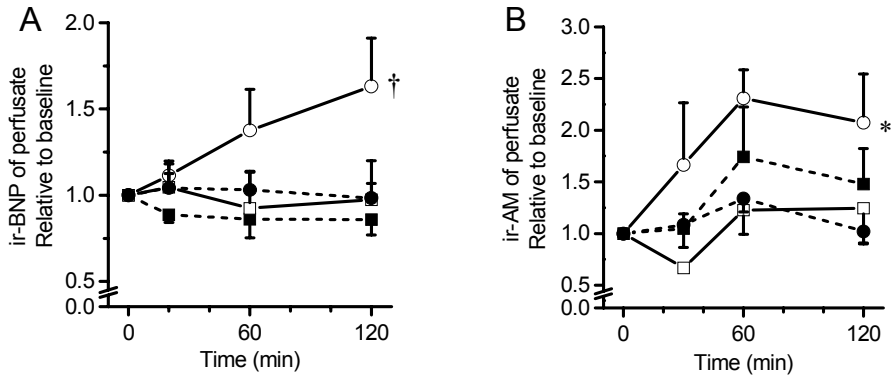

Fig. 7. Immunoreactive (ir)-BNP and ir-AM secretion during ET-1 infusion in NTG and PMCA rat hearts. Solid line, NTG rats; dashed line, PMCA rats. open box, NTG control; open circle, NTG ET-1; black box, PMCA control; black circle, PMCA ET-1. Data are means ± SEM. ****P*<0.05 vs. NTG control and vs. PMCA ET-1; † *P*<0.005 vs. SD control (repeated-measures ANOVA).

5.2 Effects of adrenomedullin on endothelin-1 induced coronary vasoconstriction (II)

As shown in study I, ET-1 induced synthesis and secretion of AM in isolated normal rat hearts. To test, whether AM is able to affect ET-1 induced coronary vasoconstriction, a separate set of experiments was performed. Again, infusion of ET-1 (1 nM) for 120 min increased perfusate AM levels by 1.6-fold (from 0.078 ± 0.012 to 0.127 ± 0.008 fmol/mL, n=6; *P*<0.05), whereas infusion of vehicle alone had no effect on ir-AM levels (n=6, *P*=NS). Administration of ET-1 at a concentration of 0.08 nM had no effect on vascular tone (Fig. 8A), but ET-1 produced a significant coronary vasoconstrictor effect at 1 nM, as previously mentioned. Despite the near-maximum dilatation of the coronary arteries induced by the relatively low coronary flow rate, infusion of AM (0.03 and 1 nM) resulted in a dose-dependent decrease in perfusion pressure. When L-NAME (300 μM), an inhibitor of NOS, was infused alone into the coronary circulation, the perfusion pressure remained constant. However, inhibition of NO synthesis augmented the constrictor effect of ET-1 at both concentrations (Fig. 8). When AM was infused into the coronary circulation in combination with L-NAME, it reduced the perfusion pressure to a similar extent as observed in the absence of inhibition of NOS. In the presence of L-NAME, AM at a concentration of 1 nM markedly reversed the pressor response to 1 nM ET-1. Similarly, under the blockade of NO synthesis, the vasoconstrictor effect of ET-1 at 0.08 nM was significantly attenuated by AM at 0.03 nM (Fig. 8).

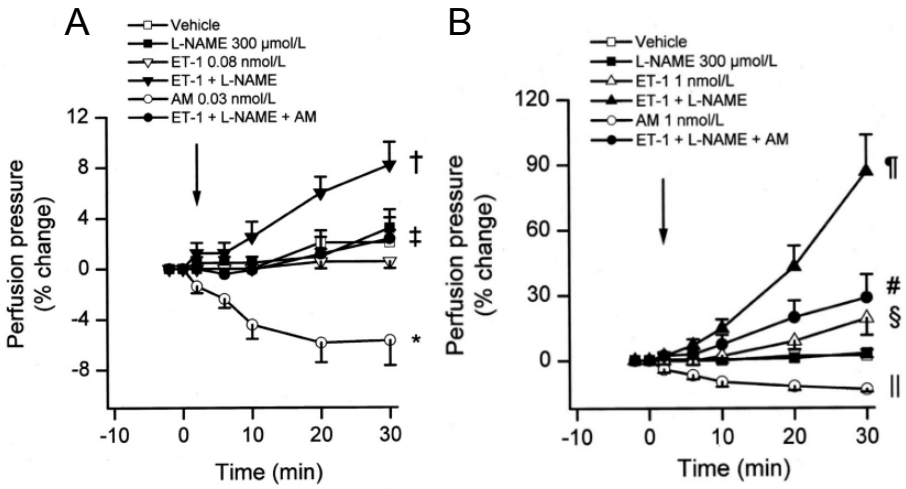

Fig. 8. Attenuation of the vasoconstrictor effect of ET-1 by AM in isolated rat hearts. After a control period, ET-1 (0.08 and 1 nmol/l) (A) and/or AM (0.03 and 1 nmol/l) was added to the perfusion fluid in the presence or absence of nitric oxide synthase inhibitor L-NAME (300 µM) (B) for 30 min. Results are expressed as % change vs. baseline values. Each point is the mean ± SEM from 6-7 separate experiments on different isolated rat hearts. *P<0.001 AM vs. vehicle; P<0.001 ET-1 + L-NAME vs. ET-1; P<0.01 ET-1 + L-NAME + AM vs. ET-1 + L-NAME; §P<0.05 ET-1 vs. vehicle; P<0.001 AM vs. vehicle; ¶ P<0.001 ET-1 + L-NAME vs. ET-1; #P<0.001 ET-1 + L-NAME + AM vs. ET-1 + L-NAME by two-way analysis of variance for repeated measurements. Note the different scale of pressure change between A and B.

5.3 Frank-Starling response in the hypertrophied double transgenic rat hearts (III)

5.3.1 Baseline characteristics of the double transgenic rats harboring human renin and angiotensinogen genes

The dTG rat line expressing human renin and angiotensinogen genes is characterized by high blood pressure and marked LVH compared to NTG littermates (Bohlender *et al.* 1997). Experiments were conducted in 7- week-old male dTG rats (n=48) and in age-matched normotensive NTG rats (n=44). The dTG rats showed no clinical signs of heart failure, such as ascites or pleural effusion. The body weights of NTG and dTG rats were 215±5 and 207±4 g, respectively (P=NS). The left ventricles of NTG and dTG rats weighed 611±12 and 890±21 mg, respectively, resulting in left ventricle/body weight ratio of 2.86±0.04 and 4.31±0.08 mg/g (P<0.0001). The ANP mRNA to 18S mRNA ratio was 9.2-fold higher in dTG than in NTG rat left ventricles (P<0.001). Also 1.6-fold

higher *c-fos* mRNA levels were detected in left ventricles of dTG rats compared with NTG rats ($P<0.05$).

When both NTG and dTG rat hearts were perfused with flow rate of 5.8 mL x gram^{-1} x min^{-1}, the dTG rat heart presented with enhanced contractility, as shown in Table 6. The perfusion pressure was higher in dTG rats (30±1 and 54±2 mmHg in NTG and dTG rat hearts, respectively, $P<0.001$), probably due to hypertension induced morphological alterations in coronary vasculature (Feigl 1983, Mervaala *et al.* 2000). As previously suggested (Strömer *et al.* 1997), the hearts were compared at similar end diastolic pressure (LVEDP=3 mmHg) and also at respective points of the Frank-Starling curve (V_B=50% of V_{max}).

Table 6. Contractile function in NTG and dTG rat hearts.

Hemodynamics at LVEDP=3 mmHg	NTG rats	dTG rats	Hemodynamics at V_B = 50% of V_{max}	NTG rats	dTG rats
DP, mmHg	29.6±1.8	44.5±1.9 §	DP, mmHg	38.8±2.8	59.8±4.1†
+dP/dt$_{max}$, mmHg/s	1216±61	1578±69‡	+dP/dt$_{max}$, mmHg/s	1450±102	1934±151*
–dP/dt$_{min}$, mmHg/s	684±34	899±34 §	–dP/dt$_{min}$, mmHg/s	820±53	1105±79*
LVEDP, mmHg	3.0±0.1	3.0±0.1	LVEDP, mmHg	11.9±1.8	23.7±4.4*

Results are mean ± SEM. LVEDP = left ventricular end diastolic pressure; DP = developed pressure; dP/dt = derivative of ventricular pressure, V_B = 50% V_{max} = left ventricular balloon volume producing 50% of the maximal DP. * $P<0.05$, † $P<0.01$, ‡ $P<0.001$, § $P<0.0001$ vs. NTG.

Left ventricles of dTG rat hearts showed 25% higher ir-ET-1 concentrations than NTG rat hearts ($P<0.05$). On the other hand, there was no difference between NTG and dTG rat left ventricles in preproET-1 mRNA levels. The rat angiotensinogen and AT_1 receptor mRNA levels were 36% and 29% lower, respectively, in left ventricles of dTG than NTG rats ($P<0.05$ and $P<0.005$). The ACE mRNA levels were similar in the left ventricles of both rat lines at this age. Further analysis showed no differences between the rat lines in the PLB mRNA levels, meanwhile the NCX mRNA levels in dTG rat left ventricles were 28±8% higher than those in NTG rat left ventricles (dTG 0.89±0.05 vs. NTG 0.69±0.02 arbitrary units, $P<0.05$).

5.3.2 Effects of loading

Adequate Frank-Starling responses were detected in both NTG and dTG hearts when the intraventricular balloon volume was stepwisely increased. Due to the concentric hypertrophy in dTG hearts, the LVEDP increased more rapidly during the stepwise increment of intraventricular balloon volume, and peak contractile values were reached at

intraventricular balloon volume of 186±18 µL compared to 234±22 µL in NTG hearts (P=0.05). When the values at LVEDP of 3 mmHg were used for comparison, maximal developed pressure was 148±8 and 156±12% of baseline in NTG and dTG rat hearts, respectively (n=8, P<0.001 vs. control for both strains), and +dP/dt to 143±11 and 139±7%, respectively (P<0.001 vs. control for both, Fig. 9).

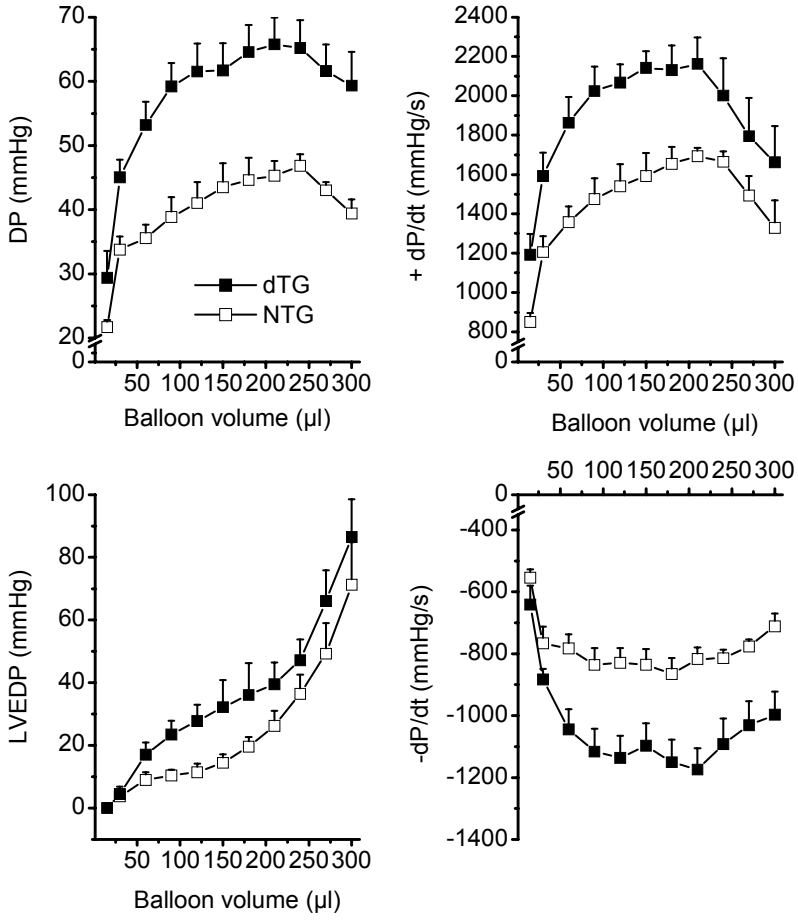

Fig. 9. Plots showing the developed pressure (DP), maximal positive and negative derivative of intraventricular pressure (+dP/dt$_{max}$, and -dP/dt$_{min}$) and left ventricular end diastolic pressure (LVEDP) in NTG and dTG rat hearts during stepwise increment of intraventricular balloon volume. NTG and dTG differ significantly in all parameters (P<0.01) (one-way ANOVA followed by Student-Newman-Keul´s post hoc test).

5.3.3 Effects of bosentan and CV-11974 on the Frank-Starling response

Bosentan (a mixed $ET_{A/B}$ antagonist) (1 μM) and CV-11974 (an AT_1 receptor antagonist) (10 nM) were used in the Langendorff perfused NTG and dTG rat hearts during the increment of intraventricular balloon volume to determine the roles of ET-1 and Ang II in Frank-Starling response. In NTG rat hearts, neither of the antagonists had any effect on the Frank-Starling response (n=8 and n=5, respectively). In contrast, bosentan decreased the Frank-Starling response by 53% in dTG rat hearts ($P<0.01$, n=8). CV-11974 did not have a significant effect (n=7, Fig. 10A). Bosentan and CV-11974 did not influence the diastolic stiffness, since the increase in LVEDP with increasing balloon volumes did not differ between differentially treated groups (Fig. 10B). Also minimal negative derivatives of intraventricular pressure, $-dP/dt_{min}$, were unaffected by the antagonists suggesting that alterations seen in the Frank-Starling response are related to systolic rather than diastolic function. Bosentan or CV-11974 had no effect on contractile parameters in control-perfused hearts (LVEDP=3 mmHg). As previously reported, these drugs did not have any effect on perfusion pressure under these experimental conditions (Magga *et al.* 1997a), indicating that there were no alterations in coronary vascular tone affecting the results.

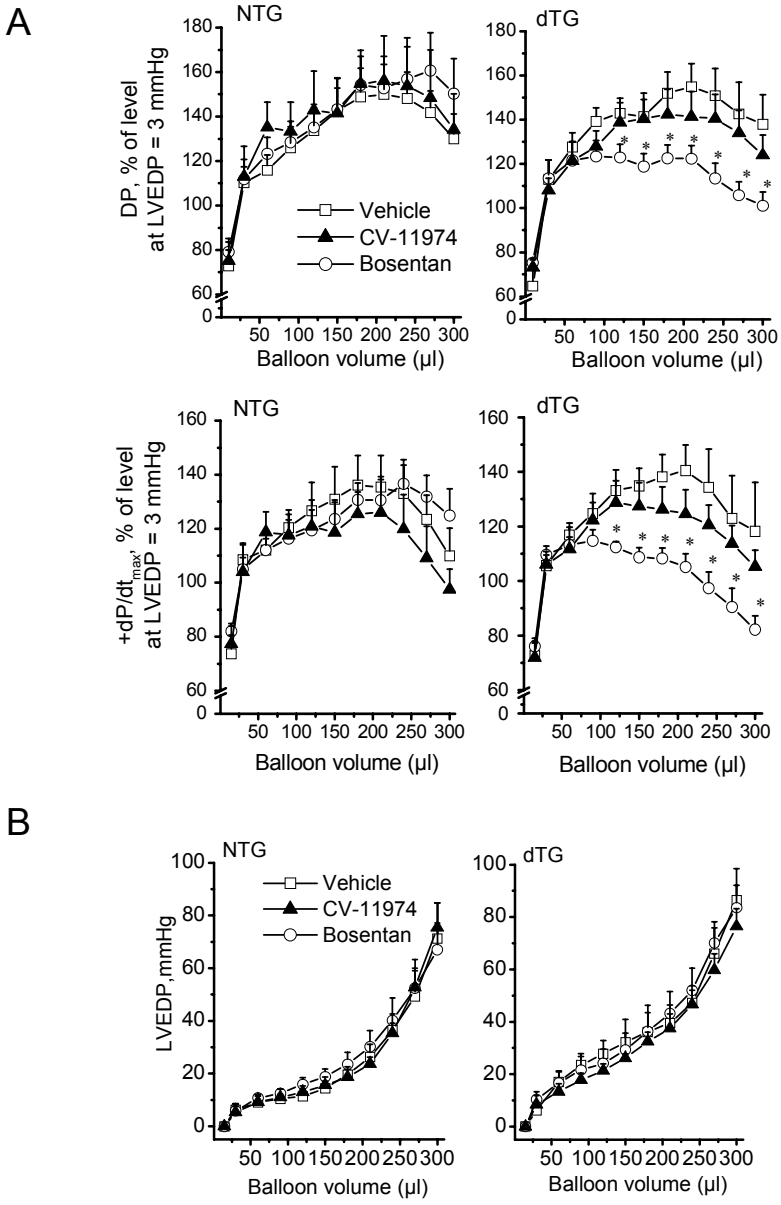

Fig. 10. Panel A. Plots showing changes in Frank-Starling responses as measured by developed pressure (DP) and maximal positive derivative of intraventricular pressure ($+dP/dt_{max}$) in response to treatment with $ET_{A/B}$ antagonist bosentan (1 µM), AT_1 antagonist CV-11974 (10 nM) or vehicle in NTG and dTG rat hearts. * *P*<0.05 vs. respective vehicle infused group. Panel B. Plots showing diastolic properties as measured by LVEDP in response to bosentan, CV-11974 or vehicle in NTG and dTG rat hearts during the stepwise increment in intraventricular balloon volume. *P*=NS vs. respective vehicle group (one-way ANOVA followed by Student-Newman-Keul´s post hoc test).

5.4. Mechanical load induced responses in mice hearts (IV)

5.4.1 Effects of atrial and ventricular loading

The method of increased coronary flow to load rat hearts has been previously described (I), (Gregg 1963, Magga *et al.* 1998a). To find the suitable level of loading in mice hearts, preliminary set of experiments with different levels of flow rate were performed. Two-hour perfusion with 4, 5 and 6 mL/min resulted in a flow-dependent increase in perfusion pressure (n=8 in each group). The 5 mL/min produced the greatest increase in contractile force (80±13 %, $P<0.05$ vs. control), and similarly, BNP mRNA levels were increased during the 2-hour experimental period 1.4±0.12, 1.9±0.13 and 1.5±0.08 -fold, for 4-, 5- and 6 mL/min, respectively ($P<0.05$ for all three vs. control). On basis of these findings, 5 mL/min flow rate was selected for future studies. Control flow rate was set to 2 mL/min, and with this flow rate the hearts were tested to be stabile for up to 5 hours of perfusion. ET-1 (1 nM, n=12) increased the perfusion pressure from 35±1 mmHg by 111±16 mmHg during the two hours perfusion, a response rather similar compared to rat hearts (Study I). Also the DT increased similarly to that in rat hearts; maximally by 40±7% ($P<0.01$).

The capillary structure was studied using light microscopy, since it previously has been shown that increasing perfusion pressure up to 200 mmHg for 10 minutes causes disruption of ECs in isolated Langendorff perfused rat hearts (McClellan *et al.* 1994). In isolated perfused mice hearts increased flow rate did not influence capillary structure even at flow rate of 6 mL/min, resulting in a perfusion pressure of 174±11 mmHg. In contrast, endothelial damage caused by Saponin treatment could be easily detected (Fig. 11).

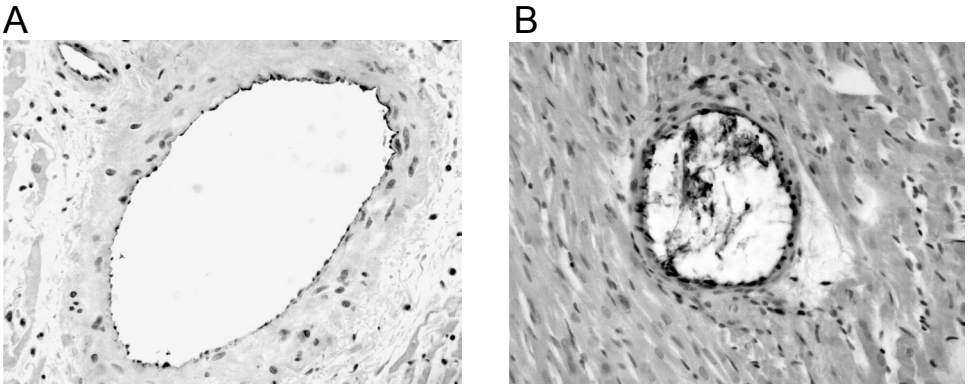

Fig. 11. Panel A. Coronary artery branch exposed to elevated arterial perfusion pressure (170 mmHg). A large coronary artery branch with preserved endothelial cell lining. Panel B. Coronary artery branch exposed to saponin (100 µg/µL). The arterial lumen is filled with detached endothelial cells (vWF, hematoxylin counterstain).

Atrial stretch system similar to that previously used for rat hearts was set up in mice (Ruskoaho *et al.* 1986, Mäntymaa *et al.* 1993). Right atrial pressure was elevated 5 mmHg above the baseline level for 2 hours by elevating the pulmonary artery cannula tip. This treatment increased the right atrial BNP mRNA levels by 60±8 % (n=6 for stretch and 11 for controls, $P<0.05$). At 1 hour, a statistically insignificant trend with a considerable variation (33±22 %) could be observed (n=5, P=NS). ANP mRNA levels remained unaltered.

5.4.2 Roles of endothelin-1 and ET_A and ET_B receptors

To analyze the role of cardiac ET-1 and Ang II systems in the Gregg effect induced contractile response, the receptor antagonists bosentan (a mixed $ET_{A/B}$ antagonist) (1 μM), CV-11974 (an AT_1 antagonist) (10 nM), BQ-123 (an ET_A antagonist) (100 nM) and BQ-788 (an ET_B antagonist) (100 nM) were used. Control experiments, performed without an increase in coronary flow, showed that during baseline conditions these drugs have no significant effect on contractile function or coronary vascular tone in the currently used experimental setup.

Perfusion pressure was elevated by 94±9 mmHg during 30 minutes of perfusion with 5 mL/min coronary flow ($P<0.001$). There was a tendency towards increased perfusion pressure during treatment with BQ-788, being statistically significant at 10 minutes ($P<0.05$) (Fig. 12). Other drugs had no significant effect on perfusion pressure.

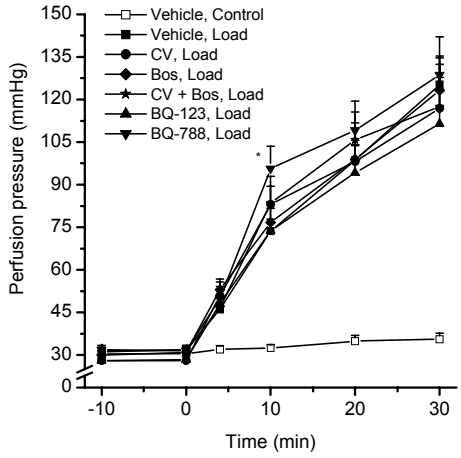

Fig. 12. Perfusion pressure during different treatments in isolated mice hearts perfused with control flow rate of 2 mL/min or loaded with flow rate of 5 mL/min. Each point is the mean ± SEM from 6-7 separate experiments on different isolated hearts. *$P<0.05$ (ANOVA followed by Student-Newman-Keul´s post hoc test).

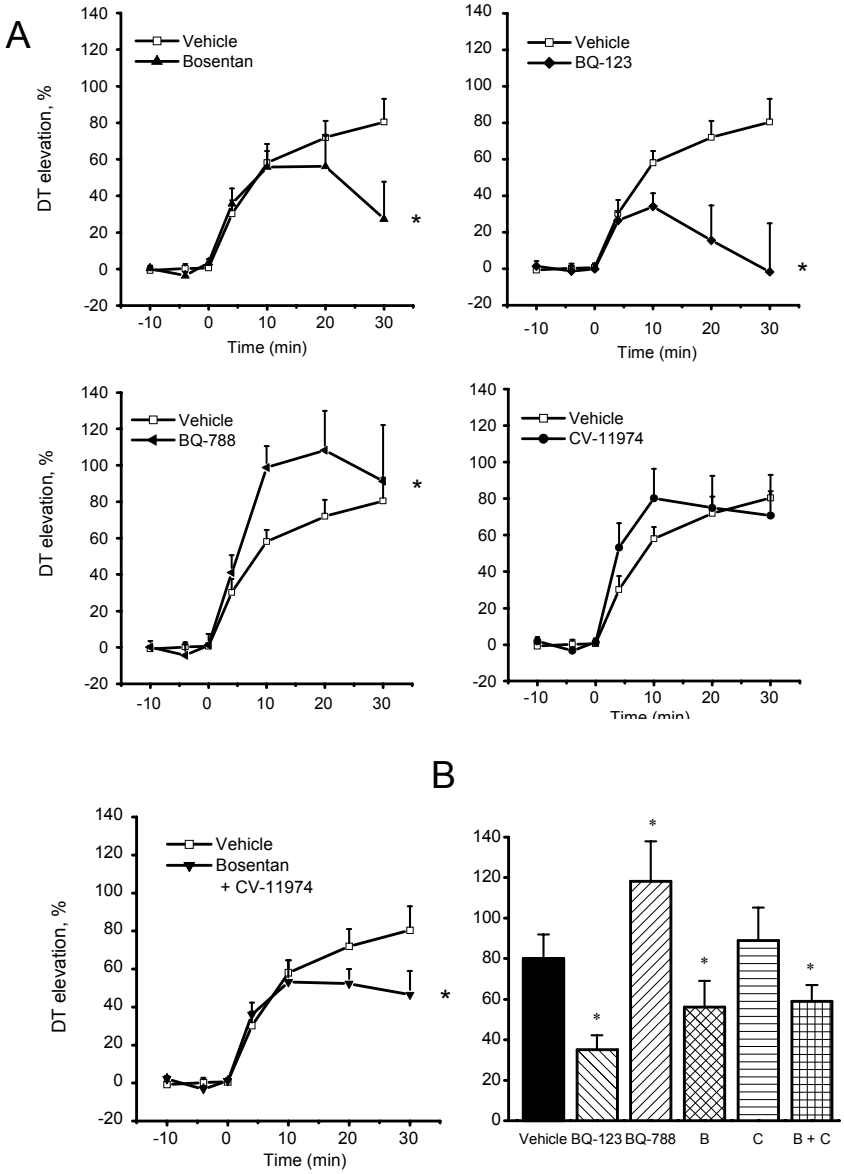

**Fig. 13. Panel A. Effect of ET- and Ang II receptor antagonists on DT in loaded mice hearts during 30-minutes loading with elevated coronary flow rate. ****P*<0.05 vs. vehicle (ANOVA). Panel B. Maximal DT elevation during loading in different groups during loading. B, bosentan; C, CV-11974; ****P*<0.05 vs. vehicle (Student's t-test).

In vehicle-perfused hearts, contractility (DT) was increased at maximum by 80±12 % (n=13, *P*<0.001) during loading (Fig. 13). Bosentan and BQ-123 significantly inhibited

the contractile response to the load, reducing the increase in DT by 34% and 56%, n=12 and 6, respectively ($P<0.05$). In contrast, BQ-788 enhanced the increase in DT by 35 % ($P<0.05$, n=6), while CV-11974 had no significant effect on contractile response to load (P=NS, n=8). When combined infusion of bosentan and CV-11974 was administered, the DT changes were similar to those with bosentan alone (P=NS vs. bosentan, $P<0.05$ vs. vehicle, n=9) (Fig. 13).

5.5 Role of type XV collagen in cardiac structure and function (V)

Northern blot hybridization was used to study the expression of type XV collagen mRNAs in the homozygous mutant mice generated by Cre/*loxP* mediated deletion of first two exons and the transcription start site. The closely spaced first and second exons of the *Col15a1* gene encode the first 33 residues of the 1,367-residue polypeptide and a split codon for the next residue (Eklund *et al.* 2000). The deletion completely abolishes *Col15a1* gene expression and no mRNA for type XV collagen was detected in the tissues.

Despite the complete lack of type XV collagen, the mice displayed no obvious alteration in phenotype, were fertile and had a normal life span. Genotyping of 345 offspring from heterozygous intercrosses showed that 21.5% were of the wild-type, 53.9% were heterozygous and 24.6% were homozygous for the null allele.

Although light microscopy did not reveal any conspicuous changes compared with the controls, electron microscopy showed abnormalities encompassing the microvessels and their endothelium. The capillaries in the wild-type mice were round and had a wide lumen, while some of those in the mutant mice were irregular in shape with intensively folded endothelial membranes. Some of the ECs were degenerated and swollen, had a pale cytoplasm with only a few cell organelles and bulged into the vessel lumen. In some cases the ECs were shrunken, showing a thin, electron-dense structure. These changes were focal and the incidence of the capillary and endothelial defects varied between samples, ranging from a few abnormal microvessels to more than 50% affected capillaries. The capillary defects were found in both the heart and skeletal muscle samples, although they were more frequent in the heart. No changes were noted in the vascular basement membranes, and no EC degeneration or changes in the capillary structure could be seen in the wild-type mice. The heart specimens containing swollen ECs showed focal ischemic changes in the cardiac myocytes, such as intracellular edema and vacuolization.

5.5.1 Effects of isoproterenol on cardiac function

Since already a mild cardiac dysfunction is known to associate with decreased responsiveness to β-AR agonists (Bristow *et al.* 1982), the hearts were stimulated with increasing doses of a β-AR agonist isoproterenol, and contractile function was analyzed. Cardiac function was studied using isolated perfused hearts from *Col15a1*$^{-/-}$ mice and wild-type mice aged six months and one year. The developed pressure as an index of

cardiac contractility was compared at the basal level and after isoproterenol stimulation. The basal contractility, as studied in isolated, paced, Langendorff perfused hearts with a soft balloon inside the left ventricle to measure contractile function, showed no difference between the mutant and wild-type mice hearts. The response to isoproterenol at concentrations of 0.01, 0.1 and 1 nM showed significantly smaller changes in the null mice (Fig. 14).

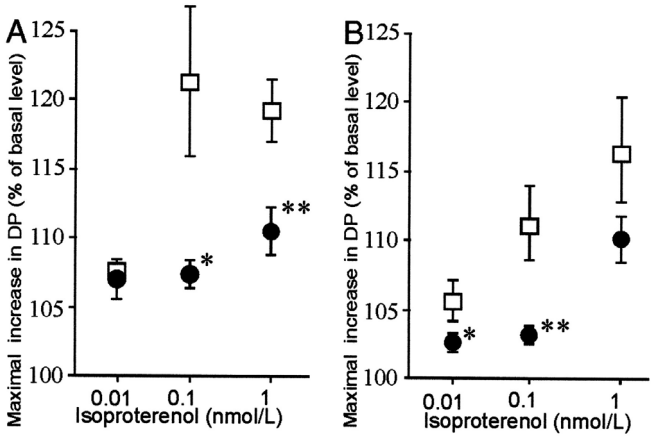

Fig. 14. Responses to low doses of isoproterenol at six months (Panel A) and one year age (Panel B) in isolated perfused hearts of *Col15a1*$^{-/-}$ mice (Closed circles) and wild-typed littermates (Open boxes) (n=7-11). The baseline developed pressure was 31.7±5.0 and 27.3±5.0 mmHg for the 6-month-old control and null mice, respectively, and 20.2±3.4 and 20.8±2.0 mmHg for the 1-year-old mice (mean ± SEM). DP; developed pressure. *P<0.05 **P<0.01 (one-way ANOVA followed by Student-Newman-Keul´s post hoc test).

Since cAMP is known to mediate the positive inotropic effect of β-adrenergic receptor stimulation, the tissue levels of cAMP was measured. A slightly diminished decrease in the left ventricular cAMP response after the administration of 0.1 nM isoproterenol was observed in the null mice compared with the wild-type mice (5.42±0.72 and 7.55±1.37 pmol/mg, respectively, P=0.098).

5.5.2 Changes in cardiac stress responses in collagen XV deficient mice

Since the abnormalities in capillary morphology observed in the mutant mice could have some consequences for blood flow and lead to pathological changes after cardiovascular stress, left ventricle samples were prepared after the exercise experiment. The possible cardiac injury was studied by assessing known markers of cardiac injury, including the extent of apoptosis, the activities of β-glucuronidase and proMMP-2 and the mRNA levels of ANP. Following exercise, statistically significant increases in the number of TUNEL-positive nuclei and in the activities of β-glucuronidase and proMMP-2 were detected in the *Col15a1*$^{-/-}$ mice. The exercise induced also highly increased ANP mRNA levels in two null mice out of eleven, although this was not statistically significant on average (P=0.06). No changes were observed in the wild-type individuals. It should be noted that the basal proMMP-2 level was lower in the null mice than in the controls.

6 Discussion

6.1 Modulation of endothelin-1 induced cardiac effects by plasma membrane Ca^{2+}-ATPase overexpression

The physiological function of the sarcolemmal calcium pump in the myocardium is unknown. Previously, Hammes *et al*. (1998) showed that there are no differences between control and PMCA rats in baseline or volume load increased cardiac performances *in vivo*. In the present study, baseline contractility was similar in NTG and PMCA hearts in isolated perfused heart preparation. Furthermore, no differences were found in baseline cardiac gene expression, secretion of ANP, AM and BNP into the perfusate, or in the left ventricular to body weight ratios between PMCA and control rats. These results along with the normal lifespan of PMCA rat line confirm that PMCA overexpressing rats have no cardiac dysfunction.

The isolated heart preparation used in the present studies allows the evaluation of regulatory processes in the whole organ level. Besides with the gene expression responses also the secretion of natriuretic peptides as well as the contractile performace can be analyzed. Naturally, the weakness compared to in vivo conditions is that the neurohumoral regulatory systems are lacking, and that the length of the experimental period is limited. However, the acute phase gene expression responses are well within the time scale of perfusion experiments. As always, the experimental model has certain limitations due to the surgical procedures and differences from the in vivo situation. However, the model allows the studying of the acute local regulatory processes independent of the systemic hemodynamic alterations.

A key finding of the present study (I) was that myocardial overexpression of the PMCA attenuated the hypertrophic response to ET-1 as shown by almost complete abolishment of the early induction of BNP, AM and *c-fos* gene expression in left ventricles as well as BNP and AM secretory responses to ET-1. The gene construct used to generate the TG ratline utilizes MLC-2 promoter, which restricts the expression of PMCA to ventricular myocardium (Hammes *et al.* 1998). ET-1 induced early activation

of BNP mRNA synthesis remained intact in atrial tissue of TG animals. This further suggests that the alterations seen in ventricular gene expression as well as AM and BNP secretion are due to increased amount of PMCA present in the ventricles. The vasoconstricting action of ET-1 was not altered in PMCA rats showing that smooth muscle cells in vascular walls of both lines responded similarly to ET-1.

Previously, PMCA has been reported to be involved in regulation of growth and differentiation processes in various cell types. In VSMCs and in ovary cells the PMCA overexpression inhibited growth and proliferation (Guerini *et al.* 1995, Husain *et al.* 1997), whereas in myoblasts PMCA overexpression resulted in acceleration of differentiation process (Hammes *et al.* 1996). Hammes et al. (1998) studied the response of cultured neonatal cardiomyocytes of this same PMCA overexpressing ratline to fetal calf serum, phenylephrine and isoproterenol. They found increased protein synthesis rate in response to all of these three different stimuli in PMCA overexpressing cells. Meanwhile, in the present study myocardial overexpression of PMCA attenuated the early induction of cardiac gene expression induced by ET-1 in perfused rat hearts. The reason for this discrepancy remains to be studied, but one possible explanation for the different effects of hypertrophic stimuli on cardiac protein synthesis in cultured cells compared with intact hearts may be due to the experimental conditions of cardiac myocytes under cell culture. For example, the data obtained in isolated neonatal and adult cardiac myocytes suggest that Ang II is a potent stimulus for cardiac *c-fos* expression (Dostal & Baker 1999), while it failed to stimulate this proto-oncogene in the intact ex vivo–perfused adult rat heart (Schunkert *et al.* 1995).

To study further the effects of PMCA overexpression to hypertrophic responses in intact adult heart, the responses in cardiac gene expression stimulated by ET-1 were compared to that produced by mechanical load in isolated, perfused heart preparation. The response to mechanical stimulus, which here was increased coronary flow, was similar to control hearts showing that there is no common alteration in myocardial gene expression in PMCA rats in response to hypertrophic stimuli. In view of unchanged baseline mRNA concentrations and peptide secretion, this suggests that there is no general alteration in synthesis and secretion of BNP and AM in PMCA overexpressing rat hearts. Furthermore, these results suggest that the signal transduction mechanisms for ET-1-induced hypertrophic responses differ from those activated by ventricular stretch. In agreement with this hypothesis, previous studies indicate that ET-1 is not obligatory for induction of BNP gene in rat ventricles in response to mechanical stimulus (Magga *et al.* 1997a), and stretch has been shown to increase the release of other growth factors, such as transforming growth factor-β (Li *et al.* 1997).

Recently, a major role was shown for PMCA 4b in the regulation of iNOS, since an inhibition of iNOS was observed with increased expression of PMCA 4b (Schuh *et al.* 2001). Although the study was carried out with embryonic kidney and neuroblastoma cell models, it suggested a novel mechanism for the PMCA in regulation of cellular function. However, in normal hearts iNOS expression is detected in conduction tissue and intracardiac neurons only (Kelly *et al.* 1996). Therefore, this mechanism is not a likely explanation for the present findings.

It has been previously shown that 20% overexpression of SERCA2 in TG mice increases the decline of calcium transients and cardiac contractility (He *et al.* 1997). SERCA2 plays a dominant role in the lowering of cytoplasmic calcium levels during

cardiac relaxation (see section 2.1.1.3.), while PMCA has only a minor role extruding calcium from cytosol after each beat (Bers 2000). In view of this, it was expected that there is no difference between NTG and PMCA hearts in baseline contractility. In the present study, the overexpression of PMCA had no effect on inotropic responses to ET-1, which suggests that the intracellular signaling mechanisms leading to hypertrophic response and to increased contractility may be different. The hypertrophic effect of ET-1 is mainly thought to be mediated by ET_A receptors (Sakai *et al.* 1996a). Activation of ET-receptors may couple to different G-protein subfamilies (Kelly *et al.* 1990, Takuwa *et al.* 1990), but major pathway for hypertrophic response is thought to involve $G\alpha_q$ and subsequent formation of IP_3 and DAG, which induce release of Ca^{2+} and activation of PKC, respectively, and lead to activation of mitogen-activated protein kinase MAPK and other signaling pathways (Clerk & Sugden 1999, Hilal-Dandan *et al.* 2000, Sugden 1999). Since ET_A receptors are known to locate in caveolae (Chun *et al.* 1994), attenuation of ET-1 response may be due to alterations in caveolar signal transduction (Smart *et al.* 1999) induced by PMCA overexpression. Several proteins involved in signal transduction have been localized to caveolae, including $G\alpha_s$, ras, PKCα, MAPK, src tyrosine kinase and channels such as IP_3-sensitive Ca^{2+} channel (Couet *et al.* 1997, Shaul & Anderson 1998, Smart *et al.* 1999). The mechanism by which PMCA overexpression could affect caveolar signaling events may involve modification of subcellular Ca^{2+} pools or direct interaction with other caveolar molecules, such as ET_A receptors or other molecules involved in ET-1 signaling process. It has been reported that in liver PMCA is regulated by G_s (Jouneaux *et al.* 1993), which can be coupled to ET_A or ET_B receptors (Jouneaux *et al.* 1993, Takagi *et al.* 1995). It has been also shown that calcium signal provoked by ETs in liver cells is not only due to activation of PLC but also to inhibition of the PMCA, PMCA being coupled to ET_A receptor by G_s (Jouneaux *et al.* 1994). In view of this, the attenuated ET-1 response in PMCA overexpressing rat hearts could be explained simply by the higher amount of PMCA present resulting in increased capacity to extrude Ca^{2+} from the specific subcellular pool. This hypothesis supports a major role for PMCA in regulating the hypertrophic ET-1 response in myocardium.

6.2 Adrenomedullin in regulation of coronary vascular tone

Previous investigations (Hayakawa *et al.* 1999) have suggested a role for NO release in the mechanisms of AM-induced vasodilation in rats. In contrast, present data (II) suggest that AM can exert a profound coronary vasodilator effect under the blockade of NO synthesis. The interplay of ET-1 and AM has been previously studied mainly at the level of the synthesis and secretion (study I), (Jougasaki *et al.* 1998, Kohno *et al.* 1995). The current findings suggest that AM may function as an endogenous modulator of ET-1-induced vasoconstriction independently of the L-arginine-NO pathway.

Administration of ET-1 *in vivo* induces a coronary constrictor effect predominantly via ET_A receptors (Haynes & Webb 1994). However, simultaneous activation of ET_B receptors, triggering the release of NO from ECs (Hirata *et al.* 1993), may limit the constrictor effect of ET-1. Accordingly, blockade of NO formation augments the effect of ET-1 on vascular tone (Lerman *et al.* 1992). In agreement, in the present study the

increase in perfusion pressure in response to ET-1 was markedly augmented in the presence of a NOS inhibitor. Whether also other vasodilator pathways may counteract the constrictor effect of ET-1 is of importance especially in situations associated with a decreased bioavailability of endothelium-derived NO.

Since AM is one of the most potent endogenous vasodilators (Jougasaki & Burnett, Jr. 2000, Samson 1999), it was of interest to study its effects on ET-1 induced vasoconstriction. AM is synthesized and secreted by ECs and VSMCs (Sugo *et al.* 1994a, Sugo *et al.* 1994b). ET-1 enhances the production of AM in cultured VSMCs (Sugo *et al.* 1995b). Stimulation of ET_B receptors increases the secretion of AM in vascular ECs (Jougasaki *et al.* 1998), suggesting that AM may compensate the vasoconstrictor effect of ET-1. In agreement with this hypothesis, the present results show that AM markedly attenuates the coronary vasoconstriction induced by ET-1. The existence of a paracrine-autocrine regulatory loop between ET-1 and AM is further supported by the finding showing that administration of ET-1 significantly increases the release of AM into the coronary effluent of the perfused rat heart (I, II). Moreover, the increase in perfusate AM levels presumably reflects only a tiny fraction of that produced and affecting locally.

Based on previous studies, stimulation of NO release appears to contribute to the vasorelaxing effect of AM in various rat vascular beds (Hayakawa *et al.* 1999). Of special importance is the finding that AM could reverse the coronary vasoconstriction induced by ET-1 under the blockade of NO synthesis. This suggests that AM may represent an alternative pathway distinct from NO to counteract the pressor response to ET-1 in the rat coronary vasculature. In previous studies (Yoshimoto *et al.* 1998) in porcine coronary artery rings, denudation of the endothelium did not modulate the relaxant effect of AM, whereas Terata et al. (2000) recently reported that in human coronary arterioles AM elicited vasodilation in part through production of NO and in part through activation of K^+ channels. In addition to the different experimental conditions, the differences in regulation of eNOS activity between the species may explain the discrepancy between these studies. Whether endogenous AM can act as a buffer for the coronary constrictor effect of ET-1 needs to be investigated further. However, there are currently no selective antagonists for AM receptors available.

The synthesis of ET-1 is regulated by numerous stimuli including ischemia (Brunner 1997), and the enhanced levels of ET-1 may contribute to the further exaggeration of coronary constriction. ET-1 can augment its own gene expression through ET_B receptors in ECs (Saito *et al.* 1995). Furthermore, the constrictor effect of ET-1 is likely to be enhanced by a simultaneous impairment of NO-dependent relaxation due to decreased bioavailability of NO in various pathophysiological conditions including atherosclerosis (Mathew *et al.* 1997). In contrast, AM synthesis and secretion has been reported to be markedly augmented in cultured ECs by hypoxia (Nakayama *et al.* 1999). Because AM suppresses the production of ET-1 (Kohno *et al.* 1995) and attenuates the coronary constrictor effect of ET-1, as shown in this study, it is tempting to speculate that AM may act against the vasoconstriction maintained by ET-1. The present observations show that exogenous AM is able to attenuate the vasoconstriction by ET-1 under conditions of low NO production typical of the coronary artery disease.

6.3 Endothelin-1 in regulation of cardiac contractile function

The key finding of the study III was the observation that $ET_{A/B}$ receptor antagonist impaired the Frank-Starling response in dTG rat hearts. This raises the question whether the effect was due to the alteration of diastolic or systolic function. Basal release of ET-1 delays relaxation in normal guinea pig hearts (Prendergast *et al.* 1997a). Similar increase in LVEDP was observed when bosentan or vehicle was infused in dTG rat hearts suggesting that the diastolic stiffness was not affected by ET-1. In addition, there was no change in maximal rate of isovolumic relaxation, $-dP/dt_{min}$, in response to bosentan. Thus the inhibition of the Frank-Starling response in dTG rat hearts by ET-1 receptor blockade appears to be mediated by alteration of the systolic function.

As previously reported (Strömer *et al.* 1997, Bartel *et al.* 2002), compensated LVH results in enhanced contractile performance in isolated hearts. In this study the rats were studied in compensated phase, showing no obvious signs of heart failure, such as ascites or pleural effusion. Further confirming this fact, the contractile performance was enhanced in dTG rat hearts. Also the perfusion pressure was slightly elevated in dTG rat hearts. This may in itself increase contractile force, as reported in studies I and IV. However, the difference in developed pressure (DP) between NTG and dTG hearts at LVEDP=3 mmHg was nearly 50 % (Table 6), while the perfusion pressure in dTG rat hearts was 54 mmHg. In experiments with increased coronary flow (studies I, IV), the perfusion pressure increased over 150 mmHg (see sections 5.1. and 5.4.).

In contrast with the results obtained with bosentan, AT_1 receptor blocker CV-11974 did not have any significant effect on the Frank-Starling response in dTG rat hearts, even though increased Ang II release has been suggested to induce ET-1 action in response to myocyte stretch in heart (Liang & Gardner 1998, Bohlender *et al.* 2000). The renin-angiotensin system components are found also in normal rat hearts, and they are induced during cardiac hypertrophy and failure (Dostal & Baker 1999). A role for Ang II in the Frank-Starling mechanisms cannot be excluded, but in this model of LVH it does not seem to be as significant regulator of contractile function as ET-1. It is also possible that local Ang II production would need blood derived renin to be completely active (Müller *et al.* 1998).

Previous studies suggest that stretch induced release of Ang II and ET-1 stimulates the NHE, and activates the NCX in its reverse mode (Ca^{2+}in -Na^{+}out) (Tavi *et al.* 2001). This has been demonstrated to be the mechanism for the slow phase of the Frank-Starling response in a normal cat papillary muscle preparation. However, inhibition of AT_1 or $ET_{A/B}$ receptors had no effect on the rapid phase of the Frank-Starling response (Perez *et al.* 2001), in agreement with the current results in normal rat heart. In the whole organ model used here it is difficult to dissect between the slow and rapid phases, and therefore the peak contractile values are a result of combination of both slow and rapid phases of the Frank-Starling response. As the slow phase only accounts for approximately 20% of the contractile response to load (Perez *et al.* 2001), alterations restricted to the slow phase cannot explain the present results. An enhanced NHE activity has been described in hypertrophied hearts (Perez *et al.* 1995). Thus it is possible that ET-1 dependent Na^{+}-H^{+} exchanger activity, compensating the acidifying mechanisms, plays a role in the Frank-Starling response in hypertrophied dTG but not in normal rat hearts. In agreement with

this, intracellular acidosis has been shown to inhibit the contractile response to stretch in a rat atrial preparation (Tavi *et al.* 1999). The increased NCX mRNA levels of the dTG rat hearts may have a role in the altered regulation of the Frank-Starling response. Furthermore, a contribution by endogenous ET-1 to the Frank-Starling mechanism of hypertrophied dTG rat hearts may also relate to the ET-1 mediated improvement of the contractile efficacy (Takeuchi *et al.* 2001). Interestingly, the increase of contractile force due to the Frank-Starling response is mediated by enhanced contractile protein responsiveness to Ca^{2+} (Kentish *et al.* 1986), while similar mechanism contributes to the positive inotropic response to ET-1 (Krämer *et al.* 1991).

The Frank-Starling mechanism is preserved in hypertrophied and even in failing hearts (Holubarsch *et al.* 1996), unlike the force-frequency relationship and the β-adrenoceptor stimulation of contractile force (Bristow *et al.* 1982, Mertens *et al.* 1992, Ohtsuka *et al.* 2000). Therefore, in these pathological conditions the cardiac function is more dependent on the Frank-Starling mechanism. Bosentan interfered with the Frank-Starling response in the compensated LVH (with modestly elevated ET-1 peptide concentrations). This suggest a greater importance in more severe hypertrophy with high ET-1 concentrations, as in failing human hearts. Interestingly, a recent study suggested that treatment with the mixed $ET_{A/B}$ antagonist LU 420627 might impair the cardiac function and survival after myocardial infarction (Nguyen *et al.* 2001). In spite of the fact that the vasodilatory effect of ET-1 receptor antagonists decreases the afterload (Spieker *et al.* 2000, Haynes & Webb 1994), and the growth inhibitory effect attenuates the level of LVH (Mulder *et al.* 1997), the impairment of the contractile performance in hypertrophied hearts under load may be one explanation for the increased number of events leading to clinical worsening in the beginning of the high-dose bosentan therapy of human CHF (Mylona & Cleland 1999). It is also possible that ET-1, even though arrhythmogenic itself secondarily due to vasoconstrictive effect (Ezra *et al.* 1989), protects from catecholamine induced arrythmogenesis by inhibition of IP_3 and cAMP generation through G_i especially during ischemia (Woodcock *et al.* 1999, James *et al.* 1994).

6.4 Distinct roles of ET_A and ET_B receptors in mice hearts

In hypertrophied dTG rat hearts loaded with intraventricular balloon, a significant contribution of ET-1 to the Frank-Starling response was observed. However, in Sprague-Dawley (SD) rat hearts the Frank-Starling response was independent of ET-1 (study III). The study with isolated mice hearts and ET receptor blockers revealed the pivotal role of ET-1 in mechanical load induced contractile response to loading with Gregg effect in normal mice hearts and distinct roles of ET_A and ET_B receptors in regulation of contractile strength (study IV). The elevation of coronary flow rate enhances contractile force and oxygen consumption in isolated perfused heart (Gregg 1963, Magga *et al.* 1998a). It activates also the expression of proto-oncogenes, stress proteins and BNP and the synthesis of total proteins (Takala 1981, Kira *et al.* 1984, Magga *et al.* 1998a). The different role of ET-1 in normal hearts of these studies may be related to at least two aspects. First, the Gregg effect involves release of ET-1 among other vasoactive factors (McClellan *et al.* 1994), and the contractile response is not dependent on only Frank-

Starling response, which may though be partially involved. Secondly, the interspecies difference between mice and rats may explain part of the results.

Previous evidence has shown a major role for ET-1 and Ang II in hemodynamic overload induced left ventricular hypertrophy and heart failure (Sakai *et al.* 1996a, Kojima *et al.* 1994). ET-1 and Ang II have been suggested to have a role in slow-phase contractile response to stretch in rat papillary muscle (Alvarez *et al.* 1999). Although ET_A receptors have been implicated in inotropic and hypertrophic responses (Kelso *et al.* 2000, Ito *et al.* 1994a), little has been known about the role of ET_B receptors. According to the present results, ET_A receptor activation accounts for ET-1 mediated increment in contractile force during load, whereas ET_B receptor activation has an opposite, inhibitory effect on contractile function. When activation of both receptor subtypes is blocked with bosentan, the net result is a reduction in contractile response to mechanical load. This is in agreement with receptor subtype amounts reported previously (Serneri *et al.* 2000).

ET_B receptor activation has been reported to produce a vasodilatory effect via NO dependent pathway (Verhaar *et al.* 1998), but especially in the lungs ET_B receptors have been implicated in clearance of ET-1 from plasma (Fukuroda *et al.* 1994). In previous study (Kinnunen *et al.* 2000), positive inotropic effect of exogenous ET-1 in isolated perfused rat heart was augmented by inhibiting NOS with L-NAME. Thus the data suggests that ET_B receptor mediated increase in NO release (Verhaar *et al.* 1998) may play an inhibitory role in regulation of contractile strength during loading. Other possible mechanism for ET_B blockade induced augmentation of contractility would be the decreased clearance of ET-1, thus inducing an increase in ET_A receptor binding of ET-1.

In the present study, there was a modest increase in perfusion pressure by BQ-788. However, the magnitude of the difference in perfusion pressures between BQ-788 and vehicle treated groups was small, and cannot explain differences in developed tension. The lack of effect on perfusion pressure by BQ-123 and bosentan shows that the contractile effects of these drugs are not related to hemodynamic factors in this model.

The data presented herein show that ET-1 contributes to the increased contractile strength in response to mechanical load. Previously, Maeda *et al*. (1998) reported increased plasma ET-1 after 30-45 minutes intensive exercise, further suggesting a physiological role for ET-1 in heart during acute hemodynamic loading. Previous report suggests that decreased contractility may be associated with ET antagonist treatment (Sakai *et al.* 1996b). Taken together the previous results (Takeuchi *et al.* 2001), the present data suggests that in certain conditions endogenous ET-1 may have a significant positive inotropic effect associated with increased contractile efficiency. It is possible that like in vessels with vasoconstriction and vasodilatation, also in the heart there is a balance between ET_A and ET_B receptor mediated regulation of contractile force. Similarly as in vasculature, also in the heart the ET_A mediated effects seem to predominate.

6.5 Collagen XV and cardiovascular structure and function

Despite the wide occurrence of type XV collagen in basement membranes throughout the body, mice lacking it are viable and fertile (V). However, the *Col15a1*$^{-/-}$ mice showed increased sensitivity to exercise-induced muscle damage. Despite the antiangiogenic role

of type XV collagen-derived endostatin (Ramchandran *et al.* 1999), no abnormalities in the number of vessels could be observed. Instead, type XV collagen appeared to play a role in the integrity of the microvessels, since its deficiency was found to lead to an apparent collapse of the capillary wall in the heart and skeletal muscle, resulting in various degrees of narrowing or obstruction of the capillary lumen and EC degeneration and swelling. Morphologically similar degenerative changes in capillary ECs have been observed in experimental models for the ischemic (Armiger & Gavin 1975) and reperfused (Ward & McCarthy 1995) myocardium and in patients with small vessel disease (Mosseri *et al.* 1991), microvascular angina, hypertrophic cardiomyopathy or dilated cardiomyopathy (Suzuki *et al.* 1995). Those previous observations suggest that the EC degeneration and swelling in *Col15a1*$^{-/-}$ mice may be caused by impaired microvasculature perfusion and ischemic damage to the endothelium. Immunostaining studies (Muona *et al.* 2002) have indicated that while type XV collagen is associated with most capillaries in adult mice, including those in the heart and the skeletal muscle, there are some tissues, including the mature lung and brain, in which it is not detected around the capillaries. The fact that the lung and brain capillaries of the null mice were normal further confirms that the defects seen in the heart and skeletal muscle capillaries are due to a lack of type XV collagen.

The exercise protocol was optimized for studying skeletal muscle injury, and the timing for analysis could cause limitations for the markers used to study cardiac injury, since the maximal responses in the expression of MMP-2 (Cleutjens *et al.* 1995) and ANP (Hama *et al.* 1995) are reached later than 48 hours after acute cardiac injury and the apoptotic effects earlier (Kajstura *et al.* 1996). Interestingly, the basal MMP-2 activities were found to be significantly lower in the *Col15a1*$^{-/-}$ mice than in the wild-type ones. Since MMP-2 is expressed by ECs (Lewalle *et al.* 1995), this could suggest loss of ECs and coincide with the identified EC degeneration.

As the organization and function of the heart as a continually contracting muscle differs from that of skeletal muscle, acute exercise is not likely to lead to similar injuries to those affecting the latter. The abnormalities in the heart microvasculature observed at the morphological level will most probably cause marked ischemic-like damage only upon loading. This has been observed with young mice lacking δ-sarcoglycan, where the primary causes of the heart phenotype is thought to be a perturbation in vascular function. In both δ-sarcoglycan (Coral-Vazquez *et al.* 1999) and type XV collagen-deficient mice acute exercise caused cardiac injury before the development of apparent cardiomyopathy. Furthermore, the preservation of the histological integrity of the heart tissue in the *Col15a1*$^{-/-}$ mice supports the hypothesis that a certain degree of vascular dysfunction may be required to reach the ischemic threshold necessary to induce myocardial necrosis, as proposed by Corel-Vazquez *et al.* (1999).

The isolated perfused hearts of *Col15a1*$^{-/-}$ mice showed decreased responses to a β -AR agonist. Reduced responsiveness to β-AR stimulation is associated with chronic heart diseases (Bristow *et al.* 1982) and with ageing (Lakatta 1999). Hearts suffering from chronic diseases have multiple changes in β-AR-mediated events, including the expression and function of β-adrenergic receptors, G-proteins, AC and G-protein receptor kinases (Post *et al.* 1999). Down-regulation of β_1-AR receptors has been suggested to occur at an early stage in the development of heart failure (Kiuchi *et al.* 1993). In humans, reduced β_1-AR mRNA levels (Engelhardt *et al.* 1996) and receptor density

(Fowler *et al.* 1986) are also detected in a mild form of cardiac dysfunction, indicating that down-regulation of β-AR receptors is not restricted to severe heart disease. Moreover, histological analysis of cardiac tissue from volume-overloaded pigs indicates that decreased responsiveness to β-AR stimulation can occur without degenerative changes such as inflammation or fibrosis (Hammond *et al.* 1992).

Microvasculature defects are involved in the initiation and progression of heart failure and cardiomyopathy in some modes of human heart disease (Gavin *et al.* 1998). Microcirculation abnormalities have been demonstrated earlier in Syrian hamsters suffering from cardiomyopathy (Factor *et al.* 1982), together with a decreased response to isoproterenol, indicating changes in β-AR signaling pathways (Feldman *et al.* 1990). The desensitization of β-AR signaling observed in *Col15a1*$^{-/-}$ mice is a hallmark of heart failure, and the microcirculation defect may be contributing to cardiac dysfunction.

In view of its collagenous primary structure, its location in the extracellular space and the consequences of the loss of its function, it could be assumed that type XV collagen functions as a structural component which is needed to stabilize cells with the surrounding connective tissue. Data also suggest that a lack of type XV collagen will cause damage to the heart in connection of induced cardiovascular stress. It is possible that this deficiency may cause mild cardiac dysfunction, detectable first as a diminished inotropic response to isoproterenol. Interestingly, these changes mimic early or mild heart disease with respect to features such as decreased inotropy and impaired response to exercise. The microvascular defects are more pronounced in the heart than in skeletal muscle and are accompanied by ischemic changes in the ECs and adjoining cardiomyocytes, and the heart phenotype may be due to impaired microcirculation.

7 Summary and conclusions

The present work underlines the importance of autocrine/paracrine ET-1 in regulation cardiac function. In detail, the findings of the present study are summarized as follows:

1. Myocardial overexpression of PMCA attenuated early induction of hypertrophic response to ET-1 but not to increased load, while baseline cardiac function remained intact. The results suggest that PMCA plays a role in regulation of myocardial function.
2. Coronary vasoconstriction response to ET-1 was augmented by pharmacological inhibition of endogenous NO formation and the enhanced constrictor effect was substantially reversed by AM. ET-1 also induced AM synthesis and release. These findings are consistent with the hypothesis that AM may play a compensatory role against excessive coronary vasoconstriction induced by ET-1.
3. ET-1 contributed significantly to the Frank-Starling response in hypertrophic dTG rat hearts. In contrast, AT_1 receptor antagonists did not seem to interfere with the Frank-Starling response, underlining the significance of endothelin system as a regulator of cardiac function in hypertrophic transgenic rat hearts with human renin and angiotensinogen genes.
4. In mice hearts, ET-1 had a dual role in contractile responses during loading with Gregg effect; ET_A receptor activation increased contractility while ET_B activation decreased it. AT_1 receptor antagonist had no effect on contractile performance, suggesting that ET-1 plays a role in regulation of contractility during load independently of Ang II.
5. Type XV collagen deficiency caused mild cardiac dysfunction, first detectable as a diminished inotropic response to isoproterenol. It is possible that the heart phenotype is due to impaired perfusion as a result of the capillary endothelial damage. Furthermore, the present findings support the idea that type XV is a structural component of the extracellular matrix needed to stabilize capillaries and muscle fibres, and is essential for the proper functioning of the heart.

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