Anna-Maria Kubin

CHARACTERIZATION OF SIGNALING MECHANISMS REGULATING CARDIAC CONTRACTILITY
ANNA-MARIA KUBIN

CHARACTERIZATION OF SIGNALING MECHANISMS REGULATING CARDIAC CONTRACTILITY

Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in the Auditorium of the Department of Pharmacology and Toxicology (Aapistie 5), on 27 May 2011, at 12 noon

UNIVERSITY OF OULU, OULU 2011
Kubin, Anna-Maria, Characterization of signaling mechanisms regulating cardiac contractility.
University of Oulu, Faculty of Medicine, Institute of Biomedicine, Department of Pharmacology and Toxicology, P.O. Box 5000, FI-90014 University of Oulu, Finland; University of Oulu, Biocenter Oulu, P.O. Box 5000, FI-90014 University of Oulu, Finland
Oulu, Finland

Abstract
The heart adapts to hemodynamic overload with cardiac hypertrophy. Initially the increase in heart mass normalizes wall stress and permits normal cardiac function. In the long term pathological growth is associated with increased heart size, loss of functional myocytes and fibrotic replacement, heart dilatation and cardiac dysfunction, which can ultimately lead to heart failure.

Vasoactive peptides participate in the regulation of cardiac contractility in an auto/paracrine way, but the peptidergic signaling pathways are largely unknown. The present study aimed to characterize the signaling mechanisms mediating the positive inotropic effect of endothelin-1 (ET-1) and the effects of prolactin releasing peptide (PrRP) on cardiac contractility in the isolated perfused rat heart preparation.

The study demonstrated that mitogen-activated protein kinases (MAPK) have opposing roles in the regulation of cardiac contractility stimulated by ET-1. Extracellular signal-regulated kinase 1/2 (ERK1/2) mediated positive inotropic response to ET-1 was found to be counterbalanced by p38-MAPK. In addition, the effect of ET-1 was partly dependent on enhanced NADPH oxidase reactive oxygen species (ROS) generation, which activated the ERK1/2 pathway. In contrast, β-adrenergic inotropic effect was limited by stimulation of ROS production via negating phospholamban phosphorylation. The positive inotropic effect of ET-1 was counterbalanced by guanylyl cyclase (GC)-cGMP-protein kinase G (PKG) pathway and neuronal nitric oxide synthase (nNOS). PrRP was found to exert a direct positive inotropic effect which was independent of cAMP and was suppressed by concurrent activation of protein kinase Cα (PKCα) and protein phosphatase 1 (PP1), and dephosphorylation of phospholamban.

In conclusion, in the present study signaling pathways in the acute regulation of cardiac contractility stimulated by ET-1 were characterized. The results suggest that the positive inotropic effect of ET-1 is mediated by ROS and ERK1/2 while p38-MAPK counterbalances the effect of ET-1. In addition, GC-cGMP-PKG pathway and nNOS modulate the response to ET-1. The study also established the previously unknown cardiac effects of PrRP. The findings provide a better understanding of molecular mechanisms involved in the regulation of cardiac contractility, and may indicate potential targets for novel therapeutic interventions.

Keywords: endothelin-1, myocardial contraction, nitric oxide, prolactin releasing peptide, reactive oxygen species, signal transduction
Kubin, Anna-Maria, Paikalliset välittäjäaineet ja niiden signalointimekanismit sydämen supistusvoiman säätelyssä.
Oulun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteellinen laitos, Farmakologia ja toksikologia, PL 5000, 90014 Oulun yliopisto; Oulun yliopisto, Biocenter Oulu, PL 5000, 90014 Oulun yliopisto

Tiivistelmä

Sydänlihaksen lisääntyvä mekaaninen kuormitus esimerkiksi verenpainetaudin tai sydäminfarktin yhteydessä voi johtaa sydämen kammion seinämän paksuuntumiseen eli hypertrofiaan. Hypertrofia auttaa sydäntä sopeutumaan lisääntyneeseen työmäärään, mutta se on myös sydän- ja verisuonitauti tapahtumien riskitekijä. Pitkään jatkuva kuormitus johtaa usein sydämen pumpausvoiman heikkenemiseen ja sydämen vajaatoimintaan.

Sydän tuottaa useita paikallisesti vaikuttavia vasoaktiivisia tekijöitä, jotka osallistuvat sydämen supistuvuuden säätelyyn. Väitöskirjatutkimuksessa tutkittiin signaalinvälitysjärjestelmiä, jotka osallistuvat endoteliini-1:n (ET-1) sydämen supistusvoimaa lisäävän eli positiivisen inotrooppisen vaikutuksen muodostumiseen sekä selvitetiin prolaktiinia vapauttavan peptidin (PrRP) vaikutuksia sydämen supistusvoimaan käyttämällä koemallina eristettyä, perfusoitua rotan sydäntä.


Asiasonat: endoteliini-1, happiradikaalit, prolaktiinia vapauttava peptidi, solunsisäinen viestinvälitys, sydänlihaksen supistuvuus, typpioksi
To my family
Acknowledgments

This work was carried out at the Department of Pharmacology and Toxicology, Institute of Biomedicine, University of Oulu. I wish to thank Professor Emeritus Olavi Pelkonen, who was the head of the department during the majority of my thesis project, for providing excellent conditions for this work and for creating an encouraging and relaxed atmosphere in which to work.

I express my deepest gratitude to my supervisor Professor Heikki Ruskoaho for providing me an opportunity to do research in his research group. In addition to his broad knowledge and experience, I highly appreciate his innovative, enthusiastic and optimistic attitude towards science and life in general.

I am much obligated to István Szokodi, MD, PhD, who taught me much about critical scientific work. His expert knowledge and methodological guidance have been of the utmost importance.

I am very grateful to Docent Eriika Savontaus and Professor Kid Törnquist for their careful review of this thesis and for their constructive criticism. I also wish to thank Anna Vuolteenaho for revising the language of the thesis.

My warmest thanks are due to Docent Pasi Tavi for scientific collaboration and valuable comments. I also want to express my gratitude to Réka Skoumal, PhD, for her essential contribution and for her friendship during these years. I also want to thank all my other co-authors, especially Docent Risto Kerkelä, Hanna Leskinen, Attila Könyi and Ábel Perjés.

I owe my gratitude to Sirpa Rutanen, Kaisa Penttilä, Marja Arbelius, Kati Lampinen and Pirjo Korpri for skilful technical assistance. I am grateful to Raija Hanni, Esa Kerttula and Marja Räinä for their much-appreciated help in all practical matters. I also wish to thank the personnel at the Laboratory Animal Center.

All the present and former members of our research group are acknowledged: it has been a privilege to work with you all. My special thanks belong to my roommates Elina Koivisto, Anne-Mari Moilanen and Marja Tölli for their cheerful company. I also want to thank Jaana Rysä, Raisa Serpi, Erja Mustonen, Tuomas Peltonen, Olli Tenhunen, Johanna Magga, Leena Kaikkonen, Laura Vainio, Annina Kelloniemi, Sini Rautio, Virva Pohjolainen, Jani Aro, Hanna Säkkinen, Anna-Maria Tolonen, Harri Pennanen, Pauli Ohukainen and the other colleagues for the good atmosphere to work in. The other staff at the Department of Pharmacology and Toxicology is most warmly acknowledged as well.
All my friends are warmly acknowledged. My special thanks go to Johanna Kiviharju and Jenni Peltonen for their long-lasting friendship.

I wish to thank my parents Irmeli and Eero Kubin for their loving care and support. They have given me an excellent basis for my life and they have always encouraged and believed in me. I also want to thank my father, as it was his scientific work that got me interested in doing research. I want to thank my brother Antti Kubin and his wife Mirja Kubin for all the delightful moments and support. My nephew and niece Atte and Veera are warmly acknowledged for bringing so much joy also into my life. My younger brother Juha Kubin deserves my sincere thanks for his interest and support. I also thank Mirja and Veli Koistinen for their care.

Finally, I want to express my gratitude to my dear husband Hannu Koistinen and our son Juho. I want to thank Hannu for his care, constant interest and encouragement, which has been essential for the completion of this work. Above all I thank him for the love and friendship we share. Our precious son Juho has brought so much joy into our lives. He is my constant source of happiness and has made everything worthwhile.

This research project has been financially supported by the Aarne Koskelo Foundation, Duodecim Society of Oulu, the Finnish Foundation for Cardiovascular Research, the Finnish Medical Foundation, the Finnish-Norwegian Medical Foundation, the Ida Montin Foundation, the Paavo Ilmari Ahvenainen Foundation and the Research and Science Foundation of Farmos.

Oulu, April 2011

Anna-Maria Kubin
Abbreviations

[Ca^{2+}]_i intracellular calcium concentration
5-HD 5-hydroxydecanoate
AC adenylyl cyclase
ACE angiotensin converting enzyme
AM adrenomedullin
Ang angiotensin
ANP atrial natriuretic peptide
AT1 angiotensin II receptor subtype 1
AT2 angiotensin II receptor subtype 2
BH4 tetrahydrobiopterin
BKCa large conductance calcium activated potassium channel
BNP B-type natriuretic peptide
CaMKII calcium-calmodulin-dependent protein kinase II
cAMP 3',5'-cyclic adenosine monophosphate
cGMP 3',5'-cyclic guanosine monophosphate
CNP C-type natriuretic peptide
Cu/Zn-SOD copper/zinc-containing superoxide dismutase
DAG 1,2-diacylglycerol
ECC excitation-contraction coupling
ECE endothelin converting enzyme
EGFR epidermal growth factor receptor
eNOS endothelial nitric oxide synthase
ERK extracellular signal regulated kinase
ET endothelin
ETα endothelin receptor subtype
GC guanylyl cyclase
GPCR G-protein coupled receptor
G-protein guanine nucleotide binding protein
GPx glutathione peroxidase
GSH glutathione
HF heart failure
H₂O₂ hydrogen peroxide
iNOS inducible nitric oxide synthase
IBMX 3-isobutyl-1-methylxanthine
IP₃ inositol-1,4,5-triphosphate
JNK  c-Jun NH2-terminal kinase
L-NAME  Nω-Nitro-L-arginine methyl ester hydrochloride
MAPK  mitogen-activated protein kinase
MAPKK  mitogen-activated protein kinase kinase
MAPKKK  mitogen-activated protein kinase kinase kinase
mitoK_{ATP}  mitochondrial K_{ATP} channel
MLCK  myosin light chain kinase
Mn-SOD  manganese containing superoxide dismutase
MnTMPyP  Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride
NCX  Na^{+}/Ca^{2+} exchanger
NF-κB  nuclear factor-kappa B
NHE  Na^{+}/H^{+} exchanger
nNOS  neuronal nitric oxide synthase
NO  nitric oxide
NPR  natriuretic peptide receptor
O_2^-  superoxide
ODQ  1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
OH  hydroxyl
PDE  phosphodiesterase
PI3K  phosphoinositide 3-kinase
PIP_2  phosphatidylinositol 4,5-diphosphate
PKA  protein kinase A
PKC  protein kinase C
PKG  protein kinase G
PLC  phospholipase C
PP  protein phosphatase
PrRP  prolactin releasing peptide
RAS  renin-angiotensin system
RNS  reactive nitrogen species
ROS  reactive oxygen species
RSK  ribosomal S6 kinase
RyR  ryanodine receptor
sarcK_{ATP}  sarcolemmal K_{ATP} channels
SEM  standard error of mean
SERCA  sarcoplasmic reticulum Ca^{2+}-ATPase
SMTC  S-Methyl-L-thiocitrulline
SOD  superoxide dismutase

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Enzyme Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TnC</td>
<td>troponin C</td>
</tr>
<tr>
<td>TnI</td>
<td>troponin I</td>
</tr>
<tr>
<td>XDH</td>
<td>xanthine dehydrogenase</td>
</tr>
<tr>
<td>XO</td>
<td>xanthine oxidase</td>
</tr>
<tr>
<td>XOR</td>
<td>xanthine oxidoreductase</td>
</tr>
</tbody>
</table>
List of original articles

The thesis is based on the following articles, which are referred to in the text by their Roman numerals.


*Equal contribution
Contents

Abstract
Tiivistelmä
Acknowledgments
Abbreviations
List of original articles
Contents

1 Introduction

2 Review of the literature

  2.1 Definition of cardiac hypertrophy and heart failure

  2.1.1 Cardiac hypertrophy

  2.1.2 Heart failure

  2.2 Regulation of cardiac contractility

  2.2.1 Excitation-contraction coupling

  2.2.2 The Frank-Starling mechanism and the slow force response

  2.2.3 The force-frequency relationship

  2.2.4 The sympathetic nervous system

  2.2.5 Circulating hormones

  2.3 Autocrine/paracrine factors regulating cardiac contractility

  2.3.1 Endothelins

  2.3.2 Angiotensin II

  2.3.3 Apelin

  2.3.4 Adrenomedullin

  2.3.5 Natriuretic peptides

  2.3.6 Prolactin releasing peptide

  2.4 NO

  2.4.1 Production of NO

  2.4.2 Effects of NO on myocardial function

  2.4.3 NO signaling

  2.4.4 Nitroso-redox interactions

  2.4.5 NO in hypertrophy and heart failure

  2.5 Reactive oxygen species

  2.5.1 Species of ROS and the antioxidant mechanisms

  2.5.2 Sources of ROS

  2.5.3 ROS in the cardiovascular system
2.6 Intracellular signal transduction .............................................................. 57
  2.6.1 G-proteins ..................................................................................... 57
  2.6.2 Protein kinase C ............................................................................ 58
  2.6.3 Mitogen-activated protein kinases ................................................ 59

3 Aims of the research 65

4 Materials and methods 67
  4.1 Materials (I-IV) ................................................................................. 67
  4.2 Experimental animals (I-IV) .............................................................. 67
  4.3 Isolated perfused rat heart preparation (I-IV) ...................................... 68
  4.4 Experimental protocols ..................................................................... 69
  4.5 Western blotting analysis (I-IV) ......................................................... 69
  4.6 p38-MAPK assay (I) ......................................................................... 69
  4.7 Detection of intracellular ROS (II) ................................................... 70
  4.8 Statistical analysis (I-IV) .................................................................. 71

5 Results 73
  5.1 The role of ERK1/2 and p38-MAPK in the regulation of cardiac contractility (I) .......................................................... 73
    5.1.1 ERK1/2 in the regulation of cardiac contractility ......................... 73
    5.1.2 Upstream activators and downstream targets of ERK1/2 .......... 74
    5.1.3 Role of p38-MAPK and downstream targets of p38-MAPK in the regulation of cardiac contractility .......................... 75
  5.2 ROS in the regulation of cardiac contractility (II) .............................. 77
    5.2.1 The role of ROS in ET-1-induced increase in cardiac contractility .......................................................... 77
    5.2.2 NADPH oxidase-derived ROS contribute to ET-1-induced inotropic response .................................................. 78
    5.2.3 Involvement of mitoKATP channels in the ET-1-induced inotropic response .................................................. 79
    5.2.4 Effects of ET-1-stimulated ROS production on ERK1/2 phosphorylation .......................................................... 80
    5.2.5 The role of ROS in the inotropic response to β-adrenergic stimulation .......................................................... 80
  5.3 ET-1-induced inotropic response, NO and GC-cGMP-PKG (III) ...... 83
    5.3.1 Contribution of nNOS to the inotropic effect of ET-1 ................. 83
    5.3.2 Effect of GC-cGMP-PKG signaling pathway on ET-1-induced inotropic response ........................................... 83
5.3.3 The effect of nNOS, GC or PKG inhibition on phospholamban and ERK1/2 phosphorylation ....................... 84
5.3.4 The role of PDE5A in the inotropic response to ET-1 ................. 85
5.4 PrRP and cardiac contractility (IV) .................................................. 85
  5.4.1 The effect of PrRP on cardiac contractility................................. 85
  5.4.2 Protein phosphatases, PKCα and PrRP signaling .................... 86

6 Discussion ................................................................................. 89
  6.1 MAPKs in the regulation of cardiac contractility ......................... 89
  6.2 ROS and cardiac contractility ...................................................... 92
  6.3 Endothelin-1, NO and GC-cGMP-PKG ............................................. 95
  6.4 The effects of PrRP on cardiac contractility ................................... 97

7 Summary and conclusions ........................................................... 99
References .................................................................................. 101
Original articles ........................................................................ 131
1 Introduction

The main function of the heart is to pump blood to supply the tissues of the body with oxygen, nutrients and other essential molecules. The heart must function under a wide variety of demands, and it is essential that cardiac output is rapidly adapted to the constantly changing needs of blood supply with various regulatory mechanisms. The basic mechanisms regulating cardiac contractility are the Frank-Starling mechanism, the force-frequency relationship and the sympathetic nervous system (Maack & O'Rourke 2007). In addition, circulating hormones contribute to cardiovascular regulation. In the heart, several peptide-ligand-G-protein coupled receptor systems exhibit high levels of expression, and locally produced vasoactive peptides, such as endothelin-1 (ET-1), also participate in the regulation of cardiac contractility in an autocrine/paracrine way (Brunner et al. 2006, Brutsaert 2003).

Cardiac hypertrophy is an early and progressive response to altered cardiac load. Pathological hypertrophy develops in many disease states, e.g. in chronic hypertension causing pressure overload or in valvular diseases causing volume overload (Bernardo et al. 2010). The development of hypertrophy is also affected by upregulated expression of various auto/paracrine factors such as ET-1 and angiotensin (Ang) II (Brunner et al. 2006, Schluter & Wenzel 2008). In mammals the majority of cardiac myocytes lose the ability to proliferate at birth or soon after, and postnatal growth occurs primarily via increase in myocyte size (Bernardo et al. 2010). The hypertrophic growth involves several events including activation of different signaling pathways, changes in gene expression, increases in the rate of protein synthesis, and the organization of contractile proteins into sarcomeric units resulting in increased cardiac mass (Bernardo et al. 2010, Heineke & Molkentin 2006).

Initially the increase in heart mass is termed compensated growth as it normalizes wall stress and permits normal cardiac function at rest (Bernardo et al. 2010). In the long term pathological growth is associated with increased heart size, loss of functional myocytes and fibrotic replacement, heart dilatation and cardiac dysfunction. Cardiac hypertrophy is an independent risk factor for myocardial infarction, arrhythmia and sudden death (Bernardo et al. 2010). In addition, hypertrophy can lead to heart failure (HF), which is a complex syndrome in which the heart is unable to maintain adequate circulation to meet the body’s normal needs (Mudd & Kass 2008). Despite current pharmacological therapy, the prognosis of the disease is poor, as the one-year mortality of HF is 17–28 %, and
overall 45–50% of patients are dead at 5 years (Mosterd & Hoes 2007; Mudd & Kass 2008).

The changes in myocyte biology are brought about by modulation of intracellular signaling pathways. Although ET-1 is a known regulator of cardiac contractility, the detailed intracellular signaling pathways mediating the cardiac effects are not well established. The aim of the study was to characterize the signaling pathways activated by the endogenous positive inotropic substance ET-1 in the mammalian adult heart. In addition, the effect of prolactin-releasing peptide (PrRP) on myocardial contractility was studied.
2 Review of the literature

2.1 Definition of cardiac hypertrophy and heart failure

2.1.1 Cardiac hypertrophy

Cardiac hypertrophy can be defined as an increase in heart mass. It is one of the cardiac remodeling mechanisms which are the early and progressive responses to different insults such as volume and pressure overload. The pathological conditions that can produce hypertrophy include hypertension, valvular diseases, myocardial infarction or ischemia associated with coronary artery disease and different cardiomyopathies (Bernardo *et al.* 2010, Lorell & Carabello 2000). Physiological cardiac hypertrophy is distinguished from pathological hypertrophy. It is the normal response of the heart to chronic exercise or pregnancy and it also includes normal postnatal heart growth. Physiological hypertrophy is associated with normal or improved cardiac function, it is reversible in the case of exercise or pregnancy-induced hypertrophy, and it does not progress to HF (Bernardo *et al.* 2010).

In mammals the majority of cardiac myocytes lose the ability to proliferate at birth or soon after, and postnatal heart growth occurs primarily via an increase in myocyte size. At the cell membrane the initiating stimuli for cardiac hypertrophy can be produced by biomechanical and stretch-sensitive mechanisms as well as neurohumoral mechanisms (Bernardo *et al.* 2010, Heineke & Molkentin 2006). It involves several events including activation of different signaling pathways, changes in gene expression, increases in the rate of protein synthesis, and the organization of contractile proteins into sarcomeric units resulting in increased cardiac mass. The changes in gene reprogramming include reactivation of immature fetal cardiac genes, including the genes for natriuretic peptides and fetal contractile proteins (Bernardo *et al.* 2010, Heineke & Molkentin 2006, Lorell & Carabello 2000).

Hypertrophy can be subdivided into concentric and eccentric hypertrophy based on changes in the shape of the hypertrophied heart (Bernardo *et al.* 2010, Heineke & Molkentin 2006, Lorell & Carabello 2000). A pathological stimulus causing pressure overload, e.g. hypertension, causes concentric hypertrophy, which is characterized by relative wall thickness and cardiac mass with a small reduction or no change in chamber volume. In contrast, volume overload,
produced by e.g. aortic regurgitation, produces an increase in diastolic wall stress and eccentric hypertrophy where there is an increase in cardiac mass with increased chamber volume. In addition, the ventricular walls are thinned (Bernardo et al. 2010, Heineke & Molkentin 2006, Lorell & Carabello 2000). Generally, eccentric cardiac hypertrophy poses a greater risk than concentric hypertrophy (Bernardo et al. 2010). Initially the increase in heart mass is termed compensated growth as it normalizes wall stress and permits normal cardiac function at rest. In the long term pathological growth is associated with increased heart size, loss of functional myocytes and fibrotic replacement, heart dilatation and cardiac dysfunction leading to HF (Bernardo et al. 2010, Heineke & Molkentin 2006). There is an association between cardiac hypertrophy and nearly all forms of HF. In addition, cardiac hypertrophy is an independent risk factor for myocardial infarction, arrhythmia and sudden death (Bernardo et al. 2010).

The signaling pathways leading to hypertrophic growth are generally complex and extensive crosstalk exists. It has been shown that the physiological and pathological stresses causing cardiac hypertrophy couple to different signaling pathways (Bernardo et al. 2010). In the pathological hypertrophy distinct signaling pathways including mitogen-activated protein kinases (MAPK), calmodulin, protein kinase C (PKC) and calcineurin play important roles while in physiological hypertrophic growth the importance of phosphoinositide 3-kinase (PI3K)-Akt has been demonstrated (Bernardo et al. 2010, Heineke & Molkentin 2006).

2.1.2 Heart failure

HF is a complex syndrome in which the heart is unable to maintain adequate circulation to meet the body’s normal needs (Mosterd & Hoes 2007, Mudd & Kass 2008). In HF numerous compensatory mechanisms occur, including retention of salt and water by the kidneys, activation of the neurohormonal systems, such as the sympathetic nervous system and renin-angiotensin-aldosterone system, and activation of intracellular signaling cascades in the heart and vasculature that alter cellular and organ morphology and function. Initially the compensatory mechanisms can improve the function of the heart, but in the disease process when the circulatory disturbance becomes chronic, the mechanisms contribute towards further worsening of cardiac function and increase the likelihood of organ failure and worsening of clinical prognosis (Mosterd & Hoes 2007, Mudd & Kass 2008).
The dysfunction can be caused by a variety of cardiovascular diseases. Generally there is an association between cardiac hypertrophy and nearly all forms of HF, and typically HF is caused by prolonged hypertension or coronary artery disease with myocardial ischemia or infarction. In addition, valvular diseases, like aortic valve stenosis, and different cardiomyopathies caused by genetic or non-genetic factors can lead to HF (Dickstein et al. 2008, Heineke & Molkentin 2006).

HF is commonly divided into systolic and diastolic HF (Dickstein et al. 2008, Mudd & Kass 2008). In systolic HF the myocardial contractility is impaired (ejection fraction ≤40%) and the heart is dilated. In diastolic HF the left ventricular ejection fraction is preserved (ejection fraction >40–50%) but the heart is often hypertrophied. The left ventricle shows impaired relaxation and/or passive elasticity and its ability to expand during diastole is diminished (Dickstein et al. 2008, Mudd & Kass 2008). Diastolic HF is more often seen in females and elderly patients and in patients with a history of hypertension, while the common etiology of systolic HF is ischemic heart disease and the patients are more often males (Chatterjee & Massie 2007, Mosterd & Hoes 2007). However, most patients with HF show evidence of both systolic and diastolic dysfunction (Dickstein et al. 2008).

In the Western world the prevalence of HF is estimated to be 1–2% (Mosterd & Hoes 2007). The prevalence increases with age, being ~8% at 75 years of age. Despite current pharmacological therapy, the prognosis of the disease is poor, as the one-year mortality of HF is 17–28% and overall 45–50% of patients are dead at 5 years (Mosterd & Hoes 2007).

### 2.2 Regulation of cardiac contractility

The main function of the heart is to pump blood to supply the body with oxygen and substrates. The heart is able to adjust its pump function to meet the demands of vital organs in the body by increasing cardiac contractility and heart rate leading to augmented cardiac output. Cardiac contractile function is regulated by a number of intrinsic and extrinsic mechanisms. The Frank-Starling mechanism and the force-frequency relationship represent two of the most important intrinsic mechanisms affecting cardiac function (Maack & O'Rourke 2007). The autonomic nervous system, various hormones and blood concentrations of O₂, CO₂ and H⁺ are also well-established regulators of cardiac contractility. In addition, autocrine/paracrine effectors such as ET-1 (Kelly et al. 1990),
adrenomedullin (AM) (Szokodi et al. 1998), nitric oxide (NO) (Prendergast et al. 1997) and Ang II (Li et al. 1994) participate in the regulation of cardiac function. With the distinct regulatory mechanisms cardiac output can be adapted to the constantly changing demands of blood supply; for instance during exertion cardiac output can be increased more than five-fold compared to resting conditions (Maack & O'Rourke 2007).

2.2.1 Excitation-contraction coupling

Calcium (Ca\(^{2+}\)) is the central second messenger in the translation of electrical signals into mechanical activity of the heart. The contraction of the heart is regulated beat-to-beat by Ca\(^{2+}\) stored and released within cardiomyocytes, in a highly coordinated process termed excitation-contraction-coupling (ECC) (Bers 2002) (Fig. 1). In the regulation of ECC Ca\(^{2+}\) can have direct effects on the myofilaments and it can activate several Ca\(^{2+}\)-dependent proteins or second messengers which modulate ECC. Such proteins include calmodulin and Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CaMKII), which are particularly important as they can modulate Ca\(^{2+}\) influx, sarcoplasmic reticulum (SR) Ca\(^{2+}\) release and SR Ca\(^{2+}\) uptake (Maier & Bers 2002).

During the cardiac action potential Ca\(^{2+}\) enters the cell mainly through voltage-dependent L-type Ca\(^{2+}\) channels. Ca\(^{2+}\) entry further triggers Ca\(^{2+}\) release from the SR via ryanodine receptors (RyR), a process termed Ca\(^{2+}\)-induced Ca\(^{2+}\) release, which was initially characterized in skinned cardiac myocytes preparation (Fabiato & Fabiato 1979, Fabiato 1985, Bers 2002). The RyR and L-type Ca\(^{2+}\) channels are located in close functional association allowing a rapid Ca\(^{2+}\) induced Ca\(^{2+}\) release to occur (Sham et al. 1995).

In the presence of diastolic levels of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) troponin I (TnI) inhibits the interaction of thin (actin) and thick (myosin) filaments (Bers 2002). The increase in [Ca\(^{2+}\)]\(_{i}\) allows Ca\(^{2+}\) to bind to the myofilament protein troponin C (TnC) resulting in the release of inhibition induced by TnI. Thereby the Ca\(^{2+}\) binding to TnC triggers a conformational change in the TnI-tropomyosin complex, which permits the initiation of myosin-actin cross-bridge cycling leading to force development and/or cell shortening (systole). The transient increase in [Ca\(^{2+}\)]\(_{i}\) plays a key role in cardiac ECC by determining the amount of Ca\(^{2+}\) ions to bind to TnC (Bers 2002).

Ca\(^{2+}\) must be removed from the cytoplasm for relaxation and ventricular filling to occur (diastole). The SR Ca\(^{2+}\)-ATPase (SERCA) and the sarcolemmal
Na\(^+/\)Ca\(^{2+}\) exchanger (NCX) are the main mechanisms for Ca\(^{2+}\) removal. In addition, sarcolemmal Ca\(^{2+}\)-ATPase and mitochondrial Ca\(^{2+}\) uniport contribute to Ca\(^{2+}\) cycling (Bers 2002). In rat and mice ventricle 92% of Ca\(^{2+}\) removal results through SERCA and 7% through NCX (Bassani et al. 1994, Hove-Madsen & Bers 1993, Li et al. 1998). In rabbit ventricular myocytes the SERCA removes 70% of the activator Ca\(^{2+}\) and NCX removes 28% (Bassani et al. 1994). The balance of Ca\(^{2+}\) fluxes in ferret, dog, cat, guinea pig and human ventricle are more like that in rabbit (Bers 2002). In all species the slow systems, sarcolemmal Ca\(^{2+}\)-ATPase and mitochondrial Ca\(^{2+}\) uniport, account for <2% of Ca\(^{2+}\) removal from the cytosol (Bers 2002, Maier & Bers 2002).

The activity of SERCA is under the control of a closely associated protein, phospholamban, which is one of the crucial regulators of cardiac contractility as it regulates both basal contractility and responses to \(\beta\)-agonists. Phospholamban is a 52-amino acid transmembrane protein located in the cardiac SR (MacLennan & Kranias 2003). Phospholamban can be phosphorylated at three distinct sites: Ser\(^{16}\) by 3',5'-cyclic adenosine monophosphate (cAMP) and 3', 5'-cyclic guanosine monophosphate (cGMP) dependent protein kinases A and G (PKA and PKG respectively), Thr\(^{17}\) by CaMKII and at Ser\(^{10}\) by PKC (Drago & Colyer 1994, Huggins et al. 1989, Movsesian et al. 1984, Simmerman et al. 1986). However, experimental evidence indicates that the last mentioned phosphorylation at Ser\(^{10}\) is not physically relevant (Edes & Kranias 1990). Protein phosphatase (PP) 1 is the main phosphatase responsible for dephosphorylating phospholamban and therefore an important mechanism regulating cardiac contractile performance (Nicolaou et al. 2009). In its dephosphorylated state, phospholamban binds to and inhibits the activity of SERCA. Phosphorylation of phospholamban relieves SERCA inhibition and enhances Ca\(^{2+}\) reuptake into the sarcoplasmic reticulum. Cardiac contractility is increased in proportion to the elevation in the size of the sarcoplasmic reticulum Ca\(^{2+}\) store and the resulting increase in Ca\(^{2+}\) release from the sarcoplasmic reticulum (MacLennan & Kranias 2003).
2.2.2 The Frank-Starling mechanism and the slow force response

The Frank-Starling mechanism plays a major role in intrinsic regulation of cardiac function in all classes of vertebrates (de Tombe et al. 2010). It describes how stretch of cardiac muscle, up to an optimum length, increases contractility thereby linking cardiac ejection to cardiac filling. The mechanism is vital for linking changes in venous return with changes in stroke volume. An increase in venous return dilates the ventricles and stretches the myocardium, thereby increasing the contractility of the heart (de Tombe et al. 2010). In healthy humans the Frank-Starling response contributes to cardiac output during submaximal exercise and during changes in posture (Drake-Holland et al. 1990, Plotnick et al. 1986). The response is augmented in the elderly, who have a diminished increase in the heart rate in response to physical exercise (Pugh & Wei 2001). The Frank-Starling response is preserved even in hypertrophied and failing hearts (Holubarsch et al. 1996, Stromer et al. 1997, Weil et al. 1998).
It has been shown that in mammalian cardiac muscle the Frank-Starling mechanism is not associated with an increase in the magnitude of the \([Ca^{2+}]\) transient (Allen & Kurihara 1982) indicating that the myofilament sensitivity for \(Ca^{2+}\) has increased. The molecular mechanisms of the augmented \(Ca^{2+}\) activation with sarcomere length are still not fully understood, but two potential mechanisms have been proposed to be underlying the Frank-Starling mechanism (Endoh 2008, Fukuda & Granzier 2005). The optimum extent of thin and thick filaments overlapping for force development is believed to play an essential role. In addition, another mechanism of an abbreviated distance between actin and myosin head with stretch, which is modulated by a giant protein, titin, has been proposed (Endoh 2008, Fukuda & Granzier 2005).

The immediate increase (Frank-Starling mechanism) in contractile force upon stretch is followed by a slowly developing increase, called the slow force response or the Anrep effect. Parmley & Chuck described the slow force response as an increase in contractility developing over approximately 10 min after the initial rapid change (Parmley & Chuck 1973). The slowly developing increase is in association with a parallel increase in \(Ca^{2+}\) transients without changes in myofilament sensitivity (Allen & Kurihara 1982).

### 2.2.3 The force-frequency relationship

When the ventricular myocardium contracts at higher frequencies over the physiological heart rate, contractile force is increased. This intrinsic regulatory mechanism of myocardial contractility is termed the positive force-frequency relationship. The positive force-frequency relationship is primarily associated with an increase in the amplitude of \(Ca^{2+}\) transients (Allen & Blinks 1978). The increase in \(Ca^{2+}\) transients is caused by an increased \(Ca^{2+}\) store in the SR. At higher frequencies cumulative depolarization duration per unit is increased, resulting in prolongation of the opening time of the voltage-dependent L-type \(Ca^{2+}\) channel and leading to increased \(Ca^{2+}\) influx and \(Ca^{2+}\) uptake by SERCA in the SR \(Ca^{2+}\) store (Pieske et al. 1995, Pieske et al. 1999b, Endoh 2004). In addition, repetitive stimulation at higher frequencies accumulates \(Na^+\) in cardiac myocytes. This may lead to an increase in \([Ca^{2+}]\), through suppression of the forward mode and/or activation of the reverse mode NCX (Cohen et al. 1982, Endoh 2004). In the induction of the positive force-frequency relationship calmodulin/CaMKII signaling may also play an important role (Endoh 2004).
In humans and larger mammals cardiac hypertrophy and HF are characterized by a change from a positive developed force-frequency relationship in normal myocardium to a flattened or negative developed force-frequency relationship in disease. In failing hearts SR Ca\(^{2+}\) uptake is reduced and altered Ca\(^{2+}\) handling could be explained by a depressed role of SERCA and enhanced systolic Ca\(^{2+}\) extrusion via NCX (Pieske et al. 1995, Pieske et al. 1999b, Rossman et al. 2004).

### 2.2.4 The sympathetic nervous system

The effectors of the sympathetic nervous system, adrenaline and noradrenaline, have effects on cardiac function via both \(\alpha\) and \(\beta\)-adrenergic receptors. Four \(\beta\)-adrenoceptor subtypes, \(\beta_1\), \(\beta_2\), \(\beta_3\) and \(\beta_4\), have been characterized. All \(\beta\)-adrenoceptors belong to the family of guanine nucleotide binding protein (G-protein)-coupled receptors (GPCRs). In cardiac myocytes \(\beta_1\)-adrenoceptor is the predominant subtype and it is coupled exclusively to activation of adenylyl cyclase (AC) through \(G_\alpha\) proteins. In addition, \(\beta_2\)-adrenoceptors, coupled to both \(G_\alpha\) and \(G_\beta\) proteins, are present in cardiac myocytes. Activation of \(\beta_1\) and \(\beta_2\) adrenoceptors results in an increase in cAMP after AC stimulation through \(G_\alpha\) proteins. The Ca\(^{2+}\) signal modulation achieved by the cAMP-PKA pathway is exerted through phosphorylation of Ca\(^{2+}\) regulatory proteins including L-type Ca\(^{2+}\) channels, phospholamban, RyR, TnI, myosin binding protein C and the NCX. \(\beta_1\)-adrenoceptor activation includes all previous processes while \(\beta_2\)-adrenoceptor stimulation is confined to activation of L-type Ca\(^{2+}\) channels with no facilitation of other processes. \(\beta_1\)- and \(\beta_2\)-adrenoceptor stimulation classically elicits effective increase in contractile function, which is associated with marked facilitation of Ca\(^{2+}\) transients and a pronounced abbreviation of twitch contraction. (Dzimiri 1999, Triposkiadis et al. 2009.) In addition, \(\beta_2\)-adrenoceptor activation may have cardioprotective and anti-apoptotic effects (Zhu et al. 2001). \(\beta_3\)-adrenoceptor activation has negative inotropic responses through the release of NO and its second messenger cGMP, perhaps coupled to \(G_\alpha\)-proteins (Emorine et al. 1989, Gauthier et al. 1996, Gauthier et al. 1998). \(\beta_4\)-adrenoceptor has been shown to be a specific affinity state of the \(\beta_1\)-adrenoceptor and it also mediates inotropic effects (Kaumann et al. 2001).

The effectors of sympathetic nervous system also act on cardiac myocytes via \(\alpha_1\)-adrenergic receptors which may mediate the positive inotropic responses. Three subtypes of \(\alpha_1\)-subtypes (\(\alpha_{1A}\), \(\alpha_{1B}\), \(\alpha_{1D}\)) exist of which \(\alpha_{1A}\) and \(\alpha_{1B}\) are the dominant ones. The hearts of most species express both \(\alpha_{1A}\)- and \(\alpha_{1B}\)-adrenergic
receptors at the protein level. The $\alpha_{1B}$-adrenergic receptor subtype predominates in rodents, whereas in the human heart $\alpha_{1A}$-adrenergic receptors are the major subtype. (Woodcock 2007.) The $\alpha_1$-adrenoceptors are GPCRs which couple to the $G_{\alpha_{q/11}}$ signaling pathway (Woodcock 2007, Xiao et al. 2006). The receptor activation results in the generation of the second messengers inositol-1,4,5-trisphosphate (IP$_3$) and 1,2-diacylglycerol (DAG), the mobilization of intracellular Ca$^{2+}$ and the activation of PKC. The maximum positive inotropic effect following $\alpha_{1A}$-adrenergic receptor stimulation is notably less than that evoked by $\beta$-adrenoceptor stimulation in the human heart (Woodcock 2007, Xiao et al. 2006).

In chronic HF sympathetic activity is increased, and it can have adverse effects on ECC and enhance apoptosis. Prolonged activation of the sympathetic nervous system diminishes the $\beta$-adrenoceptor responsiveness due to a decrease in $\beta_1$-adrenoceptor density and an uncoupling from the $G_\alpha$-AC pathway (Brodde et al. 2006, Triposkiadis et al. 2009). In such pathological settings $\alpha_1$-adrenoceptors can have an important role as a compensatory mechanism in maintaining cardiac contractility (Woodcock et al. 2008).

### 2.2.5 Circulating hormones

Several circulating hormones have an impact on cardiac contractility. Adrenaline, produced by the adrenal medulla, exerts its effects on cardiac myocytes through $\alpha$- and $\beta$-adrenoceptors (Dzimiri 1999, Triposkiadis et al. 2009). Under normal conditions the circulating catecholamines have minor effects on cardiac contractility compared to the autonomic nervous system. However, under acute stress the release of catecholamines from adrenal medulla is activated in parallel with the activation of sympathetic nervous system (Dzimiri 1999, Triposkiadis et al. 2009).

Thyroid hormone is an important regulator of cardiac function and cardiovascular hemodynamics (Kahaly & Dillmann 2005). Triiodothyronine, the physiologically active form of thyroid hormone, can exert a direct effect on cardiac myocytes by binding to nuclear triiodothyronine receptors influencing cardiac gene expression. It may also increase the sensitivity and responsiveness of the cardiac tissue to the sympathetic stimuli and the hormone may lead to hemodynamic alterations in the periphery that result in increased cardiac filling and modification of cardiac contraction (Kahaly & Dillmann 2005). In isolated heart thyroid hormones exert acutely positive inotropic effects (Segal et al. 1996).
In hyperthyroidism cardiac contractility and cardiac output are enhanced and systemic vascular resistance is decreased, while the opposite is observed in hypothyroidism (Kahaly & Dillmann 2005). Thyroid hormone-responsive cardiac genes include SERCA (Rohrer & Dillmann 1988) and phospholamban (Kiss et al. 1994). In addition, phospholamban phosphorylation is influenced by thyroid status, which correlates with the enhancement of the contractile function of the left ventricle (Ojamaa et al. 2000). Thyroid hormones regulate both the transcriptional and posttranscriptional levels of several plasma-membrane ion transporters, which together coordinate the electrochemical and mechanical responses of the myocardium. Triiodothyronine can also induce alterations in specific cardiac contractile proteins, α- and β-myosin heavy chain (Kahaly & Dillmann 2005).

Both insulin (Muniyappa et al. 2007) and glucagon (White 1999) have a positive inotropic effect, and insulin-like growth factor has also been demonstrated to improve myocardial function (Cittadini et al. 1996, Donath et al. 1996), but the physiological significance of these mechanisms is unclear.

2.3 Autocrine/paracrine factors regulating cardiac contractility

Cardiomyocytes are in intimate contact with the endothelial cells of the coronary myocardial capillaries which sense and transduce local signals, such as mechanical forces, hormones and hypoxia, in the perfusing blood (Brutsaert 2003). The finding that damaging of the endocardial endothelium of isolated cat papillary muscle decreased contraction force was the earliest evidence demonstrating that endocardial cells synthesize and secrete substances that can affect contractile performance (Brutsaert et al. 1988). Thereafter several locally acting auto/paracrine factors, such as ET-1, Ang II, apelin, AM and natriuretic peptides, have been described to be involved not only in contractile function but also in remodeling processes after myocardial injury and overload (Brutsaert 2003).
2.3.1 Endothelins

The endothelin system

ET-1 is a 21-amino acid vasoactive peptide which was originally identified in cultured porcine endothelial cells (Yanagisawa et al. 1988). Soon after the discovery of ET-1 two structurally related peptides, differing by 2 and 6 amino acids, were identified and termed endothelin-2 (ET-2) and endothelin-3 (ET-3) (Inoue et al. 1989). A fourth isoform, originally identified as vasoactive intestinal contractor in the mouse, is a species-specific analogue to human ET-2 (Bloch et al. 1991). ET-1 is produced by vascular endothelial and smooth muscle cells, cardiac myocytes, fibroblasts, airway epithelial cells, macrophages, pancreatic islets and brain neurons (Kedzierski & Yanagisawa 2001). ET-2 is expressed in the ovary and by intestinal epithelial cells, and ET-3 is expressed in endothelial cells, brain neurons, renal tubular endothelial cells and intestinal epithelial cells. In the cardiovascular system ET-1 is the predominant isoform (Kedzierski & Yanagisawa 2001).

ET-1 is synthesized from a larger preproform, prepro ET-1, consisting of 212 amino acid residues (Fig. 2). Prepro ET-1 is cleaved by furin-like endopeptidase to pro ET-1 (Blais et al. 2002, Denault et al. 1995), also called big ET-1, consisting of 38 amino acid residues. Big ET-1 is further processed into ET-1 by a family of membrane-bound zinc metalloproteinases called ET-converting enzyme 1 (Xu et al. 1994) and ET-converting enzyme 2 (Emoto & Yanagisawa 1995) (ECE-1 and ECE-2, respectively). Targeted disruption of the ECE-1 gene has indicated that ECE-1 is the main enzyme responsible for the transformation of big ET-1 into ET-1 (Yanagisawa et al. 1998). In addition to these proteases, other enzymes contribute to the final processing step, since in mice lacking both ECE-1 and ECE-2 the levels of mature endothelin peptides are reduced by only one-third (Yanagisawa et al. 2000). Exogenous big ET-1 can also be metabolized to a peptide containing 31 amino acids [ET-1(1-31)] by a chymase. However, ET-1 (1-31) is probably partly bioconverted to ET-1 in the body since its actions in vivo resemble those of ET-1 (Fecteau et al. 2005). At the gene level expression of ET-1 is stimulated by a number of physical and chemical factors. These factors include low shear stress, hypoxia, Ang II, growth factors and other cytokines. In contrast, the expression is inhibited by natriuretic peptides, NO, prostacyclin and heparin (Brunner et al. 2006). ET-1 can be rapidly secreted, implying that the peptide is
partially stored in endothelial cells rather than being synthesized *de novo* in response to stimuli of short duration (Brunner *et al.* 2006).

Soon after the discovery of ET-1 two endothelin receptors (ET\textsubscript{A} and ET\textsubscript{B}) were identified. Both receptors are G-protein coupled heptahelical transmembrane proteins with considerable sequence homology (Arai *et al.* 1990, Sakurai *et al.* 1990). In myocytes and vascular smooth muscle cells ET\textsubscript{A} is the predominant receptor while ET\textsubscript{B} is predominantly expressed in endothelial cells. Within the cardiovascular system interaction of ET\textsubscript{A} receptors with ET-1 results mostly in vasoconstriction and cell proliferation whereas ET\textsubscript{B} receptors exert opposite effects (Brunner *et al.* 2006). Additionally, ET\textsubscript{B} receptors expressed in vascular smooth muscle cells can elicit vasoconstriction (Teerlink *et al.* 1994, Brunner *et al.* 2006).

The clearance of ET-1 from plasma occurs through ET\textsubscript{B} receptor. The ET\textsubscript{B} -mediated clearance mechanism is particularly important in the lung, where 80% of the ET-1 passing through this organ is retained (Fukuroda *et al.* 1994). Cleavage can also occur by neutral endopeptidase in tissues and in the circulation (Abassi *et al.* 1992), and recent experimental evidence suggests that carboxypeptidase A (cathepsin A9) plays an important role as an endothelin-1-inactivating enzyme (Seyrantepe *et al.* 2008).
Fig. 2. Schematic presentation of biosynthesis and structure of endothelin-1. ECE, endothelin converting enzyme; NO, nitric oxide; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; CNP, C-type natriuretic peptide. Modified from Brunner et al. 2006.

Vascular effects of ET-1

All ET isopeptides share structural homology with the snake venom safarotoxin. Like safarotoxin, ETs are peptides with potent and characteristically long-lasting vasoconstrictor and vaso pressor actions (Kloog et al. 1988). ET-1 is one of the most potent vasoconstrictors identified so far (Yanagisawa et al. 1988).

In the vascular system ET-1 contributes to basal vascular tone. Both $\text{ET}_A$ and $\text{ET}_B$ receptors located on vascular smooth muscle cells mediate the potent vasoconstrictor effects, whereas endothelial cell $\text{ET}_B$ receptors exert opposite effects (Schneider et al. 2007). Administration of ET-1 to intact circulation induces a biphasic response, starting with a transient reduction in arterial pressure, which is followed by a protracted hypertensive phase (Kedzierski & Yanagisawa 2001). The depressor phase of the response involves a normally functioning endothelium with intact $\text{ET}_B$ receptor signaling (D’Orleans-Juste et al. 2002) whereas vasoconstriction and the resulting hypertensive phase are mediated by
ETα receptor activation (Bird et al. 1993, Kedzierski & Yanagisawa 2001). Activation of endothelial cell ETβ receptors stimulates the production of NO and vasodilator cyclooxygenase metabolites, which exert vasorelaxant effects on the underlying smooth muscle (Schneider et al. 2007). In normal blood vessels a balance exists between the endothelial production of ET-1 and NO. In disease states both NO and ET-1 production may be dysregulated, which can lead to an increase in arterial constrictor tone, blood pressure and exacerbation of myocardial ischemia (Brunner et al. 2006).

The effects of ET-1 on myocardial contractility

In the heart ET-1 has a positive inotropic effect in most mammalian species including rat (Kelly et al. 1990, Kinnunen et al. 2000, Kramer et al. 1991, Szokodi et al. 2002), rabbit (Chu & Endoh 2005, Wang et al. 2000), guinea pig (Ishikawa et al. 1988a, Ishikawa et al. 1988b) and man (Pieske et al. 1999a). In healthy humans, ET-1 has been reported to elicit a positive basal inotropic effect as ET receptor blockade caused a reduction in systolic contractility (MacCarthy et al. 2000). In the slowly developing part of the contractile response to increased stretch ET-1 also has a mediating role (Perez et al. 2001). Although the majority of studies in larger mammals show that ET-1 exerts a positive inotropic effect there are also studies conducted in rats and guinea pigs showing no positive inotropic effect or even negative inotropic effect in isolated hearts (Karwatowska-Prokopczuk & Wennmalm 1990, Neubauer et al. 1990) or in vivo (Baydoun et al. 1989, Beyer et al. 1994). This can be due to the vasoconstrictive effect of ET-1 limiting coronary flow and in vivo increasing afterload (Baydoun et al. 1989, Beyer et al. 1994).

In mice the effect of ET-1 on cardiac contractility has been more controversial as both positive and negative effects on contractility have been observed. ET-1 10 nmol/L has a positive inotropic effect in myocytes from S129 mice (Pi et al. 2002). However, high concentrations of exogenous ET-1 (1–300 nM) exerted a negative inotropic effect in right ventricle strips isolated from C57 mice (Izumi et al. 2000). Similarly, in ventricular cardiomyocytes from the same strain 0.01–10 nM concentrations of ET-1 have exerted a negative inotropic effect (Nishimaru et al. 2007, Nishimaru et al. 2008). In contrast, in isolated perfused whole heart preparations from C57 mice exogenous ET-1 1 nM has been shown to increase contractility (Piuhola et al. 2003). Therefore, experimental conditions (single cardiomyocytes vs. multicellular preparations, pacing frequency, loading...
conditions, etc) as well as species differences seem to influence the response to ET-1.

In cardiomyocytes both ET\textsubscript{A} and ET\textsubscript{B} receptors are expressed, with a prominence of ET\textsubscript{A} receptors (85–90\%) (Molenaar \textit{et al.} 1993) and the positive inotropic response is generally mediated via ET\textsubscript{A} receptors. However, ET\textsubscript{B} receptors also seem physiologically relevant (Brunner \textit{et al.} 2006). In mice, ET\textsubscript{A} receptor-mediated increase in contractile force is suppressed by ET\textsubscript{B} receptor stimulation (Piuhola \textit{et al.} 2003).

ET-1 binding to ET\textsubscript{A} receptors on the myocyte surface activates phospholipase C (PLC) and stimulates hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP\textsubscript{2}) to DAG and IP\textsubscript{3} (Hilal-Dandan \textit{et al.} 1992, Jones \textit{et al.} 1992, Takanashi & Endoh 1991, Brunner \textit{et al.} 2006). IP\textsubscript{3} rapidly increases intracellular calcium by mobilization from the sarcoplasmic reticulum. In human left ventricle IP\textsubscript{3} formation and changes in myofibrillar Ca\textsuperscript{2+} responsiveness have been associated with the positive inotropic effect of ET-1 (Pieske \textit{et al.} 1999a, Ponicke \textit{et al.} 1998). Downstream of DAG activation of sarcolemmal Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE)-1 contributes to the positive inotropic effect of ET-1. NHE-1 activation causes intracellular accumulation of Na\textsuperscript{+} that potentially increases intracellular Ca\textsuperscript{2+} by reversing the normal direction of NCX (Aiello \textit{et al.} 2005, Kentish 1999). In addition, NHE-1 stimulation leads to intracellular alkalinization and sensitization of contractile proteins to Ca\textsuperscript{2+} (Kramer \textit{et al.} 1991). The ET-1-induced inotropy has been suggested to be dependent on the PKC pathway (Chu \textit{et al.} 2003, Kramer \textit{et al.} 1991). However, in rabbit hearts ET-1 increased contractility via the PLC pathway, but not through the PKC pathway (Kelso \textit{et al.} 2000). In guinea pig myocytes it was found that a combination of mechanisms including an increase in transmembrane Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels and the stimulation of NHE-1 via PKC activation are involved in the response to ET-1 (Woo & Lee 1999). Although many intracellular second messengers and mechanisms are known to be involved in mediating the contractile effects of ET-1, the detailed signaling pathways are still not fully understood.

\textit{ET-1 in cardiac hypertrophy and heart failure}

ET-1 can stimulate myocyte growth and myofibrillogenesis, thereby causing cardiac hypertrophy. In cultured neonatal rat cardiomyocytes and fibroblasts ET-1 has been shown to induce hypertrophy in several studies (Brunner \textit{et al.} 2006). \textit{In vivo} aortic banding increased moderately cardiomyocyte prepro-ET-1 mRNA
expression in rat cardiomyocytes (Arai et al. 1995) and ET<sub>A</sub> receptor antagonist was shown to prevent cardiac hypertrophy in the early phase of pressure overload (Ito et al. 1994). The signaling mechanisms mediating the hypertrophic effects include activation of PLC, PKC, and extracellular signal-regulated kinase-1 and -2 (ERK1/2) leading to phosphorylation and activation of transcription factors with resulting expression of hypertrophic genes (Sugden 2003). In HF the expression of ET-1 is upregulated and it is involved in the pathophysiology of the disease. In failing heart ET<sub>A</sub> receptors are upregulated while the number of ET<sub>B</sub> receptors is decreased, and therefore it is hypothesized that the ET<sub>A</sub> isoform is responsible for the deleterious effects of ET-1 (Brunner et al. 2006).

2.3.2 Angiotensin II

In the renin-angiotensin system (RAS) angiotensinogen is released by the liver and secreted into the circulation where renin, secreted by the kidneys, catalyzes the cleavage of angiotensinogen to Ang I. Ang I is then catalyzed to Ang II by angiotensin converting enzyme (ACE)-1 on the surface of endothelial cells in the pulmonary circulation. Ang II, considered the main effector peptide of the RAS, is then acting on specific angiotensin receptors. The RAS is centrally involved in the maintenance of the body’s water and sodium balance and the regulation of hemodynamic homeostasis. In addition to the classical cascade of RAS it has been shown that local RAS exist in the heart and several other tissues. In the heart, local RAS has autocrine and paracrine activity and the functional effects of renin and Ang II in remodeling and HF are at least partially attributed to this tissue-based system. (Paul et al. 2006.) In addition to ACE-1, ACE-2, aminopeptidase A, neural endopeptidase, propylendopeptidase and chymase are also able to cleave Ang I, producing different bioactive peptides that include Ang (1-7), Ang II (1-8), Ang III (2-8), and Ang IV (Schluter & Wenzel 2008).

Ang II binds to and activates type 1 angiotensin receptors (AT1) and type 2 angiotensin receptors (AT2), which are both G-protein coupled (Schluter & Wenzel 2008). Most of the signal transduction pathways induced by Ang II in cardiomyocytes are mediated by the AT1 receptor. AT2 receptors are mainly expressed in fetal tissues. In adult hearts AT2 are barely detectable, but the receptors are up-regulated in pathophysiological conditions (Schluter & Wenzel 2008).

Ang II has been shown to modulate cardiac contractility, but the results are controversial. In ventricular feline cardiomyocytes Ang II exerts a positive
contractile effect, and the underlying mechanisms have been suggested to include enhancements of L-type Ca\(^{2+}\) currents and intracellular Ca\(^{2+}\) transients (Petroff et al. 2000), increased ET-1 (Perez et al. 2003) and production of reactive oxygen species (ROS) (Cingolani et al. 2006). In isolated adult mouse cardiomyocytes Ang II can elicit both positive (Liang et al. 2010, Rajagopal et al. 2006) and negative effects on cardiac contractility (Liang et al. 2010). The positive inotropic effect has been shown to be mediated by both G-protein/PKC and β-arrestin-2 dependent mechanisms (Rajagopal et al. 2006). Recently Liang et al. found that Ang II has complex effects on cardiac contractility in mice. In isolated hearts Ang II induced a rapid decrease in peak left ventricular-developed pressure (+dP/dt\(_{\text{max}}\)), which was followed by a marked dose-dependent increase in +dP/dt\(_{\text{max}}\) that plateaued above baseline. They observed both decreases and increases in L-type Ca\(^{2+}\) currents and contractility, with the decreases requiring PI3K and PKC (Liang et al. 2010). Interestingly, PKC was also found to be involved in the positive inotropic actions of Ang II, suggesting a dual role for PKC (Liang et al. 2010). In rat ventricular cardiomyocytes Ang II has been shown to elicit a negative inotropic effect in which PKC, tyrosine kinase and p38-MAPK, but not nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and ROS are involved (Palomeque et al. 2006). In human ventricular myocytes Ang II has no effect on contractility while in atrial myocytes Ang II elicits a positive inotropic effect (Holubarsch et al. 1993, Mollmann et al. 2007) via PKC (Mollmann et al. 2007).

In cardiomyocytes Ang II has been shown to mediate hypertrophy (Schluter & Wenzel 2008). In neonatal cardiomyocytes the hypertrophic effect is mediated via AT1 receptors and the signaling pathway involves NADPH oxidase, ROS and MAPKs. Compared to responses in neonatal cardiomyocytes, the direct hypertrophic effect of Ang II in adult ventricular cardiomyocytes is smaller and it has been linked to AT1 receptor and ERK1/2 activation. In vivo studies have demonstrated that Ang II is part of a pro-hypertrophic response to pressure overload via AT1 receptors, but its involvement is not essential (Schluter & Wenzel 2008). AT2 receptor activation has been suggested to be a countervailing mechanism in cardiac hypertrophy (Lemarie & Schiffrin 2010). Ang (1-7), acting via the Mas receptor, has been shown to antagonize the pro-hypertrophic effect of ACE-1 and Ang II (Schluter & Wenzel 2008).
2.3.3 Apelin

The APJ receptor is a GPCR that was first identified in 1993 (O’Dowd et al. 1993). The receptor remained “orphan” until apelin was identified in 1998 (Tatemoto et al. 1998). Currently there is no evidence that the apelin-APJ pathway involves more than one receptor (Chandrasekaran et al. 2008).

Apelin is secreted as a 77-amino-acid pre-proprotein which is cleaved into shorter mature biologically active forms including the 36, 17, 16, 13, and 12 amino acids peptides (Chandrasekaran et al. 2008). The degradative pathway for apelin is not fully known. ACE2 cleaves apelin-36 and apelin-13 (Vickers et al. 2002), but the produced fragments of apelin may still have functional activity (Pitkin et al. 2010). Apelin and APJ mRNA are expressed in several tissues, with particularly high concentrations in the cerebellum, vascular endothelium, heart, lung and kidney. The apelin-APJ system has a number of physiological and pathophysiological roles and the pathway includes juxtacrine, paracrine and autocrine signaling (Chandrasekaran et al. 2008).

In the cardiovascular system apelin modulates vascular tone, predominantly vasodilatation mediated by NO, although vasoconstriction has also been observed (Chandrasekaran et al. 2008). Apelin regulates cardiac function and it is among the most potent endogenous inotropic agents yet described. In isolated perfused rat hearts apelin produced a positive inotropic response and increased the peak rate of left ventricular pressure rise (peak dP/dT) (Szokodi et al. 2002), and in isolated adult rat ventricular myocytes apelin increased sarcomere shortening (Farkasfalvi et al. 2007). In vivo apelin increases cardiac contractility in rats (Atluri et al. 2007, Berry et al. 2004) and mice (Ashley et al. 2005). Apelin- or APJ-deficient mice have decreased basal cardiac contractility in vivo and the isolated ventricular myocytes have impaired sarcomeric function and reduced myocyte contractility (Charo et al. 2009). The basal cardiac function of apelin-deficient mice is normal or only slightly impaired, but their exercise capacity and maximal oxygen consumption is reduced. The knockout mice display aging-associated reduced cardiac contractility from 6 months of age and develop severe impairment in heart contractility in a pressure overload-induced HF model (Kuba et al. 2007). The effect of apelin has also been studied in human tissue, where apelin produces concentration-dependent increases in the force of contraction in paced atrial strips (Maguire et al. 2009). Moreover, acute administration of apelin increases cardiac output in vivo in humans (Japp et al. 2010). In vitro the inotropic actions of apelin are independent of ET-1, Ang II, catecholamines and
NO and may involve activation of PLC, PKC, NCX (Szokodi et al. 2002) and sarcolemmal NHE (Farkasfalvi et al. 2007, Szokodi et al. 2002).

The cardiovascular effects of the apelin-APJ system are opposite to many effects of the RAS, which has a marked role in the pathogenesis of HF. It has been suggested that the apelin-APJ system can act as a compensatory mechanism which initially ameliorates the harmful effects RAS activation, but becomes down-regulated in end-stage HF (Chandrasekaran et al. 2008).

2.3.4 Adrenomedullin

AM is a 52-amino-acid vasodilator peptide that was originally isolated from human pheochromocytoma (Kitamura et al. 1993a). AM belongs to the calcitonin gene-related peptide (CGRP) superfamily based on sequence homology. Since its discovery, the peptide has been found in plasma and a variety of tissues including the adrenal medulla, vasculature, lungs and the heart (Yanagawa & Nagaya 2007). AM is processed from a prepro-AM, which consists of 185 amino acids. In addition to AM, prepro-AM contains another bioactive peptide, proadrenomedullin N-terminal 20 peptide (PAMP) (Kitamura et al. 1993b). PAMP has been observed to have a hypotensive effect when injected intravenously, but its action is weaker than that of AM (Kato et al. 2005).

Originally AM was characterized as a vasodilatory molecule (Kitamura et al. 1993b), but it has been demonstrated to have also several other effects in the cardiovascular system. In addition to hypotensive effects AM causes natriuresis and diuresis and modulates cardiac contractility (Yanagawa & Nagaya 2007). In isolated rabbit ventricular myocytes (Ikenouchi et al. 1997) and papillary muscles (Fontes-Sousa et al. 2009) AM had a negative inotropic effect. In the latter study AM was also found induce an acute increase in myocardial distensibility suggesting that AM may modulate diastolic function and cardiac filling (Fontes-Sousa et al. 2009). AM had no effect on cardiac contractility in the rat papillary muscle or in ventricular cardiomyocytes (Stangl et al. 2000). Similar findings were made in papillary muscle preparations from sham operated rats or rats with chronic HF after coronary artery ligation (Baumer et al. 2002). In vivo in propranolol pretreated dogs with controlled heart rate (atrial pacing) AM had arterial vasodilating actions but had no effect on preload and diastolic function and did not demonstrate inotropic actions (Lainchbury et al. 2000). In isolated human myocardial trabeculae from cadaver graft right atria and left ventricle AM
did not elicit changes in contractility in either atrial or ventricular trabeculae (Saetrum Opgaard et al. 2000).

Local AM overexpression in the left ventricle improved systolic function without altering diastolic properties in the normal rat heart (Leskinen et al. 2008). In isolated rat heart AM has a positive inotropic effect (Szokodi et al. 1996) through cAMP-independent Ca\(^{2+}\) release (Szokodi et al. 1998) and the inotropic effect is not modulated by NOS inhibition (Kinnunen et al. 2000). AM produced a positive inotropic effect in rat papillary muscle where AM increased contractility through a cAMP-dependent pathway (Ihara et al. 2000). In muscle strips from human atrial or ventricular myocardium AM exerted a Ca\(^{2+}\) dependent direct positive inotropic effect, the effect being pronounced in the atrial tissue. In addition, the inotropic effects were found to be related to the elevation of intracellular cAMP levels and activation of PKA (Bisping et al. 2007). In the failing myocardium the positive inotropic effect was found to be reduced in both atria and ventricles (Bisping et al. 2007).

Overall, the effects of AM are considered beneficial. It is a possible endogenous suppressor of myocyte hypertrophy, and AM is proposed to play protective roles in cardiovascular disorders such as hypertension and HF (Ishimitsu et al. 2006, Yanagawa & Nagaya 2007).

2.3.5 Natriuretic peptides

Natriuretic peptides are structurally homologous but genetically distinct peptide hormones that have important roles in the regulation of cardiovascular homeostasis (McGrath et al. 2005, Ruskoaho 2003). The main members of the natriuretic peptide family are atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Natriuretic peptides are synthesized as prepropeptides which are first cleaved to propeptide forms. In the second step, propeptides are cleaved to form biologically active peptides. In the second cleavage also the N-terminal fragment of the respective peptide is formed and secreted into the circulation, and it can be used to measure natriuretic peptide secretion because of longer half-life (McGrath et al. 2005, Ruskoaho 2003).

In the normal heart ANP is mainly synthesized and secreted by the atrial myocytes while BNP is mainly produced by ventricular myocytes (McGrath et al. 2005, Pagel-Langenickel et al. 2007, Ruskoaho 2003). In pathological conditions the expression of both ANP and BNP by the ventricle increases. The production of ANP and BNP is primarily stimulated by wall stretch. In addition, increased
circulation or tissue levels of ET-1, catecholamines, Ang II and cytokines can directly increase the expression and release of ANP and BNP. CNP is mainly produced by the vascular endothelium. Natriuretic peptides are removed from the circulation by clearance receptors and through hydrolysis by neutral endopeptidase (McGrath et al. 2005, Pagel-Langenickel et al. 2007, Ruskoaho 2003).

The effects of natriuretic peptides are mediated by natriuretic peptide receptor (NPR)-A, NPR-B and NPR-C (Kuhn 2004, Nishikimi et al. 2006, Pagel-Langenickel et al. 2007). NPR-A and NPR-B are guanylyl cyclase (GC)-linked and utilize cGMP. Both ANP and BNP bind preferentially to NPR-A while CNP binds preferentially to NPR-B. All three peptides bind NPR-C with similar affinity. NPR-C is not linked to GC but it may mediate some of the cellular actions of natriuretic peptides by coupling to Gαi proteins. However, NPR-C mainly acts as clearance receptor and thereby modulates the circulating and local natriuretic peptide concentrations (Kuhn 2004, Nishikimi et al. 2006, Pagel-Langenickel et al. 2007).

The natriuretic peptides exert effects via circulation and they also act as autocrine and/or paracrine factors. ANP and BNP cause diuresis, natriuresis, vasodilatation and inhibition of renin secretion and aldosterone synthesis. CNP has mainly paracrine effects and it causes vasodilatation, but the peptide does not have diuretic and natriuretic properties (Baxter 2004, D'Souza et al. 2004, Nishikimi et al. 2006). ANP, BNP and CNP have been shown to modulate cardiac contractility when administered exogenously, but the effects have been diverse. Generally ANP has been shown to have a negative effect on contractility, but in some studies no functional effects have been found. With BNP similar results have been demonstrated (D'Souza et al. 2004). CNP perfusion in isolated mouse hearts has been shown to produce a biphasic, initially positive inotropic and lusitropic, then negative inotropic effect (Pierkes et al. 2002). However, it has also been reported to have a negative inotropic effect in several studies (Pagel-Langenickel et al. 2007). The effects of natriuretic peptides are mediated by cGMP and the effects are likely concentration-dependent. Low levels of cGMP may mediate the positive inotropic effects while high levels of cGMP produce negative inotropic effects (D'Souza et al. 2004). Importantly, all natriuretic peptides have antihypertrophic and antifibrotic effects and protect the heart from excessive cardiac remodeling. In the HF the expression of ANP and BNP is increased to compensate HF through natriuretic and diuretic action (Nishikimi et al. 2006, Ritchie et al. 2009).
2.3.6 Prolactin releasing peptide

Prolactin-releasing peptide (PrRP) was isolated in 1998 and it was shown to be a potential endogenous ligand for the orphan GPCR, hGR3/GPR10 (Hinuma et al. 1998). PrRP possesses two molecular forms, a 31-amino-acid peptide and the C-terminal 20 residues (PrRP20). Rat PrRP1-31 differs from human PrRP1-31 at only five positions. The peptide was named after its ability to be a potent prolactin-releasing factor for rat anterior pituitary cell (Hinuma et al. 1998). Since its discovery PrRP has been established to have several biological functions in addition to being a hypophysiotropic hormone (Sun et al. 2005). PrRP has been demonstrated to modulate the hypothalamo-pituitary-adrenal axis for the stress response (Maruyama et al. 2001), feeding behavior and energy balance (Lawrence et al. 2000, Takayanagi et al. 2008) as well as nociception (Laurent et al. 2005).

PrRP has been shown to be involved in the central control of cardiovascular function. Intracerebroventricular administration of PrRP increased significantly mean arterial blood pressure in conscious rats (Samson et al. 2000). In addition, in anesthetized rats injection of PrRP into the caudal ventrolateral medulla oblongata produced a robust increase in heart rate and renal sympathetic nerve activity (Horiuchi et al. 2002), demonstrating that the peptide is involved in central control of cardiovascular function. Peripheral tissue PrRP contents, apart from the adrenal gland (62 fmol/g wet tissue), are below 6 fmol/g wet tissue (Matsumoto et al. 1999). Specific PrRP binding sites have been studied by Satoh et al. in rat peripheral tissues. Interestingly, PrRP binding sites were found in a number of rat peripheral tissues with high levels present in the heart (Satoh et al. 2000). Taking into account that PrRP plasma levels are very low (0.13 pM) (Matsumoto et al. 1999), it is unlikely that the peptide can act as a circulating hormone. However, the knowledge of the peripheral effects of PrPRP and the potential role of the peptide in the regulation of cardiac function is limited.

2.4 NO

Furchgott and Zawadzki first demonstrated that vasodilatation in response to acetylcholine was mediated by a non-prostanoid endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki 1980). In 1987, the free radical NO was described to possess the characteristics of this factor (Palmer et al. 1987). Later it has been widely demonstrated that in the cardiovascular system NO is a key
signaling messenger and it is involved in many important biological functions in cardiovascular physiology. In addition to its fundamental role as a paracrine regulator of vascular relaxation NO also plays a major role in regulating cardiac function (Bredt & Snyder 1994).

2.4.1 Production of NO

NO is produced by nitric oxide synthase (NOS) enzymes, which catalyze the formation of NO from the amino acid L-arginine (Nathan & Xie 1994). There are three main NOS isoforms: neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3) and inducible NOS (iNOS, NOS2) which all are expressed in cardiovascular tissues. Each NOS isoform is a product of a separate gene and shares over 50% amino acid homology. In regions involved in cofactor binding they also have similar enzymatic mechanisms that involve electron transfer oxidation of the terminal guanidine nitrogen of L-arginine (Nathan & Xie 1994) (Fig. 3).

Both nNOS and eNOS are generally constitutively expressed and their activities are primarily regulated by intracellular Ca\(^{2+}\)/calmodulin levels, while iNOS expression is induced by inflammation (Hare 2003). For enzymatic activity, NOS proteins must bind cofactors and dimerize (Alderton et al. 2001). NOS proteins first bind to the cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), and the additions of L-arginine, tetrahydrobiopterin (BH4) and heme allow the NOS protein to form dimers. eNOS and nNOS formed this way are inactive and dependent on calmodulin binding stimulated by transient increases in intracellular calcium. The main switch for iNOS is at the level of transcription since the iNOS binds calcium/calmodulin and is active even at low (resting intracellular) concentrations of calcium (Alderton et al. 2001).

In the heart NO is produced in at least two major cell types: cardiac myocytes and endothelial cells (Seddon et al. 2007b). Endothelial cells, lining vasculature and endothelium, are rich in eNOS. Cardiac myocytes express both eNOS and nNOS (Seddon et al. 2007b). eNOS and nNOS are expressed in distinct subcellular compartments of cardiomyocytes; eNOS is localized to the sarcolemmal caveolae through its interaction with caveolin-3 (Barouch et al. 2002, Feron et al. 1996) while nNOS is localized to the sarcoplasmic reticulum (Xu et al. 1999) where it is associated with the RyR (Barouch et al. 2002). Importantly, the different subcellular locations of eNOS and nNOS enable them to play distinct roles in cardiac function.
2.4.2 Effects of NO on myocardial function

In addition to NO's fundamental role as a paracrine regulator of vascular relaxation, it also plays a major role in the regulation of cardiac function. The significant impact of NO on cardiac contractility was first suggested by using unselective pharmacological blockage of NOS (Balligand et al. 1993, Balligand et al. 1995, Hare et al. 1995). The distinct roles of NOS isoforms have been studied later by using selective pharmacological inhibitors and gene-modified mice.

Gyurko et al. reported that Langendorff isolated heart preparations of genetically deficient eNOS mice showed no difference in basal contractility, but inotropic and lusitropic responses to β-adrenergic agonist isoprenaline were enhanced (Gyurko et al. 2000). Similar findings were also made in isolated ventricular myocytes (Godecke et al. 2001, Wang et al. 2008b) and in vivo (Barouch et al. 2002) studies. It has also been reported that eNOS gene deletion enhanced inotropic response in basal conditions (Barouch et al. 2002). These results suggest that eNOS limits the inotropic response to β-adrenergic stimulation and its genetic absence may enhance contractility.

More variable results have been obtained from studies for phenotypes related to cardiac contractility of nNOS knockout mice. The majority of findings indicate that cardiac nNOS-derived NO plays a significant role in the autocrine regulation of myocardial contractility. Enhanced basal left ventricle contraction and enhanced inotropic response to β-adrenergic stimulation was observed in vitro in left ventricle myocytes from nNOS-/- mice (Ashley et al. 2002, Sears et al 2003, Martin et al. 2006) or in wild-type myocytes after pharmacological nNOS inhibition (Ashley et al. 2002, Sears et al 2003). Contrary to this, in an in vivo study nNOS knockout mice showed no significant differences in basal contractility, but the inotropic response to isoprenaline stimulation was decreased.
(Barouch et al. 2002). Myocardial nNOS has been demonstrated to promote left ventricular relaxation by regulating phospholamban phosphorylation and the rate of intracellular Ca$^{2+}$ decay via a cGMP-independent effect on PP activity (Zhang et al. 2008). The discrepancy may be due to the differences between experimental conditions and concentrations of β-agonists (Lim et al. 2008, Seddon et al. 2007b). In addition, the role of myocardial nNOS in the regulation of left ventricle function and β-adrenergic responsiveness in nNOS-/- models in vivo may be due to the effects of nNOS gene deletion on noradrenaline release from cardiac sympathetic nerves and on central sympathetic outflow. These mechanisms may influence indirectly both basal and β-adrenergic left ventricle function in the whole animal. In isolated cardiomyocytes nNOS-derived NO may also have a biphasic effect. In response to high concentrations of β-adrenergic stimulation nNOS-derived NO may enhance or maintain inotropy while in response to submaximal β-adrenergic receptor stimulation it may diminish the positive inotropic effect (Lim et al. 2008, Seddon et al. 2007b).

2.4.3 NO signaling

The intracellular effects of NO are mediated through at least two distinct pathways, the cGMP-dependent and cGMP-independent pathway (Fischmeister et al. 2005, Ziolo et al. 2008). In addition, NO can activate AC, which leads to increased cAMP levels and myocardial contractility. Several actions of NO are mediated through stimulation of GC leading to elevation of cGMP levels. Two different isoforms of GC exist, the soluble GC activated by NO and particulate GC activated by natriuretic peptides ANP, BNP and CNP. Increases in cGMP levels can modulate the activity of several targets such as PKG and cGMP-regulated phosphodiesterases (PDE) (Fischmeister et al. 2005, Ziolo et al. 2008). In the heart PKG can regulate contractile function (Hofmann et al. 2006). PKG activation can mediate phosphorylation of TnI leading to depression of myofilament calcium sensitivity (Layland et al. 2002). PKG has also been shown to be able to phosphorylate the α1C subunit of the L-type Ca$^{2+}$ channel in cardiac myocytes (Jiang et al. 2000, Yang et al. 2007).

PDEs are enzymes that catabolize either cAMP or cGMP (Kass et al. 2007b, Rao & Xi 2009). Currently 11 different isoenzymes with a total of 48 isoforms of PDEs have been classified based on substrate affinity, selectivity, and regulation mechanisms. In the heart five PDE isoforms have been observed: PDE1, PDE2, PDE3, PDE4 and PDE5. PDE1 and PDE2 have dual substrate affinity, PDE3 is
cGMP-sensitive but cAMP-selective, PDE4 is cAMP-specific and PDE5 is cGMP-specific (Kass et al. 2007b, Rao & Xi 2009). PDE5 was previously not thought to be important for cardiac regulation (Corbin et al. 2003). However, it has been shown that although the impact of PDE5 on resting cardiac function is relatively small, it can potentially modulate acute contractile tone in cells stimulated by external agonists. PDE5 has been shown to regulate acute adrenergic-stimulated contractility in dog (Senzaki et al. 2001), mouse (Takimoto et al. 2005a), and human heart (Borlaug et al. 2005). In cardiac myocytes PDE5 has an important role in the regulation of cGMP levels. Inhibition of PDE5 leads to elevated cGMP and increased PKG activity, which could act as a brake to contractile stimulation (Takimoto et al. 2005a).

In addition to activating cGMP-dependent signaling pathways, NO can directly modify sulfhydryl residues of proteins through S-nitrosylation. Cellular proteins that may undergo S-nitrosylation are RyR2 (Xu et al. 1998), SERCA (Lokuta et al. 2005), L-type Ca2+ channel (Campbell et al. 1996, Sun et al. 2006) and Kv1.5 channel (Nunez et al. 2006). S-nitrosylation is an important effector of NO bioactivity and regulator of cellular signal transduction (Jaffrey et al. 2001, Ziolo et al. 2008). Both NOS1 and NOS signal through cGMP dependent and independent pathways. However, cGMP independent effects could be preferentially mediated by nNOS-derived NO (Jaffrey et al. 2001, Ziolo et al. 2008), but the relative contribution of cGMP-dependent and S-nitrosylation-mediated myocardial effects of nNOS derived NO is not fully established (Ziolo et al. 2008).

### 2.4.4 Nitroso-redox interactions

Reactive nitrogen species (RNS) of biological importance include S-nitrosothiols and peroxynitrite in addition to NO. Generally, balance between RNS and ROS is defined by the idea that RNS and ROS work together in biological systems to achieve optimal signaling and imbalance can be due to increased ROS or decreased RNS levels (Zimmet & Hare 2006). The bioavailability of NO is influenced by free radical superoxide (O2−) which avidly interacts with NO and inactivates it (Seddon et al. 2007a). Peroxynitrite, which is formed when NO reacts with O2−, can influence cardiovascular function. Therefore increased superoxide generation may lead to reduced NO bioavailability and the development of endothelial dysfunction, which is an important contributor to disease pathophysiology (Seddon et al. 2007a). In addition, cross talk exists
between enzymes that produce ROS and NO as it has been shown that nNOS and xanthine oxidoreductase (XOR) are co-localized in the SR and that inhibition or absence of nNOS leads to increased XOR-dependent superoxide generation in murine left ventricular myocardium (Khan et al. 2004).

2.4.5 NO in hypertrophy and heart failure

Endogenous myocardial NO may modulate the development of maladaptive hypertrophy and HF. NOS enzymes can become uncoupled, which leads to generation of $\text{O}_2^-$ rather than NO, and it can contribute to cardiovascular pathobiology (Moens & Kass 2006). BH4, a NOS cofactor that regulates NOS function by maintaining stability of NOS dimers (Stuehr et al. 2001, Vasquez-Vivar et al. 1998, Wever et al. 1997), is redox-sensitive and its oxidative degradation is one of the main mechanisms leading to NOS uncoupling (Moens & Kass 2006).

nNOS is upregulated in response to pressure overload (Massion et al. 2003). In neonatal rat cardiomyocytes hypertrophy produced by integrin stimulation was associated with phosphorylation of nNOS and was inhibited by the non-specific NOS inhibitor L-NAME (Umar et al. 2009). Myocardial nNOS expression and activity were found to be increased following experimental myocardial infarction (Bendall et al. 2004, Damy et al. 2003, Dawson et al. 2005) and in human failing hearts (Damy et al. 2004). In failing myocardium increased nNOS expression is accompanied by its translocation from the SR to the sarcolemma (Bendall et al. 2004, Damy et al. 2003, Damy et al. 2004). It has been proposed that in cardiomyocytes nNOS has a protective role, especially in conditions of cardiac volume and pressure overload and in remodeling (Umar & van der Laarse 2010).

Normally eNOS-derived NO has antihypertrophic effects. In pressure overload eNOS may be uncoupled, which may be causally related to left ventricle hypertrophy, remodeling and dysfunction (Umar & van der Laarse 2010). Inhibition of eNOS produced increased protein synthesis and cell size in adult rat ventricular cardiomyocytes (Wenzel et al. 2007). Pressure overload has been demonstrated to trigger eNOS uncoupling leading to oxidative stress that contributes to hypertrophy, dilatory remodeling and cardiac dysfunction, and the process was prevented by supplementary BH4 (Takimoto et al. 2005b). In addition, BH4 has been shown to reverse the established hypertrophy following pressure overload (Moens et al. 2008). The role of eNOS in the regulation of myocardial function in remodeling or HF is not clear. Most studies imply that
eNOS expression and activity are reduced in the failing heart. In the remodeling process eNOS may have a protective effect (Umar & van der Laarse 2010).

### 2.5 Reactive oxygen species

#### 2.5.1 Species of ROS and the antioxidant mechanisms

Oxygen, which is vital for life, is a substrate for the generation of a variety of reactive species (Giordano 2005). ROS is a collective term for several types of reactive oxygen metabolites including radicals such as superoxide (O$_2^-$) and hydroxyl (OH), and non-radicals such as hydrogen peroxide (H$_2$O$_2$) that are either oxidizing agents and/or are easily converted into radicals. ROS can interact with a large number of molecules including other small inorganic molecules as well as proteins, lipids, carbohydrates, and DNA. Through such interactions, the function of the target molecules can be altered or destroyed (Giordano 2005).

ROS generation is generally a cascade of reactions (Fig. 4). The cascade starts with the production of O$_2^-$, which is rapidly dismutated to H$_2$O$_2$ either spontaneously, particularly at low pH, or catalyzed by superoxide dismutase (SOD). The rapid rate of dismutation keeps the concentration of O$_2^-$ extremely low, in the picomolar-nanomolar range (Hool 2006). Compared to O$_2^-$, H$_2$O$_2$ is a relative stable molecule and able to cross membranes, which makes it a biologically important candidate as a ROS and signaling molecule (Rhee et al. 2005, Sundaresan et al. 1995). H$_2$O$_2$ is metabolized to water and oxygen by catalase, which is a naturally occurring enzyme and anti-oxidant (Giordano 2005, Hool 2006). Hydrogen peroxide can react with metal ions (Fenton reaction) or with Cl$^-$, which generate highly reactive .OH or hypochlorite ions, respectively. The half-life of .OH is less than 1 ns and it is unable to diffuse more than one or two molecular diameters before it reacts. Therefore it is an extremely reactive free radical, which can in practice react with any cellular component. The cascade of ROS generation also includes the reaction of O$_2^-$ with NO to form peroxynitrite (Giordano 2005, Hool 2006).

Several cellular mechanisms counterbalance the production of ROS (Maulik & Das 2008, Nordberg & Arner 2001). Three different isoenzymes of SOD, which metabolize O$_2^-$ to hydrogen peroxide, have been identified. Manganese-containing SOD (Mn-SOD) is present in mitochondria and copper/zinc-containing-SOD (Cu/Zn-SOD) exists in the cytosol. The third isoform,
extracellular copper/zinc-containing SOD (ecSOD, SOD-3), is mainly produced and secreted by vascular smooth muscle cells (Maulik & Das 2008, Nordberg & Arner 2001).

Catalase is an intracellular antioxidant enzyme which is predominantly located in cellular peroxisomes and to some extent in the cytosol (Maulik & Das 2008, Nordberg & Arner 2001). Catalase catalyzes the dismutation of \( \text{H}_2\text{O}_2 \) to water and molecular oxygen. In addition, catalase prevents OH radical formation from \( \text{H}_2\text{O}_2 \) via the Fenton reaction. In high-level oxidative stress catalase is very effective and it protects cells from \( \text{H}_2\text{O}_2 \) produced within the cell (Maulik & Das 2008, Nordberg & Arner 2001). Catalase is especially important in the case of limited glutathione content or reduced glutathione peroxidase (GPx) activity, and it plays a significant role in the development of tolerance to oxidative stress in the adaptive response of cells (Wassmann et al. 2004).

The glutathione (GSH) system (GSH, glutathione reductase, and glutathione peroxidase) is the most abundant intracellular thiol-based antioxidant system which is present in millimolar concentrations in all living aerobic cells (Ballatori et al. 2009, Maulik & Das 2008). The GSH system acts primarily as a sulfhydryl buffer, but it is also involved in the detoxification of compounds through conjugation reactions catalyzed by glutathione S-transferase or directly. GSH is known to directly detoxify \( \text{H}_2\text{O}_2 \) in the GPx-catalyzed reaction, and GPx can also reduce other peroxides to alcohols. In the reactions oxidized glutathione is formed, which is then reduced by the NADPH-dependent glutathione reductase (Ballatori et al. 2009, Maulik & Das 2008).

The thioredoxin system is the other ubiquitously expressed thiol-reducing antioxidant system that consists of thioredoxin, thioredoxin reductase, and NADPH (Maulik & Das 2008). The system reduces the oxidized cysteine groups on proteins through an interaction with the redox-active center of thioredoxin. The oxidized thioredoxin is then reduced with the help of thioredoxin reductase and NADPH. Thioredoxin can limit oxidant stress through its direct effect as an antioxidant or through its interactions with other key signaling proteins (thioredoxin interacting proteins) (Maulik & Das 2008). In addition, thioredoxin can enhance Mn-SOD expression (Das et al. 1997).

In addition to antioxidant-scavenging enzymes, a large number of low molecular weight compounds, including vitamins C and E, different selenium compounds, lipoic acid, and ubiquinones, are considered to be antioxidants of biological importance (Maulik & Das 2008, Nordberg & Arner 2001).
2.5.2 Sources of ROS

In the heart, all cell types, including cardiomyocytes, endothelial cells, vascular smooth muscle cells, fibroblasts and infiltrating inflammatory cells, can generate ROS (Giordano 2005). ROS can be formed by several mechanisms, mainly by mitochondria, xanthine oxidase (XO) and NADPH oxidases. In mitochondria ROS can be generated during oxidative phosphorylation in the mitochondria as a by-product of normal cellular aerobic metabolism. Electron transport within the mitochondria is generally very efficient, but a small leakage of single electrons can participate in the reduction of oxygen to superoxide. Especially the complexes I and III convert a fraction of O₂ to O₂⁻. The actual amount of O₂⁻ released by the mitochondria also depends on the activity of Mn-SOD located in the mitochondrial matrix (Giordano 2005).

Xanthine oxidoreductase (XOR) has two isoforms: XO and xanthine dehydrogenase (XDH) (Berry & Hare 2004). XDH can be converted to XO by oxidation of the sulfhydryl residues or by proteolytic cleavage. Both forms catalyze the conversion of hypoxanthine to xanthine and xanthine to uric acid. XOR can reduce both oxygen and NAD⁺ but has greater affinity for the latter. XO reduces molecular oxygen, leading to the formation of both O₂⁻ and H₂O₂ as by-products of terminal steps of purine metabolism (Berry & Hare 2004).

An important source of superoxide is NADPH oxidase, which produces ROS by transferring electrons across the membrane from NADPH to molecular oxygen.
NADPH oxidase is a multi-subunit enzyme complex first identified in neutrophils (Babior et al. 1973). Compared to other sources where ROS are generated as a by-product, NADPH oxidases generate ROS in a highly regulated manner (Akki et al. 2009, Brandes et al. 2010). In mammalian cells seven Nox proteins have been identified: Nox1-Nox5 and dual oxidase (Duox) 1 and 2, each encoded by a separate gene and with distinct tissue distribution. The isoforms are distinguished by the presence of distinct Nox (for NADPH OXidase) catalytic subunits. The Nox proteins differ in expression, subcellular location, and the mode of action. In addition, the enzymatic activity of the oxidase depends on additional protein subunits which vary according to the isoform (Akki et al. 2009, Brandes et al. 2010). Although all Nox homologs should primarily release $\text{O}_2^-$ there are differences in the type of ROS produced. Nox1, Nox2 and Nox5 seem to produce primarily $\text{O}_2^-$ (Serrander et al. 2007b, Suh et al. 1999) while the biologically reactive product of Nox4 is predominately $\text{H}_2\text{O}_2$ (Serrander et al. 2007a).

Nox1, Nox2, Nox4 and Nox5 are probably the major sources of ROS in the cardiovascular system. Nox1 is predominantly expressed in vascular smooth muscle cells (Lassegue et al. 2001), Nox2 is expressed in cardiomyocytes (Bendall et al. 2002, Hingtgen et al. 2006, Xiao et al. 2002), endothelial cells (Gorlach et al. 2000, Li & Shah 2002), fibroblasts (Pagano et al. 1997) and some vascular smooth muscle cells (Lassegue et al. 2001, Touyz et al. 2002), while Nox4 is expressed in cardiomyocytes (Byrne et al. 2003, Li et al. 2006), endothelial cells (Ago et al. 2004), vascular smooth muscle cells (Hilenski et al. 2004) and fibroblasts (Hilenski et al. 2004). Nox5 is missing in rodents but it has been found in human endothelial cells and smooth muscle cells (BelAiba et al. 2007, Fulton 2009, Jay et al. 2008). There is no evidence that Nox3 or Duoxes have important roles in the cardiovascular system. The expression of Nox3 is high in the inner ear, and the expression of Duox proteins is restricted to epithelial cells (Bedard & Krause 2007).

NADPH oxidases seem to have two general physiological roles. Superoxide produced by NADPH oxidase is involved in host defense, such as respiratory burst that occurs in phagocytes (Leto & Geiszt 2006). The second role is in signaling as $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ produced by Nox enzymes can specifically and reversibly react with proteins altering their localization, activity and half-life (Akki et al. 2009, Brandes et al. 2010, Brown & Griendling 2009). Many agonists and stimuli can activate NADPH oxidase in cardiovascular cells. These include G-protein-coupled agonists (such as Ang II, ET-1), growth factors (e.g. vascular endothelial growth factor, thrombin) cytokines (such as tumor necrosis-$\alpha$,
transforming growth factor β) and metabolic factors (e.g. insulin, glucose) as well as mechanical forces and hypoxia-reoxygenation (Akki et al. 2009, Brandes et al. 2010, Brown & Griendling 2009).

NADPH oxidase-derived ROS may mediate their effects through diverse downstream signaling pathways. They can activate redox sensitive kinases (such as the MAPK family) directly or indirectly via inhibition of protein tyrosine phosphatases; alter the activity of redox-sensitive transcription factors (such as activator protein (AP-1), nuclear factor-kappa B (NF-κB), hypoxia-inducible factor-1 (HIF-1), signal transducer and activator of transcription (STAT)); and have a direct effect on enzymes, receptors and ion channels (Akki et al. 2009, Brandes et al. 2010, Brown & Griendling 2009). It has been demonstrated that NADPH oxidase-derived ROS can be involved in the regulation of myocardial intracellular Ca²⁺ homeostasis via modulation of L-type Ca²⁺ channel and the RyR. In coronary artery smooth muscle cells NADPH oxidase-derived O₂⁻ has been shown to activate the RyR/Ca²⁺ release channels on the SR to mobilize Ca²⁺ (Yi et al. 2006). NADPH oxidase activation has been shown to increase S-glutathionylation of RyR and hence SR Ca²⁺ release in cardiac SR (Sanchez et al. 2005). ET-1 together with enhanced NADPH oxidase activity increased L-type Ca²⁺ channel open probability in cardiac myocytes (Zeng et al. 2008).

Table 1. The expression of different Nox isoforms in the heart.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Expressed Nox isoform</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiomyocytes</td>
<td>Nox2, Nox4</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Nox2, Nox4, Nox5 (in humans)</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Nox2, Nox4</td>
</tr>
<tr>
<td>Vascular smooth muscle cells</td>
<td>Nox1, Nox2, Nox4, Nox5 (in humans)</td>
</tr>
</tbody>
</table>

2.5.3 ROS in the cardiovascular system

In biological organisms ROS were first identified as major contributors to damage in the aging process (Beckman & Ames 1998). The beneficial function of ROS production was first realized in host defense when deficiency in ROS generation was linked to the reduced killing ability in leukocytes (Bedard & Krause 2007). Over the last decades, it has become clear that ROS are involved not only in cellular damage and killing of pathogens, but also in a large number of reversible regulatory processes in virtually all cells and tissues (Giordano 2005). In health, there is a balance between ROS generation and the antioxidant systems that
scavenge or reduce ROS. In contrast, increased ROS production and/or reduced antioxidant reserve can cause redox imbalance and oxidative stress that enhance susceptibility of biological molecules and membranes to reaction with ROS (Giordano 2005).

There is increasing evidence that ROS are involved in the regulation of normal cardiac function. A relationship has been suggested between contractile activity, oxidative metabolism and ROS production. In isolated cardiomyocytes increases in contraction frequency are accompanied by enhanced oxygen consumption and ROS formation (Heinzel et al. 2006, Saitoh et al. 2006). In isolated rat cardiac trabeculae XO inhibitor increased Ca\(^{2+}\) responsiveness of myofilaments, suggesting that ROS suppress baseline contractility (Perez et al. 1998). ROS have been shown to influence β-adrenergic responsiveness as the antioxidant vitamin C has been reported to enhance the positive inotropic response to the β-adrenergic agonist dobutamine in patients with preserved left ventricular systolic function (Mak & Newton 2001). ET-1 has been reported to increase intracellular levels of ROS in cultured rat, mouse and cat cardiomyocytes (Cheng et al. 1999, Cingolani et al. 2006, De Giusti et al. 2008, Dong et al. 2006). However, the effect of ROS in inotropic response to ET-1 is controversial. Leptin has been reported to suppress contractility acting through ET receptors and increased ROS formation in mice ventricular myocytes (Dong et al. 2006), while Ang II induced ET-1 release and increase in ROS generation, which in turn triggered an increase in contractility in cat cardiomyocytes (Cingolani et al. 2006). In addition, it has been suggested that the positive inotropic effect of exogenous ET-1 is almost exclusively dependent on ROS production (De Giusti et al. 2008).

The impact of NADPH oxidase on cardiomyocyte contractile function is also controversial. In isolated cat cardiomyocytes ET-1 treatment for 15 minutes induced a positive inotropic effect, which was abolished by a NADPH oxidase inhibitor (De Giusti et al. 2008). On the contrary, in isolated adult mice ventricular myocytes one-hour treatment with high concentrations of leptin depressed contractility via a NADPH oxidase-dependent mechanism (Dong et al. 2006). Also transforming growth factor-β1 exposure for 3–4 hours was shown to induce contractile dysfunction through NADPH oxidase-dependent ROS generation in isolated adult rat ventricular myocytes (Li et al. 2008).

In the cardiovascular system oxidative stress is involved in numerous pathological states, such as atherosclerosis (Khatri et al. 2004), myocardial reperfusion injury (Asimakis et al. 2002, Yoshida et al. 2000), genesis of cardiac hypertrophy (Pimentel et al. 2001), apoptosis (von Harsdorf et al. 1999) and HF
In HF oxidative stress triggers a variety of changes such as interstitial fibrosis, loss of functional myocytes due to enhanced apoptosis and necrosis, ultimately leading to pump dysfunction (Giordano 2005, Murdoch et al. 2006, Sawyer et al. 2002, Zimmet & Hare 2006). Enhanced ROS production may also directly contribute to contractile dysfunction. Excessive levels of ROS can alter the activity of different proteins involved in ECC, including the SR Ca\(^{2+}\) release channel, SERCA, and the L-type Ca\(^{2+}\) channel, by modifying sulfhydryl groups of cysteine residues (Zima & Blatter 2006).

In HF oxidative stress is at least partly due to the functional uncoupling of the respiratory chain caused by inactivation of complex I (Ide et al. 1999). The antioxidant capacity, such as activity of Cu/Zn SOD and catalase, is also reduced in the failing myocardium (Dhalla & Singal 1994). In compensated hypertrophy XOR activity is not likely to be significantly increased (Murdoch et al. 2006), but in congestive HF there is a significant increase in cellular XOR levels and activity (Cappola et al. 2001). In such cases XO is capable of producing large amounts of ROS with potential pathophysiological relevance (Bergamini et al. 2009, Berry & Hare 2004). Moreover, XO can generate NO by catalyzing the reduction of nitrate to nitrite and nitrite to NO in the presence of NADH as electron donor, but the exact relevance of this particular XOR function is not clear (Berry & Hare 2004, Li et al. 2003).

In left ventricular hypertrophy the expression of p22phox, p67phox, p47phox and Nox2, and Nox activity have been shown to be up-regulated during progression of cardiac hypertrophy to failure in a guinea pig model (Li et al. 2002). In addition, in human HF enhanced p47phox staining was observed, indicating increased Nox activity (Heymes et al. 2003). Nox2 has been shown to be required for induction of cardiac hypertrophy by Ang II, but not by pressure overload in studies using Nox2 knockout mice (Murdoch et al. 2006). A recent study using cardiac-specific Nox4 knockout mice has demonstrated that Nox4 is a major source of oxidative stress, mediating mitochondrial dysfunction, apoptosis and left ventricular dysfunction after pressure overload (Kuroda et al. 2010). Importantly, mitochondrial dysfunction can facilitate electron leakage and induce ROS-induced ROS release and further increase oxidative stress (Zorov et al. 2000). In addition, ROS production from other enzymatic sources such as cyclooxygenase and nitric oxide synthase can be increased in the failing heart (Murdoch et al. 2006).
2.6 Intracellular signal transduction

2.6.1 G-proteins

GPCRs are a conserved family of receptors containing a structure of seven transmembrane α-helices (Salazar et al. 2007). For example, Ang II, ET-1 and the sympathetic nervous system mediate their effects via GPCRs. GPCRs are activated by ligand binding to extracellular site of the receptor and signaling cascade is initiated via heterotrimeric G-proteins. G-proteins consist of α, β and γ subunits of which β and γ subunits are tightly associated and are often referred to as a single entity, the βγ-subunit. Currently 15 α-subunit, 6 β-subunit and 12 γ-subunit variants are known to exist. Interaction of the GPCR receptor with an agonist results in exchange of the bound GDP to GTP in the α-subunit. The GTP-bound α-subunit then dissociates from the receptor and Gβγ subunit. Both α and βγ subunits stimulate distinct downstream effectors (Salazar et al. 2007).

The Ga-proteins are divided into four primary families (Ga_s, Ga_i, Ga_q, and Ga_11) based on similarity of α-subunits among individual family members. The most commonly studied receptors are Ga_s and Ga_i coupled β-adrenergic receptors, Ga_q coupled ET-1, Ang II and α_2-adrenergic receptors and Ga_i coupled adenosine, α_2-adrenergic and muscarinic-2 receptors (Salazar et al. 2007). The G-protein complexes differ in their affinity to downstream effectors, for example activation of Ga_q stimulates PLC leading to generation of IP_3 and DAG while the Ga_s subunit activates AC leading to increased production of cAMP and activation of PKA (Rockman et al. 2002, Salazar et al. 2007).

The Ga_q signaling pathway in vivo is known to mediate the hypertrophic response (Salazar et al. 2007). Overexpression of Ga_q results in modest cardiac dysfunction and increased hypertrophic markers, and high overexpression produces marked hypertrophy, HF and increased mortality (D'Angelo et al. 1997, Mende et al. 1998). Ga_s-coupled receptors mainly regulate cardiac contractility (Salazar et al. 2007). In response to catecholamine stimulation mice overexpressing Ga_s show increased heart rate and contractility, but they eventually develop myocardial damage such as cellular hypertrophy, fibrosis and necrosis (Iwase et al. 1996). Genetic overexpression of synthetic Ga_i-coupled receptor causing sustained Ga_i stimulation produces bradycardia and cardiomyopathy (Redfern et al. 2000). Patients with dilated cardiomyopathy and HF show increased Ga_i levels, suggesting a role in cardiomyopathies (Bohm et al. 1990, Flesch et al. 1996).
2.6.2 Protein kinase C

PKC is a group of serine/threonine protein kinases which are classified into three main subgroups (Ferreira et al. 2010, Palaniyandi et al. 2009). The classical PKCs include isoenzymes α, β1, β2, and γ, which are activated by phosphatidylserine, DAG and Ca²⁺. The novel PKCs (δ, ε, θ, η, and μ) require DAG but not Ca²⁺ for activity, and the atypical PKCs (ι, ζ and λ) are not stimulated by lipids or Ca²⁺ but by lipid derived second messengers (Ferreira et al. 2010, Palaniyandi et al. 2009). PKC activators include GPCRs, e.g., Ang II and ET-1 and among the molecular targets of PKC isoforms are mammalian target of rapamycin (mTOR), glycogen synthase kinase (GSK), MAPKs, Akt, TnT and TnI (Palaniyandi et al. 2009). In addition to the role as central physiological signaling molecules PKCs are involved in cardiac remodeling such as inflammation, cardiac hypertrophy and fibrosis, HF and ischemic heart disease. However, studying the effects of different PKC isoforms on cardiac function is challenged by their complex regulation, compensation by other isoforms and species-specific differences in expression. In addition, the effects of an isoform can vary depending on the time point studied (Palaniyandi et al. 2009).

In cardiovascular pathophysiology the functional roles of PKCα, PKCβ, PKCδ, and PKCε isoforms have been studied in more detail. In neonatal cardiomyocytes PKCα has been shown to induce hypertrophy (Dorn & Force 2005, Palaniyandi et al. 2009). However, in the adult heart the functional role of PKCα seems to differ. In vivo it has been found that PKCα overexpression had no effect on cardiac growth, while depressed cardiac contractility and deletion of PKCα increased contractility (Braz et al. 2004). In addition, it has been demonstrated that PKCα depresses contractility through phosphorylation of TnI and/or TnT (Sumandea et al. 2003). It has been suggested that PKCα is a critical determinant of systolic function but has minimal effect on cardiac hypertrophy (Dorn & Force 2005).

PKCβ has been shown to be involved in the signaling pathway that mediates cardiac hypertrophy. In several studies it has been found that PKCβ expression is upregulated in adult cardiac myocytes under hypertrophic stimuli and also in human HF (Ferreira et al. 2010). In mouse cardiomyocytes overexpression of PKCβ2 results in left ventricular hypertrophy and fibrosis (Wakasaki et al. 1997). However, PKCβ is likely not necessary for cardiac hypertrophy as the hypertrophic response to phenylephrine or aortic banding was not attenuated in PKCβ knock-out mice (Roman et al. 2001).
PKCε is activated in response to hypertrophic stimuli in both cultured rat cardiomyocytes and in vivo (Ferreira et al. 2010). Overexpression of constitutively active PKCε results in mild concentric hypertrophy with normal cardiac function (Takeishi et al. 2000), and cardiac-specific overexpression of a PKC activator (receptor of activated C kinase, ΨεRACK) also causes hypertrophic cardiomyopathy with preserved systolic function (Mochly-Rosen et al. 2000). On the other hand, PKCε is not required for pressure overload-induced hypertrophy (Klein et al. 2005). PKCδ has been shown to be involved in normal cardiomyocyte growth (Chen et al. 2001, Hahn et al. 2002). However, PKCδ likely has a more significant role in myocardial ischemia and infarction (Dorn & Force 2005).

2.6.3 Mitogen-activated protein kinases

MAPKs are a conserved family of proteins that play an integral role in distinct signaling events in all eukaryotic cells (Rose et al. 2010). MAPKs are involved in a diverse repertoire of biological events including cell proliferation and differentiation, metabolism, survival, immune response and apoptosis. The three main sub-families of MAPKs identified in the myocardium are ERK1/2, p38-MAPK and c-Jun NH2-terminal kinases (JNK). The ERK1/2 pathway is mainly found to be responsive to stimulation of growth factors while JNK and p38-MAPK are called stress-activated MAPKs as they are activated by physical, chemical and physiological stressors (Rose et al. 2010).

MAPKs are typically activated by a three-tiered kinase cascade which allows signal amplification, modulation, and specificity in response to different stimuli (Fig. 5) (Rose et al. 2010). The first MAPK kinase kinase (MAPKKK, MAP3K, MEKK, or MKKK) activates a MAPK kinase (MAPKK, MAP2K, MEK, or MKK), which in turn activates the effector MAPK through serial phosphorylation. Finally activated MAPKs can phosphorylate serine or threonine residues on a wide array of their intracellular targets. The functional specificity of MAPKs is further contributed to by positive and negative modulators and scaffold proteins and negative feedback regulation by phosphatases among other inhibitor regulators. Importantly, also significant overlap and cross-talk exists among different MAPK cascades (Rose et al. 2010).
ERK1/2

ERK1 and ERK2 are usually referred to as ERK1/2, also known as p44/42 MAPK, as they are 83% identical and share most of the signaling activities (Rose et al. 2010). ERK1/2 can be activated through phosphorylation of the Thr and Tyr residues in the Thr-Glu-Thr motif by the upstream kinase MEK1/2, which can be activated by several MAPKKKs such as Raf, which is activated by Ras, PKC and Src (Ravingerova et al. 2003, Rose et al. 2010, Sugden & Clerk 1998b).

In cardiomyocytes ERK1/2 can be activated by both extracellular and intracellular factors. Hypertrophic stimuli including GPCR-agonists such as ET-1 and phenylephrine, growth factors including insulin-like growth factor 1 and fibroblast growth factor, as well as mechanical stretch of the myocytes stimulate the ERK1/2 cascade (Rose et al. 2010). Transactivation of epidermal growth factor receptor (EGFR) has also been established to be an important mechanism that links GPCRs and ERK1/2 activation (Thomas et al. 2002). Stimulation of GPCRs induces metalloproteinase-mediated ectodomain shedding of membrane-anchored proheparin-binding epidermal growth factor (EGF). Soluble heparin-
binding EGF then binds to and activates EGFR, leading to ERK1/2 phosphorylation via recruitment of the Ras-Raf1-MEK1/2 cascade (Fuller et al. 2008, Wetzker & Bohmer 2003).

Activated ERK1/2 phosphorylates a diverse range of intracellular targets throughout the cell including transcription factors such as GATA-4 and nuclear factor activators of transcription (NFAT) (Liang et al. 2001, Sanna et al. 2005). In addition, ERK1/2 can phosphorylate the downstream kinases MAPKAPK-1 and p90 ribosomal S6 kinase (RSK). p90RSK, in turn, can regulate a variety of downstream effectors, including NHE, NF-κB and glycogen-synthase kinase 3β (GSK-3β) (Ravingerova et al. 2003).

*In vitro* ERK1/2 has been shown to be involved in cardiomyocyte hypertrophic growth and gene expression as well as resistance to apoptosis in multiple studies (Bueno & Molkentin 2002, Rose et al. 2010). Genetically engineered animal models have further elucidated the functional role of the ERK1/2 pathway in cardiac hypertrophy *in vivo*. Initially it was demonstrated that overexpression of Ras, which is an upstream regulator of ERK1/2, promoted cardiomyopathy characterized by pathological ventricular remodeling and premature death (Hunter et al. 1995). In mice transgenic overexpression of MEK1 induced stable concentric hypertrophy within 12 months of age with increased systolic function, suggesting that the signaling pathway stimulates physiologic hypertrophy. In addition, the mice were partially protected from apoptotic stimuli, suggesting that ERK1/2 may possess cardioprotective characteristics (Bueno et al. 2000). In response to pressure overload transgenic mice with cardiac-specific expression of dominant negative form of Raf-1 showed reduced phosphorylation of the MAPK target ELK1 and attenuated cardiac hypertrophy, suggesting that ERK1/2 is required for this response *in vivo* (Harris et al. 2004). Likewise, in mice cardiac-specific deletion of Raf-1 leads to left ventricular systolic dysfunction and heart dilatation without hypertrophy (Yamaguchi et al. 2004). The study of Purchell et al. indicates that reduction of ERK1/2 activity is not sufficient to prevent hypertrophy. In ERK1−/− and ERK2+/− mice, as well as in transgenic mice overexpressing ERK1/2-inactivating dual-specificity phosphatase 6 in the heart, hypertrophic growth was not attenuated after pathologic or physiologic hypertrophic stimulus. However, in long-term pressure overload the selective ablation of cardiac ERK1/2 signaling predisposed the heart to decompensation and failure in conjunction with an increase in myocyte apoptosis, suggesting that ERK1/2 signaling plays a protective role in response to pathological stimuli (Purchell et al. 2007).
Recently a new regulatory mechanism of ERK1/2 function was described involving a previously unrecognized autophosphorylation site of ERK2 at Thr188 in response to hypertrophic stimuli (Ang II, phenylephrine, neuregulin-1β and transaortic constriction) (Lorenz et al. 2009). It was found that activation of Gαq-coupled receptors mediated a protein-protein interaction between Gβγ and ERK1/2 leading to autophosphorylation of ERK2 at Thr18 and translocation of ERK1/2 to the nucleus to phosphorylate nuclear targets known to cause cardiac hypertrophy. In addition, the mechanism depends on Raf-MEK1/2-ERK1/2 cascade activation and ERK1/2 dimerization (Lorenz et al. 2009).

**p38-MAPK**

In mammals four separate genes encoding p38-MAPK have been identified (p38α, p38β, p38γ and p38δ), of which p38α is the major isoform in the adult heart (Rose et al. 2010). The upstream kinases of p38-MAPK activation include MEKK1-4, TAK1 and ASK1 at the MAPKKK level and MKK3, -6 and possibly -4 at the MAPKK level. The MAPKKs activate p38-MAPK by phosphorylation of its Thr-Gly-Tyr motif. The p38-MAPK pathway can be activated by several extracellular stimuli such as heat, osmotic shock, inflammatory cytokines and growth factors (Rose et al. 2010). In addition, p38-MAPK is activated by the GPCR-agonists ET-1, Ang II and phenylephrine (Sugden & Clerk 1998a). Downstream substrates for activated p38-MAPK include cytoplasmic proteins, transcription factors and other nuclear proteins (Rose et al. 2010). The p38-MAPK pathway has been implicated in the heart in a variety of functions including inflammatory response, myocyte hypertrophy, cardiac gene regulation, energetic metabolism, contractility, proliferation and regulation of cell death (Wang 2007).

p38-MAPK can regulate myocyte contractility, as activation of p38-MAPK has been shown to have negative inotropic effects in vitro (Chen et al. 2003, Liao et al. 2002) and in vivo (Liao et al. 2001). The underlying mechanism has been suggested to be independent of changes in intracellular Ca²⁺ homeostasis (Liao et al. 2002) but partly related to modifications of sarcomeric proteins (Chen et al. 2003). More recently, the underlying mechanism has been suggested to involve attenuated phosphorylation of α-tropomyosin (Vahebi et al. 2007). In addition, p38-MAPK may affect contractility by promoting NCX transcription (Menick et al. 2007).

In cultured cardiomyocytes p38-MAPK activation was initially suggested to promote cardiac growth and hypertrophy. In neonatal cardiomyocytes...
pharmacological inhibition of p38-MAPK was shown to attenuate myocyte growth in response to hypertrophic stimuli (Nemoto et al. 1998, Zechner et al. 1997) while overactivation of p38-MAPK was shown to induce cardiac hypertrophy (Nemoto et al. 1998, Wang et al. 1998, Zechner et al. 1997).

Studies using transgenic mice have shown different effects in vivo. Mice overexpressing MKK3 or MKK6, which produced specific activation of p38-MAPK, did not show cardiac hypertrophy but exhibited increased fibrosis and expression of fetal marker genes characteristic of cardiac failure (Liao et al. 2001). In contrast, mice expressing dominant-negative mutants of p38α, MKK3, or MKK6 produced increased cardiac hypertrophy in response to pressure overload, suggesting that p38-MAPK may have an inhibitory function on cardiac hypertrophy (Braz et al. 2003). Furthermore, mice with cardiac-specific p38α conditional knockout developed significant levels of cardiac hypertrophy, which was similar to wild-type animals, but showed increased fibrosis, apoptosis and dysfunction in response to pressure overload (Nishida et al. 2004). In rats adenovirus-mediated gene transfer of constitutively active MKK3be into the left ventricle produced cell proliferation, inflammation, and fibrosis, but no morphological signs of cardiomyocyte hypertrophy were observed (Tenhunen et al. 2006).

p38-MAPK has also been implicated in physiological hypertrophy. Loss of p38-MAPK by deletion of its upstream activator ASK1 or by conditional knockout of p38-MAPK from the myocardium produced increased hypertrophy without increased fibrosis in response to swimming indicating that the ASK1/p38-MAPK signaling pathway negatively regulates physiological hypertrophy (Taniike et al. 2008).

**JNK**

Three JNK genes, JNK1, JNK2 and JNK3, have been identified, generating ten known isoforms through alternative splicing that share >80% homology (Davis 2000). JNK1 and JNK2 are ubiquitously expressed while JNK3 is mainly found in the heart, brain and testis (Rose et al. 2010). As a stress-activated protein kinase JNK can be activated by similar stress factors as p38-MAPK, including hyperosmolarity, ischemia-reperfusion and oxidant stress. In addition, JNK can be activated by growth factors and GPCRs (Rose et al. 2010).

The MAPKKKs that can activate JNKs include MEKK1, MEKK2 and MEKK3 as well as mixed lineage kinase 2 and 3, which then activate the
MAPKs MKK4 and MKK7. MKK4/7 activates JNK by phosphorylation on a Thr-Pro-Tyr motif (Rose et al. 2010). In addition, the Wnt signaling pathway can activate JNK. Activated JNK has a number of downstream substrates including nuclear and cytoplasmic proteins. In cardiac myocytes the main targets of JNK phosphorylation include c-Jun and ATF-2 (Sugden & Clerk 1998a, Rose et al. 2010). JNK has a role in many biological processes including cell proliferation, differentiation, apoptosis and cell survival, and in the heart the JNK pathway has been related to hypertrophy, cell death and remodeling (Rose et al. 2010).

In cardiomyocytes JNK activation has been found to lead to a hypertrophic phenotype, but in vivo the role of JNK has been found to be controversial (Rose et al. 2010, Wang 2007). In transgenic animal models JNK activation has not been found to induce cardiac hypertrophy but a significant induction of fetal gene expression and restrictive cardiomyopathy (Liang & Molkentin 2003, Liang et al. 2003, Petrich et al. 2004, Sadoshima et al. 2002). The explanations for the different observations may be related to functional redundancies of different JNK isoforms and the effects of dominant-negative mutants of JNK on other hypertrophic pathways. In addition to prohypertrophic signaling molecules Akt (Shao et al. 2006) and c-Jun (Sugden & Clerk 1998a), JNK can also modulate negative regulators of hypertrophic signaling including calcineurin (Liang et al. 2003) and JunD (Hilfiker-Kleiner et al. 2006).
3 Aims of the research

The aim of the study was to characterize the signaling mechanisms regulating cardiac contractility when the heart was stimulated with different GPCR-agonists. The main agonist used was ET-1, because it is among the most potent inotropic substances identified. More specifically, the objectives where:

1. To study the role of ERK1/2 and p38-MAPK and the potential upstream regulators and the downstream effectors of MAPK signaling in the regulation of cardiac contractility stimulated by ET-1.
2. To characterize the role of endogenous ROS production in the acute regulation of cardiac contractility by using two distinct GPCR agonists, ET-1 and dobutamine, the potential cellular sources of ROS, and the molecular mechanisms by which ROS affect contractility.
3. To evaluate the role of NO in the regulation of cardiac contractility stimulated by ET-1 by studying the role of nNOS and NO-dependent cellular pathways.
4. To determine the effects of PrRP on cardiac contractility and the underlying signaling pathways.
4 Materials and methods

4.1 Materials (I-IV)

Zoniporide was supplied by Dr. Ross Tracey, Pfizer Global Research and Development, Groton, Conn; HMR 1098 by Dr. Jürgen Pünter, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany, and sildenafil by Pfizer, New York. The other chemicals and their suppliers were ET-1, PrRP-31 (Phoenix Europe GmbH, Karlsruhe, Germany); AG1478, GF-109203X (bisindolylmaleimide I), LY 294002, ML-7, phorbol 12-myristate 13-acetate, SB239063, U0126, U-73122, calyculin, 3-isobutyl-1-methylxanthine (IBMX), ocdac acid, Ro32-0432 (Merck Chemicals Ltd., Nottingham, UK); ET-1, dobutamine, N-acetylcysteine, 5-hyroxydecanoate (5-HD), paxiline, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), S-Methyl-L-thiocitruilline (SMTC), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), KT5823 (Sigma-Aldrich Co, St. Louis, MO); Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) and apocynin (Calbiochem-Novabiochem Corp. Darmstadt, Germany); dihydroethidium (Molecular Probes Inc., Eugene, OR). Anti-phospho-ERK1/2, anti-ERK1/2, anti-p38, and anti-phospho-p90 RSK (Cell Signaling Technology Inc., Hitchin, Hertfordshire, UK), anti-phospho-p38 (Chemicon International Inc., Temecula, Calif.), anti-EGFR, anti-phospho(Ser16)-phospholamban (Santa Cruz Biotechnology, Santa Cruz, Calif.), anti-phospho(Ser16)-phospholamban (Badrilla Ltd, Leeds, West Yorkshire, UK), and anti-phospho(Thr17)-phospholamban (Santa Cruz Biotechnology, Santa Cruz, Calif) and anti-phospho-tyrosine (Upstate Biotechnology, Lake Placid, NY). Isoform-specific PKC antibodies (anti-PKCα, δ and ε) were from Sigma (Saint Louis, Mo).

4.2 Experimental animals (I-IV)

Male 6- to 7-week-old Sprague-Dawley rats from the Center for Experimental Animals at the University of Oulu were used for isolated perfused rat heart experiments. The animals were housed in plastic cages with free access to tap water and normal rat chow in a room with a controlled humidity of 40% and temperature of 22°C. A 12 h light and 12 h dark environmental light cycle was maintained. All procedures in the study conformed to the principles outlined in
the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health (NIH Publication No.85-23, revised 1996). The experimental design was approved by the Animal Use and Care Committee of the University of Oulu.

4.3 Isolated perfused rat heart preparation (I-IV)

The isolated, perfused rat heart preparation used in this study was a modification of the previously described experimental setup (Ruskoaho et al. 1986, Szokodi et al. 2002). The isolated heart preparation method is a well-defined tool when studying e.g. basic heart physiology in the form of myocardial function (Bell et al. 2011). Briefly, rats were decapitated after sedation with CO₂. The abdominal cavity was immediately opened, the diaphragm transected and lateral incisions made along both sides of the rib cage. The heart was cooled, the aorta was cannulated above the aortic valve and retrograde perfusion was begun with a modified Krebs-Henseleit bicarbonate buffer, pH 7.40, equilibrated with 95% O₂ and 5% CO₂ at 37°C. The composition of the buffer was (in mmol/L) NaCl 113.8, NaHCO₃ 22.0, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.1, CaCl₂ 2.5 and glucose 11.0. Hearts were perfused at a constant flow rate of 5.5 mL/min with a peristaltic pump (Minipuls 3, model 312, Gilson, Villiers, France). Variation in perfusion pressure reflecting coronary vascular resistance was measured by a pressure transducer (model MP-15, Micron Instruments, Los Angeles, Calif) situated on a side arm of the aortic cannula. Heart rate was maintained constant (300 beats/min) by atrial pacing using a Grass stimulator (model S88, Grass Instruments, West Warwick, RI) (11 V, 0.5 ms). Contractile force (apicobasal displacement) was obtained by connecting a force displacement transducer (FT03, Grass Instruments, West Warwick, RI) to the apex of the heart and recorded with a Grass polygraph (model 7DA, Grass Instruments, Quincey, A, USA). The hearts were put under a resting tension of 2.0 g. The drugs were infused via an infusion pump (Secan PSA 55, Sky Electronics S.A). When inhibitors were used, the lowest effective dose was chosen. The hearts were allowed to stabilize for 35–40 minutes before any interventions.
4.4 Experimental protocols

Table 2. Summary of the experimental protocols in isolated perfused heart.

<table>
<thead>
<tr>
<th>Study</th>
<th>Methods</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Western blotting</td>
<td>ET-1, AG1478, GF-109203X, SB239063, LY294002, ML-7, phorbol 12-myristate 13-acetate, U-73122, U0126, zoniporide</td>
</tr>
<tr>
<td>II</td>
<td>Western blotting</td>
<td>ET-1, dobutamine, N-acetylcysteine, MnTMPyP, apocynin, 5-HD, paxilline, HMR 1098</td>
</tr>
<tr>
<td>III</td>
<td>Western blotting</td>
<td>ET-1, L-NAME, SMT, ODQ, KT5823, sildenafil</td>
</tr>
<tr>
<td>IV</td>
<td>Western blotting</td>
<td>PrRP-31, dobutamine, calyculin A, IBMX, okadaic acid, Ro32-0432</td>
</tr>
</tbody>
</table>

4.5 Western blotting analysis (I-IV)

At the end of each experiment left ventricles were blotted dry, weighed, immersed in liquid nitrogen and stored at -70°C until assayed. The tissue was broken in liquid nitrogen, and homogenized in lysis buffer containing in mmol/L: 20 Tris (pH 7.5), 10 NaCl, 0.1 EDTA, 0.1 EGTA, 1 β-glycerophosphate, 1 Na3VO4, 2 benzamidine, 1 phenylmethylsulfonyl fluoride (PMSF), 50 NaF, 1 dithiothreitol (DTT) and 10 μg/mL each of leupeptin, pepstatin and aprotinin. The tissue homogenates were centrifuged at 2000 rpm in +4°C for 1 min. To separate the total protein fraction, 5xNEB (20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4) was added to the tissue homogenate, following by centrifugation at 12500 rpm for 20 min. The supernatant was frozen in liquid nitrogen and stored in -70°C. For Western blots, protein samples (30 μg) were loaded onto SDS-PAGE and transferred to nitrocellulose filters. The membranes were blocked in 5% nonfat milk and incubated with the indicated primary antibody overnight. For a second staining, the membranes were stripped for 30 min in 60°C in stripping buffer (62.5 mmol/L Tris (pH 6.8), 2% SDS, 100 mmol/L mercaptoethanol). Protein levels were detected using enhanced chemiluminescence.

4.6 p38-MAPK assay (I)

Left ventricle tissue was homogenized in lysis buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L β-
glycerophosphate, 2.5 mmol/L sodium pyrophosphate, 1% Triton X-100, 1 mmol/L Na3VO4, 2 mmol/L benzamidine, 1 mmol/L PMSF, 50 mmol/L NaF, 1 mmol/L DTT, and 10 μg/mL each of leupeptin, pepstatin, and aprotinin. Tissue homogenates were clarified at 14,000 x g for 10 minutes and subjected to p38-MAPK activity assay by using ATF-2 as substrate. Briefly, 250 μg of protein extract was incubated for 4 hours with immobilized phospho-p38 antibody. The pellets were washed twice with lysis buffer and once with kinase buffer consisting of 25 mmol/L Tris (pH 7.5), 5 mmol/L β-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na3VO4, and 10 mmol/L MgCl2. Finally, the pellets were suspended in 40 μL of kinase buffer, including 200 μmol/L ATP and 2 μg of ATF-2 fusion protein (Cell Signaling Technology Inc., Hitchin, Hertfordshire, UK). The kinase reaction was conducted at +30°C for 30 minutes and stopped by placing the samples on ice and adding 30 μL of 2 x SDS. Next, the samples were boiled, microcentrifuged, and levels of phospho-ATF-2 were determined with immunoblot analysis as previously described (Tenhunen et al. 2004).

4.7 Detection of intracellular ROS (II)

ROS were detected using ethidium fluorescence as previously described (Sovershaev et al. 2006). In these experiments rat hearts were perfused with Krebs-Henseleit bicarbonate buffer containing dihydroethidium (10 μmol/L) with or without the studied drugs for 10 min followed by a washout of dihydroethidium for 5 min. Dihydroethidium enters the cells and is oxidized by intracellular ROS to produce fluorescent ethidium that subsequently intercalates into DNA. Increase in dihydroethidium oxidation to ethidium and the subsequent increase in fluorescence is directly proportional to the levels of ROS, primarily superoxide anion. By the end of the treatment hearts were rapidly excised and a vertical section of the left ventricle was cut. The sample was embedded in Tissue Tec O.C.T. (Sakura Finetek Europe B.V, Zoeterwoude, NL) compound and frozen (-70°C) until 20 μm cryosections were prepared for microscopy. Ethidium fluorescence was measured with Olympus Fluoview 1000 confocal inverted microscope. Ethidium was excited at 488 nm and the emitted light was collected with a spectral detector from 560 to 660 nm through a 20x objective lens. From each histological sample a randomly selected area of 635 x 635 μm (1024 x 1024 pixels) was scanned with a fixed pixel time of 40 μs and at constant 488 nm laser power. Each image was further analyzed with Fluoview 1.04a software. To get an
estimate of ROS activity of the cells in each image the background subtracted nuclear fluorescence intensity was measured from 5–10 randomly selected nuclei.

4.8 Statistical analysis (I-IV)

Results are presented as mean±standard error of mean (SEM). Two-way repeated-measures analysis of variance (ANOVA) was used to evaluate the statistical significance of difference among groups for cardiac contractility. When significant differences were detected for the treatment-by-time interactions, least significance difference (LSD) (II and III) or Bonferroni post hoc test (I, IV) was used for specific comparisons. The other parameters were analyzed with one-way ANOVA followed by LSD (II and III) or Bonferroni (I, IV) post hoc test. A value of P < 0.05 was considered statistically significant.
5 Results

5.1 The role of ERK1/2 and p38-MAPK in the regulation of cardiac contractility (I)

5.1.1 ERK1/2 in the regulation of cardiac contractility

Intracoronary infusion of ET-1 produced a slowly developing and sustained increase in contractility in isolated perfused rat heart (P < 0.001). Western blotting showed that infusion of ET-1 for 10 minutes increased ERK1/2 phosphorylation in isolated hearts (P < 0.01). U0126, an inhibitor of MEK1/2 which is the upstream regulator of ERK1/2, was used to examine how ERK1/2 contributes to the positive inotropic action of ET-1. U0126 (1.5 µmol/L) significantly reduced the inotropic effect of ET-1 (P < 0.001) and the levels of phospho-ERK1/2 (P < 0.01) in the ET-1-stimulated left ventricles (Fig. 6).

Fig. 6. A. In isolated rat hearts, infusion of ET-1 (1 nmol/L) for 10 minutes increased developed tension (DT), and U0126 (1.5 µmol/L), an MEK1/2 inhibitor, attenuated ET-1-enhanced contractility. Results are expressed as a percentage change versus baseline values. Data are mean±SEM, n=4–6. *P < 0.05, †P < 0.001 vs. control and U0126; ‡P < 0.001 vs. ET-1. B. ET-1 increased phospho-ERK1/2 levels and U0126 abolished ET-1-induced ERK1/2 phosphorylation. Results in the bar graph are expressed as the ratio of phospho-ERK1/2 and total ERK1/2. Data are mean±SEM, n=4. **P < 0.01 vs. control; #P < 0.05, ##P < 0.01 vs. ET-1.
5.1.2 Upstream activators and downstream targets of ERK1/2

The translocation of PKC isoforms between cytosolic and particulate fraction was determined to study the activation of PKC. In Western analysis no consistent increases in the translocation of PKCα, PKCδ, or PKCε into the particulate fraction were seen in response to ET-1 infusion. In addition, PKC inhibitor GF109203X (90 nmol/L) had no effect on ET-1-enhanced contractility (P = NS) or ET-1-stimulated increase in phospho-ERK1/2 levels. The positive inotropic effect of ET-1 was not altered either when PLC was inhibited with U-7312213 (100 nmol/L) (P = NS).

ET-1 increased total tyrosine phosphorylation of EGFR in the left ventricle indicating EGFR transactivation, and the effect was abolished by the EGFR tyrosine kinase inhibitor AG1478 (1 µmol/L). In addition, AG1478 significantly reduced ET-1-induced ERK1/2 phosphorylation (P < 0.05) and the inotropic response to ET-1 (P < 0.001). Infusion of AG1478 alone had no effect on developed tension (P = NS) (Fig. 7).

Sarcolemmal NHE1 was studied as a potential downstream target of ERK1/2 signaling. NHE-1 inhibitor zoniporide (1 µmol/L) attenuated the ET-1-induced inotropic response (P < 0.001), but had no effect on cardiac contractility alone (P = NS). Western blot analysis showed that ET-1 increased the phospho-p90RSK levels in the membrane fraction which was inhibited by both U0126 and AG1478.
Fig. 7. A. EGFR phosphorylation was measured by immunoprecipitation with anti-EGFR antibody followed by immunoblotting of immunoprecipitates with anti-phosphotyrosine (P-Tyr) antibody. ET-1 increased EGFR tyrosine phosphorylation and EGFR tyrosine kinase inhibitor AG1478 (1 µmol/L) abolished it. B and C. AG1478 reduced the ET-1-induced increases in ERK1/2 phosphorylation. Results in the bar graph are expressed as the ratio of phospho-ERK1/2 and total (Tot) ERK1/2. Data are mean±SEM, n=3–6. *P < 0.05 vs. control; #P < 0.05 vs. ET-1. D. AG1478 attenuated the ET-1-induced increase in contractility. DT, developed tension. Data are mean±SEM, n=4–6. †P < 0.001 vs. control and AG1478, ‡P < 0.001 vs. ET-1.

5.1.3 Role of p38-MAPK and downstream targets of p38-MAPK in the regulation of cardiac contractility

To assess the involvement of p38-MAPK in the positive inotropic effect of ET-1, SB239063 was used. Western Blot analysis showed that ET-1 treatment increased p38-MAPK phosphorylation while SB239063 (3 µmol/L) decreased the effect measured by immunocomplex kinase assay with ATF-2 as a substrate. In isolated
hearts administration of SB239063 augmented the ET-1-induced inotropic response (P < 0.05) while infusion of SB239063 alone had no effect on developed tension (P = NS) (Fig. 8).

ET-1 significantly increased phospholamban phosphorylation at Ser-16 in the presence of SB239063, but ET-1 alone had no effect on phospholamban phosphorylation. Infusion of SB239063 alone did not increase phospholamban phosphorylation significantly (P = NS) (Fig. 9). In addition, U0126 did not alter phospholamban phosphorylation.

ET-1-induced ERK1/2 phosphorylation was not altered by inhibition of p38-MAPK with SB239063. Similarly, administration of U0126 had no effect on phospho-p38-MAPK levels. AG1478 markedly reduced ET-1-induced increases in phospho-p38-MAPK levels in addition to its effect on ERK1/2 phosphorylation.

**Fig. 8.** A. ET-1 increased p38-MAPK activity and the effect was decreased by p38-MAPK inhibitor SB239063 (SB) (3 µmol/L). Results are mean±SEM, n=4. **P < 0.01 vs. control; #P < 0.05, ##P < 0.01 vs. ET-1. B. ET-1-enhanced contractility was augmented by SB239063. DT, developed tension. Data are mean±SEM, n=4–6. †P < 0.001 vs. control and SB239063; ‡P < 0.05 vs. ET-1.
Fig. 9. Western blot analysis shows that ET-1 increased phospholamban (PLN) phosphorylation at Ser-16 in the presence of the p38-MAPK inhibitor SB239063 (SB) (3 µmol/L). Results are mean±SEM, n=3–5. **P < 0.01 vs. control; #P < 0.05, and ##P < 0.01 vs. ET-1+SB239063.

5.2 ROS in the regulation of cardiac contractility (II)

5.2.1 The role of ROS in ET-1-induced increase in cardiac contractility

ROS production was studied in isolated perfused adult rat hearts by ROS-dependent oxidation of dihydroethidium to ethidium, and the ethidium florescence was detected from left ventricles with confocal microscopy. Hearts exposed to ET-1 (1 nmol/L) and dihydroethidium (10 µmol/L) produced significantly greater ethidium fluorescence intensity as compared to control hearts (P < 0.01) demonstrating increased ROS levels. The antioxidant N-acetylcysteine (500 µmol/L) blunted the ET-1-induced increase in ethidium fluorescence in isolated hearts (P < 0.001), whereas the antioxidant alone had a small effect on fluorescence intensity (P < 0.05) (Fig. 10A).

In the isolated perfused rat heart preparation, intracoronary infusion of ET-1 (1 nmol/L) for 10 min produced a 43-percent increase (P < 0.001) in cardiac contractility. Infusion of N-acetylcysteine (500 µmol/L) alone had no effect on
developed tension \( (P = \text{NS}) \), but when it was infused in combination with ET-1, the inotropic effect of ET-1 was significantly attenuated \( (P < 0.001) \) (Fig. 10B).

Similarly, the SOD mimetic MnTMPyP \( (10 \, \mu\text{mol/L}) \) decreased the inotropic effect of ET-1 \( (P < 0.05) \) while infusion of MnTMPyP alone had no effect on cardiac contractility \( (P = \text{NS}) \).
5.2.3 Involvement of mitoK\textsubscript{ATP} channels in the ET-1-induced inotropic response

The role of mitochondrial K\textsubscript{ATP} (mitoK\textsubscript{ATP}) channels was studied by using 5-HD (200 μmol/L), a mitoK\textsubscript{ATP} channel blocker. Opening of mitoK\textsubscript{ATP} channels has previously been shown to trigger ischemic preconditioning by increased production of ROS (Forbes et al. 2001, Pain et al. 2000). When 5-HD was infused in combination with ET-1, it attenuated the positive inotropic response to ET-1 (P < 0.001), but infusion of 5-HD alone had no effect on developed tension (P = NS). ROS measurements showed that 5-HD alone attenuated fluorescence intensity (P < 0.05); however, ET-1 was still able to increase ethidium fluorescence in the presence of 5-HD (P < 0.05). In addition to mitoK\textsubscript{ATP} channels, the role of other K\textsuperscript{+} channels in the inotropic response to ET-1 was assessed. Inhibition of mitochondrial large conductance calcium activated potassium (BK\textsubscript{Ca}) channels with paxilline (1 μmol/L) did not alter baseline contractility, but it attenuated the ET-1-induced inotropic response (P < 0.01). In contrast,
administration of sarcolemmal \( \text{K}_{\text{ATP}} \) (sarcK\text{ATP}) channel inhibitor HMR 1098 (3 \( \mu \text{mol/L} \)) failed to alter the ET-1-enhanced contractility (P = NS) (Fig. 4D). These data indicate that mitochondrial BK\text{Ca} channels, but not sarcK\text{ATP} channels, are involved in the response to ET-1.

### 5.2.4 Effects of ET-1-stimulated ROS production on ERK1/2 phosphorylation

Administration of ET-1 (1 nmol/L) for 10 min increased ERK1/2 phosphorylation (P < 0.001), as in study I. Administration of N-acetylcysteine (500 \( \mu \text{mol/L} \)) (Fig. 12A), MnTMPyP (10 \( \mu \text{mol/L} \)) or apocynin (100 \( \mu \text{mol/L} \)) (Fig. 12B) significantly attenuated ET-1-induced ERK1/2 phosphorylation (P < 0.01, P < 0.001 and P < 0.05, respectively). The inhibitors alone had no effect on the phosphorylation state of ERK1/2 (P = NS) (Fig. 12).

![Fig. 12. ET-1-induced increase in ERK1/2 phosphorylation (phospho-ERK/total-ERK) was attenuated by N-acetylcysteine (NAC) (A) and apocynin (B) in the left ventricles. Data are mean±SEM, n=4–9. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. control; *P < 0.05, **P < 0.01 vs. ET-1.](image)

### 5.2.5 The role of ROS in the inotropic response to \( \beta \)-adrenergic stimulation

In isolated heart the \( \beta \)-adrenergic agonist dobutamine (250 nmol/L) produced a rapidly developing positive inotropic effect, which was significantly augmented
by N-acetylcysteine (500 μmol/L) (P < 0.01) (Fig. 13A). In addition, the NADPH oxidase inhibitor apocynin (100 μmol/L) strongly enhanced the inotropic response to dobutamine (P < 0.05) (Fig. 13B).

Infusion of dobutamine for 2 minutes at the dose of 250 nmol/L increased phospholamban phosphorylation at Ser-16 and Thr-17 sites (P < 0.05 and P < 0.01, respectively), and the effect was augmented in the presence of apocynin (100 μmol/L) (P < 0.01 and P < 0.05, respectively). Infusion of apocynin alone had no effect on the phosphorylation state of phospholamban (P = NS) (Fig. 13 C and D). Infusion of dobutamine for 10 minutes had a modest effect on phospholamban phosphorylation, while at the same time point phospholamban phosphorylation at both Ser-16 and Thr-17 sites was still markedly increased in the dobutamine- and apocynin-treated hearts. Dobutamine treatment for 2 and 10 min had no effect on ERK1/2 phosphorylation alone or in the presence of apocynin.
Fig. 13. A and B. Dobutamine (Dob, 250 nmol/L) increased developed tension (DT), and N-acetylcysteine (NAC, 500 μmol/L) (A) and apocynin (Apo, 100 μmol/L) (B) increased the dobutamine-enhanced contractility. Data are mean±SEM, n=3–5. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control and NAC (A) or apocynin (B); †P < 0.05, ‡P < 0.01 vs. dobutamine. C and D. Dobutamine-increased phospholamban (PLN) phosphorylation was augmented by apocynin at Ser-16 (C) and Thr-17 (D). Total (Tot) ERK1/2 levels are shown as loading controls. Data are mean±SEM, n=2–3. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. control; *P < 0.05, **P < 0.01 vs. dobutamine.
5.3 ET-1-induced inotropic response, NO and GC-cGMP-PKG (III)

5.3.1 Contribution of nNOS to the inotropic effect of ET-1

In the isolated perfused rat heart preparation, intracoronary infusion of ET-1 (0.5 nmol/L) for 10 min produced an increase in cardiac contractility (P < 0.001). When the non-selective nNOS inhibitor L-NAME (300 μmol/L) was infused in combination with ET-1, the ET-1 induced inotropic effect was augmented (P < 0.05). Similarly, the effect of ET-1 was increased by the nNOS inhibitor SMTC (3 μmol/L) (P < 0.01). When L-NAME or SMTC were infused alone into the coronary circulation they had no influence on developed tension (P = NS) (Fig. 14).

![Fig. 14. ET-1 (0.5 nmol/L) increased developed tension (DT), and the NOS inhibitor L-NAME (300 μmol/L) (A) and the nNOS inhibitor SMTC (3 μmol/L) (B) augmented the ET-1-enhanced contractility. Data are mean±SEM, n=4–7. ***P < 0.001 vs. control and L-NAME (A) or SMTC (B); #P < 0.05, ##P < 0.01 vs. ET-1.]

5.3.2 Effect of GC-cGMP-PKG signaling pathway on ET-1-induced inotropic response

The role of GC in the positive inotropic effect of ET-1 was investigated by using the GC inhibitor ODQ. Intracoronary infusion of ET-1 (1 nmol/L) increased cardiac contractility by 46% (P < 0.001). ODQ (10 μmol/L) augmented the ET-1-
induced positive inotropic effect (P < 0.05) while infusion of ODQ alone had no
effect on developed tension (P = NS). Similarly, inhibition of PKG with KT5823
(100 nmol/L) significantly increased the ET-1-induced positive inotropic effect
(P < 0.01). Infusion of KT5823 alone had no effect on contractile force (P = NS)
(Fig. 15).

Fig. 15. GC inhibitor ODQ (10 μmol/L) (A) and PKG inhibitor KT5823 (100 nmol/L)
increased the ET-1 (1 nmol/L)-enhanced contractility. DT, developed tension. Data are
mean±SEM, n=4–9. ***P < 0.001 vs. control and ODQ (A), KT5823 (B); #P < 0.05,
##P < 0.01 vs. ET-1.

5.3.3 The effect of nNOS, GC or PKG inhibition on phospholamban and ERK1/2 phosphorylation

SMTC alone or in combination with ET-1 had no effect on phospholamban phosphorylation at Ser-16 or Thr-17 sites in left ventricles. In addition, administration of ODQ or KT5823 alone or in combination with ET-1 had no effect on phospholamban phosphorylation. ET-1 infusion in isolated hearts for 10 min increased ERK1/2 phosphorylation (P < 0.05 vs. control), as in studies I and II. Inhibition of NO production by L-NAME or SMTC had no effect on ET-1-induced ERK1/2 phosphorylation. In addition, inhibition of GC-PKG pathway by ODQ or KT5823 did not alter ET-1-induced ERK1/2 phosphorylation.
5.3.4 The role of PDE5A in the inotropic response to ET-1

The contribution of PDE5A, which catabolizes cGMP, in the inotropic response to ET-1 was studied by using its inhibitor sildenafil. When given together with ET-1, sildenafil (1 μmol/L) significantly attenuated the ET-1-induced positive inotropic effect (P < 0.001). Infusion of sildenafil alone had no effect on contractile force (P = NS). In addition, sildenafil attenuated the ET-1-induced ERK1/2 phosphorylation (P < 0.01). Infusion of sildenafil alone had no effect on contractile force or ERK1/2 phosphorylation (P = NS) (Fig. 16).

5.4 PrRP and cardiac contractility (IV)

5.4.1 The effect of PrRP on cardiac contractility

In the isolated rat heart preparation PrRP-31 (1 to 100 nM) induced a dose-dependent, gradual increase in cardiac contractility, and the maximal response to PrRP was observed at the concentration of 10 nM (P < 0.001) (Fig.17).
The involvement of the cAMP-PKA pathway in PrRP signaling was studied by inhibiting cAMP catabolism with a non-selective phosphodiesterase inhibitor, IBMX. The effect of PrRP (10 nM) on contractility was not affected by IBMX (10 μM) (P = NS), but IBMX markedly enhanced the inotropic response to dobutamine (250 nM) (P < 0.001).

5.4.2 Protein phosphatases, PKCa and PrRP signaling

The contribution of protein phosphatases to PrRP signaling was studied by using the protein phosphatase type 1 (PP1) and type 2A (PP2A) inhibitor calyculin A (1 nM) and the PP2 inhibitor okadaic acid (10 nM). Calyculin A augmented the inotropic response to PrRP (P < 0.001) while okadaic did not alter the response to PrRP (P = NS) (Fig. 17A).

To study the effect of PKCa, its inhibitor Ro32-0432 (100 nM) was used. Ro32-0432 markedly increased the inotropic response to PrRP (P < 0.001) (Fig. 17B). In addition, Western blot analysis showed that in the presence of Ro32-0432 PrRP significantly increased phospholamban phosphorylation at Ser-16 (P < 0.01), but PrRP alone had no effect on phospholamban phosphorylation (Fig. 18).
Fig. 17. PrRP-31 (10 nmol/L) increases cardiac contractility. Effects of PP1/PP2A inhibitor calyculin A (1 nmol/L), a PP2A inhibitor, okadaic acid (10 nmol/L) (A) and a PKCα inhibitor, Ro32-0432 (100 nmol/L) (B) on PrRP-induced inotropic response. DT, developed tension. Data are mean±SEM, n=4–8. *P < 0.001 vs. control; †P < 0.001 vs. PrRP.

Fig. 18. PrRP increases phospholamban (PLN) phosphorylation at Ser-16 in the presence of Ro32-0432 (100 nmol/L). Tot-p38 is shown as loading control. Results in bar graph are mean±SEM, n=3–4. *P < 0.05 vs. control; #P < 0.01 vs. PrRP.
6 Discussion

6.1 MAPKs in the regulation of cardiac contractility

The MEK1/2-ERK1/2 pathway is known to constitute an important adaptive mechanism in the myocardium (Bueno & Molkentin 2002, Rose et al. 2010, Wang 2007). The role of ERK1/2 has been studied in many transgenic models (Bueno et al. 2000, Harris et al. 2004, Purcell et al. 2007), but although the systolic function of the heart has been affected in these models, it has not been established whether ERK1/2 can directly modulate cardiac contractile function. An important finding of the present study was that MEK1/2-ERK1/2 signaling regulates myocardial contractility. In the isolated heart infusion of ET-1 increased ERK1/2 phosphorylation, and inhibition of ERK1/2 activation by U0126, a potent MEK1/2 inhibitor, attenuated the positive inotropic effect of ET-1. The finding shows that MEK1-ERK1/2 signaling regulates myocardial contractility in addition to influencing cell growth and survival.

There are several mechanisms by which GPCR-mediated activation of the Raf-MEK1/2-ERK1/2 cascade can take place. Previously, ET-1 has been suggested to increase cardiac contractility (Chu et al. 2003) and ERK1/2 phosphorylation (Bogoyevitch et al. 1994) via a PKC-dependent pathway. However, PKC is unlikely to mediate the inotropic effect of ET-1 in the isolated rat heart. After 10 minutes of infusion of ET-1 translocation of PKCα, PKCδ, or PKCε into the particulate fraction was not observed and inhibition of PKC with GF-109203X did not alter the ET-1-stimulated increase in phospho-ERK1/2 levels or the inotropic response to ET-1. In addition, pharmacological inhibition of PLC, the upstream regulator of PKC, did not alter the inotropic effect of ET-1.

The functional importance of the heparin binding-EGF-EGFR pathway has previously been demonstrated in hypertrophic and developmental growth processes. EGFR transactivation has been indicated to be an important pathway that links GPCR and ERK1/2 activation (Thomas et al. 2002). Stimulation of GPCRs induces metalloproteinase-mediated ectodomain shedding of proheparin-binding EGF-like growth factor, which can activate EGFR, leading to ERK1/2 phosphorylation via the Ras-Raf1-MEK1/2 cascade (Wetzker & Bohmer 2003). Previous studies have shown that in vivo inhibition of heparin-binding EGF shedding and EGFR transactivation can inhibit GPCR agonist-induced left ventricle hypertrophy and cardiac dysfunction (Asakura et al. 2002). In the
present study ET-1 was found to increase total tyrosine phosphorylation of EGFR in the left ventricles. In addition, inhibition of EGFR transactivation by the EGFR tyrosine kinase inhibitor AG1478 attenuated the ET-1-induced increase in phospho-ERK1/2 levels and the inotropic response to ET-1. The results show that EGFR can be involved in the regulation of myocardial contractility, acting as a proximal component of MEK1/2-ERK1/2 signaling (Fig. 19).

ERK1/2 phosphorylation has previously been reported to be redox-sensitive in cultured cardiomyocytes (Clerk & Sugden 2006, Tanaka et al. 2001) and therefore it was examined whether ROS modulates ERK1/2 phosphorylation in the intact adult rat heart. The results indicate that activation of ERK1/2 signaling is mainly redox-sensitive because ET-1-induced ERK1/2 phosphorylation was significantly suppressed by the ROS scavengers N-acetylcysteine and MnTMPyP. Moreover, inhibition of NADPH oxidase by apocynin attenuated ERK1/2 phosphorylation in response to ET-1 (Fig. 19). ROS can potentially modulate the GPCR-mediated activation of the Raf-MEK1/2-ERK1/2 cascade by several mechanisms. GPCR-mediated ROS production may inactivate protein-tyrosine phosphatases, resulting in increased tyrosine phosphorylation of EGFR, which then signal through Ras to the ERK1/2 cascade (Clerk & Sugden 2006, Wetzker & Bohmer 2003). In addition, ROS can activate Ras through direct modifications of redox-sensitive cysteine residues that lead to the activation of the Raf-MEK1/2-ERK1/2 pathway (Clerk & Sugden 2006, Pimentel et al. 2006).

The downstream signaling of ERK1/2 was also studied. Sarcolemmal NHE1 has previously been shown to be involved in the positive inotropic effect of ET-1 (Chu et al. 2003, Kramer et al. 1991, Sugden 2003, Takeuchi et al. 2001, Wang et al. 2000). In addition, it has been demonstrated that activation of ERK1/2 can result in phosphorylation of the NHE1, either directly by ERK1/2 itself (Moor & Fliegel 1999) or indirectly through p90RSK (Takahashi et al. 1999). In isolated heart, inhibition of NHE1 with zoniporide decreased the inotropic response to ET-1, which is in agreement with the previous studies. In addition, ET-1 increased phospho-p90RSK levels in the membrane fraction and both AG1478 and U0126 attenuated it. Therefore, the results indicate that membrane-associated p90RSK can mediate the effect of ET-1 on NHE1 activity (Fig. 19). Stimulation of NHE1 can lead to accumulation of intracellular Na\(^+\), which can indirectly promote an increase in intracellular Ca\(^{2+}\) levels via increased Ca\(^{2+}\) influx via reverse mode of operation of the NCX (Aiello et al. 2005, Kentish 1999). In addition, NHE1 can mediate intracellular alkalization and sensitization of cardiac myofilaments to Ca\(^{2+}\) (Kramer et al. 1991).
The finding that \( \approx 40\% \) of the inotropic response to ET-1 remained unaffected after ERK1/2 inhibition with U0126 indicates the existence of further ERK1/2-independent signaling mechanisms. In the preliminary experiments myosin light-chain kinase (MLCK) inhibition significantly reduced the ET-1-mediated increase in contractility, which is in line with previous findings (Andersen et al. 2002, Chu & Endoh 2005) and suggests that MLCK may be an important mediator of the positive inotropic effect of ET-1.

p38-MAPK signaling has been associated with various pathological conditions in the heart, including hypertrophy and cardiac decompensation (Rose et al. 2010, Wang 2007). Previously it has been found that activation of p38-MAPK results in reduction of contractility in cultured rat cardiomyocytes (Liao et al. 2002). In addition, pharmacological inhibition of p38-MAPK increased contractile force in a dose-dependent manner (Liao et al. 2002). In the present study it was found that inhibition of p38-MAPK had no effect on baseline cardiac contractility. However, inhibition of p38-MAPK augmented the inotropic effect of ET-1, suggesting that activation of p38-MAPK provides a negative feedback to the ET-1-mediated inotropic response. Similarly, it has been demonstrated that \( \beta_2 \)-adrenoceptor-stimulated contractile response is enhanced by pharmacological inhibition of p38-MAPK in isolated mouse cardiomyocytes (Zheng et al. 2000).

Although p38-MAPK is known to regulate cardiac function (Liao et al. 2002, Zheng et al. 2000), the molecular mechanisms by which p38-MAPK contributes to cardiac contractility are still elusive. It has been shown that p38-MAPK activation can directly decrease ERK1/2 activation through a protein phosphatase 2A-dependent mechanism in cardiac ventricular myocytes (Liu & Hofmann 2004). However, the findings of this study do not support the hypothesis of a direct cross-talk between the ERK1/2 and p38-MAPK pathways, as inhibition of p38-MAPK did not further increase ET-1-induced ERK1/2 phosphorylation. Previously, \( \beta \)-adrenergic-stimulated phospholamban phosphorylation has been shown to be attenuated by adenosine A1 receptor activation in a p38-MAPK-sensitive manner (Liu & Hofmann 2003). In this study it was found that in the presence of p38-MAPK inhibition, ET-1 increased the phospholamban phosphorylation at Ser-16 site. In its dephosphorylated state, phospholamban binds to and inhibits the activity of SERCA. Phosphorylation of phospholamban alters the interaction between phospholamban and SERCA and enhances \( Ca^{2+} \) reuptake into the sarcoplasmic reticulum. Subsequently, cardiac contractility is increased in proportion to the elevation in the size of sarcoplasmic reticulum \( Ca^{2+} \) store and the resulting increase in \( Ca^{2+} \) release from the sarcoplasmic reticulum.
Therefore, the results imply that p38-MAPK may limit increases in contractility via dephosphorylation of phospholamban in the myocardium (Fig. 19).

![Diagram of signaling pathways](image)

**Fig. 19.** Theoretical presentation of the proposed signaling pathways involved in the positive inotropic effect of ET-1. Binding of ET-1 on ET$_{	ext{A}}$-receptor leads to the activation of the MEK1/2-ERK1/2 pathway, which can be mediated by EGFR. NADPH oxidase can be activated by ET-1 and increased ROS are involved in the activation of ERK1/2. The exact organization of the cascade upstream of ERK1/2 is still elusive. Downstream of ERK1/2 p90RSK and NHE are activated, leading to changes in intracellular $[\text{Ca}^{2+}]_i$. ET-1 also activates the p38-MAPK, which may lead to attenuation of the ET-1-induced increase in contractility via dephosphorylation of phospholamban. SL, sarcolemma; SR, sarcoplasmic reticulum; L-$\text{Ca}^{2+}$ ch, L-type $\text{Ca}^{2+}$ channel; NCX, $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger; SERCA, sarcoplasmic reticulum $\text{Ca}^{2+}$-$\text{ATP}$ase; PLN, phospholamban; RyR, ryanodine receptor; NHE, $\text{Na}^{+}/\text{H}^{+}$ exchanger.

### 6.2 ROS and cardiac contractility

Excessive ROS levels contribute to the development of contractile dysfunction in many pathological states including ischemia-reperfusion injury (Adlam *et al.* 2005, Murphy & Steenbergen 2008) and HF (Giordano 2005, Murdoch *et al.* 2006, Saavedra *et al.* 2002). In contrast, a moderate increase in ROS production has been proposed to trigger cardioprotection against subsequent ischemia-
reperfusion injury (Chen et al. 1995, Forbes et al. 2001, Murphy & Steenbergen 2008). However, the functional importance of ROS under physiological conditions in the myocardium is largely unknown. The present study shows that ROS are involved in the acute regulation of cardiac contractility in the intact rat heart. ET-1-induced slowly developing but sustained increase in cardiac contractility is in part mediated by ROS. In contrast, the inotropic response to dobutamine, which develops rapidly, is counterbalanced by simultaneous activation of endogenous ROS production. These data support the hypothesis that ROS act as signaling molecules in the modulation of cardiac function in a physiological milieu.

Previous studies have produced conflicting results regarding the effect of ROS and ET-1 on cardiac contractile function. In mice ventricular myocytes leptin has been shown to suppress contractility via ET receptors and increased ROS formation (Dong et al. 2006). On the contrary, in cat cardiomyocytes Ang II has been shown to induce ET-1 release and an increase in ROS generation, which triggered an increase in contractility (Cingolani et al. 2006). In the same model the positive inotropic effect of exogenous ET-1 was almost exclusively dependent on ROS production (De Giusti et al. 2008). In this study it was found that ROS partially mediate the ET-1-induced increase in contractile force in the intact adult rat heart (Fig. 19). Administration of ET-1 enhanced ROS production, which was prevented by the antioxidant N-acetylcysteine. In addition, the inotropic response to ET-1 was significantly attenuated by the ROS scavengers N-acetylcysteine and MnTMPyP. In the myocardium the NADPH oxidase family of enzymes are major sources of O$_2^-$ (Akki et al. 2009, Murdoch et al. 2006). The present results show that NADPH oxidase-derived ROS can partially mediate the contractile response, because both ET-1-induced increase in contractility and ethidium fluorescence were markedly attenuated by a NADPH oxidase inhibitor, apocynin. However, the finding that more than 60% of the ET-1-induced positive inotropic effect remained unaffected after effective ROS scavenging indicates the existence of further ROS-independent signaling mechanisms.

Opening of mitoK$_{ATP}$ channels has previously been shown to trigger ischemic preconditioning by increased production of ROS (Forbes et al. 2001, Pain et al. 2000). In the experiments of the present study inhibition of mitoK$_{ATP}$ channels with 5-HD attenuated the positive inotropic effect of ET-1. ROS measurements showed that 5-HD has only a small effect on ET-1-induced ROS production, which indicates that the involvement of mitoK$_{ATP}$ channels in the positive inotropic effect of ET-1 is independent of ROS production. Opening of mitoK$_{ATP}$
channels has previously been demonstrated to support inotropy by maintaining efficient energy transfer between mitochondria and cytosol (Garlid et al. 2006). The finding that the mitochondrial BK,

channel inhibitor paxilline, but not the sarcK,

ATP channel inhibitor HMR1098, attenuated the response to ET-1 similarly to 5-HD demonstrates the importance of this other mitochondrial potassium channel as well as supports the hypothesis that mitochondrial matrix K+ influx is needed for an appropriate response to positive inotropic stress.

It has been shown that the antioxidant vitamin C enhances the positive inotropic response to the β-adrenergic agonist dobutamine in patients with preserved left ventricular systolic function (Mak & Newton 2001). In the present study it was found that the β-adrenergic inotropy is suppressed by endogenous ROS production as ROS scavenging with N-acetylcysteine potentiated the positive inotropic effect of dobutamine. The data also demonstrate that NADPH oxidase is the likely source of ROS as the effect was also enhanced by the NADPH oxidase inhibitor apocynin.

Stimulation of cardiac β1-adrenergic receptors with dobutamine activates the Gaα1c-AC-cAMP-PKA pathway. Stimulation of PKA leads to phosphorylation of many proteins that are important for cardiac function, including phospholamban (Dzimiri 1999, Luo et al. 1994). Phospholamban, in turn, has an important role in mediating the heart’s contractile responses to β1-adrenergic agonists (Luo et al. 1994). Importantly, the present results demonstrate that the effect of dobutamine on phospholamban phosphorylation was markedly enhanced at both Ser-16 and Thr-17 sites in the presence of apocynin, suggesting that NADPH oxidase-derived ROS may counterbalance the impact of β-adrenergic stimulation via limiting phospholamban phosphorylation in the heart. Therefore, β-adrenergic activation of endogenous ROS formation may be an important determinant of the sympathetic response to stress.

The effect of dobutamine on EKR1/2 phosphorylation was also examined. These studies demonstrate that, in contrast to ET-1 signaling, β-adrenergic stimulation does not necessarily activate the ERK1/2 pathway. Infusion of dobutamine at a dose of 250 nmol/L for 2 or 10 minutes did not increase ERK1/2 phosphorylation. Previously, noradrenaline and isoprenaline have been shown to increase ERK1/2 phosphorylation only at very high concentrations (50 μmol/L) in isolated rat hearts (Lazou et al. 1994). This may explain why dobutamine infused at a much lower concentration failed to stimulate the ERK1/2 pathway in the present study.
Of particular interest was the finding that the inotropic responses to ET-1 and dobutamine were differentially regulated by inhibiting NADPH oxidase ROS production ex vivo under identical experimental conditions. The results suggest that the distinct signaling pathways activated by these agonists are modulated differentially by ROS. ET-1-induced ROS formation may enhance contractility via increased ERK1/2 signaling, whereas the β-adrenergic agonist-induced ROS generation may suppress contractility through reducing phospholamban phosphorylation.

In vitro the effect of NADPH oxidase on cardiomyocyte contractile function is controversial. Treatment of isolated cat cardiomyocytes with ET-1 for 15 minutes produced a positive inotropic effect, which was abolished by a NADPH oxidase inhibitor (De Giusti et al. 2008). On the contrary, short-term (1 hour) treatment with high concentrations of leptin depressed contractility by a NADPH oxidase-dependent mechanism in isolated adult murine ventricular myocytes (Dong et al. 2006). Similarly, NADPH oxidase-dependent ROS generation induced contractile dysfunction in isolated adult rat ventricular myocytes which were exposed to transforming growth factor-β1 for 3–4 hours (Li et al. 2008). NADPH oxidase is a multi-subunit enzyme with five oxidase isoforms, which are distinguished by the presence of distinct catalytic subunits (Nox1-Nox5). The enzymatic activity of the oxidase also depends on additional protein subunits, which vary according to the isoform (Akki et al. 2009). The different agonists may activate distinct NADPH oxidase isoforms at different subcellular locations with dissimilar temporal patterns (transient versus sustained activation). Thus, differences may exist in the amount of ROS produced by different agonists, which can in turn activate or inhibit distinct downstream signaling molecules resulting in differing functional responses.

6.3 Endothelin-1, NO and GC-cGMP-PKG

NO is an important modulator of myocardial contractile function (Massion et al. 2003), and the impact of NO and different NOS isoforms has been widely studied. The cardiac response to β-adrenergic stimulation has been consistently reported to be limited by eNOS (Barouch et al. 2002, Godecke et al. 2001, Gyurko et al. 2000, Wang et al. 2008b), but the effect of nNOS on contractile function is more controversial (Barouch et al. 2002, Khan et al. 2003, Martin et al. 2006, Sears et al. 2003). The results of the present study show that nNOS suppress the positive inotropic effect of ET-1, as the inotropic effect of ET-1 was enhanced by non-
specific NOS inhibitor L-NAME and nNOS selective inhibitor SMTC. The finding agrees with the previous studies where cardiac nNOS was demonstrated to have a significant limiting role in the autocrine control of cardiac contractility (Ashley et al. 2002, Burkard et al. 2007, Sears et al. 2003).

In the cardiovascular system stimulation of soluble GC leading to the elevation of cGMP has been shown to be a main target of NO (Fischmeister et al. 2005). In the present study the positive inotropic effect of ET-1 was enhanced by inhibition of GC with ODQ, indicating that soluble GC-cGMP is involved in the response to ET-1. PKG, in turn, is known to be an important target of cGMP (Wegener et al. 2002), and in the experiments the response to ET-1 was also increased by the PKG inhibitor KT5823. Recent studies have revealed the importance of PDE5A for cardiac regulation. Although the impact of PDE5A on resting cardiac function is relatively small, it can modulate acute contractile responses in cells stimulated by external agonists (Kass et al. 2007a). PDE5A has an important role in the regulation of cGMP levels in cardiomyocytes. Inhibition of PDE5A leads to elevated cGMP levels and increased PKG activity, which could then act as a brake for contractile stimulation (Takimoto et al. 2005a). PDE5A has been shown to regulate acute adrenergic-stimulated contractility (Senzaki et al. 2001, Borlaug et al. 2005, Takimoto et al. 2005a). In this study it was found that PDE5A can also be involved in the inotropic effect of ET-1 as the PDE5A inhibitor sildenafil decreased the inotropic response to ET-1, and the finding agrees with a GC-cGMP-PKG-dependent mechanism acting as a negative modulator of cardiac inotropic responses.

NO has been shown to inhibit ET-1-induced cardiomyocyte hypertrophy via suppression of ERK1/2 signaling through the NO-cGMP-PKG pathway in rat neonatal ventricular myocytes (Cheng et al. 2005). In the present study ET-1 treatment increased ERK1/2 phosphorylation significantly. PDE5A inhibition with sildenafil attenuated both ET-1-induced inotropic response and ERK1/2 phosphorylation. However, NOS inhibitor L-NAME or nNOS inhibitor SMTC did not alter ET-1-induced ERK1/2 phosphorylation, although both inhibitors significantly augmented the contractile response to ET-1. Similarly, GC or PKG inhibition had no effect on ET-1-increased ERK1/2 phosphorylation. Therefore the data suggest that the augmented inotropic response to ET-1 after inhibition of the nNOS and GC-cGMP-PKG pathway is not mediated by ERK1/2.

nNOS is localized predominantly in the sarcoplasmic reticulum and therefore ion channels and transporters involved in the regulation of Ca²⁺ handling are potential targets for nNOS-based signaling (Fukuda et al. 2008). It has previously
been demonstrated that phospholamban is one of the targets of nNOS signaling (Wang et al. 2008a, Zhang et al. 2008); however, the effect was independent of cGMP-PKG signaling (Zhang et al. 2008). Based on the findings of the present study, phospholamban is likely not involved in the nNOS-mediated regulation of ET-1-induced inotropic response, as inhibition of nNOS with SMTC had no effect on phospholamban phosphorylation in the presence of ET-1. In addition, inhibition of GC or PKG did not have any effect on phospholamban phosphorylation. This suggests that the augmented inotropic response to ET-1 after inhibition of nNOS and GC-cGMP-PKG pathway is mediated via alternative pathways. Interestingly, it has been suggested that NO may influence myocardial contractility by myofilament-based control in addition to modulating [Ca^{2+}]. (Fukuda et al. 2008). In preliminary experiments reported in study I it was found that ET-1-mediated increase in contractility was significantly attenuated by inhibition of myosin light chain kinase; however, whether the NO signaling pathway can modulate MLCK remains to be determined.

6.4 The effects of PrRP on cardiac contractility

PrRP has previously been shown to be involved in the central control of cardiovascular function (Horiuchi et al. 2002, Samson et al. 2000). The present study showed that PrRP has a modest direct, dose-dependent, slowly developing positive inotropic effect in the intact rat heart, suggesting that PrRP may be involved in the regulation of cardiovascular homeostasis.

PrRP was found to modulate cardiac contractility in the low nanomolar range (1-10 nM) in agreement with the reported affinity (IC50 of 6.6 ± 0.7 nM) of the specific binding sites of PrRP in the myocardium (Satoh et al. 2000). However, it is unlikely that PrRP can act as a circulating hormone because the plasma levels of the peptide are very low (0.13 pM) (Matsumoto et al. 1999). Currently it is not known whether PrRP is synthesized and/or released in the myocardium. There are studies suggesting that PrRP may act as paracrine factor that regulates catecholamine secretion. PrRP is coexpressed with tyrosine hydroxylase, which is the rate-limiting enzyme in noradrenaline synthesis in A1 and A2 noradrenergic neurons of the rat medulla oblongata (Horiuchi et al. 2002, Maruyama et al. 2001), and PrRP and noradrenaline cooperatively modulate the hypothalamo-pituitary-adrenal axis (Maruyama et al. 2001). PrRP was found to be produced in the cultured adrenal gland where it was colocalized with tyrosine hydroxylase and phenylethanolamine N-methyltransferase, which converts noradrenaline to
adrenaline (Fujiwara et al. 2005). In addition, PrRP has been reported to increase catecholamine secretion from rat PC12 adrenal pheochromocytoma cells (Nanmoku et al. 2003). It can be hypothesized that PrRP may modulate noradrenaline release from sympathetic nerve terminals in the myocardium in a similar fashion to other neuropeptides such as neurotensin, substance P and calcitonin gene-related peptide (Katori et al. 2005, Osadchii et al. 2005, Seyedi et al. 1999); however, further studies are needed to elucidate this issue.

The positive inotropic effect of PrRP was found to be modest and therefore it was hypothesized that the effect could be suppressed by simultaneous activation of counter-regulatory mechanisms. Activation of PDEs, which hydrolyze cAMP, can limit the inotropic response of agents that utilize cAMP. In this study it was found that the positive inotropic effect of the β1-adrenergic receptor agonist dobutamine was enhanced by the non-selective PDE inhibitor IBMX. In contrast, IBMX had no effect on the cardiac effect of PrRP, showing that PDEs are not involved. In addition, the data suggest that the AC-cAMP-PKA pathway does not mediate the inotropic effect of PrRP.

Cardiac contractility can be regulated by protein phosphatases, which promote dephosphorylation of various target proteins. The inotropic effect of PrRP was found to be counterbalanced by activation of PP1, but not PP2A. Interestingly, it has been shown that cardiac-specific overexpression of PKCα reduces cardiac contractility by increasing PP1 activity (Braz et al. 2004). Although PKCα has been demonstrated to be a negative modulator of cardiac function (Braz et al. 2004, Hambleton et al. 2006), its role in the acute regulation of contractility is unclear. Ro32-0432, a PKCα inhibitor (Hambleton et al. 2006), was found to significantly augment the inotropic effect of PrRP, suggesting that activation of PKCα provides a negative feedback to the PrRP-mediated inotropic response. In addition, PrRP promoted the phosphorylation of phospholamban at Ser-16 in the presence of PKCα inhibition. Based on this data it can be hypothesized that activation of PKCα may have an important homeostatic function by counterbalancing inotropic stimulation in the heart.
7 Summary and conclusions

In the present study the signaling mechanisms involved in the acute regulation of cardiac contractility in the isolated perfused rat heart preparation were characterized. The main findings of the study can be summarized as follows:

1. MAPKs have opposing roles in the acute regulation of cardiac contractility. ERK1/2-mediated positive inotropic response to ET-1 was counterbalanced by activation of p38-MAPK. EGFR was found to be the upstream regulator and the p90RSK-NHE1 pathway the downstream effector of ERK1/2 signaling. p38-MAPK activation suppressed ET-1-increased contractility by dephosphorylating phospholamban.

2. NADPH oxidase-derived ROS play a physiological role in the acute regulation of cardiac contractility. The ET-1-induced, slowly developing but sustained increase in cardiac contractility was partly dependent on enhanced ROS generation, which in turn activated the ERK1/2 pathway. The rapidly developing β-adrenergic effect was limited by concurrent stimulation of ROS production via negating phospholamban phosphorylation.

3. The inotropic response to ET-1 was counterbalanced by nNOS. In addition, the effect of ET-1 was negatively modulated by the GC-cGMP-PKG pathway. The augmented inotropic response to ET-1 was not accompanied by altered ERK1/2 or phospholamban phosphorylation. PDE5A inhibition depressed the contractility response to ET-1 by decreasing ERK1/2 phosphorylation.

4. PrRP exerts a direct positive inotropic effect in the intact heart. The effect was independent of cAMP and was suppressed by concurrent activation of PKCα and PP1 leading to dephosphorylation of phospholamban.
References


111


Original articles


Reprinted with permission from Lippincott, Williams & Wilkins (I) and Elsevier Inc. (II, IV).

Original articles are not included in the electronic version of the dissertation.
1083. Rasi, Karolina (2010) Collagen XV as a matrix organizer: its function in the heart and its role together with laminin α4 in peripheral nerves


1085. Mäkelä, Kari Antero (2010) The roles of orexins on sleep/wakefulness, energy homeostasis and intestinal secretion


1089. Miettinen, Johanna (2011) Studies on bone marrow-derived stem cells in patients with acute myocardial infarction


1091. Stefanini, Karoliina (2011) Colorectal carcinogenesis via serrated route

1092. Männistö, Tuja (2011) Maternal thyroid function during pregnancy: effects on pregnancy, perinatal and neonatal outcome and on later maternal health

1093. Uutela, Toini (2011) Health-related quality of life and functional ability as patient-reported outcomes in rheumatoid arthritis: A study from two Finnish hospital-based populations

1094. Solyom, Szilvia (2011) BRCA/Fanconi anemia pathway genes in hereditary predisposition to breast cancer


1096. Lunnela, Jaana (2011) Internet-perusteinen potilasohjauksen ja sosiaalisen tuen vaikutus glaukomapotilaan hoitoon sitoutumisessa

1097. Koskela, Ritva (2011) Microscopic colitis: Clinical features and gastroduodenal and immunogenetic findings

1098. Kurola, Paula (2011) Role of pneumococcal virulence genes in the etiology of respiratory tract infection and biofilm formation

Book orders:
Granum: Virtual book store
http://granum.uta.fi/granum/
CHARACTERIZATION OF SIGNALING MECHANISMS REGULATING CARDIAC CONTRACTILITY

Anna-Maria Kubin