Characterization of inotropic signaling induced by endogenous peptides apelin and endothelin

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To my father

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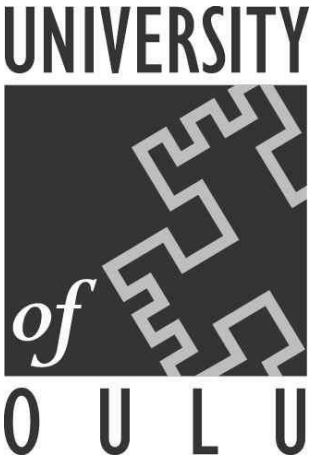

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LIST OF ABBREVIATIONS

5-HD	5-hyroxy-decanoate
AC	adenylate cyclase
ACE-2	angiotensin-converting enzyme type 2
ADP	adenosine diphosphate
Ang II	angiotensin II
AR	adrenoreceptor
AT1-R	angiotensin II type-1 receptor
ATP	adenosine triphosphate
Bis	Bisindolylmaleimide I
BK_{Ca}	mitochondrial large conductance calcium activated potassium channel
cAMP	cyclic adenosine monophosphate
DMSO	dimethyl-sulfoxide
DTT	dithiothreitol
EC_{50}	half-maximal effective concentration
EGFR	epidermal growth factor receptor
ERK1/2	extracellular signal-regulated kinases 1 and 2
ET	endothelin
GPCR	G-protein coupled receptors
GRK	G-protein coupled receptor kinase
GTP	guanidine triphosphate
Gα	G protein α-subunit
Gβγ	heterodimer of G protein β and γ-subunit
HF	heart failure
IP3	inositol-triphosphate
LTCC	L-type calcium channel
LV	left ventricular
MAPK	mitogen-activated protein kinase
$mitoK_{ATP}$	mitochondrial ATP-dependent potassium channel

MLCK	myosin light chain kinase
MnTMPyP	Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride
NAD(P)H	nicotinamide dinucleotide phosphate
NCX	Na^+-Ca^{2+} exchanger
NE	noradrenaline
NO	nitric oxide
NHE	Na^+-H^+ exchanger isoform 1
$O_2\bullet^-$	superoxide
p90RSK	p90 ribosomal S6 kinase
PAGE	polyacrylamide gel electrophoresis
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PTX	pertussis toxin
RAAS	renin-angiotensin-aldosterone system
RLC	myosin regulatory light chain
ROS	reactive oxygen species
sarcK_{ATP}	sarcolemmal K^+-ATP channel
SERCA	sarcoplasmic reticulum calcium-ATPase
SOD	superoxide dismutase
TnI	troponin I

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1 GENERAL INTRODUCTION

Heart failure (HF) is the condition, in which the amount of blood circulated by the heart at a given time (cardiac output) fails to match the demand of the peripheral organs. It leads to decreased physical exercise capacity, peripheral organ damage, poor quality of life, and a reduction in life expectancy. Parallel with the aging in the Western world, the incidence of acute and chronic heart failure have been constantly increasing, making chronic heart failure one of the leading causes of mortality and the most costly medical illness in Europe and in the USA. It is estimated that 26 million people have HF worldwide, and 1 million people are newly diagnosed with HF every year in the USA and the European Union alone (Rich, 2001; López-Sendón, 2011; McMurray *et al.*, 2012). Incidence of HF is estimated to increase by approximately 30 % in the next 15 years. (Heidenreich *et al.*, 2011). Although HF is still considered as having comparable 5-year survival rate to many malignancies, recent development in understanding the pathomechanism of underlying neurohumoral activation and the continuous improvement of therapy via implementing results of clinical trials lead to a dramatic reduction of more than 50 % in the mortality of patients with chronic HF since the early '90-s (McMurray, 2011). On the contrary, survival rate of acute HF episodes hasn't changed at all in the past three decades, and mortality and rehospitalization rates remain high (Tavazzi *et al.*, 2013). In chronic HF the sympathetic nervous system and the renin-angiotensin-aldosterone system (RAAS) are activated in a vicious cycle and are responsible for the majority of pathophysiological processes. They are therefore addressed by current therapies, like β-adrenerg blockade or RAAS antagonists. Medical management of heart failure also involves treatment of volume overload by diuretics for symptom relief and neurohumoral modification and heart rhythm normalization to reduce mortality.

It is well known, however, that the long term survival of HF patients is strongly related to the proper and timely management of the acute exacerbation events of the disease. In the acute condition impaired cardiac hemodynamics is the root-cause of symptoms and its management determines clinical outcome. A major difficulty in HF therapy is that traditional inotropic agents, although improving cardiac hemodynamics, have detrimental or no effect on long-term survival. Due to their proarrhythmogenic effect, β-adrenerg agonist (Tacon *et al.*, 2012) and phosphodiestherase inhibitors (Packer *et al.*, 1991) are restricted to short-term palliation at

intensive care or as bridge to cardiac surgery. Cardiac glycosides are recommended as second-line treatment only, since they fail to improve mortality (Lindenfeld *et al.*, 2010). As such, there is an unmet need in HF therapy for a novel agent that would improve cardiac contractility and also increase patient survival. To achieve that, it is essential to get a better understanding of the endogenous regulation of contractility.

The primary role of the heart is to sustain circulation within the organism by pumping blood beat by beat through the vasculature. This is vital in order to supply organs with oxygen and nutrients. Depending on the actual state of the body, the demand of different organs for resources may show a broad variety through time. Among others, one key mechanism to meet those altering demand is adapting the strength of cardiac contractions.

Cardiac muscle contracts as a result of the spreading of the action potential, which initiates calcium ion entry into the cardiomyocytes through the L-type calcium channels (LTCC). This calcium signal induces further release of Ca^{2+} from the sarcoplasmic reticulum via ryanodine receptors (calcium-induced calcium release). The intracellular free calcium binds to troponin C, triggering such a conformational change in the troponin-tropomyosin complex that makes the myosin binding site on actin accessible to myosin, leading ultimately to myosin moving along the actin filament. Upon repolarization, intracellular calcium is transferred back to the sarcoplasmic reticulum by the sarcoplasmic reticulum calcium-ATPase (SERCA) or excreted from the cell by the sodium-calcium exchanger. Myosin detaches from actin and regenerates to its active, binding-ready state by hydrolyzing adenosine triphosphate (ATP) to adenosine diphosphate (ADP), so the cardiac muscle relaxes.

Any mechanism aiming to improve the strength of cardiac contractions has to result in at least one of the following:

i. increase intracellular free Ca^{2+} levels at excitation
ii. enhance the sensitivity of myofibrillar proteins to Ca^{2+}
iii. increase the transition rate of myosin into the strongly actin-bound force-generating state

The contractile force of the heart is constantly under regulation of neural, endocrine, paracrine and autocrine factors. The vegetative nervous system innervates the heart, increasing

heart rate and cardiac contractility through the release of the neurotransmitter noradrenaline during sympathetic stimuli. Adrenocorticotropic hormone and sympathetic activation induces release of adrenaline and noradrenaline from the adrenal medulla, providing the endocrine regulation of inotropy. Cardiac tissues like cardiomyocytes, fibroblasts and vascular endothelial and smooth muscle cells also release a wide array of humoral factors, among which some peptides, like endothelin (ET-1) (Ishikawa *et al.*, 1988), apelin (Szokodi *et al.*, 2002) and adrenomedullin (Szokodi *et al.*, 1996) are identified as positive inotropic agents.

As discussed above, chronic stimulation of β-adrenergic signaling leads to increased mortality. The more recently discovered endogenous cardiac peptides like ET, apelin or adrenomedullin, however, represent novel targets of therapy, as their inotropic effect is significantly different to that of β-adrenergic stimulus in both characteristics and underlying signaling mechanisms. Therefore, this thesis focuses on exploring the signaling pathways induced by the peptides apelin and ET.

2 INTRODUCTION

2.1 G protein-coupled receptor signaling

Interestingly, most of the endocrine and paracrine substances that regulate cardiac inotropy function via binding to G-protein coupled receptors (GPCR) on the cardiomyocytes. G-protein coupled receptors are a conserved family of cell surface receptors containing a structure of seven transmembrane α-helices (Ding *et al.*, 2013). They are the largest family of membrane proteins and mediate most cellular responses to hormones and neurotransmitters, as well as being responsible for vision, olfaction and taste. Individual GPCRs have unique combinations of signal-transduction activities involving multiple G-protein subtypes, as well as G-protein-independent signaling pathways and complex regulatory processes. (Rosenbaum *et al.*, 2009). Heterotrimeric G proteins are the molecular switches that turn on intracellular signaling cascades in response to the activation of GPCRs by extracellular stimuli. Agonists bind to the extracellular surface of a GPCR and induce a conformational change that leads to G protein activation. G proteins are composed of three subunits, α, β and γ. The G-protein α-subunit (Gα) family is divided into four groups based on subunit sequence homology: G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ (Kostenis *et al.*, 2005), and GPCR signaling is determined by the type of Gα the

receptor is coupled to (Oldham and Hamm, 2008). The receptor stimulates G-protein activation by catalyzing the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Gα and by dissociation of the GTP-bound Gα from the $G_{\beta\gamma}$ heterodimer. After dissociation, free Gα-GTP and Gβγ subunits activate various enzymatic effectors, like adenylate cyclase (AC), phospholipase C (PLC) isoforms and ion channels, to induce other small molecules, the so-called second messengers (Ding *et al.*, 2013). β_1-adrenoreceptor (AR), for example, activates stimulatory G_s proteins, whereas β_2-ARs use both G_s and inhibitory G_i proteins. G_s signaling stimulate the effector enzyme, AC, resulting in dissociation of ATP into the second messenger cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA). Targets of PKA-mediated phosphorylation in the heart are, among others, LTCC, ryanodine receptors, phospholamban, troponin I (TnI), myosin binding protein-C and the phospholemman subunit of Na^+/K^+-ATPase. Activated PKA acts on its targets in a manner that increases heart rate, cardiac contractility, conductivity and relaxation. G_i-protein-coupled receptor signaling inhibits AC, consequentially decreasing cAMP level and leading to a reduction in L-type Ca^{2+} currents, which inhibits the force of contraction in myocytes. Heart rate is also slowed down by G_i mediated effects on pacemaker cells (Salazar *et al.*, 2007). G_i proteins activate mitogen-activated protein kinases via small G-proteins Ras (Chiloeches *et al.*, 1999), and contribute to the regulation of receptor signaling and activation of nuclear transcription (Triposkiadis *et al.*, 2009). Ligand-stimulated receptor signaling via G_q is initiated by the membrane-recruitment and activation of PLCβ. PLCβ is responsible for hydrolyzation of phosphatidylinositol-biphosphate into two second messengers: diacylglycerol and inositol-triphosphate (IP3). Diacylglycerol activates several isoforms of the protein kinase C (PKC) family. IP3 binds receptors on the sarcoplasmic reticulum to induce the release of calcium stores into the cytoplasm. Unlike the short bursts of contraction and relaxation induced by calcium induced calcium release, IP3-mediated Ca^{2+} signaling results in a sustained Ca^{2+} release that triggers calcineurin activation and consequent targeting of the nuclear transcription factors (Salazar *et al.*, 2007). Upon agonist stimulation, the dissociation of $G_{\beta\gamma}$ from the heterotrimeric G-protein promotes translocation and targeting of G-protein-coupled receptor kinases (GRKs) to the membrane. GRKs are responsible for the phosphorylation and subsequent downregulation and desensitization of GPCRs. The phosphorylated receptors are internalized by the cells, thereby reducing the available number of receptors on the cell surface. Another mechanism of desensitization is mediated via. Binding to GRK-phosphorylated receptors, the scaffolding β-arrestin proteins shut off G-protein signaling by blocking further G-protein coupling. β-arrestins

also activate signaling pathways such as ERK and tyrosine kinases, independently of G-protein coupled signaling (Salazar *et al.*, 2007).

2.2 Endothelin in the cardiovascular system

2.2.1 Structure

As it became clear in the early 1980's that endothelial cells release vasoactive agents, intense research focused on identifying these factors. ET-1 -firstly isolated in 1988 (Yanagisawa *et al.*, 1988)- has been found to be the most potent and long lasting endogenous vasoconstrictor known so far (Hillier *et al.*, 2001). Its half-maximal effective concentration (EC_{50}) of 0.2–0.6 nmol/L, is about 10 fold less than necessary for comparable contractions induced by angiotensin II or neuropeptide Y and about 100 fold higher concentration of noradrenaline is required for the same vascular contraction (Yanagisawa *et al.*, 1988). ET-1 belongs to a family of highly homologous peptides in mammalian systems (for review see (Rubanyi and Polokoff, 1994; Masaki, 1995)). ET-1, ET-2, and ET-3 are encoded by distinct genes on chromosomes 6, 1 and 20, respectively (Inoue *et al.*, 1989). The isoforms are all 21-amino-acid residues with two intrachain disulfide bonds, a hairpin loop consisting of polar amino acids and a hydrophobic C-terminal tail. ET-2 and ET-3 differs from ET-1 in 2 and 6 amino acids, respectively (Yanagisawa and Masaki, 1989).

2.2.2 Expression

ET-1, the predominant and biologically most relevant isoform in humans, is produced by the vascular endothelium and smooth muscle cells, cardiac myocytes, fibroblasts, macrophages, airway epithelial cells, macrophages, pancreatic islets and brain neurons among others.

ET-2 is expressed by epithelial cells of the intestines, lung, heart, kidney and ovaries (Ling *et al.*, 2013). Recent studies suggest it has a role during ovulation (Ko *et al.*, 2006), and is associated with human breast tumor growth, invasion and possibly in inflammatory disease (Grimshaw *et al.*, 2004).

ET-3 can be found in endothelial cells, brain neurons, intestinal epithelial cells and renal tubular epithelial cells and it is involved in release of vasodilators such as NO and prostacyclin (Barton and Yanagisawa, 2008)

The concentration of ET-1 in plasma in many species is ~1 pM, two orders of magnitude below the pharmacological threshold, and plasma ET-2 and ET-3 are found at even lower concentrations. Therefore, under normal physiological conditions, ETs are not circulating hormones; rather they act as autocrine and paracrine factors at multiple sites in the body (Kedzierski and Yanagisawa, 2001).

2.2.3 Function in the cardiovascular system

ET-1 has multiple functions in the heart. It is involved in controlling of coronary vascular tone, cardiomyocyte growth and fibroblast proliferation. In addition, ET-1 has been established as an important regulator of cardiac contractility (Sugden, 2003; Brunner *et al.*, 2006; Endoh, 2006). ET-1 has a positive inotropic effect in most mammalian species including rat (Kelly *et al.*, 1990; Krämer *et al.*, 1991; Kinnunen *et al.*, 2000), rabbit (Wang *et al.*, 2000; Chu and Endoh, 2005), guinea pig (Ishikawa *et al.*, 1988), cat (Cingolani *et al.*, 2006; De Giusti *et al.*, 2008), dog (Chu *et al.*, 2003; Czóbel *et al.*, 2009) and man (Pieske *et al.*, 1999; Maccarthy *et al.*, 2000), although the magnitude of the responses differs among species. In mice the effect of ET-1 on cardiac contractility has been more controversial. In isolated cardiomyocytes, ET-1 has a positive inotropic effect in S129 mice (Pi *et al.*, 2002), whereas the peptide exerts a negative inotropic effect in C57 mice (Nishimaru *et al.*, 2007, 2008). In contrast, ET-1 has been shown to increase contractility using isolated perfused heart preparations from C57 mice (Piuhola *et al.*, 2003a). Therefore, experimental conditions (single cardiomyocytes vs. multicellular preparations, pacing frequency, loading conditions, etc.) as well as species (or even strain) differences seem to influence the response to exogenous ET-1. Of particular importance, endogenous ET-1 has been shown to contribute to the Gregg effect (enhanced contractility due to an increase in coronary flow rate) in mice(Piuhola *et al.*, 2003a), the Frank–Starling response (Piuhola *et al.*, 2003b), and the slow force response (Anrep effect) to stretch in rats (Pérez *et al.*, 2001).

2.2.4 Receptor

ET-1 binds to two subtypes of GPCRs, ET_A and ET_B receptors, which are responsible for the actions of the peptide (Masaki, 1995). Both ET_A and ET_B receptors are expressed in cardiomyocytes, with a dominance of ET_A receptors (85−90%) (Molenaar *et al.*, 1993). The ET_A receptor is responsible for the positive inotropic effect of ET-1 in rats (Takeuchi *et al.*, 2001), cats (De Giusti *et al.*, 2008) and mice (Piuhola *et al.*, 2003a), while the ET_B receptor appears to counterbalance the action of the ET_A receptor (Piuhola *et al.*, 2003a).

2.2.5 Signaling

In vitro studies have suggested that ET-1 exerts most of its positive inotropic effect by increasing myofilament Ca^{2+} sensitivity, but the inotropic response is also associated with a moderate increase in intracellular Ca^{2+} transients too (Watanabe and Endoh, 1999; Yang *et al.*, 1999; Talukder *et al.*, 2001). However, the exact subcellular mechanisms have not been fully elucidated. ET_A receptor is commonly considered to signal through G_q protein-dependent activation of the PLC–protein kinase C (PKC) cascade in cardiomyocytes (Sugden, 2003; Brunner *et al.*, 2006). Previous studies suggest that ET-1 increases cardiac contractility via a PKC-dependent activation of NHE (Krämer *et al.*, 1991; Chu *et al.*, 2003; Zolk *et al.*, 2004). Stimulation of NHE can lead to intracellular alkalization and consequent sensitization of cardiac myofilaments to intracellular Ca^{2+} (Krämer *et al.*, 1991; Goldberg *et al.*, 2000). On the other hand, NHE-mediated accumulation of intracellular Na^+ can indirectly promote a rise in intracellular levels of Ca^{2+} via a reverse-mode NCX (Yang *et al.*, 1999; Pérez *et al.*, 2001). In addition to NCX, ET-1 can enhance intracellular Ca^{2+} transients by increasing L-type Ca^{2+} current (Watanabe and Endoh, 1999). Although PKC has been proposed to play a central role in ET-1 signaling, our recent data indicate that PKC is unlikely to mediate the inotropic effect of ET-1 (Szokodi *et al.*, 2008). In the intact adult rat heart, ET-1 failed to induce translocation of various PKC isoforms (PKCα, PKCδ, or PKCε) to the membrane fraction from cytosol. Moreover, GF-109203X, a specific PKC inhibitor, did not attenuate the inotropic response to ET-1, although it markedly reduced the inotropic effect of phorbol 12-myristate 13-acetate, a direct activator of PKC. Furthermore, pharmacological inhibition of PLC, the upstream regulator of PKC, also failed to alter the inotropic action of ET-1 (Szokodi *et al.*, 2008).

Our group established that activations of the extracellular signal-regulated kinases 1 and 2 (commonly referred to as ERK1/2), members of the mitogen-activated protein kinase (MAPK) superfamily are -in contrast to the PLC–PKC cascade- critically involved in the inotropic response to ET-1 (Szokodi *et al.*, 2008). The basic arrangement of the cascade includes the small G protein Ras working upstream of a core module consisting of a sequence of successively acting kinases: the serine/threonine kinase Raf that phosphorylates and activates two MAPK kinases, MEK1 and MEK2, which directly phosphorylate the dual site in the activation loop (Thr-Glu-Tyr) of the target kinases ERK1 and ERK2 (Bueno and Molkentin, 2002; Fuller *et al.*, 2008; Rose *et al.*, 2010). We also demonstrated that ET-1 produced a rapid increase in phospho-ERK1/2 levels, and inhibition of ERK1/2 activation by U0126, a potent MEK1/2 inhibitor, markedly attenuated the ET-1–induced increase in contractile force in the intact rat heart (Szokodi *et al.*, 2008).

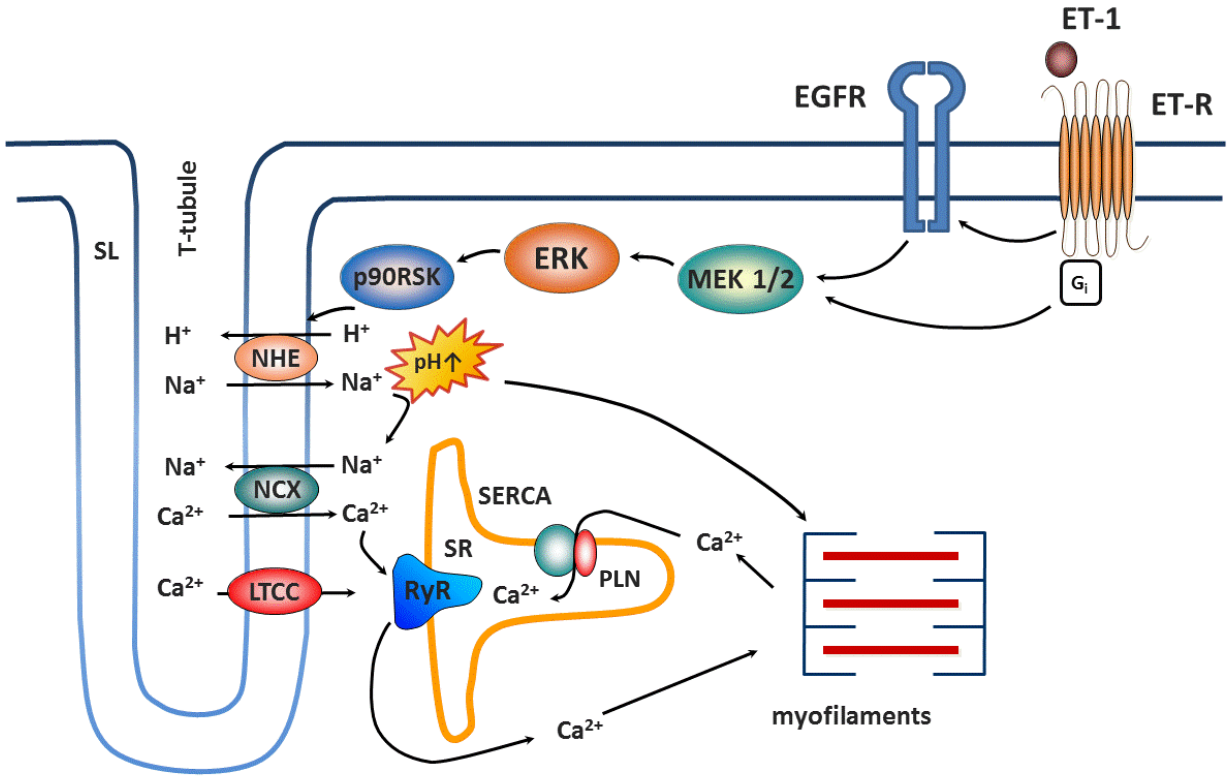

Figure 1: Inotropic signaling pathway of ET-1

Epidermal growth factor receptor (EGFR) transactivation is an important pathway that links GPCRs and ERK1/2 activation via recruitment of the Ras–Raf1–MEK1/2 cascade (Wetzker and Böhmer, 2003; Fuller *et al.*, 2008). Our results demonstrated that ET-1 induced

EGFR transactivation and inhibition of this process is accompanied by attenuation of the ET-1–induced increase in phospho-ERK1/2 levels and the inotropic response to ET-1, suggesting that EGFR acts as a proximal component of MEK1/2–ERK1/2 signaling (Szokodi *et al.*, 2008). Activation of ERK1/2 can result in phosphorylation of the C-terminal regulatory domain of the NHE1, either directly by ERK1/2 itself (Moor and Fliegel, 1999) or indirectly through the p90 ribosomal S6 kinase (p90RSK) (Takahashi *et al.*, 1999). Importantly, our group showed that ET-1 increased p90RSK phosphorylation in the membrane fraction, and inhibition of EGFR transactivation and MEK1/2 attenuated this increase in phosphor-p90RSK levels. Therefore, it is conceivable that membrane-associated p90RSK mediates the effect of ET-1 on NHE1 activity (Szokodi *et al.*, 2008).Collectively, our results have revealed a crucial role for the EGFR–ERK–p90RSK–NHE1 pathway in the ET-1–mediated positive inotropic response in isolated rat hearts (Szokodi *et al.*, 2008). Figure 1 illustrates our understanding of the ET-1-induced inotropic signaling pathways.

2.2.6 Modulation of ET-1 signaling by reactive oxygen species

Excessive ROS production is characteristic for various pathological conditions, including congestive heart failure. It has been proven that oxidative stress triggers a variety of changes in heart failure, including cardiomyocyte hypertrophy, apoptosis, necrosis, and interstitial fibrosis ultimately leading to pump dysfunction. Moreover, excessive levels of ROS can alter the activity of different proteins involved in excitation–contraction coupling; therefore oxidative stress seems to directly contribute to the development of contractile dysfunction (Penna *et al.*, 2009; Canton *et al.*, 2011; Heusch and Schulz, 2011; Santos *et al.*, 2011). In contrast, recently it has been revealed that endogenously produced reactive oxygen species (ROS), acting as signaling molecules, can regulate the positive inotropic response to ET-1. Acute administration of ET-1 enhanced ROS production, measured by oxidation of dihydroethidium to ethidium in isolated rat cardiomyocytes (Zeng *et al.*, 2008). In agreement, an increase in $O_2^{\bullet -}$ formation after ET-1 stimulus was detected by lucigenin-enhanced chemiluminescence in isolated cat cardiomyocytes (De Giusti *et al.*, 2008). Administration of antioxidants prevented the ET-1-induced increase in ROS production in all of these models (De Giusti *et al.*, 2008; Zeng *et al.*, 2008). Moreover, it has been shown that the positive inotropic effect of exogenous ET-1 is abolished by ROS scavengers, suggesting that the inotropic response is almost exclusively

dependent on ROS production in isolated adult cat cardiomyocytes (Cingolani *et al.*, 2006; De Giusti *et al.*, 2008). Using the same model, Ang II has been reported to induce the release of endogenous ET-1, which in turn triggers an increase in contractility through enhanced ROS generation (Cingolani *et al.*, 2006). However, the functional importance of ROS under physiological conditions in the myocardium remains obscure.

2.3 Apelin in the cardiovascular system

2.3.1 Structure

In 1993 a novel GPCR called APJ was identified by homology cloning. It shares greatest sequence identity (30 % in total, 54% in the transmembrane regions) with the angiotensin II type-1 receptor (AT1-R) but does not bind angiotensin II (Ang II) (O'Dowd *et al.*, 1993) The APJ remained "orphan" until 1998, when its endogenous ligand was isolated from bovine stomach extract. The ligand was identified as a 36 amino acid peptide named apelin (for APjEndogenous LIgaNd) (Tatemoto *et al.*, 1998), and later on the receptor was renamed "apelin receptor" by international consensus (Pitkin *et al.*, 2010).

The human apelin gene is located on chromosome Xq25– 26.1 and it contains 3 exons. Exon 1 and 2 include the coding regions, encoding a 77 amino acid preproprotein that is then cleaved to shorter active peptides. The human apelin gene shows great sequence homology across all species examined, including cattle, rats, and mice among others, with the last 23 residues of the C terminus being identical in mammals. Sequence analysis of the mature peptide revealed identity, albeit limited, to angiotensin II (Lee *et al.*, 2000).

The apelin gene encodes a 77 amino acid preproprotein, and the mature peptide was identified at first as the C-terminal 36 amino acid fragment of the preproapelin (apelin-36). The pathway of apelin metabolism has not been precisely described yet, but the 77 amino acid prepropeptide contains a number of basic residues that are potential cleavage sites for peptidases. Cleavage at these sites produces a family of C-terminal fragments, including apelin-36, apelin-17, apelin-13, apelin-12 and the post-translationally modified (Pyr1)apelin-13 , which are all agonists at the apelin receptor, but the binding affinity and biological efficacy differ from isoform to isoform, with the shorter fragments being more potent (Tatemoto *et al.*, 1998). (Pyr1)apelin-13 is the most potent and abundant form in cardiac tissue (Maguire *et al.*,

2009). The lack of cysteine residues in these C-terminal fragments suggests that the mature peptides are monomeric. Fragments shorter than 12 amino acids are biologically inert (Tatemoto *et al.*, 2001). Apelin has a brief plasma half-life of less than 5 min in man (Japp *et al.*, 2010).

Little is known about the degradation pathways of the apelin peptides. The only enzyme, yet identified to hydrolyze both apelin-36 and apelin-13 with high efficacy is angiotensin-converting enzyme type 2 (ACE-2), a carboxypeptidase which also cleaves Ang II to biologically inactive isoforms (Vickers *et al.*, 2002). However, the exact role of ACE-2 in the elimination of apelin is not yet entirely clarified, since the cleaved fragments still possess some cardiovascular activity in certain experimental setups.

2.3.2 Expression

Apelin and apelin receptor are expressed widely through the organism. In humans, preproapelin and apelin receptor mRNA are abundant in the central nervous system, heart, lung, kidney, placenta and mammary gland. In human vasculature, both apelin and its receptor, apelin receptor, are detectable immunhistologically in endothelial cells and vascular smooth muscle cells in human large conduit vessels, small arteries and veins. In the heart, apelin receptor-like immunoreactivity was present in the endocardial endothelium and, in lesser extent, also in the myocardium - reviewed by (Kleinz and Davenport, 2005). The apelin receptor density in human myocardium is comparable to that of AT1-R II receptor, but it is much lower than that for ET receptors (Katugampola *et al.*, 2001). The apelin peptide is present in normal human plasma, the amount of immunoreactive apelin was found to be in the magnitude of 100 pg/ml. Apelin-like immunoreactivity was detected in the heart, being over 200-fold higher in the right atria than in the left ventricles (Földes *et al.*, 2003). Immunhistology studies localized apelin peptide to the endocardial endothelium, while it was not detectable in cardiomyocytes (Kleinz and Davenport, 2004). This distribution pattern, the low plasma level and short lifespan of the circulating peptide suggest an autocrine or paracrine way of action for apelin in the cardiovascular system.

2.3.3 Function in the cardiovascular system

Soon after its discovery, potent vasodilator and positive inotropic effects of the peptide were revealed; a rare combination among endogenous agents. Further investigations reported that the peptide may play a role in regulation of cardiovascular development and angiogenesis.

The positive inotropic effect of apelin has been established in the intact (Szokodi *et al.*, 2002) and failing (Dai *et al.*, 2006) rat heart and in humans as well (Japp *et al.*, 2010). Being active in the subnanomolar range, apelin appears to be one of the most potent endogenous positive inotropic agents yet identified, augmenting cardiac contractility by approximately 70% of the increased force observed with isoproterenol. This inotropic effect is comparable in magnitude to the results seen previously in isolated rat hearts with other endogenous inotropic peptides ET-1 (Szokodi *et al.*, 2008) and adrenomedullin (Szokodi *et al.*, 1996)

2.3.4 Receptor

The human apelin receptor, originally named as APJ, has the characteristic 7-transmembrane domain structure of a GPCR and it shares close sequence homology with the angiotensin receptor-1. However, angiotensin-II has no affinity to the apelin receptor.). The different sized apelin fragments exert different binding characteristics to the apelin receptor, with the length of the isoform being reciprocally related to its binding affinity (Tatemoto *et al.*, 1998).

The human apelin receptor gene was located to 11q12 chromosome and genomical analysis suggested that the receptor has no subtypes (O'Dowd *et al.*, 1993). It has to be mentioned, that several apelin-mediated responses, just like many other GPCR-linked signals, showed signs of desensitization, probably due to receptor internalization following activation. Interestingly, the length of the apelin fragment linking to the receptor not only determines the peptide's affinity to its receptor, but it was found to determine the characteristics of receptor internalization. The apelin receptor also exhibits nuclear localization suggesting the possibility that apelin could directly take part in transcriptional regulation -reviewed in (Japp and Newby, 2008).

2.3.5 Signaling

Apelin inhibits forskolin-induced cAMP production in cells expressing apelin receptor. This finding suggests that apelin receptor couples to inhibitory G_i proteins. Apelin peptides activate p70S6 kinase, a regulator of cell cycle progression, in umbilical endothelial cells through two separate phosphorylation signals. One regulator is the phosphatidylinositide 3-kinases - Akt cascade and the other pathway is mediated via ERKs. These apelin-induced signaling cascades are pertussis toxin (PTX) sensitive, strengthening the hypothesis that the apelin receptor is linked to G_i proteins (Masri *et al.*, 2004).

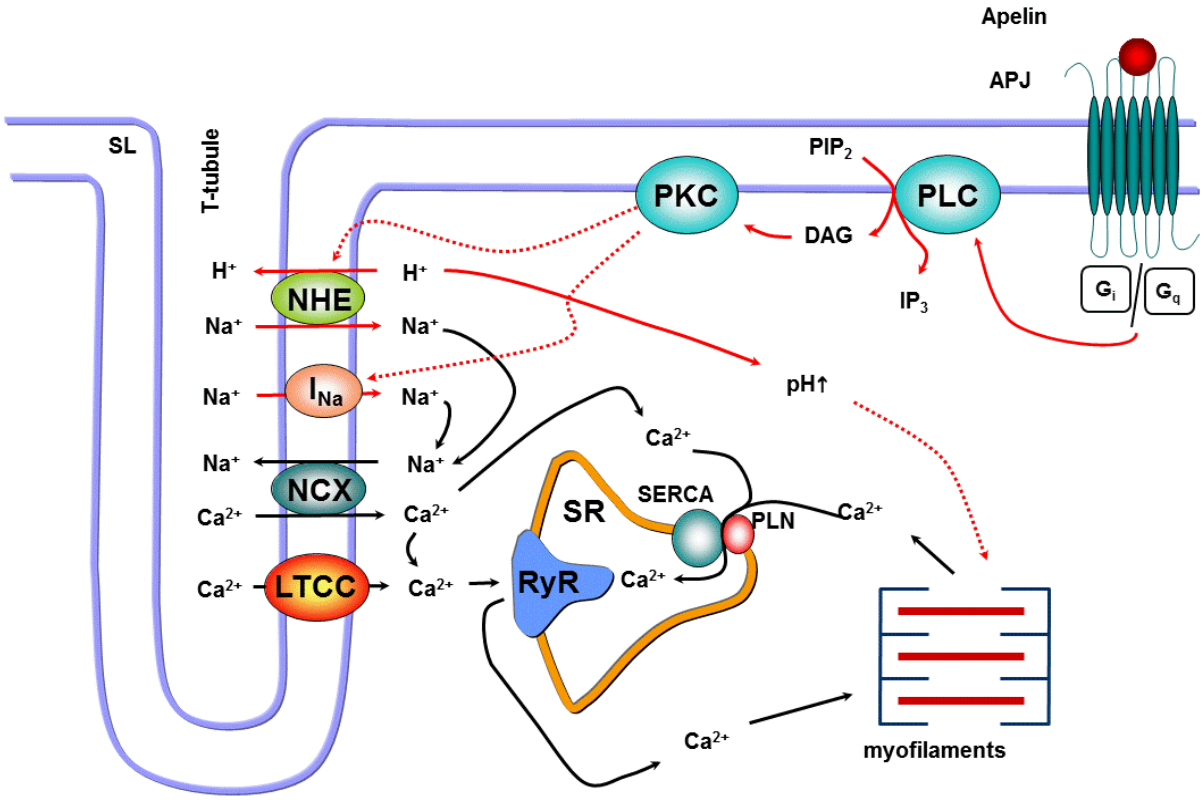

Figure 2: Inotropic signaling pathway of apelin.

Apelin may exert positive inotropic effect via both G_q and G_i proteins. Protein kinase C can induce NHE activity. Stimulation of the NHE can lead to intracellular alkalization and consequent sensitization of cardiac myofilaments to intracellular Ca^{2+}. Furthermore, NHE-mediated accumulation of intracellular Na^+ can indirectly promote a rise in intracellular levels of Ca^{2+} via reverse mode NCX. Continuous red lines show experimentally proven pathways, dotted red lines show speculated ones.

Apelin may induce cardiac contractility via both PTX–insensitive G_q and PTX–sensitive G_i proteins. Phospholipase C, PKC, NHE and NCX have been identified as mediators of the apelin-induced inotropic signaling (Szokodi *et al.*, 2002; Wang *et al.*, 2008). Apelin-induced activation of NHE leads to intracellular alkalization and thereby it can directly sensitize cardiac myofilaments to intracellular Ca^{2+} (Farkasfalvi *et al.*, 2007). On the other hand, NHE-mediated accumulation of intracellular Na^+ can indirectly promote a rise in intracellular levels of Ca^{2+} via reverse mode NCX. However, whether apelin directly increases intracellular calcium currents or acts by solely sensitizing myofilaments to calcium remains controversial (Dai *et al.*, 2006; Farkasfalvi *et al.*, 2007; Wang *et al.*, 2008).

Early data suggested that PKC-mediated increase in cardiac contractility may result from increased NHE activity and subsequent intracellular alkalization (Krämer *et al.*, 1991). On the contrary, more recent evidence demonstrated that PKC-dependent positive inotropic response was not associated with alteration of intracellular pH (Kang and Walker, 2006). It is possible however, that PKC activation induces cardiac contractility by enhancing myofibrillar Ca^{2+} sensitivity via phosphorylation of myosin regulatory light chain (RLC) (Venema *et al.*, 1993) or TnI (Pi *et al.*, 2003; Westfall and Borton, 2003), and PKC was also reported to enhance Ca^{2+} transients via LTCC (He *et al.*, 2000; Huang *et al.*, 2001). The exact PKC isoenzyme contributing to the apelin-induced contractile response has not been identified yet. Figure 2 summarizes the already established inotropic signaling pathways of apelin.

3 AIMS OF THE THESIS

Excessive data supports the idea that ET-1 and apelin are important regulators of cardiac homeostasis and play significant role in cardiovascular pathology. Proper understanding of their role in regulation of cardiac contractility may offer novel targets of heart failure therapy. We aimed to explore the underlying signaling mechanism of these inotropic peptides with focus on:

1) the role of endogenous ROS production,
2) MAPK activation,
3) identifying PKC isoforms that are involved in the signaling
4) and looking for downstream mechanisms by which the peptides induce inotropy.

4 MATERIALS AND METHODS

4.1 Animals

Male 7-week-old Sprague-Dawley rats from the Center for Experimental Animals at the University of Oulu were used (n=316). The rats were housed in plastic cages 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 protocols were reviewed and approved by the Animal Use and Care Committee of the University of Oulu and 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).

4.2 Materials

Drugs used were ET-1, dobutamine, N-acetylcysteine, 5-hyroxy-decanoate (5-HD), ML-7 and paxilline (Sigma-Aldrich Co, St. Louis, MO); Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP) and apocynin (Calbiochem–NovabiochemCorp. Darmstadt, Germany); HMR 1098 (generously supplied by Dr. Jürgen Pünter, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany); dihydroethidium (Molecular Probes Inc., Eugene, OR), Apelin-16 (Phoenix Europe GmbH, Karlsruhe, Germany); Bisindolylmaleimide I (Bis) (Merck Chemicals Ltd., Nottingham, UK); U0126 (LC Laboratories, Woburn, MA, USA). ET-1, N-acetylcysteine, 5-HD, MnTMPyP, HMR 1098 and dobutamine were dissolved in distilled water; Apelin-16 was dissolved in 0,6 % acetic acid; apocynin, Bis, dihydroethidium, ML-7, paxilline and U0126 were dissolved in dimethyl-sulfoxide (DMSO). The final DMSO concentration was < 0.15 % in the perfusion buffer. DMSO and acetic acid were added to each vehicle control experiments in volumes equal to those used for diluting the drugs in parallel experiments.

4.3 Isolated Perfused Rat Heart Preparation

Rats were decapitated and hearts were quickly removed and arranged for retrograde perfusion by the Langendorff technique as described previously (Szokodi *et al.*, 2008). The hearts were perfused with a modified Krebs-Henseleit bicarbonate buffer, pH 7.40, equilibrated with 95% O_2-5% CO_2 at 37°C. Hearts were perfused at a constant flow rate of 5.5 mL/min

with a peristaltic pump (Minipuls 3, model 312, Gilson, Villiers, France). Heart rate was maintained constant (305±1 beats per minute) by atrial pacing using a Grass stimulator (model S88, Grass Instruments, West Warwick, RI, USA) (11 V, 0.5 ms). Contractile force (apicobasal displacement) was obtained by connecting a force displacement transducer (FT03, Grass Instruments, West Warwick, RI, USA) to the apex of the heart at an initial preload stretch of 20 mN. Perfusion pressure reflecting coronary vascular resistance was measured by a pressure transducer (model BP-100, iWorx Systems, Inc., Dover, NH, USA) situated on a side arm of the aortic cannula. Data were recorded using IX-228 Data Acquisition System and LabScribe recording and analysis software (iWorx Systems, Inc., Dover, NH, USA). An equilibration period (40 ±4 min) and a 5-minute control period were followed by the addition of various drugs to the perfusate for 5, 10, 15 or 20 minutes. The drugs Bis (90 nmol/L), ML-7 (1 μmol/l), U0126 (5 μmol/l) N-acetylcysteine (500 μmol/l), MnTMPyP (10 μmol/l), 5-HD (200 μmol/l), HMR 1098 (3 μmol/l), apocynin (100 μmol/l) and paxilline (1 μmol/l) were chosen because they are known to suppress PKC activity, (Hu and Nattel, 1995), myosin light chain kinase (Aoki *et al.*, 2000), ERK1/2 activation (Tenhunen, 2004), to act as a ROS scavenger (Cheng *et al.*, 1999) and a superoxide dismutase mimetic (Amin *et al.*, 2001), to inhibit mitochondrial ATP-dependent potassium channels (mitoK_{ATP}) (Pain *et al.*, 2000), sarcolemmal K_{ATP} (sarcK_{ATP}) channel (Gok *et al.*, 2006), NAD(P)H oxidase (Dong *et al.*, 2006), and mitochondrial large conductance calcium activated potassium (BK_{Ca}) channel (Cao *et al.*, 2005), respectively. After the end of experiments, hearts were rapidly dissected, left ventricular (LV) samples were frozen in liquid nitrogen and they were stored in – 70 °C.

4.4 Western blot analysis for PKC isoform translocation

We followed the protocol found to be the most effective in preserving PKC isoforms by Hunter and Korzick (Hunter and Korzick, 2005) for sample preparation. Briefly, frozen LV tissues were grinded in liquid nitrogen and were dissolved and homogenized in ice-cold lysis buffer (20 mmol/L Tris, pH 7.5; 2 mmol/L each of EDTA and EGTA, pH 7.5–8.0; 5 mmol/L sodium fluoride; 5 μg/ml each of leupeptin and aprotinin; 0.5 μg/ml pepstatin A; 0.3 mmol/L phenylmethylsulfonyl fluoride (PMSF); 1 AM vanadate; 3 mmol/L dithiothreitol) using a glass-glass tissue grinder. Samples were then centrifuged at 100 000 x g for 1 h at 4 °C, and the supernatant was defined as the soluble fraction. Pellets were resuspended in ice-cold lysis

buffer containing 1% TritonX for 30 min. This fraction was then cleared with a 1 h 100 000 x g centrifugation (4 °C), and the resulting supernatant was defined as the particulate fraction. Protein concentrations were determined by the method of Bradford. Protein extracts were matched for protein concentration and stored denaturated in SDS loading buffer at -70 °C. Equal protein volumes (7 µg) of particulate and soluble fraction were loaded onto conventional 7.5 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked in 50 % Odyssey blocking buffer (LI-COR GmbH, Bad Homburg, Germany) in TBS-tween and incubated with indicated primary antibody overnight. Protein levels were detected using fluorescence by Odyssey CLx infrared imaging System (LI-COR GmbH, Bad Homburg, Germany). Isoform-specific anti-PKCα antibody was from Sigma (Saint Louis, MO, USA) and anti-PKCε antibody was from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

4.5 Western blot analysis for RLC phosphorylation

LV samples were subjected to urea/glycerol- polyacrylamide gel electrophoresis (PAGE) to separate phosphorylated and nonphosphorylated RLC as described previously (Hidalgo *et al.*, 2006; Ding *et al.*, 2010). We used a slightly modified protocol of Hidalgo et al. for protein isolation. Briefly, frozen LV tissues were grinded in liquid nitrogen and were dissolved in freshly prepared ice-cold urea sample buffer (9M urea, 50 mmol/L Tris, 300 mmol/L glycine, 5 mmol/L dithiothreitol (DTT), and 0.001% bromophenol blue, pH 8.6. containing of 50 % glycerol and 1:100 Protease inhibitor cocktail and Phosphatase Inhibitor cocktail 3 (Sigma-Aldrich, St. Louis, MO, USA)). Then the samples were transferred to a water bath at 60 °C and were shaken there for 4 min. Samples were then centrifuged at 1300 x g for 5 min at 4 °C. The supernatant was collected and protein concentrations were determined by the method of Bradford. Samples were set to standard concentration by addition of lysis buffer and were stored at -70 °C. Urea glycerol PAGE was used. The mobility of proteins in the non-denaturating urea glycerol PAGE varies with the electrical charge of the protein, phosphorylation and diphosphorylation result in additional migration of RLC in the urea-PAGE system, and thus the different grades of RLC-phosphorylation are represented by separate bands in the gel. Equal volumes (7 µg) of proteins were loaded onto the urea-glycerol gels. The resolving gel consisted of 10 % acrylamide, 40 % glycerol, and the stacking gel of 5 %

acrylamide, 20 % glycerol both in 25 mmol/L glycine, 20 mmol/L Tris, pH 8.6. The running buffer contained 122 mmol/L glycine, 20 mmol/L Tris, pH 8.6. Proteins were and transferred to nitrocellulose membranes. The membranes were blocked in 5 % nonfat milk in TBS-Tween and incubated overnight with anti-RLC (cardiac isoform) primary antibody (a most generous gift from Prof. James Stull from UT Southwestern). Protein levels were detected by enhanced chemiluminescence using an Amersham ECL Plus kit (GE Healthcare Life Sciences, Buckinghamshire, England) and Fujifilm LAS-3000 Imager (Fuji Photo Film Co., Tokyo, Japan).

4.6 Western blot analysis for MAPK and PLN phosphorylation

Frozen LV tissues were grinded in liquid nitrogen and were dissolved and homogenized in ice-cold lysis buffer containing of 20 mmol/L Tris, (pH 7.5), 10 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, supplemented with 1 mmol/L β-glycerophosphate, 2 mmol/L DTT, 1 mmol/L Na3VO4, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 2 μg/mL pepstatin, 2 mmol/L benzamidine, 1 mmol/L PMSF and 20 mmol/L NaF. Samples were then centrifuged at 1300 x g for 5 min at 4 °C and the supernatant was collected. Protein concentrations were determined by the method of Bradford. Protein extracts were matched for protein concentration and stored denaturated in SDS loading buffer at -70 °C. Equal volumes (30 μg) of protein samples were loaded onto 10 % SDS-PAGE and transferred to nitrocellulose membranes. Protein levels were detected using fluorescence as described above. Quantification of the blots was done by using the Quantity One Basic 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA). The primary antibodies used were anti-phospho-ERK1/2, anti-ERK1/2, anti-p38, anti-phospho-p38 (Cell Signaling Technology Inc., Danvers, MA, USA) and anti-phospho(Ser16)-phospholamban (Badrilla Ltd, Leeds, West Yorkshire, UK), and antiphospho(Thr17)-phospholamban (Santa Cruz Biotechnology, SantaCruz, CA) antibodies.

4.7 Detection of intracellular ROS

ROS were detected using ethidium fluorescence as previously described (Sovershaev *et al.*, 2006). In these experiments rat hearts were perfused with KHB containing dihydroethidium (10 μmol/l) with or without 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 are directly proportional to the levels of ROS, primarily superoxide anion (Kevin *et al.*, 2003). By the end of the treatment hearts were rapidly excised and 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 20× objective lens. From each histological sample a randomly selected area of 635×635 μm (1024×1024 pixels) was scanned with a fixed pixel time of 40 μs and at the 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

Results are presented as mean±SEM. Repeated-measures ANOVA test was used to evaluate the statistical significance of differences among groups for cardiac contractility. The two factors were treatment group (with 4 categories: vehicle control; inhibitor; apelin / ET-1 and apelin / ET-1 + inhibitor) and time (with 19 or 24 categories, from -3 to 15 or 20 min) as the repeated measure. When significant differences were detected in 2-way repeated measures ANOVA for the treatment-by-time interactions, a Bonferroni post hoc test was used for specific comparisons. In cases of 2 groups per comparison unpaired Student's t test was used; all other parameters were analyzed with 1-way ANOVA followed by Bonferroni post hoc test. Differences were considered statistically significant at the level of $P<0.05$.

5 RESULTS

5.1 Role of reactive oxygen species in the ET-1-induced inotropic signaling

5.1.1 ET-1 increases intracellular ROS production in the myocardium

Previously, 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; Dong *et al.*, 2006; De Giusti *et al.*, 2008). To study whether ET-1 has any effect on ROS production in isolated perfused adult rat hearts, we evaluated ROS-dependent oxidation of dihydroethidium to ethidium in cryosections of left ventricles by fluorescence microscopy (Sovershaev *et al.*, 2006). Ethidium fluorescence was detectable in all examined images. Hearts exposed to ET-1 (1 nmol/L) and dihydroethidium (10 μmol/L) produced significantly greater ethidium fluorescence intensity as compared to control hearts ($P<0.01$, Figure 3 A and B). Moreover, the antioxidant N-acetylcysteine (500 μmol/L) blunted the ET-1-induced increase in ethidium fluorescence in isolated hearts ($P<0.001$), whereas the ROS scavenger alone had a small effect on fluorescence intensity ($P<0.05$, Figure 3 C)

5.1.2 ET-1 increases cardiac contractility via enhanced ROS generation

To assess whether increased ROS production modulates the positive inotropic effect of ET-1, the antioxidant N-acetylcysteine and the superoxide dismutase mimetic MnTMPyP (Amin *et al.*, 2001) were used. In the isolated perfused rat heart preparation, intracoronary infusion of ET-1 (1 nmol/L) for 10 min produced a slowly developing but sustained increase (43%, $P<0.001$) in cardiac contractility, as reported previously (Piuhola *et al.*, 2003a; Szokodi *et al.*, 2008). Infusion of N-acetylcysteine (500 μmol/L) alone had no effect on developed tension (P=NS). When N-acetylcysteine was infused in combination with ET-1, it significantly attenuated the ET-1-induced inotropic effect, the reduction being 33% at the end of 10 min infusion time ($P<0.001$, Figure 4 A). Similarly, when ET-1 was infused in the presence of MnTMPyP (10 μmol/L), the inotropic effect was decreased by 35% ($P<0.05$, Figure 4 B). Infusion of MnTMPyP alone had no effect on cardiac contractility (P=NS).

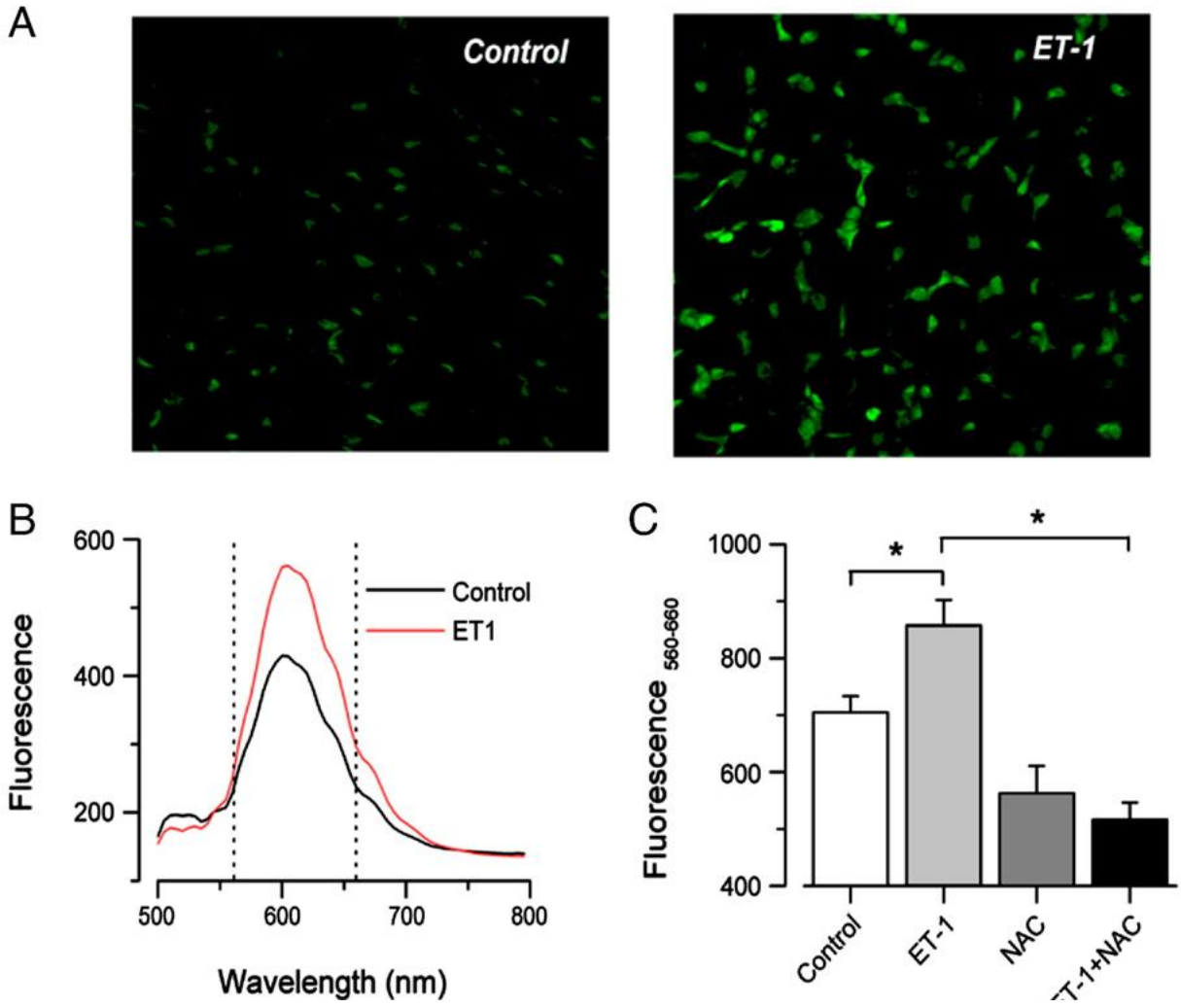

Figure 3: Estimation of ROS production by ethidium fluorescence in the myocardium

A. Representative images from ventricular histological sections taken from control and ET-1 (1 nmol/L) perfused rat hearts pre-labeled with dihydroethidium (10 μmol/L). **B.** Ethidium fluorescence spectrum elicited by 488 nm laser excitation from control (black line) and ET-1 treated (red line) ventricular samples. Dotted lines indicate the spectral area used for detecting the ethidium specific fluorescence. **C**. Averaged nuclear ethidium fluorescence (560–660 nm) from controls compared to the fluorescence from ET-1, N-acetylcysteine (NAC) or ET-1+NAC treated ventricular sections (n=4 for each group). Data are reported as mean±SEM. *P<0.01 by 1-way ANOVA followed by LSD post hoc test.

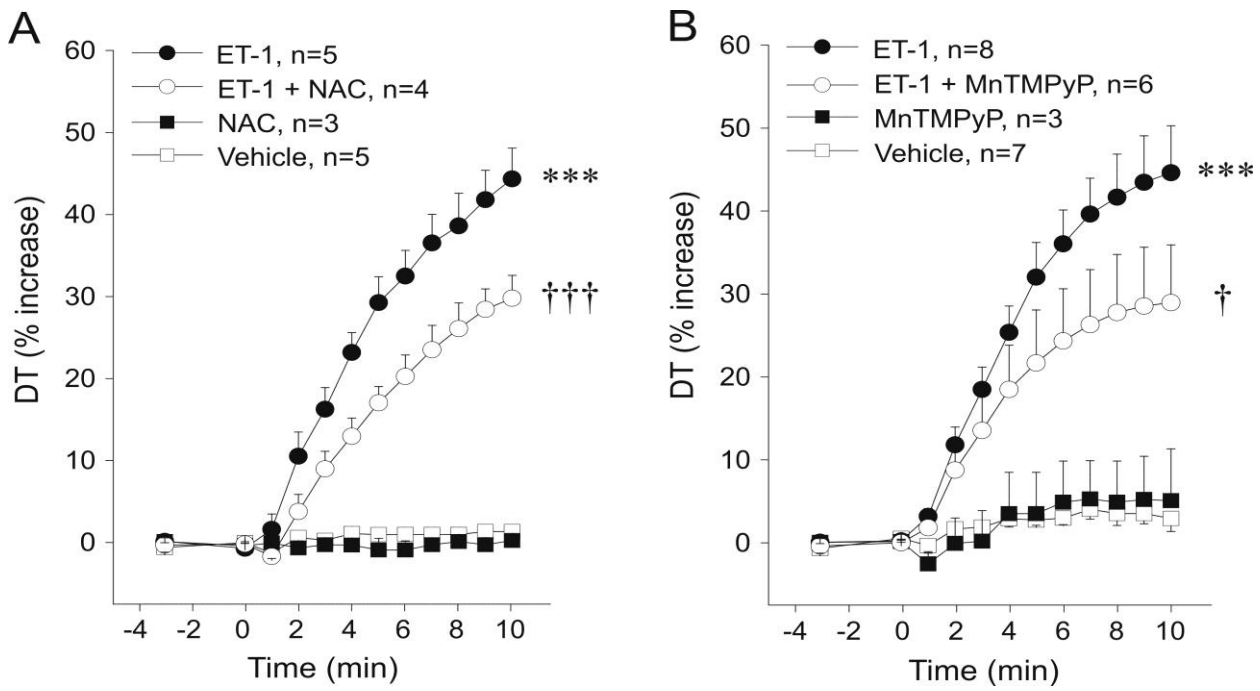

Figure 4: ROS formation contributes to ET-1-mediated increase in contractility

A and B In isolated perfused hearts infusion of ET-1 (1 nmol/L) increased developed tension (DT), and the antioxidant N-acetylcysteine (NAC, 500 µmol/L) (**A**) and the superoxide dismutase mimetic MnTMPyP (10 µmol/L) (**B**) attenuated ET-1-enhanced contractility. Results are expressed as a percent change versus baseline values. Data are mean±SEM. ***: P< 0.001 vs. vehicle control, †: P<0.05; †††: P<0.001 vs. ET-1 by repeated measures ANOVA followed by LSD post hoc analysis.

5.1.3 NAD(P)H oxidase-derived ROS contribute to ET-1-induced inotropic response

The membrane-associated NAD(P)H oxidases are important sources of $O_2^{\bullet -}$ in the myocardium (Griendling *et al.*, 2000; Murdoch *et al.*, 2006). Previously it has been shown that ET-1 activates NAD(P)H oxidase and induces ROS production in cultured rat cardiomyocytes (Yang *et al.*, 2005). To assess the contribution of NAD(P)H oxidase in mediating the inotropic effect of ET-1 we used apocynin (Dong *et al.*, 2006). When given together with ET-1, apocynin (100 µmol/L) significantly attenuated ET-1- induced positive inotropic effect throughout the entire experimental period, the reduction being 36% at the end of 10 min infusion time (P<0.001, Figure 5 A). Infusion of apocynin alone had no effect on contractile force (P=NS, Figure 5 A). ROS measurements revealed that apocynin eliminated the ET-1-induced increase in ethidium fluorescence in isolated hearts (P<0.001, Figure 5 B), whereas the drug alone had no significant effect on fluorescence intensity (P=NS, Figure 5 B).

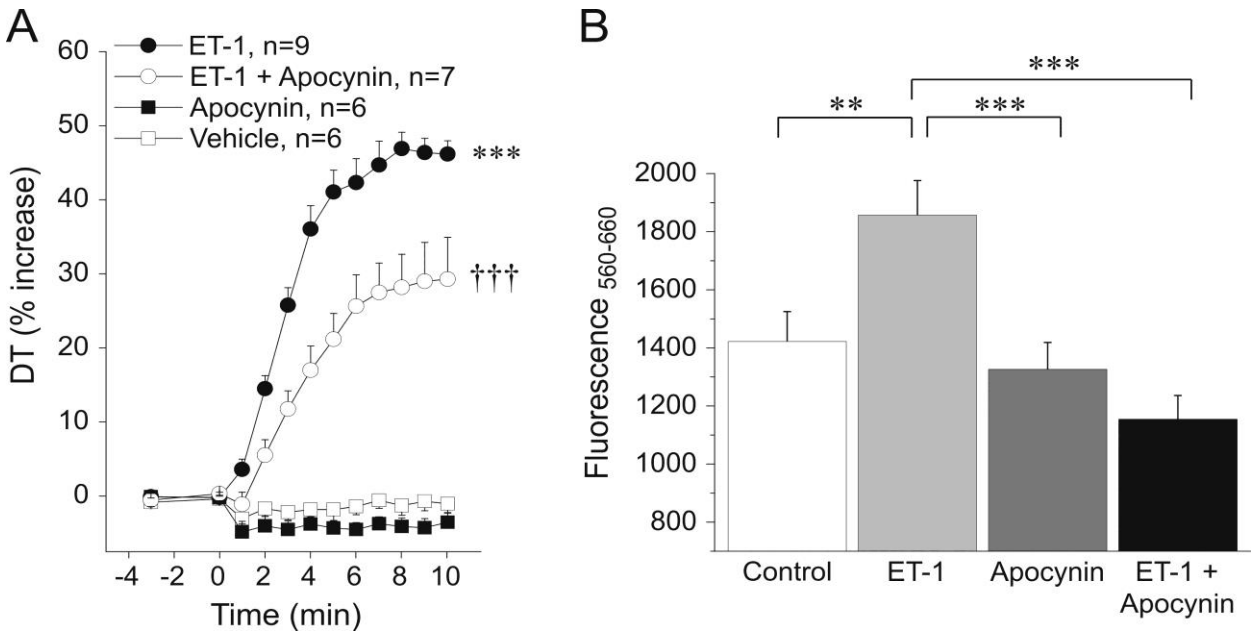

Figure 5: NAD(P)H oxidase is involved in the inotropic response to ET-1

A. Apocynin (100 μmol/L), a NAD(P)H oxidase inhibitor, attenuated ET-1-induced increase in contractility. Results are expressed as a percent change versus baseline values. DT indicates developed tension. Data are mean±SEM. ***: P<0.001 vs. control and apocynin; †††: P<0.001 vs. ET-1 by repeated measures ANOVA followed by LSD post hoc analysis. **B**. Averaged nuclear ethidium fluorescence (560–660 nm) from controls (n=3) compared to the fluorescence from ET-1 (n=3), apocynin (n=4) or ET-1+apocynin (n=4) treated ventricular sections. Data are reported as mean±SEM. **: P<0.01; ***: P<0.001 by 1-way ANOVA followed by LSD post hoc test.

5.1.4 Inhibition of mitoK$_{ATP}$ channel opening attenuates ET-1-induced inotropic response

Opening of mitoK$_{ATP}$ has been shown to increase mitochondrial production of ROS in the myocardium (Pain *et al.*, 2000; Forbes *et al.*, 2001; Heinzel *et al.*, 2005). Therefore we asked if mitoK$_{ATP}$ are involved in the inotropic response to ET-1 via increased ROS production. The role of mitoK$_{ATP}$ was studied by using 5-HD (200 μmol/L), a mitoK$_{ATP}$ blocker (Pain *et al.*, 2000). Infusion of 5-HD had no effect on developed tension (P=NS). When 5-HD was infused in combination with ET-1, it attenuated the positive inotropic response to ET-1 by 43% at 10 min time point (P<0.001) (Figure 6 A). ROS measurements showed that 5-HD alone decreased fluorescence intensity (P<0.05); however, ET-1 was still able to increase ethidium fluorescence in the presence of 5-HD (P<0.05) (Figure 6 B). These results suggest that opening

of mitoK$_{ATP}$ is required for the development of a full inotropic response. However, ROS production is not involved in this effect.

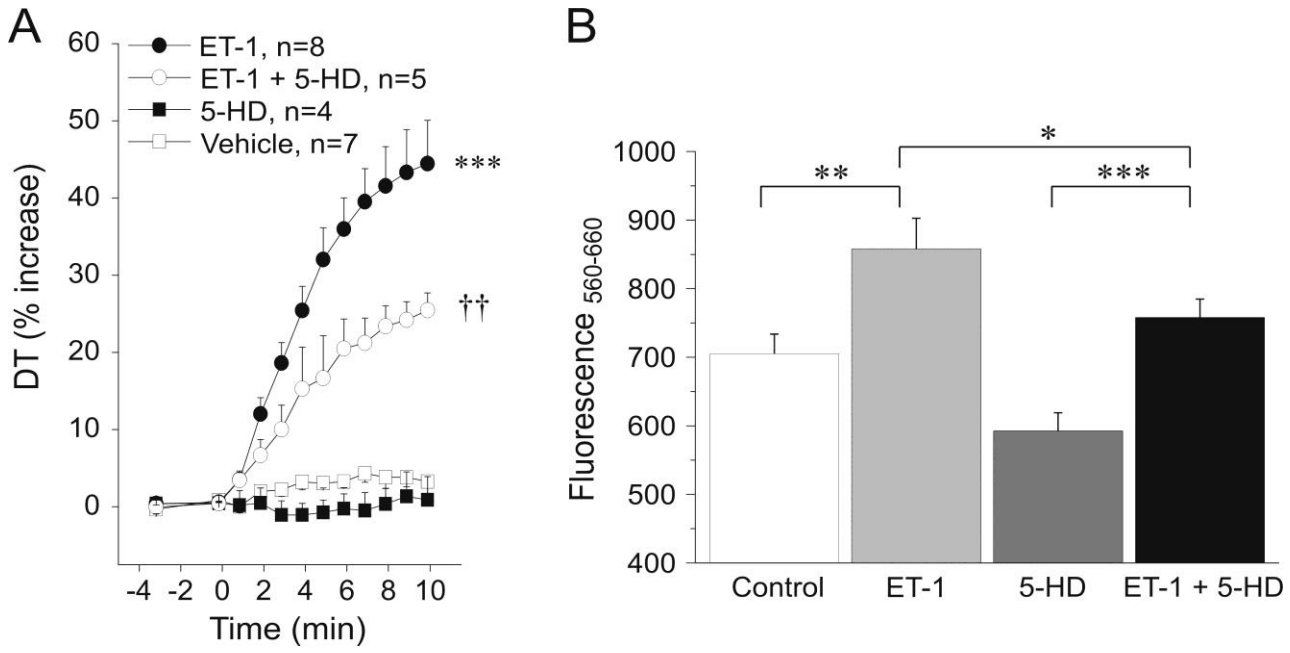

Figure 6: mitoKATP is required for ET-1-induced inotropic effect

A. 5-HD (200 µmol/L), an inhibitor of mitoKATP channels, attenuated ET-1-induced increase in contractility. Results are expressed as a percent change versus baseline values. DT indicates developed tension. Data are mean±SEM. . ***: P<0.001 vs. control; ††: P<0.01 vs. ET-1 by repeated-measures followed by ANOVA LSD post hoc analysis. **B**. Averaged nuclear ethidium fluorescence (560–660 nm) from controls compared to the fluorescence from ET-1, 5-HD or ET-1+5-HD treated ventricular sections (n=4 for each group). Data were analyzed by 1-way ANOVA followed by LSD post hoc test and are reported as mean±SEM. *: P<0.05, **: P<0.01, ***: P<0.001.

5.1.5 Involvement of BK$_{Ca}$ channels but not sarcK$_{ATP}$ channels in ET-1-induced inotropic response

In addition to mitoK$_{ATP}$, we assessed the role of other K^+ channels in the inotropic response to ET-1. The role of mitochondrial large conductance calcium activated potassium channels (BK$_{Ca}$) and sarcolemmal K^+-ATP channels (sarcK$_{ATP}$) in mediating the inotropic response to ET-1 was studied by using the inhibitors paxilline (Cao *et al.*, 2005) and HMR1098 (Gok *et al.*, 2006), respectively. Infusion of paxilline (1 µmol/L) alone did not alter contractility, but it attenuated the ET-1-induced inotropic response by 41% at 10 min time point (P<0.01, Figure 7 A). In contrast, administration of HMR 1098 (3 µmol/L) failed to alter the ET-1- enhanced

contractility (P=NS) Figure 7 B). These data indicate that mitochondrial BK_{Ca} channels, but not $sarcK_{ATP}$ channels, are involved in the response to ET-1.

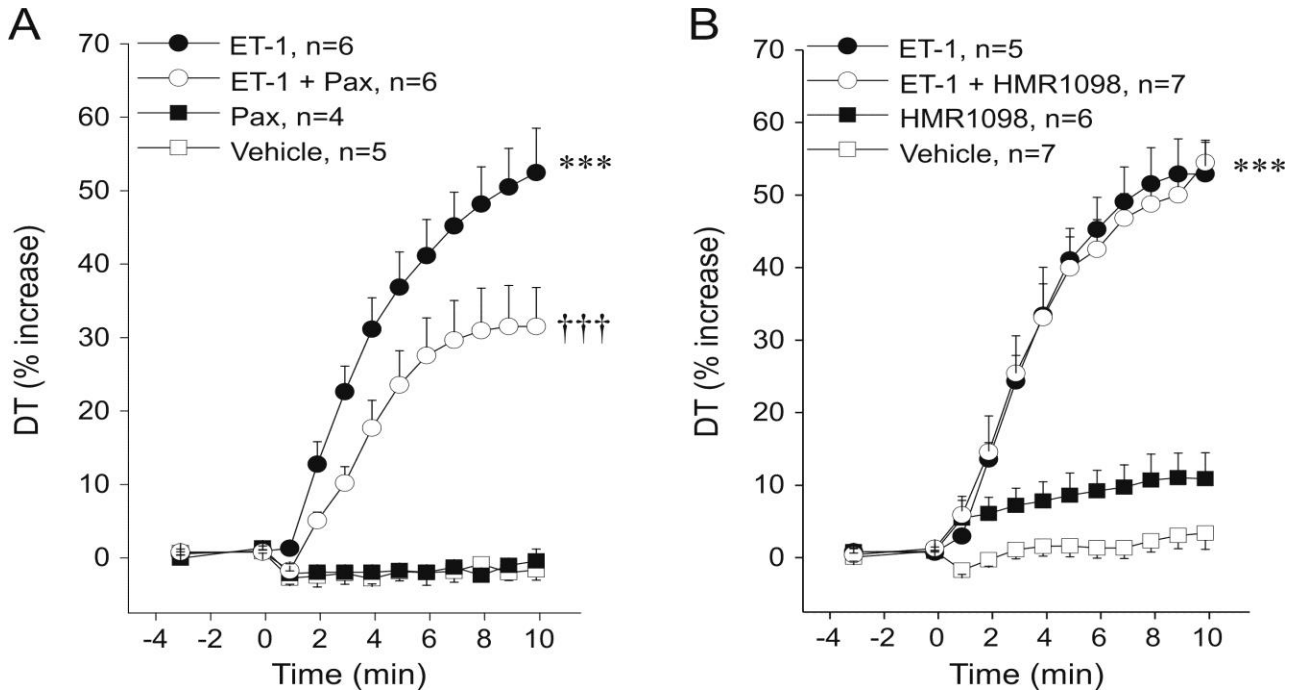

Figure 7: Other K^+ channels' contribution to the inotropic response to ET-1

A. Infusion of the BK_{Ca} channel inhibitor paxilline (Pax) attenuated ET-1-induced increase in contractility. **B.** HMR1098, an inhibitor of $sarcK_{ATP}$ channels, did not alter ET-1-enhanced contractility. Results are expressed as a percent change versus baseline values. Data are mean±SEM. ***: P<0.001 vs. control; ††: P<0.01 vs. ET-1 repeated-measures ANOVA followed by LSD post hoc analysis.

5.1.6 ET-1-stimulated ROS production enhances ERK1/2 phosphorylation

We have recently demonstrated that activation of ERK1/2 plays a crucial role in the positive inotropic effect of ET-1 (Szokodi *et al.*, 2008). Since ERK1/2 phosphorylation has been reported to be redox-sensitive in cultured cardiomyocytes (Tanaka *et al.*, 2001; Clerk and Sugden, 2006),we examined whether ROS modulates ERK1/2 activation in the intact adult rat heart. In agreement with our previous data (Szokodi *et al.*, 2008), administration of ET-1 (1 nmol/L) for 10 min increased phopho-ERK1/2 levels (P<0.001 Figure 8). Administration of N-acetylcysteine (500 μmol/L), MnTMPyP (10 μmol/L) or apocynin (100 μmol/L) significantly attenuated ET-1-induced ERK1/2 phosphorylation (P<0.01, P<0.001 and P<0.05, respectively) (Figure 8 A-C). The inhibitors alone had no effect on the

phosphorylation state of ERK1/2 (P=NS Figure 8 A-C). These results indicate that ROS can act as the upstream activator of the ERK1/2 pathway to mediate the inotropic effect of ET-1.

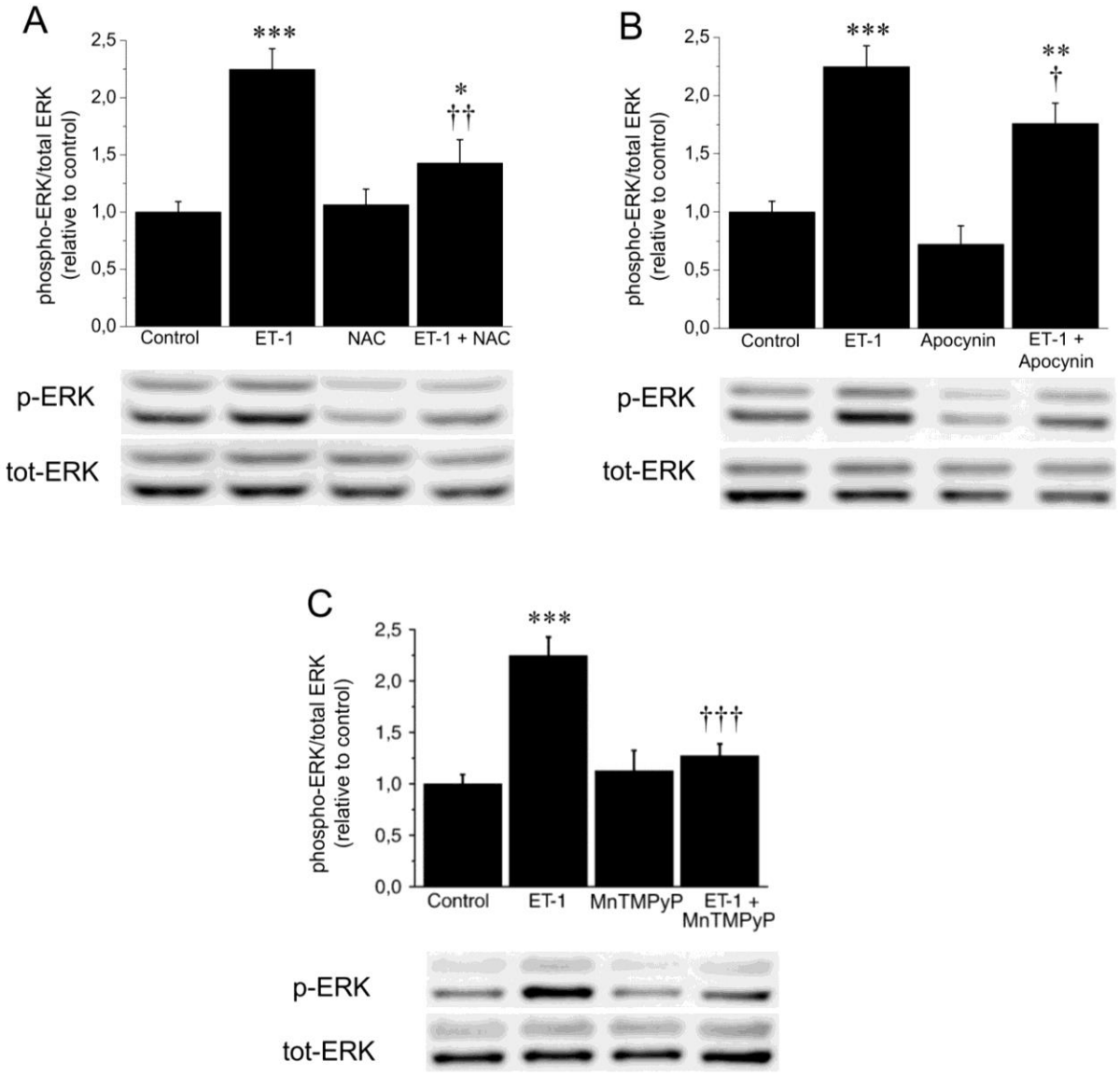

Figure 8: ROS signaling regulates ERK1/2 phosphorylation

A–C. Western blot analysis shows that ET-1-induced increase in ERK1/2 phosphorylation (phospho-ERK/total-ERK) was attenuated by N-acetylcysteine (NAC) (A), apocynin (B) and MnTMPyP (C) in the left ventricles. Data are mean±SEM. Data were analyzed by 1-way ANOVA followed by LSD post hoc test. *: P<0.05, **: P<0.01, ***: P<0.001 vs. control; †: P<0.05, ††: P<0.01, †††: P<0.001 vs. ET-1.

5.2 Characterization of apelin-induced inotropic signaling mechanisms

5.2.1 Positive inotropic effect of apelin is mediated through specific PKCε isoform

In the isolated perfused rat heart preparation, administration of apelin (2 nmol/L) for 20 min induced a slowly developing and sustained increase in cardiac contractility (27±3%, $P<0.001$; Figure 9 A), in line with our former results demonstrating that this apelin isoform has a pronounced inotropic effect in the range of 0.1-10 nmol/L concentration (Szokodi *et al.*, 2002). To test the effect of different apelin isoforms on cardiac contractility, we performed pilot experiments with [Pyr^{1}]apelin-13, apelin-13 and apelin-16, and found that the positive inotropic responses to these isoforms were on the same magnitude in our model (at 2 and 10 nmol/L concentrations, data not shown). These data are in agreement with the findings of Maguire and co-workers demonstrating that [Pyr^{1}]apelin-13, apelin-13 and apelin-36 have comparable potency and efficacy in inducing positive inotropic effect in human paced atrial strips (Maguire *et al.*, 2009).

Our former experiments suggested that apelin may act via PLC–PKC cascade(Szokodi *et al.*, 2002). In line with this, infusion of Bis (90 nmol/L), a selective PKC inhibitor, decreased apelin-induced inotropic response by 42% ($P<0.05$; Figure 9 A), the same inhibitory effect we described previously (Szokodi *et al.*, 2002). Infusion of Bis alone had no effect on contractile force (P=1.0 vs. vehicle; Figure 9 A).

To provide further evidence that PKC contributed to apelin signaling, we examined the activation of PKCα and PKCε, the isoforms most important to the regulation of cardiac contractility (Braz *et al.*, 2004; Kang and Walker, 2005). PKC isoforms show rapid translocation from the soluble to the particulate fraction of the cardiomyocyte upon stimulation (Clerk *et al.*, 1994). When compared to controls, apelin treatment for 5 min produced a significant increase in the particulate partitioning of PKCε in the adult rat LV (Figure 9 B). However, during a more prolonged, 10-min apelin infusion, the subcellular distribution of PKCε returned to those in control hearts (data not shown), suggesting a transient increase in PKCε activation. In contrast to PKCε, no consistent PKCα translocation could be detected upon apelin administration (Figure 9 B and data not shown).

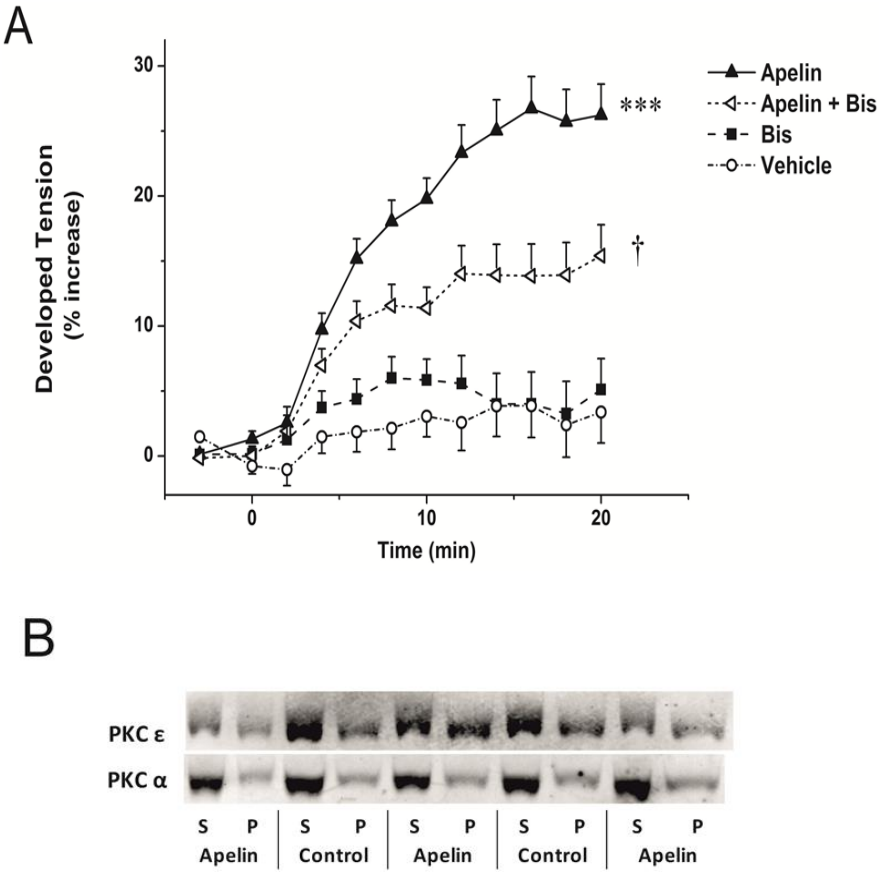

Figure 9: Positive inotropic effect of apelin mediated through PKCε

A. Effect of apelin (Apelin-16 2 nmol/L) and Bis (90 nmol/L) on developed tension in isolated perfused, paced rat hearts. After a control period vehicle or drugs were infused for 20 minutes. Results are expressed as a percent change vs. baseline values. Data are mean ± SEM (n=5). *** P< 0.001 vs. vehicle control, † p< 0.05 vs. apelin by repeated measures ANOVA and Bonferroni post-hoc test. **B.** Representative Western blot detection of translocation of PKCε and α isoforms from the soluble (S) to the particulate (P) fraction of left ventricular proteins in apelin treated and control animals.

5.2.2 Apelin-induced inotropy is mediated through RLC

Our previous findings suggest that apelin exerts its positive inotropic effect primarily through increasing the sensitivity of myofilaments to Ca^{2+} rather than increasing intracellular Ca^{2+} concentrations (Farkasfalvi *et al.*, 2007). Increased phosphorylation of RLC by MLCK leads to an increase in the Ca^{2+} sensitivity of force development and improved cross-bridge kinetics in cardiac myofibrils (Colson *et al.*, 2010).

To examine whether MLCK contributes to the positive inotropic effect of apelin, we used ML-7, a potent and selective inhibitor of MLCK, in the perfused adult rat heart. ML-7 (1 μmol/L) significantly attenuated the inotropic response to apelin, the maximal reduction being 52.5 % (P<0.01). Infusion of ML-7 alone had no significant effect on contractile force when compared to vehicle control (P=1.0; Figure 10 A).

Next, we performed urea-glycerol PAGE to separate phosphorylated and nonphosphorylated RLC in the apelin treated rat LV myocardium.

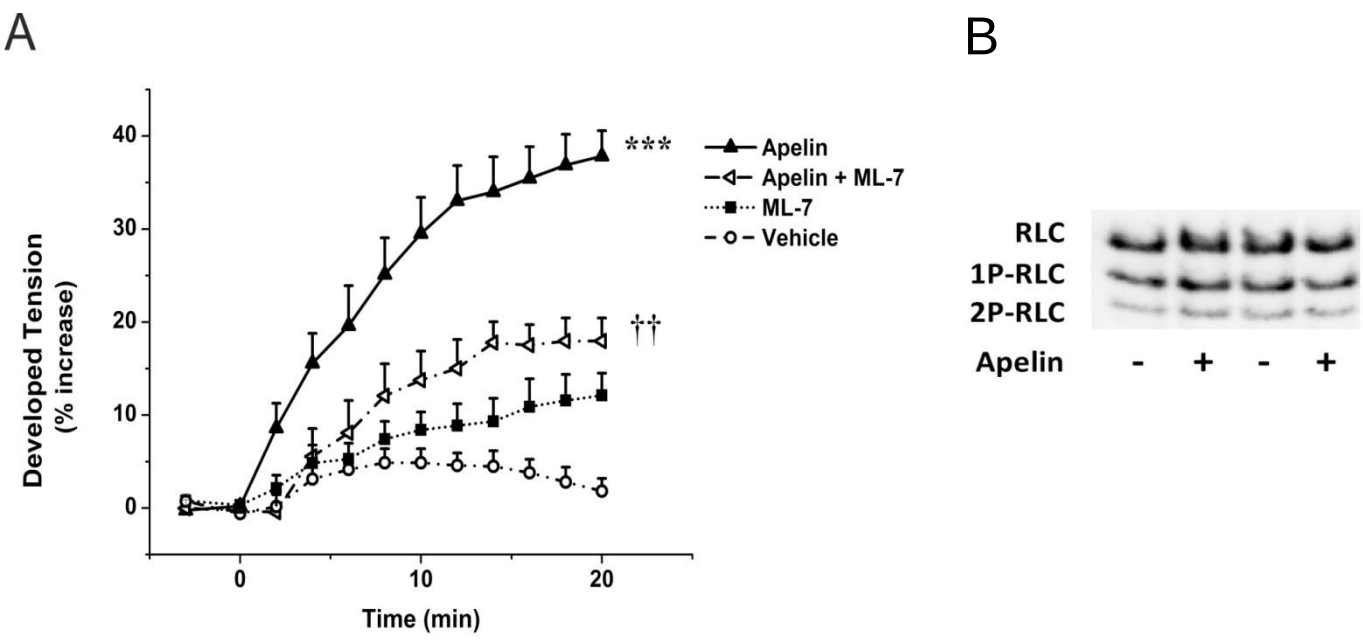

Figure 10: Positive inotropic effect of apelin mediated through RLC

A. Effect of apelin (Apelin-16 2 nmol/l) and ML-7 (1 µmol/l) on developed tension in isolated perfused, paced rat hearts. After a control period vehicle or drugs were infused for 20 minutes. Results are expressed as a percent change vs. baseline values. Data are mean ± SEM (n=5). ***: P< 0.001 vs. vehicle control, ††: P< 0.01 vs. apelin by repeated measures ANOVA and Bonferroni post-hoc test. **B.** Representative Western blot detection of RLC phosphorylation in left ventricular proteins of apelin treated and control animals using non-denaturating urea gel electrophoresis. RLC: non-phosphorylated RLC; 1P-RLC: 1x phosphorylated RLC; 2P-RLC: 2x phosphorylated RLC.

The level of basal RLC phosphorylation was found to be comparable to the results presented by others using the same technique (Hidalgo *et al.*, 2006; Ding *et al.*, 2010), but apelin treatment failed to induce detectable increase in RLC phosphorylation under our experimental conditions (Figure 10 B).

5.2.3 Apelin and MAPK signaling

To explore the potential involvement of MAPK signaling in modulating the inotropic response to apelin, we assessed the apelin-induced alterations in ERK1/2 and p38-MAPK phosphorylation. Immunoblotting revealed that apelin induced a sustained increase in LV ERK1/2 phosphorylation (P<0.01 at 5 min, *P*<0.05 at 10 and 20 min vs. controls), with a maximum increase of 99±23 % at 10 min (Figure 11 A and B). Phosphorylation of p38-MAPK showed a clear but non-significant trend for an increase after 5 min. On the contrary, by 10

min of infusion, apelin significantly decreased p38-MAPK phosphorylation (-65±3 % vs. control, *P*<0.05; Figure 11 C and D).

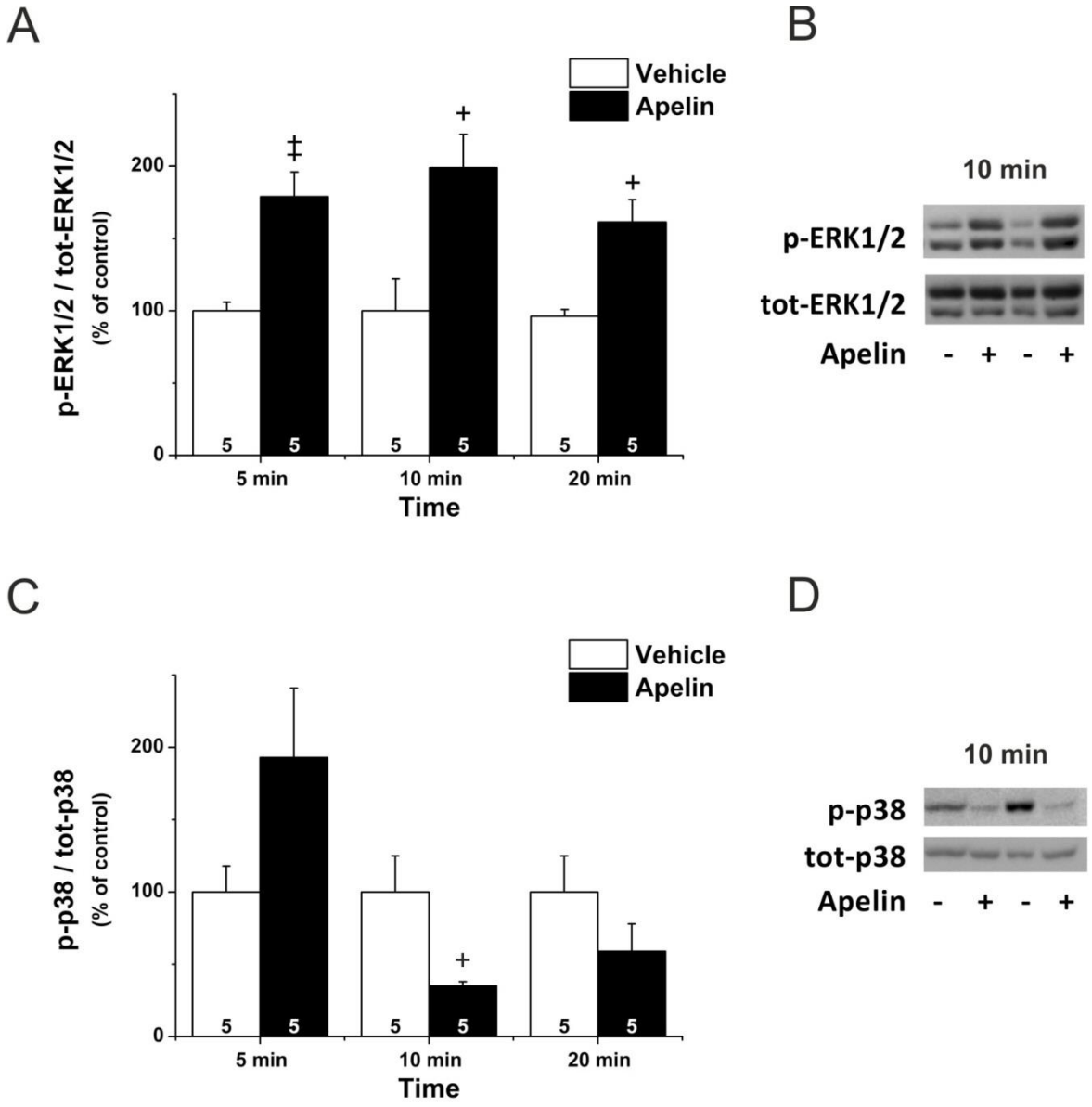

Figure 11: Apelin and MAPK signaling

A. Western blot analysis of time-dependent ERK1/2 phosphorylation in left ventricles. Results in the bar graph are expressed as the ratio of phospho-ERK1/2 (p-ERK 1/2) and total (tot) ERK1/2 in percent of values in vehicle-treated control animals (n=5-6). **B.** ERK1/2 phosphorylation in a representative blot of left ventricular proteins from hearts treated with apelin or vehicle for 10 minutes. **C.** Western blot analysis of time-dependent p38 phosphorylation in left ventricles. Results in the bar graph are expressed as the ratio of phospho-p38 (p-p38) and total (tot) p38 in percent of values in vehicle-treated control animals (n=4-5). **D.** p38 phosphorylation in a representative blot of left ventricular proteins from hearts treated with apelin or vehicle for 10 minutes. **+**: P< 0.05 vs. vehicle, ‡: P< 0.01 vs. vehicle by unpaired Student's t test.

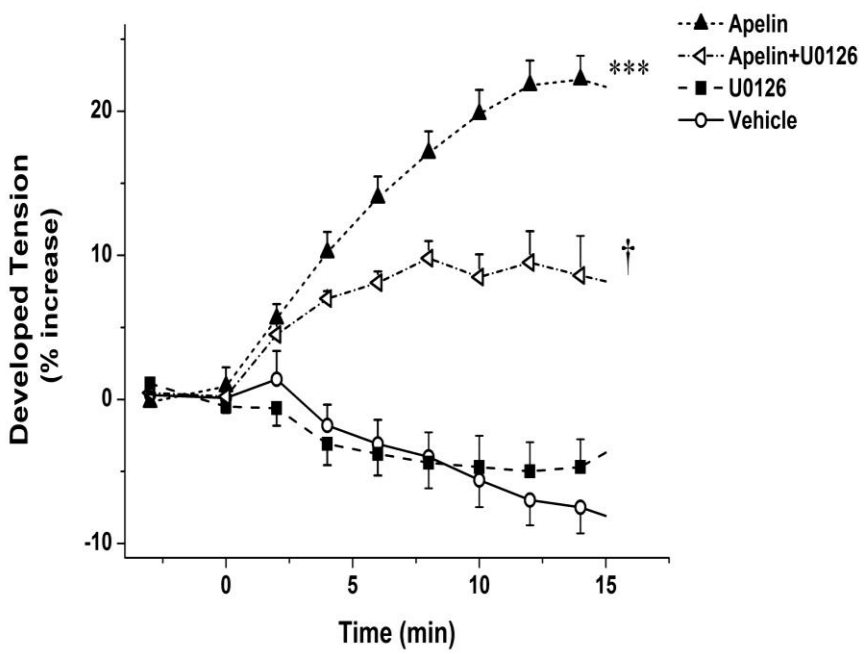

Figure 12: Positive inotropic effect of apelin mediated through ERK1/2

Effect of apelin (Apelin-16 12 nmol/l) and U0126 (5 µmol/l) on developed tension in isolated perfused, paced rat hearts. After a control period vehicle or drugs were infused for 15 minutes. Results are expressed as a percent change vs. baseline values. Data are mean ± SEM (n=4). ***: P< 0.001 vs. vehicle, †: P< 0.5 vs. apelin by repeated measures ANOVA and Bonferroni post-hoc test.

To demonstrate that ERK1/2 activation is necessary to the development of apelin-induced inotropic response, we used U0126, which is a potent selective inhibitor of MAPK kinases 1 and 2 (MEK1/2), the upstream regulator of ERK1/2. The inotropic effect of apelin was significantly attenuated by U0126 (5 µmol/L), the maximal reduction being 56 % ($P<0.05$). Infusion of U0126 alone had no significant effect on contractile force ($P=1.0$; Figure 12). Immunoblotting of LV lysates showed that U0126 almost completely abolished ERK1/2 phosphorylation after 15 min of perfusion, either administered alone (31±15 % of control, $P<0.01$) or in combination with apelin (4±8 % of the apelin-treated group, $P<0.001$; Figure 13 A and B).

Particulate partitioning of PKCε in neonatal rat ventricular myocytes is accompanied by subsequent activation of ERK1/2 (Heidkamp et al., 2001). Since apelin significantly increased PKCε translocation and ERK1/2 phosphorylation in the intact rat heart, we examined whether PKC is an upstream activator of ERK1/2 in apelin signaling. Interestingly, we found that the PKC inhibitor Bis, which potently attenuated the apelin-enhanced contractility, had no effect on the apelin-induced increase in ERK1/2 phosphorylation (Figure 14 A and B), demonstrating that ERK1/2 and PKC represent independent pathways mediating the inotropic effect of apelin.

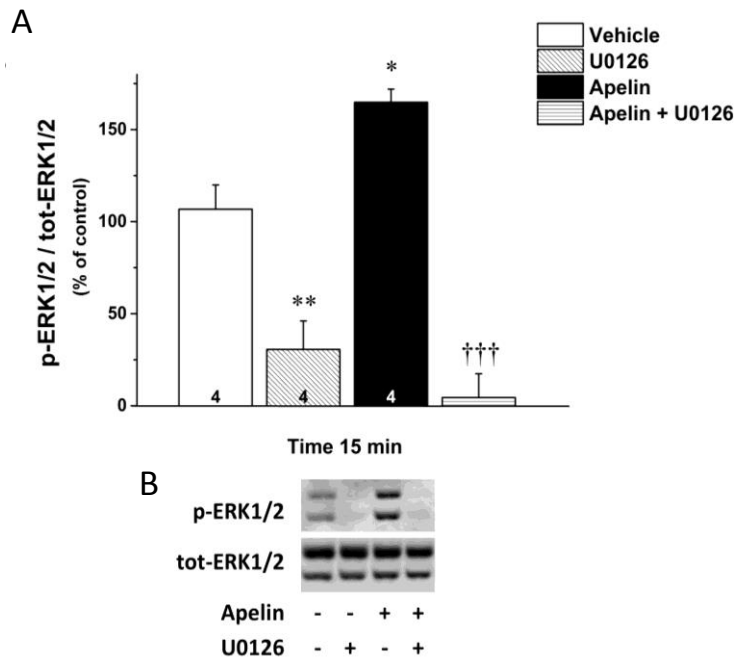

Figure 13: Apelin-mediated ERK1/2 activation

A. Western blot analysis of ERK 1/2 phosphorylation in left ventricles of hearts treated with vehicle, apelin and U0126, or their combination for 15 minutes. Results in the bar graph are expressed as the ratio of phospho-ERK1/2 (p-ERK 1/2) and total (tot) ERK1/2 (n=5). **B.** ERK1/2 phosphorylation in a representative blot of left ventricular proteins from hearts treated with vehicle, apelin and U126, or their combination for 15 minutes. *: P< 0.5; **: P< 0.01 vs. vehicle, †††: P< 0.001 vs. apelin by one-way ANOVA and Bonferroni post-hoc test.

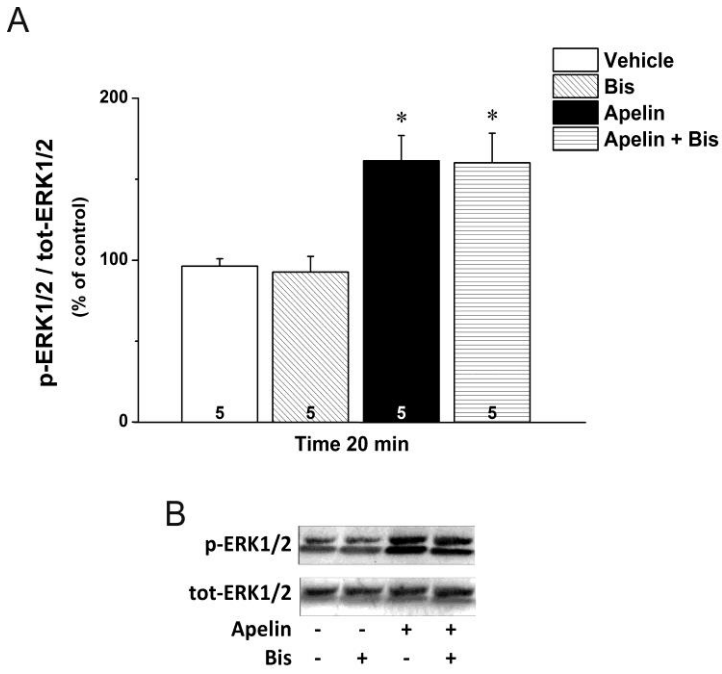

Figure 14: Relation of apelin-induced ERK1/2 phosphorylation to PKCs

A. Western blot analysis of ERK 1/2 phosphorylation in left ventricles of hearts treated with vehicle, apelin and Bis, or their combination for 20 minutes. Results in the bar graph are expressed as the ratio of phospho-ERK1/2 (p-ERK 1/2) and total (tot) ERK1/2 (n=5). *: $p < 0.05$ vs. vehicle by one-way ANOVA followed by a Bonferroni post hoc test. **B.** ERK1/2 phosphorylation in a representative blot of left ventricular proteins from hearts treated with vehicle, apelin and Bis, or their combination for 20 minutes.

6 DISCUSSION

6.1 ET-1 and ROS

We provide here evidence that ROS are critically involved in the acute regulation of cardiac contractility in the intact rat heart. Our results show that ET-1, which activates ERK1/2−p90 ribosomal S6 kinase−Na^+-H^+ exchanger-1 pathway (Szokodi *et al.*, 2008), enhances cardiac contractility in part via increased ROS generation. These data strongly support the hypothesis that ROS serve as signaling molecules in the modulation of cardiac function in a physiological milieu.

Prior studies have produced conflicting results regarding the role of ROS and ET-1 in the regulation of contractile function in isolated cardiomyocytes. Leptin has been reported to suppress contractility acting through ET-1 receptors and increased ROS formation in murine ventricular myocytes (Dong *et al.*, 2006), while angiotensin II induces ET-1 release and an increase in ROS generation, which in turn triggered an increase in contractility in cat cardiomyocytes (Cingolani *et al.*, 2006). Moreover, it has been suggested that the positive inotropic effect of exogenous ET-1 is almost exclusively dependent on ROS production in this model (De Giusti *et al.*, 2008). Our results demonstrate that ROS can partially mediate the ET-1-induced increase in contractile force in the intact adult rat heart. Acute administration of ET-1 enhanced ROS production, measured by oxidation of dihydroethidium to ethidium, a reaction primarily dependent on intracellular levels of $O_2^{\bullet -}$ (Sovershaev *et al.*, 2006). Moreover, the antioxidant N-acetylcysteine prevented the ET-1-induced increase in ethidium fluorescence. Importantly, the inotropic response to ET-1 was significantly attenuated by the ROS scavengers N-acetylcysteine and MnTMPyP.

6.1.1 Role of NAD(P)H and mitochondrial K^+ channels

The NAD(P)H oxidase family of enzymes is a major source of $O_2^{-\bullet}$ in the myocardium (Geiszt, 2006; Murdoch *et al.*, 2006). G_qPCR ligands like Ang II and ET-1 are known activators of NAD(P)H oxidases (Callera *et al.*, 2003; Giordano, 2005; Kimura *et al.*, 2005). Notably, our data suggest that NAD(P)H oxidase-derived ROS partially mediate the contractile response, because the ET-1-induced increase in contractility and ethidium

fluorescence was markedly suppressed by a NAD(P)H oxidase inhibitor apocynin. Moreover, a superoxide dismutase (SOD) mimetic had similar effect on cardiac contractility as the NAD(P)H oxidase inhibitor, proposing that $O_2\bullet^-$ is far more relevant in mediating the inotropic response than H_2O_2. (Superoxide dismutase generates H_2O_2 from $O_2\bullet^-$, therefore a SOD mimetic would reduce $O_2\bullet^-$ levels.)

NAD(P)H oxidase-derived ROS may trigger a larger release of ROS from the mitochondria via opening the mitoK_{ATP} (Kimura *et al.*, 2005), the phenomenon called "ROS-induced ROS release" (Zorov *et al.*, 2006). Mitochondrial ROS formation after mitoK_{ATP} opening was demonstrated by several studies (Pain *et al.*, 2000; Oldenburg, 2003; Kimura *et al.*, 2005). Andrukhiv et al. have shown that an increase in mitochondrial matrix pH, induced by mitochondrial K^+ influx through mitoK_{ATP}, is responsible for this effect. Moreover, it has been suggested that $O_2\bullet^-$ is produced in complex I of the electron transport chain after mitoK_{ATP} opening (Andrukhiv *et al.*, 2006). It is well established that mitoK_{ATP} play a crucial role in cardioprotection against ischemia–reperfusion injury (Pain *et al.*, 2000; Forbes *et al.*, 2001). However, the physiological function of mitoK_{ATP} in the heart is still elusive. Tian et al. have demonstrated the contribution of mitoK_{ATP} opening and increased mitochondrial ROS formation to the inotropic action of ouabain in isolated rat cardiomyocytes (Tian *et al.*, 2003). In our experiments, 5-HD, a mitoK_{ATP} blocker, significantly attenuated the inotropic response to ET-1 in intact rat myocardium. Interestingly, ROS measurements revealed that 5-HD had minimal effect on ET-1-induced increase in ethidium fluorescence, indicating that the involvement of mitoK_{ATP} in the inotropic effect of ET-1 is likely to be independent of ROS generation. In contrast, De Giusti et al have suggested that ET-1–induced increase in contractility is dependent on mitochondrial ROS formation. In cat ventricular myocytes, both ET-1–induced $O_2^-\bullet$ production and positive inotropic response was abolished by the non-selective K_{ATP} channel blocker glibenclamide. Notably, the selective mitoK_{ATP} blocker 5-HD markedly attenuated the positive inotropic action of ET-1, while it had no statistically significant effect on ROS formation (De Giusti *et al.*, 2008). Garlid et al. have reported that mitoK_{ATP} inhibition decreases the ability of the heart to respond to inotropic stress induced by dobutamine, ouabain or calcium (Garlid *et al.*, 2006). They have proposed that the opening of mitoK_{ATP} adds a parallel K^+ conductance to prevent stress-induced contraction of mitochondrial matrix volume and expansion of intermembrane space volume, thereby

maintaining efficient energy transfer between mitochondria and cytosol. The hypothesis is that mitochondrial matrix K^+ influx is crucial for an appropriate response to positive inotropic stress (Garlid *et al.*, 2006). Moreover, our results demonstrating that the mitochondrial BK_{Ca} channel inhibitor paxilline, but not sarcK_{ATP} channel inhibitor HMR1098, attenuated the response to ET-1, support the hypothesis that mitochondrial matrix K^+ influx is crucial for an appropriate response to positive inotropic stress. The observation that ROS can induce the opening of mitoK_{ATP} in isolated rat heart mitochondria (Queliconi *et al.*, 2011), raises the intriguing possibility that NAD(P)H oxidase-derived ROS may orchestrate the activation of these channels to maintain a high-work state of the myocardium. Whether such mechanism may operate under physiological conditions, remains to be established.

6.1.2 ROS and signaling

Our group demonstrated previously that activation of the ERK1/2−p90RSK−NHE1 pathway plays a critical role in the positive inotropic effect of ET-1 (Szokodi *et al.*, 2008). Our recent results indicate a mainly redox-sensitive activation of ERK1/2 in the intact adult rat heart, because the ET-1−induced ERK1/2 phosphorylation was markedly suppressed by ROS scavengers and inhibition of NAD(P)H oxidases. GPCR-dependent activation of the Raf–MEK1/2–ERK1/2 cascade can occur through multiple mechanisms (Wetzker and Böhmer, 2003; Clerk and Sugden, 2006). For instance, G_q-mediated PKC activation can stimulate Raf, the first member of the ERK1/2 cascade. EGFR transactivation, which is an alternative mechanism that couples GPCRs and ERK1/2 activation (Wetzker and Böhmer, 2003), contributes to the ET-1-induced increase in contractility, acting as a proximal component of MEK1/2–ERK1/2 signaling (Szokodi *et al.*, 2008). 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 mechanisms (Wetzker and Böhmer, 2003; Clerk and Sugden, 2006). Moreover, ROS can also enhance Ras activity, via direct modifications of redox-sensitive cysteine residues (specifically Cys118), leading to activation of the Raf–MEK1/2–ERK1/2 pathway (Clerk and Sugden, 2006; Pimentel *et al.*, 2006). Additionally ROS can directly activate G proteins. The βγ-subunit liberated by that activation can initiate ERK activation (Nishida *et al.*, 2000). According to this finding, one may speculate that ET-1−induced ROS production may have a feedback effect on G proteins linked to ET receptor to increase ERK signaling. ET-1−enhanced endogenous ROS production

may facilitate NHE activity via increased phosphorylation of ERK1/2 and p90RSK. Consequent alkalization can directly enhance myofibrillar Ca^{2+} sensitivity, but the increased Na^{+} influx can also trigger the reverse-mode function of NCX, thereby increasing Ca^{2+} influx. There is evidence that NCX activity can be directly modulated by free radicals, although the involvement of ROS in the reverse-mode NCX activation remains on a speculative level (Reeves *et al.*, 1986; Goldhaber, 1996). Ca^{2+} influxes could be modified by ROS in another way too: ET-1 has been reported to increase L-type Ca^{2+} channel open-state probability via ET_A receptors in isolated rat cardiac myocytes and this effect was significantly attenuated by antioxidants or NAD(P)H oxidase inhibition. These data demonstrate a mechanism of activation of Ca^{2+} influx via stimulation of NAD(P)H-derived $O_2\bullet^-$ production (Zeng *et al.*, 2008), which can also contribute to the ROS-dependent positive inotropic effect of ET-1. For a summary of ROS influence on ET-1 signaling, please check Figure 15.

The diversity of previous (Cingolani *et al.*, 2006; Dong *et al.*, 2006; De Giusti *et al.*, 2008) and present findings may be related to a number of factors. ET-1 has been shown to increase contractility in several species such as rat, rabbit, cat, guinea pig and human(Brunner *et al.*, 2006; Endoh, 2006). However, ET-1 can elicit both positive and negative inotropic effects in murine models. ET-1 induces a negative inotropic effect in isolated mouse cardiomyocytes (Nishimaru *et al.*, 2007), in line with the findings of Dong et al. (Dong *et al.*, 2006). In contrast, ET-1 has been shown to increase contractility in perfused whole heart preparations using the same strain of mice (Piuhola *et al.*, 2003a).Therefore, in addition to species differences, the experimental conditions (single cardiomyocytes vs. multicellular preparations, temperature, pacing frequency, loading conditions, etc.) seem to have a major influence on the response to ET-1.

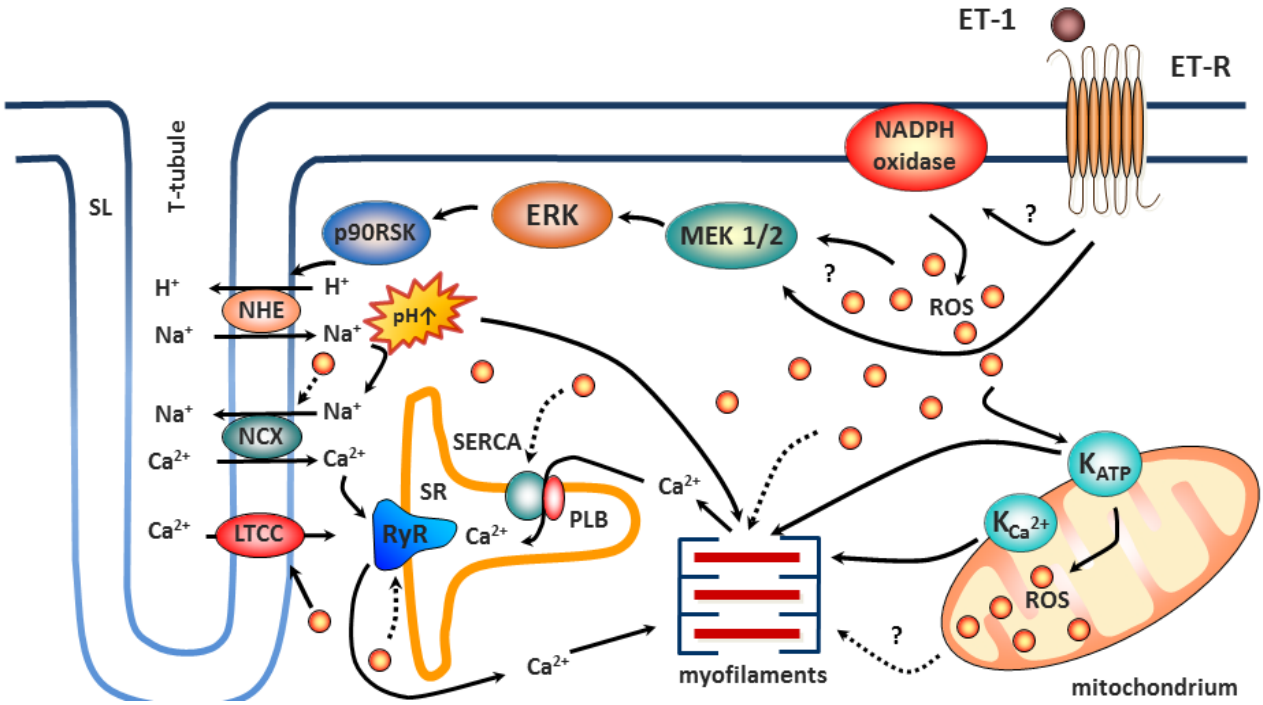

Figure 15: Role of ROS in the modulation of the positive inotropic response of ET-1

ET-1 activates the MEK1/2–ERK1/2–p90RSK pathway via G protein-coupled receptor (ET-R). Sarcolemmal (SL) NHE is activated by this pathway, resulting in increased NCX activity in its reverse mode. Opening of mitoK$_{ATP}$ (K_{ATP}) and BK$_{Ca}$ (K_{Ca}) channel also contribute to the ET-1–induced inotropy. Endogenous ROS production is enhanced after ET-1 by NAD(P)H oxidase activation and by increased mitochondrial ROS formation. There is evidence for ROS dependent ERK1/2 and LTCC activation during ET-1 stimulus. Modulation of the sarcoplasmic reticulum (SR) Ca^{2+} channel (RyR) and SERCA theoretically is possible by ROS while phospholamban (PLB) seemed to be unaffected during ET-1 signaling. Black lines mean activation. Continuous lines show experimentally proven pathways, dotted lines show speculated ones. Question marks refer to unknown way of action.

Strikingly, we have found that the inotropic responses to ET-1 and dobutamine were differentially regulated by inhibiting NAD(P)H oxidase-dependent ROS production under identical *ex vivo* experimental conditions (Kubin *et al.*, 2011). Our present 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. Of note, phospholamban phosphorylation has no major role in the positive inotropic effect of ET-1 (Szokodi *et al.*, 2008), while dobutamine failed to activate ERK1/2 (Kubin *et al.*, 2011). The impact of NAD(P)H oxidase on cardiomyocyte contractile function is similarly controversial *in vitro*, as, depending on the

stimulus, species and experimental conditions, NAD(P)H oxidase inhibition can abolish positive inotropic effect (De Giusti *et al.*, 2008), or prevents decrease of contractile function (Dong *et al.*, 2006; Li *et al.*, 2008). It is an intriguing question, how specific downstream events can be achieved via a seemingly identical upstream trigger. NAD(P)H oxidase is a multi-subunit enzyme with five oxidase isoforms, which are distinguished by the presence of distinct catalytic subunits (Nox1-Nox5). In addition, the enzymatic activity of the oxidase depends on additional protein subunits, which vary according to the isoform (Akki *et al.*, 2009). Thus, various agonists may activate distinct NAD(P)H oxidase isoforms at discrete subcellular locations with dissimilar temporal pattern (transient versus sustained activation). In association with spatiotemporal variations, large differences may exist in the amount of ROS produced by different agonists, which in turn may activate/inhibit divergent downstream signaling molecules resulting in differing functional responses.

6.2 The inotropic signaling of apelin

Apelin is among the most potent endogenous inotropes yet identified (Szokodi *et al.*, 2002); however, the cellular mechanisms underlying its inotropic effect are not fully clarified. This study provides several important findings regarding the signaling network activated by apelin in the adult rat heart. The present results demonstrate that pharmacological inhibition of PKC significantly reduces the positive inotropic effect of apelin, confirming previous data from our (Szokodi *et al.*, 2002) and other laboratories (Wang *et al.*, 2008). The PKC family consists of a variety of isoenzymes, e.g. classical (α, βI, βII, and γ), novel (δ, ε, θ, and η) and atypical PKCs (ζ, ι/λ). Individual isoenzymes can have different, even opposing functions (Churchill *et al.*, 2008) and they are each localized to distinct subcellular sites following activation(Mochly-Rosen *et al.*, 1990). Various PKC isoforms are considered to regulate cardiac contractility (Braz *et al.*, 2004; Kang and Walker, 2005). However, the exact PKC isoenzyme that contributes to the apelin-induced contractile response has not been identified yet. Our present data indicate that apelin promotes PKCε but not PKCα translocation to the particulate fraction. Specific PKCε anchoring proteins are localized at the Z-lines and intercalated discs in cardiomyocyte (Robia *et al.*, 2001). Upon activation, PKCε is known to accumulate in these very specific regions of ventricular myocytes, resulting in a strong positive inotropic effect (O-Uchi *et al.*,

2008). These findings locate activated PKCε to the close vicinity of apelin receptor, the cognate receptor of apelin (Farkasfalvi *et al.*, 2007).

RLC controls myofilament cross-bridge properties and thereby modulates the force of contractions in the heart. RLC is phosphorylated by the cardiac MLCK (Ding *et al.*, 2010) which is counterbalanced by the activity of myosin light chain phosphatase (Rajashree *et al.*, 2005). Increased RLC phosphorylation results in an increase of the Ca^{2+} sensitivity of myofilaments (Colson *et al.*, 2010). The phosphate turnover rate of cardiac RLC is much slower than that of skeletal or smooth muscle cells, suggesting that cardiac RLC plays a sustained, fine-tuning role in adjusting the kinetic properties of the contractions (Kamm and Stull, 2011). Since the force development in response to apelin is comparable in timescale to that of RLC phosphorylation in the heart, one may speculate that apelin improves myofilament function through activation of MLCK. In line with that, we demonstrate here that MLCK inhibition diminishes the apelin-enhanced contractility. The pharmacological inhibitor used here is known to act with high selectivity on MLCK, an enzyme that functions solely as a kinase for RLC. It has been reported that ML-7 inhibits sarcomeric organization in rat cardiomyocytes in a similar fashion to cardiac MLCK RNA interference (Gu *et al.*, 2010) and reduces RLC phosphorylation in isolated rat ventricular strips (Riise *et al.*, 2008). The 1 μmol/L ML-7 dose applied in our experiments provide even higher selectivity to MLCK than the 10 or 20 μmol/L doses used by the above mentioned studies, based on the Kinase Inhibitor Database of the MRC Protein Phosphorylation Unit at Dundee (http://www.kinase-screen.mrc.ac.uk/kinase-inhibitors). Therefore it is plausible to assume that the apelin-mediated increase in cardiac contractility is partly dependent on MLCK activation. Nevertheless, no significant apelin-induced increase in RLC phosphorylation was detected by urea-glycerol PAGE. One should consider, however, that given the rate of approximately 40 % of RLC phosphorylation under basal physiological conditions (Kamm and Stull, 2011), only modest increase in phosphorylation is conceivable. Still, a subtle change can be sufficient to have a significant effect on contractility. It was demonstrated in isolated rat papillary muscles that even a less than 10 % increase in the overall RLC phosphorylation level can be attributed to a 70 % increase in contractile force (Riise *et al.*, 2008). One limitation of the current study is that such small changes may remain undetectable in the intact heart under our *ex vivo* experimental conditions.

The exact mechanisms of cardiac MLCK activation remain elusive. Contrasting smooth- and skeletal muscle isoforms, cardiac MLCK was found to be Ca^{2+}/calmodulin-independent. On the other hand, potential phosphorylation sites for PKC were identified on cardiac MLCK (Chan *et al.*, 2008). Some studies demonstrated PKC-dependent RLC phosphorylation in the heart (Venema *et al.*, 1993; Kanaya *et al.*, 2003), but others provided evidence challenging the role of PKC in triggering RLC regulation (Russell and Molenaar, 2004; Grimm *et al.*, 2006). Although MLCK is controlled remarkably differently in cardiac and smooth muscle tissues, it is noteworthy that the inhibition of PKC markedly attenuated the apelin-induced RLC phosphorylation in vascular smooth muscle cells (Hashimoto *et al.*, 2006). Therefore, cardiac MLCK and RLC are potential downstream targets of PKC, mediating apelin-triggered positive inotropic response.

The MAPKs are well known regulators of diverse processes in the heart under physiological and pathophysiological conditions (Rose *et al.*, 2010), but only a few reports demonstrated that MAPKs can regulate cardiac contractility (Liao *et al.*, 2002; Szokodi *et al.*, 2008). Our study provides evidence that apelin activates ERK1/2 in the myocardium, and suppression of ERK1/2 signaling significantly attenuates the apelin-mediated increase in the contractile force. Previously we have demonstrated that activation of NHE contributes to the inotropic effect of apelin (Szokodi *et al.*, 2002; Farkasfalvi *et al.*, 2007). Since ERK1/2 is a recognized activator of NHE (Malo *et al.*, 2007), we propose here a functional ERK1/2-NHE axis in apelin signaling. ERK1/2 can be activated, among many others, by PKCs (Heidkamp *et al.*, 2001). Knowing that PKC is involved in the inotropic effect of apelin, one can speculate that PKC is an upstream regulator of ERK1/2. Our finding, that PKC inhibition, which is sufficient to reduce the inotropic response to apelin, does not decrease apelin-induced ERK1/2 phosphorylation indicates that apelin activates ERK1/2 via a PKC-independent mechanism. Of note, approximately 50 % of the inotropic response to apelin remained unaffected even if ERK1/2 phosphorylation was practically undetectable. Thus, PKC and ERK1/2 are parallel and independent signaling pathways mediating the effect of apelin on cardiac contractility. In contrast to ERK1/2, apelin significantly reduced p38-MAPK phosphorylation in the intact rat heart. Activation of p38-MAPK appears to have an important homeostatic function by counterbalancing excess inotropic stimulation. β_2-adrenergic receptor or ET receptor stimulation increases p38-MAPK activation, and pharmacological inhibition of p38-MAPK

activation augments β_2-adrenergic receptor - or ET-mediated increases in cardiac contractility (Zheng *et al.*, 2000; Szokodi *et al.*, 2008). Moreover, p38-MAPK activation has a crucial role in delivering the negative inotropic effect of tumor necrosis factor- α (Bellahcene *et al.*, 2006). However, the impact of reduced p38-MAPK activity on cardiac contractility is controversial. Some studies suggest that pharmacological (Szokodi *et al.*, 2008) or genetic inactivation of p38-MAPK (Liao *et al.*, 2002) or its upstream kinase MKK3 (Bellahcene *et al.*, 2006) alone had no effect on baseline cardiac contractility, whereas others propose that reducing p38-MAPK activity by chemical or genetic approaches may indeed augment contractile force (Liao *et al.*, 2002; Kaikkonen *et al.*, 2014). Whether the observed decrease in p38-MAPK phosphorylation may contribute to the apelin-mediated increase in cardiac contractility remains to be defined.

Accumulating lines of evidence suggest that the activation of PKCε and MEK1/2–ERK1/2 cascades constitute important adaptive mechanisms in the myocardium under pathological conditions. PKCε and ERK1/2 signaling have been reported to confer cardioprotection in vivo against ischemia-reperfusion injury by reducing cell death (Dorn *et al.*, 1999; Lips *et al.*, 2004). Using a genetic model, loss of apelin exacerbated myocardial ischemia-reperfusion injury associated with compromised activation of the MEK1/2–ERK1/2 signaling pathway (Wang *et al.*, 2013). In addition to regulating cell survival, PKCε and ERK1/2 also control the pattern of LV remodeling. Inhibition of PKCε translocation triggered LV enlargement and wall thinning with depressed contractile function in $G\alpha_q$-overexpressing mice. Reciprocally, enhanced PKCε activation resulted in a more favorable LV geometry with improved LV performance in $G\alpha_q$ mice, displaying concentric instead of eccentric remodeling (Wu *et al.*, 2000). Moreover, it has recently been demonstrated in genetically modified mice that the MEK1/2–ERK1/2 signaling pathway directly regulates the balance between eccentric and concentric growth of the heart. Constitutive ERK1/2 activation promotes concentric cardiomyocyte hypertrophy, whereas ERK1/2 deficiency leads to pronounced eccentric hypertrophy in response to increased mechanical load or neurohumoral stimulation (Kehat *et al.*, 2011). Although the underlying signaling mechanisms remain to be explored, apelin deficiency in chronic pressure overload resulted in severe heart failure characterized by LV dilation and impaired cardiac performance (Kuba *et al.*, 2007). Our current results raise the intriguing possibility that stimulation of the apelin–apelin receptor system with concomitant activation of ERK1/2 and PKCε signaling, besides inhibiting eccentric growth and apoptosis, may also alleviate hemodynamic stress in

the injured heart by directly improving cardiac contractility. For summary, please check Figure 16.

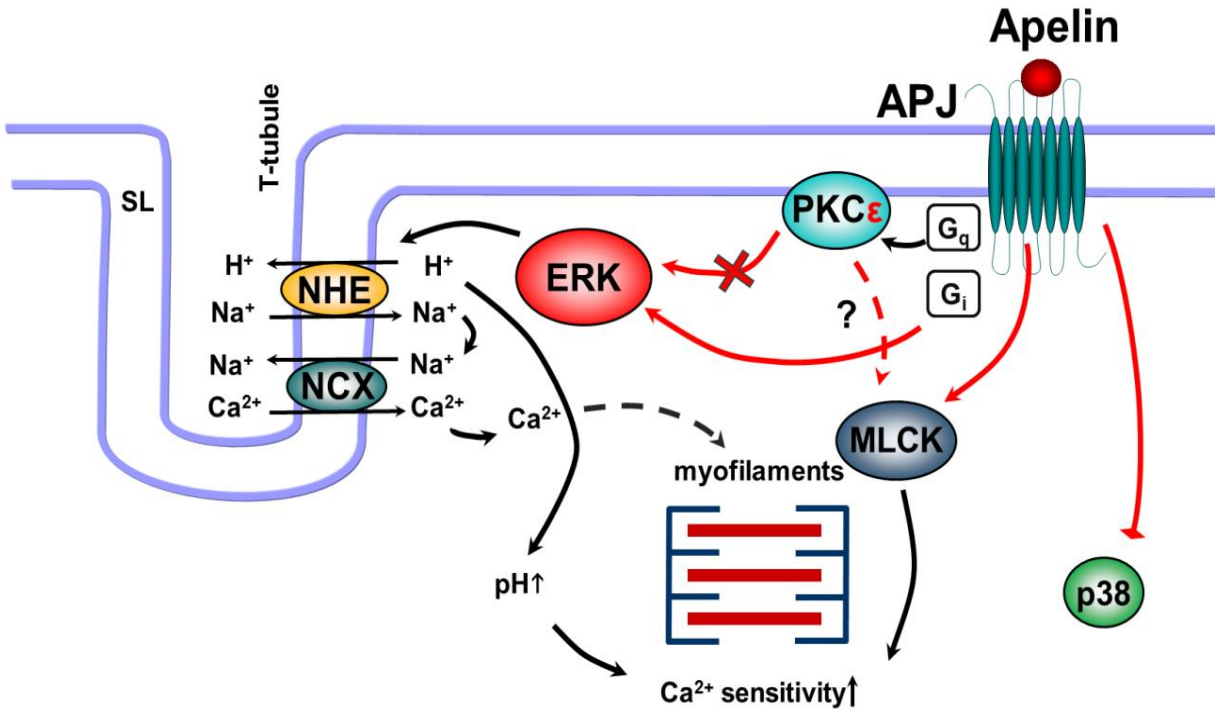

Figure 16: Summary figure of identified inotropic signaling mechanisms of apelin.
Red continuous lines show newly identified pathways, question mark refers to hypothetical connection. Pointed arrows represent activation, blunt-ending line shows inhibition.

6.3 Perspectives of our research

6.3.1 ROS

The concept that excessive levels of ROS contribute to the development of contractile dysfunction in various pathophysiological states, including congestive heart failure, is well accepted. Therefore, there are numerous attempts to introduce antioxidants as therapeutic agents both in clinical trial and also by the nutrition supplement industry. However, emerging body of evidence suggests important role of endogenously generated ROS to regulate cardiac function under physiological conditions. Recent studies, besides ours, have proved that endogenous ROS production can modulate the inotropic response to ET-1 (De Giusti *et al.*, 2008), β-AR stimulation (Mak and Newton, 2001; Andersson *et al.*, 2011; Kubin *et al.*, 2011),

Ang II (Cingolani *et al.*, 2006; Palomeque *et al.*, 2006), leptin (Dong *et al.*, 2006), and transforming growth factor-β_1 (Li *et al.*, 2008). Moreover, it has been shown that ROS-dependent mechanisms are crucial for the development of the slow force response (Anrep effect) to stretch (Caldiz *et al.*, 2007; Villa-Abrille *et al.*, 2010). Recognition of the complex role played by ROS in physiological regulation may provide an explanation why antioxidant treatment failed to improve cardiac function in heart failure patients in clinical trials (Yusuf *et al.*, 2000; Devaraj and Jialal, 2005; Hare *et al.*, 2008), despite substantial evidence for the detrimental effects of ROS. Those trials underline the importance of understanding the mechanisms related to endogenous ROS production and their precise role in cardiac signaling under both physiological and pathophysiological conditions.

Although there is an enormous literature on the role of ROS in ischemia and reperfusion injury and in the development of chronic heart failure, little is known about regulatory function of ROS on beat-to-beat contractions under these pathological conditions. It is reported though that β-adrenergic inotropy was enhanced by antioxidant treatment in stunned myocardium of isolated working guinea pig hearts (Tejero-Taldo *et al.*, 1999). The multiple effects that ROS exert in cardiovascular disease states make it difficult to study the pure inotropic potential in many cases, since ROS modifies contractile function also via triggering cardiac hypertrophy and remodeling. The existing data are also quite controversial. Shinke et al. reported that acute administration of the antioxidant vitamin C enhanced the contractile response to dobutamine and improved myocardial efficiency in patients with moderate heart failure (Shinke *et al.*, 2007), which is in line with our *ex vivo* findings with β-stimulation and ROS-inhibition (Kubin *et al.*, 2011). On the contrary, the positive effect of vitamin C on dobutamine-induced inotropic response was not present in other studies with heart failure patients (Mak and Newton, 2004). It is remarkable that the heart failure patient groups had a marked difference in the severity of cardiac dysfunction at the studies of Shinke et al. or Mak and Newton, the latter being more severe (Mak and Newton, 2004; Shinke *et al.*, 2007). This difference might serve an explanation to the different outcomes of antioxidant treatment, suggesting that normal redox responsiveness, still present in mild heart failure is lost in more progressed disease state. Of note, that vitamin C alone was not able to modulate cardiac contractility in either study. In contrast, reduced ROS generation by xanthine oxidase-inhibitor allopurinol alone lead to an improvement in resting cardiac contractile performance in dogs with chronic heart failure.

Allopurinol also enhanced the inotropic response to both pharmacological β-adrenergic stimulation and exercise in the heart failure group; however it had no effect on baseline contractility or β-adrenergic responsiveness in healthy animals (Ukai *et al.*, 2001). These data reveal that ROS can acutely influence cardiac contractile performance also in the failing heart. However, they also highlight possible differences between physiological and pathophysiological states. For instance, the xanthine oxidase system seems not to contribute to ROS signaling under normal conditions, on the contrary it becomes a significant ROS source and an important regulator of cardiac inotropy in heart failure. Thus the results from physiological models should be implemented to disease states with caution.

6.3.2 HF therapy

ET-1 plasma level is elevated in chronic HF patients, and the plasma concentration has prognostic value for survival (Pacher *et al.*, 1996). ET-1 may contribute to the progression of HF in multiple ways: the vasoconstrictor effect directly increases peripheral resistance, but ET-1 can augment other vasoconstrictor neurohormonal mechanisms like the renin-angiotensin-aldosterone system (RAAS) and adrenaline. Moreover, ET-1 has mitogenic properties and can induce cardiac hypertrophy and remodeling (Love and McMurray, 1996). For those reasons there was a great enthusiasm in the early 2000s towards ET-receptor antagonist as novel treatment opportunity in HF. Various clinical trials (ENABLE, EARTH, HEAT, VERITAS, etc.) tested the effect of different ET receptor antagonists on acute or chronic HF patients, but the final results were disappointing: despite promising results in animal models and early-phase clinical trials, ET-receptor blockade failed to improve long-term outcomes in phase III clinical trials (Kalra *et al.*, 2002; Luscher *et al.*, 2002; Anand *et al.*, 2004; McMurray *et al.*, 2009). There are different theories that try to explain the reason for that failure. One consideration is that HF patients in trials received ET-blockade on the top of conventional heart failure therapy, which included inhibition of the RAAS and also beta-adrenergic inhibition. If two neurohumoral systems are blocked already, inhibition of a third system may provide little additional benefit (Kelland and Webb, 2006). Additionally, endogenous ET-1 contributes to the intrinsic regulator mechanism of cardiac contractility known as the Frank-Starling response (Piuhola *et al.*, 2003b). It was demonstrated that, unlike the down-regulated beta-adrenergic response and the blunted force-frequency relationship, the Frank-Starling mechanism remains intact even in failing hearts (Holubarsch *et al.*, 1996). Thus, one can speculate that one reason

for the unsatisfactory results of ET-receptor blockade in long-term treatment of HF is that it inhibits the failing heart's remaining mechanism to adopt to increased loading conditions (Szokodi *et al.*, 2003).

As HF epidemic emerges worldwide (López-Sendón, 2011), there is an increasing need to develop treatment methods that increase patient survival and relieve symptoms by restoring or maintaining cardiac pump function at the same time. It is therefore very interesting to notice to compare apelin to ET-1. Although both are endogenous, GPCR-mediated positive inotropic peptides that share tissue distribution, induce similar signaling pathways and mutually act in an autocrine/paracrine manner, there are a few, but significant differences in their profiles. Probably the most important difference is in their effect on vasculature: ET-1 is a potent vasoconstrictor (Yanagisawa *et al.*, 1988), apelin, on the contrary, has strong vasodilator effects on both arterial and venous side of the circulation (Lee *et al.*, 2000; Tatemoto *et al.*, 2001; Cheng *et al.*, 2003). This sole difference raises the possibility that apelin may be beneficial for certain HF patients: by dilating arteries and veins, apelin mechanically offloads the heart, as peripheral resistance and mean circulatory filling pressure, and consequentially the cardiac afterload and preload are decreased. Taken this together with the positive inotropic effect of the peptide, we propose that apelin maintains cardiac output in an energetically favorable manner, since the same or increased pumping work is done against lower resistance. This hypothesis is supported by the fact that chronic administration of apelin (two-week continuous infusion) to mice, while clearly increasing cardiac output and inotropy, did not induce left ventricular hypertrophy, which is otherwise a frequently seen result after chronic administration of other positive inotropic substances (Ashley *et al.*, 2005). This might be due to some other beneficial properties of apelin: in contrast to ET-1, the apelin-apelin receptor system antagonizes the RAAS (Ishida *et al.*, 2004; Chun *et al.*, 2008), a major contender in the development of hypertension and cardiac remodeling. Apelin is also likely to decrease cardiac fibrosis (Siddiquee *et al.*, 2011) and ROS-linked cardiac hypertrophy (Foussal *et al.*, 2010). These characteristics brought the apelin-apelin receptor system into the focus of HF research. Apelin plasma levels are elevated significantly in humans in early stages, but tendentiously fall below baseline level in patients suffering from severe HF (Chen *et al.*, 2003), simultaneously cardiac apelin receptor expression is downregulated in chronic HF patients (Földes *et al.*, 2003). Downregulation of the apelin receptor could be maladaptive, since lower expression

level could lead to lower receptor density and to consequent impaired apelin efficacy. Thus, while the apelin-apelin receptor signaling cascade can be beneficial in HF, this pathway is likely to be endogenously downregulated in severe heart disease. (It is noteworthy, that the apelin receptor is one of the most upregulated genes in the myocardium following the placement of a left ventricular assist device. Moreover, mechanical offloading of the failing left ventricle also increases cardiac tissue apelin levels (Chen *et al.*, 2003).) Hence the apelin-apelin receptor pathway represents a potential target for heart failure therapy (for summary check Figure 17).

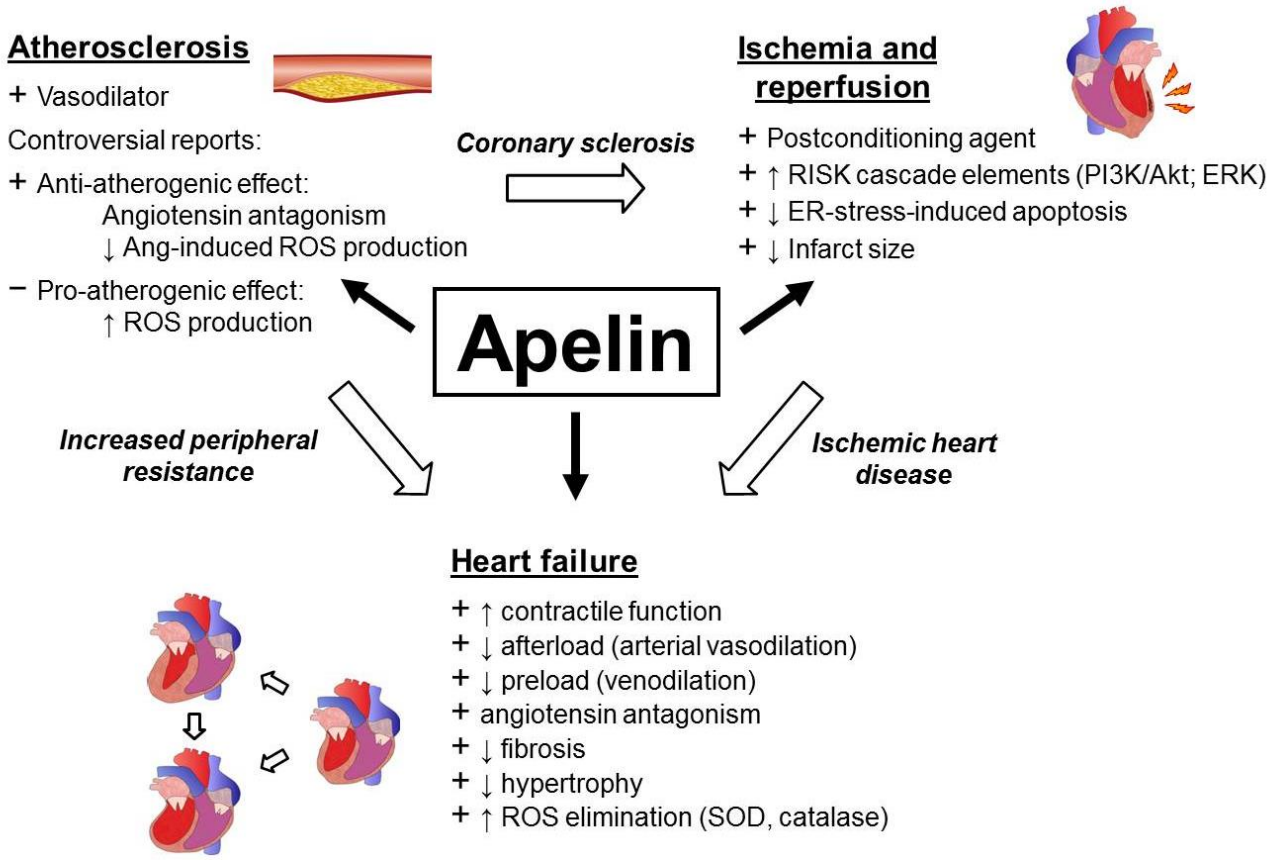

Figure 17: Potential role of apelin at pathological cardiovascular conditions

"+" and "–" mark beneficial and deleterious effects, respectively. Empty arrows represent connections between pathological entities.

We therefore found it especially important to increase understanding of the apelin-apelin receptor pathway in the heart. Since peptide therapy is quite complicated and not cost-effective on the long run, the possible way of pharmacological interaction would be some small molecule that mimics apelin's effect, either on the receptor or on the intracellular level. Proper design of such intervention requires detailed knowledge of the intracellular signaling

mechanisms. Our current data demonstrate some similarities (ERK1/2, MLCK) between ET-1 and apelin signaling, but we also identified marked differences in the two pathways, namely the PKC dependence of apelin-induced inotropy and the lack of p38 activation during apelin stimulus.

7 CONCLUSION

The present work studied the underlying signaling mechanisms of the apelin- and ET-1-induced positive inotropic response in isolated adult rat hearts. As the main findings of our studies, (1) we present evidence that -1-induced increase in cardiac contractility is dependent on enhanced NAD(P)H oxidase-derived ROS generation, which in turn, (2) activates the ERK1/2 pathway. (3) Opening of mitochondrial potassium channels (mitoK_{ATP} and BK_{Ca}) is necessary for the inotropic response to ET-1, however, this effect appears to be independent of ROS generation. (4) We have identified PKCe as the specific PKC isoenzyme activated by apelin. (5) The current study also showed that apelin stimulates ERK1/2 phosphorylation and ERK1/2 activity is required to the fully developed positive inotropic effect of apelin. (6) Moreover, our data demonstrates that ERK1/2 activation occurs independently of PKC signaling. (7) We also provided evidence for the first time that activation of MLCK contributes to the apelin-induced inotropic response. Thereby we link an additional effector mechanism to the apelin signaling, strengthening our hypothesis that apelin's main way of action is sensitizing myofilaments to intracellular Ca^{2+}.

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a. Publications related to this thesis

Perjés Á, Skoumal R, Tenhunen O, Kónyi A, Simon M, Horváth IG, Kerkelä R, Ruskoaho H, Szokodi I. Apelin Increases Cardiac Contractility via Protein Kinase Cε- and Extracellular Signal-Regulated Kinase-Dependent Mechanisms. *PLoS One* 9(4):e93473, (2014) **IF: 3.730**[*]; Cited: 0

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