Characterization of inotropic signaling induced by endogenous peptides apelin and endothelin

Ph.D. Thesis

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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 [1–3]. Although there has been a significant improvement in the efficacy of chronic HF therapy [4], survival rate of acute HF episodes hasn't changed at all in the past three decades [5]. 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 [6] and phosphodiestherase inhibitors [7] 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 [8]. 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 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 the peptides endothelin (ET) [9], apelin [10] and adrenomedullin [11] are identified as positive inotropic agents. 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.

INTRODUCTION

Endothelin in the cardiovascular system

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 [12]- has been found to be the most potent and long lasting endogenous vasoconstrictor known so far [13]. ET-1 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. Under normal physiological conditions, ETs are not circulating hormones; rather they act as autocrine and paracrine factors at multiple sites in the body [14]. 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 positive inotropic regulator of cardiac contractility [15–17]. 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[18], the Frank–Starling response [19], and the slow force response (Anrep effect) to stretch in rats [20].ET-1 binds to two subtypes of GPCRs, ETA and ETB receptors, which are responsible for the actions of the peptide [21]. Both ETA and ETB receptors are expressed in cardiomyocytes, with a dominance of ETA receptors (85−90%) [22]. The ETA receptor is responsible for the positive inotropic effect of ET-1[23].

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 as well[24–26]. However, the exact subcellular mechanisms have not been fully elucidated. ETA receptor is commonly considered to signal through G_q protein-dependent activation of the protein kinase C (PKC) cascade in cardiomyocytes [15,16]. Previous studies suggested that ET-1 increases cardiac contractility via a PKC-dependent activation of sodium-proton exchanger (NHE) [27–29]. Stimulation of NHE can lead to intracellular alkalization and consequent sensitization of cardiac myofilaments to intracellular Ca^{2+} [27,30]. 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 sodium-calcium exchanger (NCX) [20,25]. In addition to NCX, ET-1 can enhance intracellular Ca^{2+} transients by increasing L-type Ca^{2+} current [24]. 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 [31].

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 [31]. Activation of ERK1/2 can result in phosphorylation of NHE, either directly by ERK1/2 itself [32] or indirectly through the p90 ribosomal S6 kinase (p90RSK) [33]. Importantly, our group showed that membrane-associated p90RSK is likely to mediate the effect of ET-1 on NHE activity [31].

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 [34–37]. In contrast, it has recently 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 in isolated rat [38] and cat [39].cardiomyocytes. Administration of antioxidants prevented the ET-1-induced increase in ROS production in all of these models [38,39]. 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 dependent on ROS production [39,40]. However, the functional importance of ROS under physiological conditions in the myocardium remained obscure.

Apelin in the cardiovascular system

In 1993 a novel GPCR called APJ was identified by homology cloning. It shares greatest sequence homology with the angiotensin II type-1 receptor (AT1-R) but does not bind angiotensin II (Ang II) [41] 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 [42], and later on the receptor was renamed "apelin receptor" by international consensus [43]. (Pyr1)apelin-13 is the most potent and abundant isoform in cardiac tissue [44]. 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. Both apelin and its receptor are detectable immunhistologically in endothelial cells and vascular smooth muscle cells along the whole human vasculature. In the heart, apelin receptor-like immunoreactivity was present in the endocardial endothelium and, in lesser extent, also in the myocardium[45]. 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 [46]. Immunhistological studies localized apelin peptide to the endocardial endothelium [47]. 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.

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 [10] and failing [48] rat heart and in humans as well [49]. 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 [31] and adrenomedullin [11].

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 [42]. Apelin peptides activate at least 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, supporting the hypothesis that the apelin receptor is linked to G_i proteins [50].

Apelin may induce cardiac contractility via both PTX–insensitive G_q and PTX–sensitive G_i proteins. PLC, PKC, NHE and NCX have been identified as mediators of the apelin-induced inotropic signaling [10,51]. However, whether

apelin directly increases intracellular calcium currents or acts by solely sensitizing myofilaments to calcium remains controversial [48,51,52].

Early data suggested that PKC-mediated increase in cardiac contractility may result from increased NHE activity and subsequent intracellular alkalization [27]. On the contrary, more recent evidence demonstrated that PKC-dependent positive inotropic response was not associated with alteration of intracellular pH [53]. It is possible however, that PKC activation induces cardiac contractility by enhancing myofibrillar Ca^{2+} sensitivity via phosphorylation of myosin regulatory light chain (RLC) [54] or TnI [55,56], and PKC was also reported to enhance Ca^{2+} transients via LTCC [57,58]. The exact PKC isoenzyme contributing to the apelin-induced contractile response has not been identified yet.

AIMS OF THE THESIS

Excessive data supports the idea that ET 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 positive inotropic signaling mechanism of these endogenous 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 subcellular mechanisms by which the peptides induce inotropy.

MATERIALS AND METHODS

Isolated Perfused Rat Heart Preparation

Male 7-week-old Sprague-Dawley rats from the Center for Experimental Animals at the University of Oulu were used (n=316). All protocols were reviewed and approved by the Animal Use and Care Committee of the University of Oulu. Rat hearts were isolated for retrograde perfusion by the Langendorff technique as described previously [31]. 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. 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. 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.

Western blot analysis

Frozen LV tissues were grinded in liquid nitrogen and were dissolved and homogenized in ice-cold lysis buffer. Samples were then centrifuged and the supernatant was collected. Protein extracts were matched for protein concentration and equal volumes (30 μg) of protein samples were loaded onto 10 % SDS-PAGE and transferred to nitrocellulose membranes. Protein levels were detected using fluorescence or chemiluminescence as described previously[31]. Quantification of the blots was done by using the Quantity One Basic 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA).

Detection of intracellular ROS

ROS were detected using ethidium fluorescence as previously described [59]. 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 [60]. 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.

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. 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 $P<0.05$.

RESULTS

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 [39,40,61,62]. 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 [59]. 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,). 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)

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 [63] 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 [18,31]. 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). Similarly, when ET-1 was infused in the presence of MnTMPyP (10 μmol/L), the inotropic effect was decreased by 35% (P<0.05). Infusion of MnTMPyP alone had no effect on cardiac contractility (P=NS).

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 [64,65]. Previously it has been shown that ET-1 activates NAD(P)H oxidase and induces ROS production in cultured rat

cardiomyocytes [66]. To assess the contribution of NAD(P)H oxidase in mediating the inotropic effect of ET-1 we used apocynin [61]. 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). Infusion of apocynin alone had no effect on contractile force (P=NS). ROS measurements revealed that apocynin eliminated the ET-1-induced increase in ethidium fluorescence in isolated hearts (P<0.001), whereas the drug alone had no significant effect on fluorescence intensity (P=NS).

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

Opening of mitochondrial ATP-dependent potassium channels (mitoK$_{ATP}$) has been shown to increase mitochondrial production of ROS in the myocardium[67–69]. 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 [67]. 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). 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). 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.

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 [70] and HMR1098 [71], 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). In contrast, administration of HMR 1098 (3 μmol/L) failed to alter the ET-1-enhanced contractility (P=NS). These data indicate that mitochondrial BK$_{Ca}$ channels, but not sarcK$_{ATP}$ channels, are involved in the response to ET-1.

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 [31]. Since ERK1/2 phosphorylation has been reported to be redox-sensitive in cultured cardiomyocytes [72,73],we examined whether ROS modulates ERK1/2 activation in the intact adult rat heart. In agreement with our previous data [31], administration of ET-1 (1 nmol/L) for 10 min increased phopho-ERK1/2 levels ($P<0.001$). 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). The inhibitors alone had no effect on the phosphorylation state of ERK1/2 (P=NS). These results indicate that ROS can act as the upstream activator of the ERK1/2 pathway to mediate the inotropic effect of ET-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%, $P<0.001$), 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 [10]. Our former experiments suggested that apelin may act via PLC–PKC cascade[10]. In line with this, infusion of Bis (90 nmol/L), a selective PKC inhibitor, decreased apelin-induced inotropic response by 42% ($P<0.05$), the same inhibitory effect we described previously [10]. Infusion of Bis alone had no effect on contractile force (P=1.0 vs. vehicle).

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 [74,75]. PKC isoforms show rapid translocation from the soluble to the particulate fraction of the cardiomyocyte upon stimulation [76]. When compared to controls, apelin treatment for 5 min produced a significant increase in the particulate partitioning of PKCε in the adult rat LV. However, during a more prolonged, 10-min apelin infusion, the subcellular distribution of PKCε returned to those in control hearts, suggesting a transient increase in PKCε activation. In contrast to PKCε, no consistent PKCα translocation could be detected upon apelin administration.

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 [52]. Increased phosphorylation of RLC by MLCK[77] leads to an increase in the Ca^{2+} sensitivity of force development and improved cross-bridge kinetics in cardiac myofibrils [78].

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).

Next, we performed urea-glycerol PAGE to separate phosphorylated and nonphosphorylated RLC in the apelin treated rat LV myocardium. The level of basal RLC phosphorylation was found to be comparable to the results presented by others using the same technique [77,79], but apelin treatment failed to induce detectable increase in RLC phosphorylation under our experimental conditions.

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. 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).

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). 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, $P<0.001$).

Particulate partitioning of PKCε in neonatal rat ventricular myocytes is accompanied by subsequent activation of ERK1/2[80]. 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, demonstrating that ERK1/2 and PKC represent independent pathways mediating the inotropic effect of apelin.

DISCUSSION

ET 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−p90RSK−NHE pathway [31], 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. 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 $O_2^{\cdot -}$ [59]. 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.

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 [64,81]. 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.

NAD(P)H oxidase-derived ROS may trigger a larger release of ROS from the mitochondria via opening the mitoK$_{ATP}$ [82], the phenomenon called "ROS-induced ROS release" [83]. 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 [84]. It is well established that mitoK$_{ATP}$ play a crucial role in cardioprotection against ischemia–reperfusion injury [67,68]. However, the physiological function of mitoK$_{ATP}$ in the heart is still elusive. In our experiments, 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 [39]. 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 [85]. 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 [85]. 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 [86], 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.

ROS and signaling

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 [73,87]. For instance, G_q-mediated PKC activation can stimulate Raf, the first member of the

ERK1/2 cascade. Epidermal growth factor receptor (EGFR) transactivation, which is an alternative mechanism that couples GPCRs and ERK1/2 activation [87], contributes to the ET-1-induced increase in contractility, acting as a proximal component of MEK1/2–ERK1/2 signaling [31]. 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 [73,87]. Moreover, ROS can also enhance Ras activity, via direct, leading to activation of the Raf–MEK1/2–ERK1/2 pathway [73,88]. Additionally, ROS can directly activate G proteins. The βγ-subunit liberated by that activation can initiate ERK activation [89]. 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 [90,91]. 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 [38], which can also contribute to the ROS-dependent positive inotropic effect of ET-1.

The inotropic signaling of apelin

The present results demonstrate that pharmacological inhibition of PKC significantly reduces the positive inotropic effect of apelin, confirming previous data from our [10] and other laboratories [51]. 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 [92] and they are each localized to distinct subcellular sites following activation[93]. Various PKC isoforms are considered to regulate cardiac contractility [74,75]. 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 cardiomyocytes [94]. Upon activation, PKCε is known to accumulate in these very specific regions of ventricular myocytes, resulting in a strong positive inotropic effect [95]. These findings locate activated PKCε to the close vicinity of apelin receptor [52].

RLC controls myofilament cross-bridge properties and thereby modulates the force of contractions in the heart. Increased RLC phosphorylation by MLCK results in an increase of the Ca^{2+} sensitivity of myofilaments [78]. 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 [96]. 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. 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 [96], 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 [97]. 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 [98]. Some studies demonstrated PKC-dependent RLC phosphorylation in the heart [54,99], but others provided evidence challenging the role of PKC in triggering RLC regulation [100,101]. 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 [102], but only a few reports demonstrated that MAPKs can regulate cardiac contractility [31,103]. 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 [10,52]. Since ERK1/2 is a recognized activator of NHE [104], we propose here a functional ERK1/2-NHE axis in apelin signaling. ERK1/2 can be activated, among many others, by PKCs [80]. Knowing that PKC is involved in the inotropic effect of apelin, one could 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. Thus, PKC and ERK1/2 are parallel and independent signaling pathways mediating the effect of apelin on cardiac contractility.

CONCLUSION

The present work studied the underlying signaling mechanisms of the apelin- and ET-induced positive inotropic response in isolated adult rat hearts. As the main findings of our studies, (1) we present evidence that ET-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 could identify a specific PKC isoenzyme that gets activated by apelin stimulus. (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 intact myosin light chain kinase activity is necessary for the fully developed apelin-induced contractile 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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PUBLICATIONS OF THE AUTHOR

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**[1]; Cited: 1

Perjés Á, Farkasfalvi K, Ruskoaho H, Szokodi I, Chapter 187 - Apelin, In: Abba Kastin, Editor(s), *Handbook of Biologically Active Peptides (Second Edition)*, Academic Press, Boston, pp. 1377-1385, (2012)

Perjés Á, Kubin AM, Kónyi A, Szabados S, Cziráki A, Skoumal R, Ruskoaho H, Szokodi I Physiological regulation of cardiac contractility by endogenous reactive oxygen species *Acta Physiol* 205:(1) pp. 26-40. (2012) **IF: 4.382**; Cited(dep): 13(0)

Kubin AM, Skoumal R, Tavi P, Kónyi A, Perjés Á, Leskinen H, Ruskoaho H, Szokodi I Role of reactive oxygen species in the regulation of cardiac contractility *J Mol Cell Cardiol* 50:(5) pp. 884-893. (2011) **IF: 5.166**; Cited(dep): 10(1)

b. Publications not closely related to this thesis

Kaikkonen L, Magga J, Ronkainen VP, Koivisto E, Perjes Á, Chuprun JK, Vinge LE, Kilpiö T, Aro J, Ulvila J, Alakoski, Bibb JA, Szokodi I, Koch WJ, Ruskoaho H, Kerkelä R. p38α regulates SERCA2a function. *J Mol Cell Cardiol* 67:86-93, (2014) **IF: 5.148***; Cited(dep): 1(1)

Vainio L, Perjés Á, Ryti N, Magga J, Alakoski T, Serpi R, Kaikkonen L, Piuhola J, Szokodi I, Ruskoaho H, Kerkelä R Neuronostatin, a novel peptide encoded by somatostatin gene, regulates cardiac contractile function and cardiomyocyte survival *J Biol Chem* 287:(7) pp. 4572-4580. (2012) **IF: 4.651**; Cited(dep): 4(0)

Kónyi A, Skoumal R, Kubin AM, Füredi G, Perjés Á, Farkasfalvi K, Sárszegi Z, Horkay F, Horvath IG, Tóth M, Ruskoaho H, Szokodi I Prolactin-releasing peptide regulates cardiac contractility *Regul Peptides* 159:(1-3) pp. 9-13. (2010) **IF: 2.473**; Cited(dep): 1(0)

c. Presentations, posters, conference abstracts

Perjés Á, Skoumal R, Tenhunen O, Kónyi A, Horváth IG, Kerkelä R, Ruskoaho H, Szokodi I Protein kinase C and extracellular signal regulated kinase have distinct effects on apelin-induced inotropy (2014) *Faculty of Medicine Science Day,* Oulu, Finland - *oral presentation*

1Impact factors of 2012

Perjés Á, Skoumal R, Tenhunen O, Kónyi A, Horváth IG, Kerkelä R, Ruskoaho H, Szokodi I Protein kinase C and extracellular signal regulated kinase have distinct effects on apelin-induced inotropy- poster presentation (2013) *7th Annual Meeting of the European Council for Cardiovascular Research,* Nice, France - *poster*

Perjés Á, Ezer P, Skoumal R, Ruskoaho H, Szokodi I Map kinases and apelin-induced inotropy (2012) *The 7h Oulu Symposium*, Oulu, Finland - *poster*

Perjés Á, Vainio L, Kneifel Z, Ryti N, Magga J, Alakoski T, Ruskoaho H, Szokodi I, Kerkela R Neuronostatin regulates cardiac contractile function and cardiomyocyte survival (2012) *Annual Meeting of the Hungarian Cardiologists Society*, Balatonfüred, Hungary - *oral presentation*

Perjés Á, Kneifel Z, Scheich B, Kubin A M, Kónyi A, Szabados S, Tóth M, Ruskoaho H, Skoumal R, Szokodi I Reactive oxygen species have distinct effect on different inotropic stimuli in the isolated rat heart *Acta Physiol* 202: (Suppl. 684.) pp. 94-95. (2011) - *abstract*

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