CARDIOVASCULAR EFFECTS OF ORAL CALCIUM SENSITIZERS ON EXPERIMENTAL HEART FAILURE

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List of original publications

This thesis is based on the following original publications (Studies I-IV) and some unpublished data:


Main abbreviations

ACE  angiotensin converting enzyme
AIF  apoptosis inducing factor
ANP  atrial natriuretic peptide
ATP/ADP  adenosine triphosphate/adenosine diphosphate
AT1/2  angiotensin II receptor 1/2
Bel  B-cell leukemia/lymphoma 2
BNP  brain natriuretic peptide
CASINO  Calcium Sensitizer or Inotrope or None in Low Output Heart Failure
CTGF  connective tissue factor
DISC  death-inducing signalling complex
EMSA  electrophoretic mobility shift assay
ERK  extracellular signal-regulated protein kinase
E2F-5  transcription factor 5
FU  follow up
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
GK  Goto Kakizaki
HF  heart failure
IL-6  interleukin 6
i.p  intraperitoneally
JNK  c-Jun N-terminal kinase
KATP  adenosine triphosphate-sensitive potassium channel
LAD  left anterior descending coronary artery
LEVO  levosimendan
LS  low salt
LV  left ventricular
MAPK  mitogen activated kinase
MCP-1  monocyte chemoattractant protein 1
MI  myocardial infarction
NRF-1  nuclear respiratory factor 1
NR4a3  nuclear receptor 4a3
NCX  sodium/calcium exchanger
NFAT  nuclear factor of activated T cell
NO  nitric oxide
NYHA  New York Heart Association
PPAR  peroxisome proliferators-activated receptor family
PCG-1α  PPARγ coactivator-1
PDE  phosphodiesterase
PI3k  phosphoinositide 3-kinase
PKC  protein kinase C
PLN  phospholamban
RAS  renin-angiotensin system
ROS  reactive oxygen species
RT-PCR  reverse transcriptase-polymerase chain reaction
RyR  ryanodine receptor
SERCA2  sarco/endoplasmic reticulum Ca2+-ATPase 2a
s.c  subcutaneous
Smac/DIABLO  second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
SNS  central nervous system
SS  salt sensitive
TFAM  transcription factor A, mitochondria
TNF  tumour necrosis factor
TUNEL  terminal deoxynucleotidyl transferase mediated dUTP nick end labelling
WIS  Wistar
Abstract

Calcium sensitizers are a novel class of inotropic agents that appear advantageous for treating acute decompensated heart failure and other low output heart failure situations relative to conventional inotropic drugs, which act primarily by increasing intracellular calcium mobilization in cardiac myocytes. Levosimendan, the only calcium sensitizer in clinical use at the moment, mediates its cardiac effect by the calcium sensitization of the contractile protein troponin C. Levosimendan binds to the calcium-saturated N-terminal domain of troponin C in cardiac muscle and stabilizes the troponin molecule, with subsequent prolongation of its effect on the contractile proteins. Besides increasing the strength of cardiac contractions, levosimendan exerts vasodilatory effects through opening of the sarcolemmal and mitochondrial ATP-sensitive potassium channels. The major elimination route of levosimendan is conjugation with glutathione to cysteine and cysteinylglycine conjugates. A minor metabolism route is reduction of levosimendan in intestine by bacteria into metabolite OR-1855, which is further acetylated to OR-1896. Although this is minor pathway, it is pharmacologically very important since OR-1896 is a long-lasting metabolite sharing the pharmacological properties of the parent compound. Levosimendan is currently used only as a 24-h systemic infusion.

This study aimed at exploring the cardiovascular effects of oral calcium sensitizers in two different animal models of heart failure, namely in hypertensive and salt-sensitive Dahl/Rapp SS rats on a high-salt diet (a model of hypertensive heart failure with preserved systolic function) and in spontaneously diabetic Goto-Kakizaki rats with experimental myocardial infarction induced by coronary artery ligation (a model of post-infarct heart failure with impaired systolic functions).

We were able to demonstrate that in Dahl/Rapp SS rats both levosimendan and OR-1896 prevented mortality, produced a transient decrease in blood pressure, prevented cardiac hypertrophy and improved cardiac contractility. These beneficial effects were associated with decreased cardiac atrial natriuretic peptide mRNA expression, a marker of pressure/volume overload, attenuation of oxidative stress, inflammatory response and cellular senescence. In Goto Kakizaki rats, levosimendan and OR-1896 prevented MI-induced systolic heart failure, pronounced cardiac hypertrophy in the remote area, and sustained cardiomyocyte apoptosis. The beneficial effects of calcium sensitizers were associated with decreased myocardial atrial natriuretic peptide, inflammatory interleukin 6 and fibrogenic connective tissue growth factor mRNA expressions, and corrections of MI-induced disturbances in calcium-handling proteins (sarco/endoplasmic reticulum Ca2+-ATPase 2a, Na+/Ca2+-exchanger).

In conclusion, our findings suggest a therapeutic role for oral calcium sensitizers in the prevention of hypertension-induced heart failure with preserved systolic function as well as in the prevention of post-infarct heart failure with decreased systolic function.
1 Introduction

Heart failure, also known as congestive heart failure, is a serious condition where the heart is unable to maintain sufficient blood flow for body’s needs in different situations. Compensating mechanisms can initially hide failure in cardiac pumping capacity, but eventually full-blown syndrome will severely hinder the ability to cope with everyday tasks. Approximately 1-2% of the population in the Western world is afflicted by heart failure, with the prevalence strongly increasing with age. In the year 2020 prevalence of heart failure is estimated to increase by 20%, with a 40-50% increase in in-hospital time. (American heart association, 2004, Kupari and Lommi, 2004) Since most of the costs caused by heart failure are due to repeated hospital admissions, the burden to an already heavily encumbered health care system will be marked.

Survival after a cardiac event is impaired in the presence of preceding diabetes. Nowadays, the most common form of diabetes mellitus, type 2 diabetes, affects over 170 million people worldwide, with prevalence increasing dramatically (Campbell, 2009, Ginsberg and MacCallum, 2009). Type 2 diabetes is described as a metabolic syndrome in which the body develops a resistance to insulin and changes occur in fat metabolism. This leads to elevated blood glucose levels, quite often combined with dyslipidemias, and finally to chronic tissue damage and target organ dysfunction. Type 2 diabetes is known to be a major risk factor for cardiovascular diseases; the risk for cardiovascular disease is enhanced 2- to 5-fold in type 2 diabetic patients, more than 50% of whom will die from cardiovascular causes. (Laakso, 2001) For example, mortality for myocardial infarction is higher and survival worse in diabetic than non-diabetic patients despite similar-sized infarctions (Klamann et al., 2000, Haffner et al., 1998, Mukamal et al., 2001).

Heart failure is not an isolated condition; the underlying causes for heart failure are cardiovascular diseases, such as coronary artery disease, myocardial infarction, hypertension and valvular disease, and rarely some other conditions, such as viral infections, genetic disorders or intoxications. Hypertension, diabetes and myocardial ischaemia are common risk factors for the heart failure with preserved systolic function, which is pathophysically characterized with excessive fibrosis and myocyte hypertrophy, leading to impaired left-ventricular filling and diastolic stiffness. (Krum and Abraham, 2009) Systolic heart failure results after damage to the cardiac muscle, such as myocardial infarction, impairs heart’s ability to maintain sufficient pumping.

Acute heart failure develops when sudden cardiac event, such as myocardial infarction, myocarditis or pumping disorder caused by severe arrhythmia, hinders cardiac pumping capacity. Patients suffer from severe shortness of breath because of pulmonary oedema due to compromised pumping of the left ventricle. In the worst case, acute heart failure can be fatal, but normally it is a treatable condition, sometimes even with full recovery. (Heikkilä et al., 2008)

Chronic heart failure can occur suddenly, but typically it develops over a long period of time. At first mild, hardly detectable symptoms manifest only during stress. Symptoms then become progressively
more severe, with shortness of breath occurring even at rest. Chronic heart failure often develops rapidly to acute pulmonary oedema due to excess cardiac stress, such as infectious disease, physical or emotional stress or arrhythmia. In spite of development in modern pharmacology, chronic heart failure remains a fatal disease. (Heikkilä et al., 2008)

Approximately half of the heart failure patients have contractile failure and dilated heart, known as systolic heart failure, and the remaining patients have heart failure with preserved ejection fraction, which includes relatively normal contraction and non-dilated but often hypertrophied heart. Heart failure can also be divided into systolic and diastolic heart failure, depending on the failing ventricle. Independent diastolic heart failure is a rare condition; generally heart failure is considered to be failure in systolic functions, potentially combined with simultaneous diastolic failure. (Heikkilä et al., 2008)

Despite marked improvements, such as cardiac transplant and promising stem cell treatments, (for review see (Daneshmand and Milano, 2009, Vertesaljai et al., 2008)) In past decades, treatment of heart failure remains relatively ineffective and existing damage to the heart can not be repaired. The purpose of this study was to clarify the underlying mechanisms in development of heart failure during hypertensive heart disease and in the diabetic post-infarct remodelling. This study also aimed to evaluate the effects of oral levosimendan treatment and its potential preventive role in development of heart failure.
2 Review of the literature

2.1 Heart failure

2.1.1 Epidemiology and prevalence

The most common underlying causes of heart failure in the Western world are coronary artery disease and hypertension. In newly diagnosed patients with heart failure, 50% have coronary artery disease, 50% hypertension and 80% coronary artery disease, hypertension or both. Myocardial infarction is also major risk factor for heart failure; approximately 17% of patients develop heart failure. Other important risk factors are valvular disease, cardiomyopathies and diabetes. Predominant risk factors for rare, independent right ventricular failure are chronic pulmonary disease, myocardial infarction in the right ventricle, recurrent pulmonary embolus and primary pulmonary hypertension. (Kupari and Lommi, 2004)

Heart failure is a rarely manifested in patients younger than 50 years, but its prevalence grows rapidly with age. Due to ageing of the global population prevalence of heart failure is on the rise, especially in developing countries with limited health and social care resources. According to the Framingham study, in individuals over 40 years the lifetime risk of developing heart failure is 1 to 5. (Lloyd-Jones et al., 2002)

Heart failure remains a fatal disease; the one-year mortality of newly diagnosed cases is roughly 25%. The long-term survival is poor, with 70% of those under 65 years diagnosed with heart failure dying over the next eight years. Total-mention mortality in 2004 was over 280,000 in USA alone, 1 in 8 deaths had heart failure mentioned in death certificates. (Rosamond et al., 2008) Treatment of heart failure is expensive, according to The social insurance institution of Finland heart failure related drug costs alone were over 200 million € in year 2005.

2.1.2 Clinical symptoms and diagnosis

Shortness of breath during physical activity is one of the first symptoms of the heart failure; however, it does not necessarily indicate failure in cardiac functions. Other symptoms include fatigue, swelling of feet and ankles as well as related nocturia. In addition, changes in psychological functions, such as memory problems, restlessness and confusion, are common, especially in the elderly. Swelling of the upper abdomen, nausea, lack of appetite and changes in bowel functions are caused by congestion in systemic veins, liver and bowel. (Heikkilä et al., 2008)

Diagnosing heart failure is challenging; half of heart failure diagnoses made in primary care are incorrect (Remes et al., 1991). Diagnosis of heart failure is based on careful clinical observations, including verification of failing heart functions, clarification of underlying causes, such as lifetime habits or storage diseases, and associated diseases. (Jessup et al., 2009, Schocken et al., 2008, Hunt et al., 2009, Heliö et al., 2003)
Simplified diagnostic criteria for heart failure in primary care includes existing cardiovascular disease and three of the following symptoms (Kupari and Lommi, 2004):

1. Shortness of breath or fatigue under ordinary stress
2. Third heart sound or resting pulse over 90 bpm
3. Increased heart size in chest X-ray
4. Elevated pressure in vena jugularis or congestion of pulmonary veins in thorax X-ray.

The diagnosis can be supplemented with determination of serum brain natriuretic peptide (BNP), electrocardiogram, echocardiography and experimental treatment with diuretics. (Kupari and Lommi, 2004)

Severity of heart failure is often described using the NYHA (New York Heart Association) classification (Levin et al., 1994) as follows:

1. NYHA I: No limitations in activities, no symptoms in ordinary activities
2. NYHA II: Mild limitation of activity, mild symptoms in ordinary activities
3. NYHA III: Severe limitation in activities, symptoms during light activities
4. NYHA IV: Severely compromised capacity in all activities, symptoms manifest also during rest.

2.1.3 Treatment

Prevention of development or deterioration of existing heart failure and available treatment options for a failing heart are based on five major components (for review, see (Ruvinov et al., 2008)):

- Cardioprotection: prevention of progressive loss of cardiomyocytes via anti-apoptotic approaches or induction of mechanisms promoting survival.
- Inflammation: modulation of pro- and anti-inflammatory responses in induction of tissue healing while avoiding negative inflammatory effects.
- Extracellular matrix remodelling and cardiac fibrosis: prevention of deleterious remodelling and excessive fibrosis formation.
- Angiogenesis: enhancement of tissue healing and prevention of ischaemic damage by increasing blood supply to ischaemic areas.
- Cardiomyogenesis: induction of cardiomyocyte regeneration in an attempt to repair existing damage, such as stem cell transplants or growth factor-based therapies.

One of the main factors in treating heart failure patients is lifestyle change. Suitable physical activity, weight loss in obese patients, decreased salt in food, cessation of smoking and moderate alcohol consumption are cornerstones in the daily life of heart failure patients. The underlying cause for heart failure should also be treated when possible with revascularization, valve surgery and correction of arrhythmias. Treatment of other associated diseases, such as kidney failure, chronic obstructive pulmonary disease and atrial fibrillation, must be addressed. Maintaining adequate cardiovascular
nursing is crucial in monitoring the effectiveness of treatment and patient compliance. (Kimmelstiel et al., 2004, Koelling et al., 2005)

Several pharmacological methods are available for treating the chronic heart failure (Figure 1). In an acute situation, the focus is on increasing cardiac contractility and improving haemodynamics. When carried out for a longer time, inotropic support is considered harmful, and mechanisms responsible for the failing heart are then treated with following goals (Kupari and Lommi, 2004):

1. To suppress increased activity of the sympathetic nervous system and renin-angiotensin system (RAS) by β-blockers, ACE-inhibitors, AT₁-receptor blockers and aldosterone antagonists in different combinations.
2. To decrease workload by lowering blood pressure to the lowest possible level with ACE-inhibitors, AT₁-receptor blockers, vasodilating β-blockers or in selected cases calcium channel blockers felodipine and amlodipine.
3. To decrease elevated filling pressure with diuretics and nitrates.
4. To prevent of cardiomyocyte ischaemia by β-blockers and nitrates.

![Figure 1. Possible pharmacological treatment options for chronic heart failure. Modified from (Heikkilä et al., 2008).](image-url)
Drugs affecting to activated RAS, such as ACE-inhibitors and AT$_1$-receptor blockers, should be given to all patients with decreased function of the left ventricle (ejection factor less than 40-45 %), even though patient would be asymptomatic. They are especially useful to patients with history of myocardial infarction and hypertension. ACE-inhibitors have been found to improve prognosis in all states of heart failure, in non-responding patients with severe heart failure AT$_1$-receptor blockers can be combined into treatment. (Swedberg et al., 1999, Pfeffer et al., 2003) Aldosterone antagonism, eplerenone or spironolactone, can be useful in treating patients with non-responding, severe heart failure but that requires plasma potassium monitoring. (Pitt et al., 2005, Pitt et al., 1999)

β-blockers, with adequate dosing, should be used on all heart failure patients, despite of the aetiology, due to the harmfulness of increased sympathetic activity to the heart. They improve prognosis and prevent mortality, especially beneficial they are to the patients with ischemic heart disease or hypertension. (Packer et al., 1991, Lichstein et al., 1990) The beneficial, long-term effects of β-blockers are mediated by downregulation of the β-adrenergic receptors, increased inhibitory G-protein and G-protein-coupled receptor kinase 2 signalling and amelioration of the abnormalities of excitation-contraction-coupling proteins. (Lowes et al., 2002, Brodde, 2007) Alleviation of the symptoms with β-blocker treatment is slow; the beneficial effects may take 1-2 months to come forward. In elderly patients (<75-80 years), β-blockers is less beneficial than in younger patients.

Diuretics are used in the treatment of heart failure in patients with liquid retention. To prevent harmful effects, diuretic dose should lowest effective. Diuretics and ACE inhibitors have synergistic effect, and administration of diuretics on the patients with ACE inhibitors should be started with care. (Kupari and Lommi, 2004)

Anticoagulants are used on patients with increased risk for thromboembolic complications; however evidence in effectiveness of warfarin treatment in prevention of thromboembolia is contradictory. (for review (Ahnert and Freudenberger, 2008)) Acetylsalicylic acid (ASA) can be given to patients with coronary artery disease or type 2 diabetes. (Kupari and Lommi, 2004)

Nitrates or calcium channel blockers do not have evidence-based effect on treatment of heart failure. However, they can be used heart failure patients with coronary artery disease or hypertension. (Kupari and Lommi, 2004)

Digoxin was the only effective drug for treatment of heart failure for many centuries. Nowadays its use has declined and it is mainly used in combination with β-blockers for the patients with atrial fibrillation. Digoxin has no effect on mortality but it increases time out of hospital. (The Digitalis Investigation Group, 1997)

The asymptomatic ventricular extrasystole is a common phenomenon in heart failure and normally requires no treatment. Of currently available drugs used for arrhythmias, only amiodarone is suitable
for the treatment of heart failure, because it has no detrimental effect on left ventricular functions. Amiodarone has been shown to reduce severe arrhythmias and mortality, but it has no effect on all-cause mortality. (Singh et al., 1995) However, atrial fibrillation should be prevented actively with β-blockers and amiodarone.

2.1.4 Mechanisms of heart failure

Fundamental understanding of the biology of heart failure is lacking, mainly due to the heterogeneity of underlying mechanisms. Several mechanisms have been proposed to result in failing cardiac functions, with many of them manifesting in combination in clinical settings (Figure 2).

**Figure 2** Mechanisms related to development of heart failure with possible pharmacological treatment options. Modified from (Heikkilä, et al 2008).

2.1.4.1 Single gene mutations and genetic polymorphisms

Several single gene mutations subject patients to heart failure. The most common inherited mutations leading to cardiomyopathies are in genes coding sarcomeric proteins, such as beta myosin heavy chain, myosin binding protein C and cardiac troponin C (hypertrophic cardiomyopathy, HCM) and actin and dystrophin (dilated cardiomyopathy, DCM). Also mutations in genes coding energy production and regulation, mainly in mitochondria, Ca²⁺ cycling and transcriptional regulators, have been reported. (for review see (Hershberger et al., 2009))

Genetic differences affect not only the severity of heart failure but also the responsiveness to treatment. For example, among heart failure patients with either arginine or glycine in position 389 of β1-receptor protein, those with arginine-encoding variants have lower mortality and fewer hospitalizations, whereas patients with glycine-encoding variants show little response to therapy (Mialet Perez et al., 2003). Ethnicity also plays a role in differences between individuals.
Pathophysiologic bases of heart failure in black Americans have been postulated to be rather endothelial dysfunction and/or reduced nitric oxide bioavailability than neurohumoral activation. (Yancy, 2005) This is further supported by black American’s better response to a combined treatment of hydralazine and isosorbide than Caucasian patients (Yancy et al., 2007, Taylor et al., 2004).

2.1.4.2 Renin-angiotensin-aldosterone-system

In severe heart failure the RAS system is activated to maintain efficient tissue perfusion and glomerular filtration. Besides weakened kidney perfusion, other activating factors for the RAS system are increased sympathetic activity and decreased sodium level in distal tubules. In normal situation, activity of the RAS system is controlled via atrial baroreflex and natriuretic peptides, but this inhibitory capacity declines in a failing heart producing a positive shift towards RAS activation. Over time, the originally beneficial RAS activation becomes detrimental to the heart. (Danilczyk and Penninger, 2006)

Responses to the activated RAS system are mediated through binding of angiotensin II to angiotensin receptors, AT\textsubscript{1} and AT\textsubscript{2}. AT\textsubscript{1} receptor is a dominant form in cardiac tissue, and its activation promotes vasoconstriction, hypertrophy, fibrosis, thrombosis and atherosclerosis. Activation of the AT\textsubscript{2} receptor mediates counterbalancing functions such as vasodilatation and antihypertrophic and antifibrotic actions. Activated RAS system also increases both local and systemic aldosterone concentration, which further elevates water and sodium retention, increases sympathetic-activated peripheral responses, stiffness of large vessels, fibroblast proliferation and cardiac and smooth muscle cell collagen synthesis and diminishes baroreflexes. (Danilczyk and Penninger, 2006)

Although systemic RAS is activated at later stage of heart failure, the local cardiac RAS system is activated long before any increase can be seen in plasma levels (Bader and Ganten, 2008, Krop and Danser, 2008, Kumar et al., 2009).

2.1.4.3 Increased sympathetic signalling

Cardiac adrenergic system regulates cardiac functions, allowing heart to adapt to dramatically different workloads in a matter of seconds. This system, presumably evolved as a survival response to trauma and blood loss, gives a considerable advantage to short-term survival. However, these compensatory mechanisms are also activated when heart function declines for any reason in an attempt to maintain sufficient organ perfusion. (Bohm et al., 1997)

In the early stages of developing heart failure, increased activity of the sympathetic nervous system compensates the decline in cardiac functions. However, a high level of adrenergic stimulation is extremely cardiotoxic (Rona, 1985). As the heart becomes progressively less sensitive to stimulatory sympathetic activity in a protective manner, chronic stimulation by circulatory catecholamines leads
to a vicious circle that worsens the clinical condition and contributes to increased cardiac damage. (Bohm et al., 1997)

Especially damaged by sympathetic signalling is the peripheral vasculature, where, unlike in the heart, no protective mechanisms, like β-receptor desensitization or uncoupling of β-receptor-mediated signalling are present. Increased vasoconstriction leads to hypoperfusion and anoxia of the tissues, increasing anaerobic metabolism and workload of the left ventricle, which further stresses a failing heart. Increased noradrenalin levels also stimulate development of hypertrophy (for review, see (Osadchii, 2007)), mainly via α-receptors and fibrosis making the heart more prone to arrhythmias. Sympathetic activity also has a synergistic effect on the renin-angiotensin-aldosterone system; by increasing renin excretion (Osadchii, 2007).

In patients with heart failure, plasma noradrenalin concentration is increased compared with normal controls due to leakage from nerve endings and decreased clearance. Increased plasma concentration correlates strongly with severity and prognosis of the disease; increased noradrenalin concentration already in an asymptomatic functional disorder of the left ventricle predicts development of heart failure, ischaemic heart events and increased risk of death. (Benedict et al., 1996) In a failing heart, also the β-receptor subtypes undergo down regulation resulting in a suboptimal ratio of β₁:β₂ receptors of 50:50 instead of the normal 75:25 (Port and Bristow, 2001).

2.1.4.4 Ca²⁺ handling and regulation of excitation-contraction coupling

Altered calcium handling plays a major role in development of heart failure. In many cases, defects in calcium handling precede depressing function; for example, in compensated hypertrophy this is seen as impaired relaxation in the presence of normal or increased systolic parameters. (Gwathmey et al., 1987a) Reduced peak systolic Ca²⁺ concentration, elevated diastolic Ca²⁺ concentration and prolonged duration of Ca²⁺ transient are common calcium handling features in a failing heart. Defects in calcium handling are due to abnormalities in function or expression of calcium handling proteins, increase in space between L type Ca²⁺ channels and ryanodine receptors (RyRs) and velocity of synchrony of Ca²⁺ release. (Gwathmey et al., 1987b, Gwathmey and Hajjar, 1990b, Gwathmey and Hajjar, 1990a, Gwathmey and Hajjar, 1990c, Piacentino et al., 2003)

Impaired relaxation and abnormal force-frequency in both isolated muscles and myocytes from failing hearts suggest a deficiency in Ca²⁺ reuptake to the sarcoplasmic reticulum. A reduction in SERCA2 mRNA and protein level or SERCA2 protein activity or altered SERCA2 to NCX ratio has been found in both human and experimental studies (Zarain-Herzberg et al., 1996, Seki et al., 2003a, Schmidt et al., 1998, Okayama et al., 1997, Meyer et al., 1995, Aoyagi et al., 1999). Hypophosphorylation of phospholamban (PLN) via desensitization of the β-adrenergic signalling pathway or activation of the RAS system further reduces SERCA2 activity, decreasing contractility (Dash et al., 2001, Zheng et al., 2004). Interestingly, improvement in cardiac functions and calcium
handling can be achieved via gene transfer of SERCA2 in different experimental models (Miyamoto et al., 2000, del Monte et al., 1999, del MonteF. et al., 1999).

2.1.4.5 Cardiac hypertrophy

Cardiac hypertrophy, characterized by increased cell volume and cardiac mass, is an adaptive mechanism in case of increased volume or pressure load. Although cardiac hypertrophy, especially left ventricular hypertrophy, is a powerful risk factor for cardiac events (Artham et al., 2009), all forms of hypertrophy are not pathological; during development hypertrophy is major determinant of growth. Also exercise-induced hypertrophy mediates positive effects via the increased cardiac output required by physical activity. In developmental and physiological hypertrophy, the growth between wall thickness and chamber volume is balanced, while pathological hypertrophy is characterized by a thickened ventricular wall with a concomitant decrease in ventricular chamber dimension. (Dorn, 2007) Hence, pathological overgrowth can severely hinder cardiac pumping potential.

Cardiac hypertrophy can be divided into two separate forms, concentric and eccentric hypertrophy (Figure 3). Concentric hypertrophy, induced by pressure overload, is characterized by increased wall thickness with thickened myocytes but without a change in chamber radius. However, in eccentric hypertrophy, mediated by volume overload, chamber volume grows with increased length of individual myocytes and decreased wall thickness. Eccentric hypertrophy is typically induced by myocardial infarction, while chronic hypertension and aortic stenosis are the main risk factors for concentric hypertrophy. Neurohumoral factors, such as angiotensin II, aldosterone, adrenalin and endothelin-1, are also important stimulators of hypertrophic responses, and the corresponding receptor antagonists and inhibitors can blunt their effects (Gupta et al., 2007, Lorell and Carabello, 2000).
Mechanisms of cardiac hypertrophy. Modified from (Gupta et al., 2007)

2.1.4.5.1 Mechanisms involved in heart failure-related cardiac hypertrophy

Hypertrophic stimuli, such as wall stress, vasoactive peptides, neurohormones, and growth factors, activate a complicated signalling network eventually leading to hypertrophic response. Mechanical stress sensory system, G-protein activation and several signalling cascades, such as calcineurin or mitogen activated protein kinase (MAPK)-mediated pathways are all important regulators for cardiac hypertrophy, and their roles are discussed below. (For review see (Gupta et al., 2007, Barry et al., 2008, Heineke and Molkentin, 2006, Pikkarainen et al., 2004))

Mechanical stress of the ventricular wall is detected via specified internal sensory systems. Integrins connect the intracellular cytoskeleton to the extracellular matrix, and they have been speculated to mediate mechanical stress signals (Brancaccio et al., 2003, Donker et al., 2007). Two classes of molecules, structural and signalling proteins, mediate integrin-actin associations. The structural proteins consist of the integrins and cytoplasmic molecules such as vinculin and talin. The signaling proteins include a number of enzymes and adaptor molecules, responsible for initiation of downstream molecule activation, such as Akt, RAS and ERK1/2, which execute the hypertrophy program. (Brancaccio et al., 2006) Impairment of this sensory system can protect from pathologic hypertrophy as mice lacking the four-and-a-half LIM domains 1 (Fhl1), element of sensory apparatus
of sarcomeres, represent blunted response to hypertrophic stimuli and conserved cardiac capacity after pressure overload caused by aortic banding (Sheikh et al., 2008).

G protein activation plays an important role in the cardiovascular system. Several neurohormonal mediators, such as angiotensin II, endothelin-1 and adrenoreceptors, are coupled with different G proteins of Gaq/α11 subclass. Gaq/α11 coupling seems to be involved specifically in pathological cardiac hypertrophy, as overexpression of wildtype Gaq leads to severe cardiac hypertrophy and heart failure (D'Angelo et al., 1997), and its inactivation counteracts from these detrimental effects (Wettschureck et al., 2001). However, activation of Gaq/α11 can also promote prosurvival and anti-apoptotic effects through Akt activation, which is independent of hypertrophy-promoting functions (Howes et al., 2006). Inhibition of beta-adrenergic receptor signalling has been shown to reduce hypertrophic response induced by chronic overload (Plante et al., 2004, Paradis et al., 2000). Also transition from cardiac hypertrophy to chronic heart failure in the presence of simultaneous chronic pressure overload and loss of cardiac mass in ischaemic hearts is affected by Gαi-mediated signalling pathways (Kouchi et al., 2000).

Phosphoinositide 3-kinases (PI3K) and Akt regulate cardiomyocyte growth, survival and metabolism (Matsui et al., 2003). Activation of PI3K increases the amounts of phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4,5-triphosphate in a cell membrane, resulting in phosphorylation and activation of Akt. Akt is an important mediator of cardiomyocyte growth. Indeed, transgenic overexpression of Akt has been demonstrated to induce hypertrophic growth in the heart (Matsui et al., 2003, Shioi et al., 2002).

Calcineurin, a Ca2+-dependent serine/threonine protein-phosphatase, activates a nuclear factor of activated T cell (NFAT)-dependent hypertrophic response, which has been speculated to be an important regulator of maladaptive hypertrophy (Heineke and Molkentin, 2006). Calcineurin, which is activated by elevated intracellular calcium, binds to NFAT. Once dephosphorylated, NFAT translocates into cytoplasm and initiates prohypertrophic gene expression. Calcineurin-NFAT signalling is controlled by several kinases, such as p38 and c-Jun N-terminal kinases (JNKs), which phosphorylate NFAT and reduce hypertrophic response. (Braz et al., 2003, Liang et al., 2003) Inhibition of calcineurin activity has been shown to have beneficial effects on hypertrophy both in humans and in experimental models, but severe side-effects limit its use for treating larger populations (Wilkins and Molkentin, 2004).

Mitogen-activated protein kinases (MAPK) play an important role in hypertrophic overgrowth. MAPK family members are involved in major biological processes such as cell growth, differentiation and apoptosis, and MAPK activation has been discovered in the heart in several different disease stages, e.g. hypertrophies with a different aetiologies and ischaemic/reperfusion injury (Kyriakis and Avruch, 2001a). The MAPK superfamily is grouped on following three categories: extracellular signal-regulated protein kinase (ERKs), JNKs and p38 MAPKs. In cardiomyocytes ERKs are activated by great variety of hypertrophic stimuli, including angiotensin II, endothelin 1 and several
growth factors, as well as mechanical stress. JNKs and p38 MAPKs are activated mainly via cellular stresses such as heat shock, oxidative stress, ischaemia and inflammatory cytokines. (Kyriakis and Avruch, 2001b, Muslin, 2008) Their actions are speculated to be mediated in prosurvival manner, and they might actually be negative regulators of hypertrophy (Heineke and Molkentin, 2006, Opie et al., 2006).

Protein kinase C (PKC) is universally important mediator; functioning downstream of almost all membrane-associated transduction pathways (Nishizuka, 1986). PCK isozymes are involved in several cardiac diseases, as well as cardiac injuries and ischemic postconditioning (Palaniyandi et al., 2009). Expression of PKC subtypes, α, β and δ, is elevated throughout the transition from the hypertrophic state to heart failure, but expression of PCKε is reduced in chronic failure (Koide et al., 2003).

2.1.4.6 Oxidative stress

Mammalian heart is an obligate aerobic organ; at rest, it consumes 2- to 5-fold and during vigorous exercise over 20-fold more oxygen than the brain. Constant supply of oxygen is fundamental for cardiac functions. Severe ischaemic attacks will cause permanent damage to heart muscle. Oxygen level is also an important regulator of myocardial gene expression, nitric oxide (NO) bioavailability and generation of reactive oxygen species (ROS). (Giordano, 2005)

ROS consist of free radicals, such as superoxide radical, hydroxyl radical and hydrogen peroxide. ROS can also be produced from NO, reaction between NO and superoxide radical forms peroxynitrite, a highly reactive radical. ROS can be generated via several mechanisms: xanthine oxidase, several different NADPH oxidases, cytochrome P450, cyclo-oxygenases and uncoupled NO synthases. An electron leak from mitochondrial respiratory chain and inflammatory cells are the main source of organelles for ROS. Production of ROS can also be induced by cytokines and growth factors, such as angiotensin II and tumour necrosis factor α (TNF-α). (Giordano, 2005)

Several cellular mechanisms exist for prevention of ROS-induced damage, including enzymatic pathways such as catalase and glutathione peroxidases, superoxide dismutases and the thioredoxin system as well as non-enzymatic mechanisms of intracellular antioxidants such as vitamins E and C, lipoic acid and glutathione. These protective mechanisms maintain the balance between ROS and antioxidative defence, the so-called redox state of the cell. The pathological state of excess ROS production is known as oxidative stress (for review see (Giordano, 2005, Seddon et al., 2007)).

ROS are an important mediator of numerous cellular signalling pathways. At low concentrations, local, targeted production is crucial for normal functioning of the cell. ROS serve as second messengers in response to specific stimuli, such as angiotensin II and endothelin, and modulate activity of specific transcription factors altering physiological responses like inflammation. However, when ROS are produced in excessive amounts, saturating the cellular antioxidant capacity, they can directly react with cellular components. The main pathological effects of oxidative stress are
summarized in Figure 4 (Seddon et al., 2007). ROS can damage cellular membranes by lipid peroxidation, induce mutagenesis of DNA and damage to chromatin structures and modify activity of enzymes critical to normal function of the cell. Also general aging and age-related alterations in the cardiovascular system are suggested to be linked to long-term effects of ROS. (Sam et al., 2005, Suematsu et al., 2003, Sun et al., 2005, Wenzel et al., 2008, Hemnani and Parihar, 1998, Ide et al., 2001)

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

**Figure 4.** Main pathological effects of increased ROS production. Modified from (Seddon et al., 2007).

ROS have a strong influence in myocardial calcium handling. ROS can target sarcolemmal L-type calcium channels and suppress Ca\(^{2+}\) current, inhibit SERCA2 activity and alter the function of cardiac sodium channels, potassium channels and ion exchangers, such as Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) (Guerra et al., 1996, Goldhaber, 1996, Kaplan et al., 2003). ROS also has an affect on contractile protein machinery; phosphorylation of troponin T via ROS-related kinase apoptosis signal-regulated kinase 1 (ASK-1) diminishes contractility and impairs calcium handling (He et al., 2003).

Despite clear preclinical evidence of benefit of preventing ROS-induced cardiac damage, studies attempting to treat or prevent this in humans have so far provided disappointing results as ROS-preventive effect of vitamin supplementations have showed no cardioprotective effects. (Heart Protection Study Collaborative Group, 2002, Yusuf et al., 2000)
2.1.4.7 Apoptosis

Apoptosis is an important mechanism during development and elimination of injured or infected cells, but in excessive amounts or with longer exposure it can result in failing organ function. In apoptosis, cells die in a tightly controlled manner, opposite to cell lysis and inflammatory response in necrotic events, representing several molecular characteristics such as cellular and nuclear shrinkage, chromatin condensation, cell membrane blebbing, formation of apoptotic bodies and DNA fragmentation (for review see (Movassagh and Foo, 2008, van Empel et al., 2005)).

Apoptosis can be divided into two main categories, the intrinsic pathway, which involves mitochondria and endoplasmic reticulum, and the cell membrane death receptor activated extrinsic pathway (Movassagh and Foo, 2008, van Empel et al., 2005). The extrinsic pathway is activated via binding of death ligands, such as Fas ligand, TNF-α, TNF-related apoptosis inducing ligand (TRAIL) or TNF superfamily member 10 (TNFSF10), into their corresponding death receptors. Binding of ligand to receptor results in activation of adaptors, recruitment of pro-caspases 8 and 10 and finally formation of death-inducing signalling complex (DISC). In DISC, pro-caspases 8 and 10 are processed via autoproteolysis and released as activated proteases. Activation of apoptosis can result directly from activation of the extrinsic pathway or it can be linked to the intrinsic pathway. (Movassagh and Foo, 2008, van Empel et al., 2005)

The intrinsic pathway is promoted via a great variety of stimuli, both extra- and intracellular, such as ischaemia/reperfusion, oxidative stress, hypoxia, radiation and DNA damage, resulting in activation of death machinery located in the mitochondria and endoplasmic reticulum. One of the main characteristics in apoptosis is mitochondrial outer membrane permeabilization. Thus, pro-apoptotic factors, located between mitochondrial membranes, are released into the cytoplasm activating the irreversible apoptotic pathway. Mitochondrial permeability transition pore, a protein complex in both mitochondrial membranes, is considered to mediate the increased permeability of mitochondrial membranes and the release of apoptotic mediators, including cytochrome c, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), endonuclease G, Omim/Htr and apoptosis-inducing factor (AIF), into cytoplasm. Once released into cytoplasm, cytochrome c activates several caspases through “apoptosome” formation, Smac/DIABLO binds caspase-inhibitory proteins, and endonuclease G and AIF translocate into the nucleus and facilitate DNA damage. (van Empel et al., 2005)

Mitochondrial outer membrane permeabilization also activates the Bcl-2 family of critical apoptosis regulators, which results in release of cytochrome c from the intermembrane space and activation of further apoptotic cascades. (van Empel et al., 2005)

Beside mitochondrial and death receptor pathways, also other possible apoptotic mechanisms exist, since mice defective for both caspase 8 (crucial for intrinsic pathway) and 9 (crucial for extrinsic pathway) still have apoptosis (Wang and Lenardo, 2000).
Heart failure-related increased apoptosis has been demonstrated in both humans and several animal models (Narula et al., 1996, Olivetti et al., 1997a, Kang and Izumo, 2000). Although discovered rates of ongoing apoptosis have been low at certain time-point, chronic low-grade apoptosis has been shown to result in significant cardiomyocyte loss, which negatively influences the progression of heart failure (Abbate et al., 2003, Wencker et al., 2003, Sarkar et al., 2004). For example, in patients with dilated cardiomyopathy the prevalence of apoptosis is 0.08-0.25%, approximately 80–250 times more than in healthy controls (Saraste et al., 1999, Olivetti et al., 1997b). After myocardial infarction, acute rates of apoptosis are even greater, apoptotic rates reaching 2-12% in the border zone of human myocardial infarcts (Olivetti et al., 1996, Ottaviani et al., 1999). Of current treatment options for heart failure, suppression of effects of angiotensin II or increased β-adrenergic drive by AT₁ blockers or β-blockers, respectively, has been shown to decrease cardiomyocyte apoptosis (Kajstura et al., 1997, Groholm et al., 2004, Sabbah et al., 2000), but further pharmacological treatment options are required. For example, reduction of oxidative stress via antioxidants, prevention of mitochondrial membrane permeabilization, caspase inhibition or ATP-dependent potassium channel openers provide exciting possibilities for future treatment options.

2.1.4.8 Dysfunctional energy utilization

Evidence has emerged of alterations in energy metabolism and mitochondrial functions as a cause and effect of heart failure. Proper cardiac functions are strongly related to a balance between energy demand and production since the heart has a limited capacity for substrate storage. Development of heart failure, regardless of its aetiology, is associated with progressive loss of mitochondrial respiratory functions, leading to diminished ATP production capacity. Depressed mitochondrial functions and oxidative capacity have been reported in a failing rat heart. (Garnier et al., 2003) Lack of energy further hinders the cardiac functions by depressing Ca²⁺ handling and contractility, leading to a vicious cycle of ever-worsening heart failure.

The human heart requires a constant supply of energy and daily consumption of approximately 30 kg. Mitochondrial oxidation of fatty acids and glucose is a major source for ATP in the adult heart. Fatty acids are the preferred fuel providing about 70-90% of total ATP. (Huss and Kelly, 2005)

In the early stages of heart failure, cardiac energy metabolism has foetal-like features; fatty acid utilization remains unchanged or slightly increases with increased glucose utilization with normal ATP and decreased phosphocreatine and total creatine levels (for review see (Murray et al., 2007, Neubauer, 2007)). In patients with heart failure, a low phosphocreatine/ATP ratio correlates with clinical symptoms and predicts mortality with a high accuracy (Neubauer et al., 1997, Conway et al., 1998). Especially during hypoxia or ischaemia, the utilization of glucose is favoured because it yields more ATP per consumed oxygen. However, in the late stages of the disease both fatty acid and glucose utilization are diminished, resulting in deleterious energy depletion. Reduced activity of mitochondrial creatine kinase and myofibrillar creatine kinase results in loss of high-energy
phosphates and ATP impairing both availability and transfer of energy to myofibrils and thus provoking diminished inotropic reserve. Increased catecholamine signalling often found in heart failure further impairs contractile functions by elevating free ADP concentration. Mitochondrial dysfunction is an especially important mechanistic factor in diabetes-related cardiovascular complications, where cardiomyocyte energetics is already compromised by increased blood glucose and lipid values (for review see Sack, 2009).

Peroxisome proliferators-activated receptor family (PPAR) is a powerful modulator of cardiac energetics (for review (Madrazo and Kelly, 2008)). PPARα appears to be the main regulator of cardiac lipid metabolism; it controls several genes involved in fatty acid oxidation. Downregulation of PPARα, found in hypertrophy of both human and animal hearts, is thought to be the main mechanism controlling substrate switch from fatty acid to glucose in the failing heart. Since cellular uptake of fatty acids is dependent only on fatty acid plasma level, decreased fatty acid uptake to mitochondria leads to accumulation of lipid intermediates and cardiotoxicity. Indeed, PPARα-null mice exhibit decreased cardiac fatty acid oxidation rates with increased glucose utilization, cardiac fibrosis and decreased contractile reserve (for review, see (Murray et al., 2007, Neubauer, 2007, Madrazo and Kelly, 2008)). PPARα is regulated by PPARγ coactivator-1 (also known as PCG-1α), which activates also several other genes involved in fatty acid metabolism and oxidative phosphorylation. PCG-1α is speculated to be a protective element in the heart since PCG-1α deficient mice exhibit accelerated development of heart failure (Arany et al., 2006).

PPARs and PCG-1α provide an interesting option for modulating cardiac energetics in heart failure, but so far data on their exact roles in energy metabolism of the failing heart are controversial. However PPARα-agonist fibrates are currently used as a lipid-lowering medication. (Huss and Kelly, 2005)

2.1.4.9 Cardiomyocyte senescence

The heart has a limited capability to renew, and because of this cardiac functions and reserve decline with age. Ageing myocytes exhibit the following typical pattern of changes (for review see (Bernhard and Laufer, 2008)):

- Reduced ability to cope with stress via reduced expression of heat shock proteins and antioxidative defences.
- Electron leakage and oxidative stress from altered mitochondrial respiratory chain.
- Reduced excitation/contraction coupling related to down regulation on SERCA2, increased expression of cytoskeletal proteins and transcriptional switch of contractile protein isoforms.
- Shift in the survival signalling balance towards cell death signalling

Senescent cardiomyocytes show changes in β-adrenergic signalling and angiotensin-signalling pathways and eventually lose their ability to compensate cell lose by hypertrophic growth. Finally,
aged cardiomyocytes enter an irreversible cell cycle arrest that is characterized by upregulation of p16\(^{\text{INK4A}}\). (Chimenti et al., 2003, Domenighetti et al., 2005, Urbanek et al., 2003)

Two products of \(^{\text{INK4a/ARF}}\) locus, p16\(^{\text{INK4A}}\) and p19\(^{\text{ARF}}\), create a unique system for mediating senescence and suppressing malignancy. Despite having a partially shared nucleic acid sequence, they are encoded in different reading frames and have neither amino acid homology nor similarity in molecular functions (for review, see (Sharpless, 2004, Collado et al., 2007)). Their actions seem to be the opposite; p16\(^{\text{INK4A}}\) has been shown to promote cellular senescence while tumour suppressor p19\(^{\text{ARF}}\) appears to mediate anti-promoting effects on cellular ageing (Baker et al., 2008b).

Normally, the \(^{\text{INK4a/ARF}}\) locus is expressed in very low amounts in young tissues, but its expression is upregulated in both rodents and human tissues over time (Chkhotua et al., 2003, Krishnamurthy et al., 2004, Ressler et al., 2006). Increased expression of p16\(^{\text{INK4A}}\) has also been shown to be involved in several cancer diseases, native kidney disease and experimental hypertension (Chimenti et al., 2003, Sharpless, 2004, Collado et al., 2007, Westhoff et al., 2008). Human hearts with premature cardiac ageing and heart failure show an accumulation of p16\(^{\text{INK4A}}\)-positive primitive cells and myocytes combined with shortened telomeres, representing an imbalance between cellular growth and death (Chimenti et al., 2003) and hypertension induced expression of p16\(^{\text{INK4A}}\) has been suggested to mediate tissue damage associated with elevated blood pressure (Bergmann et al., 2008).

### 2.2 Experimental models of heart failure

There are several different experimental heart failure models available. These models are ranging from naturally occurring heart failure to experimentally or genetically induced disease types. Commonly used experimental models of heart failure are presented in Table 1.
Table 1. Experimental models used for heart failure. Modified from (Klocke et al., 2007, Monnet and Chachques, 2005, Muders and Elsner, 2000).

<table>
<thead>
<tr>
<th>Techniques</th>
<th>Species</th>
<th>Features of heart failure (HF)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Naturally occurring models</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>Hamster, dog, turkey</td>
<td>Dilated HF, neurohormonal activation</td>
</tr>
<tr>
<td>Salt-sensitive hypertension</td>
<td>Rat</td>
<td>Hypertension-induced HF</td>
</tr>
<tr>
<td><strong>Experimentally induced models</strong></td>
<td></td>
<td></td>
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<tr>
<td>Myocardial ischaemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary ligation</td>
<td>Mouse, rat, dog, pig, rabbit</td>
<td>HF after MI, no confounding atherosclerosis, widely used</td>
</tr>
<tr>
<td>Coronary embolism</td>
<td>Dog, pig</td>
<td>HF with acute coronary syndrome or diffuse coronary artery disease</td>
</tr>
<tr>
<td>Partial coronary occlusion</td>
<td>Dog, pig</td>
<td>HF after MI, hibernating myocardium</td>
</tr>
<tr>
<td>Chronic rapid cardiac pacing</td>
<td>Dog, pig, rabbit</td>
<td>Low output biventricular HF, reversible</td>
</tr>
<tr>
<td><strong>Pressure overload</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic banding</td>
<td>Mouse, rat, guinea pig</td>
<td>Diastolic HF with LV hypertrophy, no neurohumoral activation</td>
</tr>
<tr>
<td>Monocrotaline</td>
<td>Dog, rat, mouse</td>
<td>Pulmonary hypertension-associated hypertrophy of right ventricle</td>
</tr>
<tr>
<td><strong>Volume overload</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arteriovenous shunt</td>
<td>Rat, dog</td>
<td>Increased LV end-diastolic volume, no increased end-diastolic pressure</td>
</tr>
<tr>
<td>Mitral regurgitation</td>
<td>Dog</td>
<td>Asymmetric LV dilatation</td>
</tr>
<tr>
<td>Aortic regurgitation</td>
<td>Rabbit</td>
<td>LV dilatation</td>
</tr>
<tr>
<td><strong>Drug-induced heart failure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Mouse, rat, dog, rabbit, pig</td>
<td>Bilateral ventricular dilatation</td>
</tr>
<tr>
<td>Propranolol</td>
<td>Mouse, rat, turkey</td>
<td>Acute, stable HF, no ventricular dilatation</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>Rat, mouse</td>
<td>Catecholamine-induced LV dilatation and hypertrophy, widely used</td>
</tr>
<tr>
<td><strong>Genetically modified animals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>Mouse, rat, hamster</td>
<td>Large variety of several genetic models for different types of HF</td>
</tr>
</tbody>
</table>
2.3 Levosimendan

2.3.1 Mechanism of action

Levosimendan, the (-) enantiomer of \([4-(1,4,5,6\text{-tetra-hydro-4-methyl-6-oxo-3-pyridazinyl})\text{phenyl}]\text{hydrazono}\text{-propanedinitrile}\) (Figure 5), is a novel inotropic agent used in the management of acute decompensated heart failure (for review see (Lehtonen and Poder, 2007)). The less active (+) enantiomer is dextrosimendan (Kaheinen et al., 2006).

![Chemical structure of levosimendan.](image)

Levosimendan increases cardiac contractility by sensitizing the contractile proteins to calcium (Innes and Wagstaff, 2003). Levosimendan binds to the calcium-saturated N-terminal domain of troponin C in cardiac muscle in a calcium-dependent manner and stabilizes the troponin molecule with subsequent prolongation of its effect on the contractile proteins (Innes and Wagstaff, 2003, Haikala et al., 1995, Sorsa et al., 2001, Sorsa et al., 2004). Stabilization of the troponin C molecule results in an increased amount of actin-myosin interactions, and therefore, increased force for contraction. Levosimendan binds to troponin C in a calcium-dependent manner especially, in the beginning of systole, and detaches in diastole, increasing the force of contraction without shortening the relaxation time or impairing diastolic function. (Haikala et al., 1995) Compared to traditional drugs used for acute heart failure, such as dobutamine, levosimendan has one major benefit: it does not elevate intracellular calcium concentration (Lancaster and Cook, 1997) and make the heart susceptible to arrhythmias.

Levosimendan has a triple pharmacological mechanism of action; in addition to calcium sensitization, it also opens mitochondrial and sarcolemmal ATP-dependent potassium channels (KATP channel) (Kopustinskie, 2004, Yokoshiki et al., 1997b). Opening of sarcolemmal KATP channels by levosimendan relaxes smooth muscle and dilates all blood vessels such as coronary arteries or mesenteric arteries (Bowman et al., 1999, Ozdem et al., 2006). Dilatation of resistance arteries leads to decreased systemic resistance, which often can be seen as a transient decrease in blood pressure during levosimendan treatment. Dilatation of coronary arteries leads to increased coronary flow, which improves tissue perfusion of the heart, preventing ischaemic attacks. Decreased left ventricular
filling pressure caused by decreased pre- and afterload also reduce the expression of neurohormones and natriuretic peptides. (Parissis et al., 2008)

Opening of mitochondrial channels has been shown to be involved in ischaemic preconditioning and prevention of apoptosis (Wang and Lenardo, 2000, Wakiyama et al., 2002, Ardehali and O'Rourke, 2005). Levosimendan has been shown to induce pre- and post-conditioning, protect the heart during an ischaemic attack, reduce infarct size and decrease apoptosis in a KATP channel –dependent manner (du Toit et al., 2008, Kersten et al., 2000).

Levosimendan is also a selective phosphodiesterase (PDE) 3 inhibitor in vitro, but in clinical doses the relevance of PDE inhibition is considered to be somewhat insignificant (Kaheinen et al., 2006, Ajiro et al., 2002, Szilagyi et al., 2005).

2.3.2 Pharmacokinetics of levosimendan

Levosimendan shows a linear pharmacokinetic in a therapeutic dose of 0.05-0.2 µg/kg/min. It has a volume of distribution of 0.2 l/kg. Levosimendan binds strongly (97-98%) to plasma proteins, mainly albumin, and has a clearance of 3.0 ml/min/kg. (Antila et al., 2007)

Levosimendan is rapidly metabolized, with elimination half-life of approximately 1 h; only small proportion (less than 0.05%) of the levosimendan dose is excreted unmetabolized in urine. Levosimendan is mainly excreted as metabolites, in urine (54%) and faeces (44%). (Antila et al., 2007) About 5% of the total dose of levosimendan is secreted into the small intestine where it is reduced by the internal bacteria to OR-1855 (R)-6-(4-Amino-phenyl)-5-methyl-4,5-dihydro-2H-pyridazin-3-one), which is further metabolized with N-acetyltransferase 2 to biologically active OR-1896 (N-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenyl]acetamide).

2.3.3 Pharmacodynamics of levosimendan

Levosimendan dose dependently increases force of contraction in cardiac muscle without impairing relaxation of ventricles. It also dilates systemic and coronary resistance arteries and systemic capacitance veins. Levosimendan infusion increases cardiac output, stroke volume, ejection fraction and pulse rate, and decreases systolic and diastolic blood pressure, pulmonary wedge pressure, pressure in the right atrium and systemic resistance. (De Luca et al., 2006)

2.3.3.1 Clinical use of levosimendan

Levosimendan is used for hospitalized treatment of acute heart failure when standard treatment fails to provide a sufficient inotropic effect. It is administered parenterally via the peripheral or central vein as a single 24-h infusion based on individual characteristics of the patient, e.g. other inotropic or vasodilating drugs.
Levosimendan infusion is generally started with a bolus, 6-12 µg/kg over 10 minutes, and infusion is continued at a dose of 0.1 µg/kg/min. Haemodynamic response is monitored carefully to evaluate appropriate dosing, and the infusion dose is set between 0.05-0.2 µg/kg/min. Due to possible accumulation of the active, long-lived metabolite of levosimendan, OR-1896, levosimendan infusion should not be continued beyond of 24 h, however positive haemodynamic effects can be seen as long as 9 days after infusion. (Archan and Toller, 2008) Preliminary data indicate the safety and efficacy of repeated levosimendan infusions, but further studies are warranted to evaluate continuous or repeated levosimendan administration (Parle et al., 2008, Parissis et al., 2006b, Nanas et al., 2005).

Contraindications include possible allergies to levosimendan or auxiliary substances of the product, severe hypotension and tachycardia, severe renal (creatinine clearance <30 ml/min) or hepatic failure and a previous episode of torsades de pointes tachycardia. Levosimendan has been found to be teratogenic in animal experiments, and it should be used during pregnancy only when the maternal benefit exceeds the potential harm to the foetus. The effect of levosimendan on lactation remains unknown in humans. In rats, levosimendan is excreted in milk, and therefore women receiving levosimendan probably should not breastfeed.

In heart failure patients, levosimendan has a positive inotropic and vasodilatory effect, which leads to increased cardiac contraction force and perfusion of cardiac muscle and decreased pre- and afterload without impaired diastolic function (for review see (Kasikcioglu and Cam, 2006)). With patients recovering from cardiac surgery or procedure, such as balloon angioplasty, or patients treated with thrombolytic therapy, levosimendan activates the stunned myocardium and increases coronary flow (Zangrillo et al., 2009). Levosimendan infusion increases neither cardiac oxygen demand nor intracellular calcium concentration, while plasma concentrations of natriuretic peptides and endothelin-1 significantly decrease. Levosimendan does not elevate plasma catecholamine concentration with therapeutic dosing. (Antila et al., 2007)

Limited amount of data are available on the cost efficacy of levosimendan in treating heart failure. Levosimendan is more expensive than dobutamine, but the overall costs of levosimendan appear to be lower or comparable with those of placebo or dobutamine due to a shorter in-hospital time (Oliveira et al., 2005, Cleland et al., 2004, de Lissovoy et al., 2009).

2.3.3.2 Adverse effects

Levosimendan is generally well tolerated. The clinically most relevant adverse events are mainly originated by hypotension and arrhythmias. (Table 2)
Table 2  Adverse effects related to clinical use of levosimendan. “Common” indicates adverse effect in 1 of 100 patients, “very common” 1 of 10 patients. (Fimea, 2009)

<table>
<thead>
<tr>
<th>Organ system</th>
<th>Prevalence</th>
<th>Adverse effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic and nutritional disorders</td>
<td>Common</td>
<td>Hypokalemia</td>
</tr>
<tr>
<td>Psychic disorders</td>
<td>Common</td>
<td>Insomnia</td>
</tr>
<tr>
<td>Nervous system</td>
<td>Very common</td>
<td>Headache</td>
</tr>
<tr>
<td></td>
<td>Common</td>
<td>Vertigo</td>
</tr>
<tr>
<td>Heart</td>
<td>Very common</td>
<td>Tachycardia</td>
</tr>
<tr>
<td></td>
<td>Common</td>
<td>Atrial fibrillation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tachycardia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heart failure</td>
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<tr>
<td></td>
<td></td>
<td>Cardiac ischaemia</td>
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<tr>
<td></td>
<td></td>
<td>Extra systole</td>
</tr>
<tr>
<td>Vasculature</td>
<td>Very common</td>
<td>Hypotension</td>
</tr>
<tr>
<td>Gastrointestinal-track</td>
<td>Common</td>
<td>Nausea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Constipation</td>
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<tr>
<td></td>
<td></td>
<td>Diarrhoea</td>
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<tr>
<td></td>
<td></td>
<td>Vomiting</td>
</tr>
<tr>
<td>Laboratory markers</td>
<td>Common</td>
<td>Decrease in blood haemoglobin</td>
</tr>
</tbody>
</table>

2.3.3.3  Clinical studies on levosimendan

Intravenous levosimendan has been assessed in several medium- and large-scale clinical studies of treatment of heart failure (Table 3). Levosimendan treatment improves symptoms of heart failure, such as dyspnoea or fatigue and plasma BNP, relative to placebo (Packer, 2005, Slawsky et al., 2000). Compared with dobutamine, levosimendan improves symptoms equally or slightly better and is associated with a shorter hospital stay (Packer, 2005).
### Table 3. Clinical studies of treatment of heart failure with levosimendan. Modified from (Lehtonen and Poder, 2007)

<table>
<thead>
<tr>
<th>Study</th>
<th>Study model</th>
<th>Study population total/levo</th>
<th>Treatment</th>
<th>End-points</th>
<th>Results</th>
<th>Mortality</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>European dose finding study</td>
<td>Randomized, placebo-controlled, parallel group</td>
<td>151/95</td>
<td>Levo infusion (bolus 3-36 µg/kg, 0.05-0.6 µg/kg/min) vs placebo or 6 µg/kg/min dobutamine for 24 h. FU for 7 d</td>
<td>Primary: Haemodynamic improvement. Secondary: Individual haemodynamic parameters.</td>
<td>Levo significantly improved haemodynamic status with good tolerability</td>
<td>No difference</td>
<td>1</td>
</tr>
<tr>
<td>US dose duration study</td>
<td>Randomized double-blind, placebo-controlled, parallel group</td>
<td>148/96 patients with decompensated HF</td>
<td>Repeated bolus of 6 µg/kg, 0.1-0.4 µg/kg/min for 24 or 48 h. FU for 14 d</td>
<td>Primary: Haemodynamic improvement. Secondary: Individual haemodynamic parameters, symptoms of HF, 14-d outcome</td>
<td>Levo significantly improved haemodynamic status</td>
<td>No difference</td>
<td>2</td>
</tr>
<tr>
<td>REVIVE II</td>
<td>Randomized, double-blind, placebo-controlled</td>
<td>600/299 patients with primary or secondary HF with EF&lt;35%</td>
<td>Levo infusion (bolus 6-12 µg/kg, 0.1-0.2 µg/kg/min) vs placebo for 24 h. FU for 90 d</td>
<td>Primary: Death or worsening of HF with patients self-assessment combined with physician’s assessment of clinical deterioration. Secondary: Mortality at 90 d, duration of hospital stay, BNP at 24 h, patient global assessment and dyspnoea at 6 h</td>
<td>Levo significantly prevented worsening of HF and dyspnoea and improved patient’s global assessment score</td>
<td>No difference</td>
<td>3</td>
</tr>
<tr>
<td>SURVIVE</td>
<td>Randomized double-blind, placebo-controlled, parallel group, multicenter trial</td>
<td>1327/664 patients with acute decompensated HF (EF&lt;30%) not responding to standard treatment</td>
<td>Levo infusion (bolus 12 µg/kg, 0.1-0.2 µg/kg/min) vs dobutamine infusion (5 µg/kg/min) for 24 h. FU for 180 d</td>
<td>Primary: All-cause mortality in 180 d. Secondary: All cause mortality in 31 d, cardiovascular mortality in 180 d, unhospitalized days alive at 180 d, BNP at 24 h, HF symptoms</td>
<td>No difference in mortality</td>
<td>No difference</td>
<td>4</td>
</tr>
<tr>
<td>LIDO</td>
<td>Randomized, double-blind, parallel group</td>
<td>203/100 patients on levo and 103 on dobutamine</td>
<td>Levo infusion (bolus 24 µg/kg, 0.1 µg/kg/min) vs dobutamine infusion 5 µg/kg/min) for 24 h FU for 31 d</td>
<td>Primary: Haemodynamic improvement. Secondary: Symptoms of HF, 31-day outcome</td>
<td>Levo improved haemodynamic performance and prevented mortality more effectively than dobutamine</td>
<td>Levo superior to dobutamine</td>
<td>5</td>
</tr>
<tr>
<td>Study</td>
<td>Type</td>
<td>Patients</td>
<td>Intervention</td>
<td>Primary</td>
<td>Secondary</td>
<td>Tertiary</td>
<td>Results</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td>----------</td>
<td>--------------</td>
<td>---------</td>
<td>-----------</td>
<td>----------</td>
<td>---------</td>
</tr>
<tr>
<td>RUSSLAN</td>
<td>Randomized, placebo-controlled, parallel group</td>
<td>504/402 patients with inotropic support requiring HF after acute MI</td>
<td>Levo infusion (bolus 6, 12 or 24 µg/kg, 0.1, 0.2 or 0.4 µg/kg/min) for 6 h FU for 14 d</td>
<td>Safety in regards of clinically significant ischaemia and hypotension, Symptoms of HF, mortality at 14 d</td>
<td>No differences in hypotension, development of ischaemia or mortality</td>
<td>Levo superior to placebo</td>
<td></td>
</tr>
<tr>
<td>BELIEF</td>
<td>Non-randomized, open label</td>
<td>Levo infusion (bolus 6-12 µg/kg, 0.1-0.2 µg/kg/min) for 24 h Follow up for 31 d</td>
<td>Primary: Hospital discharge without inotropic therapy Secondary: Haemodynamics, clinical parameters, BNP</td>
<td>Primary: Levo infusion found to be a safe alternative therapy Secondary: Significant improvement in quality of life testing</td>
<td>Primary: Levo infusion found to be a safe alternative therapy Secondary: Significant improvement in quality of life testing</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CASINO</td>
<td>Randomized</td>
<td>299/100 heart failure patients hospitalized with NYHA class IV and EF&lt;35%</td>
<td>Levo infusion (bolus 16 µg/kg, 0.1-0.4 µg/kg/min) vs placebo/dobutamine infusion (10 µg/kg/min) for 24 h. FU 1 year</td>
<td>Primary: Mortality at 1 and 6 months and 1 year. Secondary: Death or rehospitalization due to worsening HF.</td>
<td>Study prematurely halted by data safety monitoring board due to superior results of levo</td>
<td>Levo superior to placebo or dobutamine</td>
<td></td>
</tr>
<tr>
<td>PERSIST</td>
<td>Randomized, double-blind</td>
<td>307/102 1 mg once daily, 103 1 mg twice daily</td>
<td>Oral levo 1 mg once or twice daily. FU at least 180 d</td>
<td>Primary: Patient Journey, repeated symptom assessment. Secondary: Time to death or worsening of HF, days alive and out of hospital, all-cause cardiovascular mortality, NYHA class, BNP, patient assessment</td>
<td>No improvement of Patient Journey Improved quality of life and decreased BNP</td>
<td>No difference</td>
<td></td>
</tr>
</tbody>
</table>

Levosimendan reduced mortality in the LIDO (Efficacy and safety of intravenous levosimendan compared with dobutamine in severe low-output heart failure), RUSSLAN (Safety and efficacy of a novel calcium sensitizer, levosimendan, in patients with left ventricular failure due to an acute myocardial infarction. A randomized, placebo-controlled, double-blind study) and CASINO (Calcium Sensitizer or Inotrope or None in Low Output Heart Failure) trials, and a positive effect on mortality was still seen at 6 months after the infusion (Follath et al., 2002, Moiseyev et al., 2002, Zairis et al., 2004). In REVIVE (The Randomized multicenter Evaluation of Intravenous leVosimendan Efficacy vs. placebo in the short-term treatment of decompensated heart failure) trials, levosimendan was associated with a slightly higher mortality than placebo, but this difference was not statistically significant (Packer, 2005). However, it is noteworthy that neither the LIDO, RUSSLAN, CASINO, nor REVIVE trials were powered for assessment of mortality, and therefore, any conclusions on a matter should be drawn with care. In the larger SURVIVE (Survival of Patients With Acute Heart Failure in Need of Intravenous Inotropic Support) trial (total of 1327 patients), levosimendan and dobutamine showed similar results for mortality at any time-point or comparisons between treatments (Mebazaa et al., 2007). In conclusion, data on levosimendan in the short- or long-term survival of patients with acute decompensated heart failure remain inconclusive.

Levosimendan has also been investigated extensively in smaller scale clinical studies. Heart failure is associated with changes in inflammatory functions, oxidative stress and induction of apoptosis, which all of worsen survival (for review see (Giordano, 2005, Huss and Kelly, 2005, Foo et al., 2005)). Levosimendan infusion has been shown to reduce markers of oxidative and nitrosative stress and inflammatory and apoptotic pathways in patients with advanced heart failure without severe adverse effects (Adamopoulos et al., 2006b, Avggeropoulou et al., 2005, Parissis et al., 2004b, Parissis et al., 2007, Trikas et al., 2006). Levosimendan is also speculated to beneficially affect extracellular matrix remodelling based on the finding that levosimendan reduces serum levels of matrix metalloproteinases in patients with decompensated heart failure (Tziakas et al., 2005). The use of levosimendan has been evaluated in several diseases other than heart failure, and it has been shown to have beneficial effects after aortic valve replacement for aortic stenosis and on refractory cardiogenic shock, renal functions on heart failure, acute respiratory distress syndrome and pulmonary failure and stunned myocardium (Sonntag et al., 2004, Yilmaz et al., 2007, Morelli et al., 2006, Jorgensen et al., 2008, Fuhrmann et al., 2008). Levosimendan administered in a preconditioning manner before coronary artery bypass grafting protects from myocardial damage induced by the procedure; this finding may be related to levosimendan ion channel opening properties (Tritapepe et al., 2006).

To summarize, results from levosimendan trials suggest that levosimendan improves symptoms and clinical course compared with placebo and is similar to dobutamine. According to a very recent meta-analysis, levosimendan has a positive trend towards improved survival relative to placebo. Compared with dobutamine, levosimendan is associated with improved survival. (Delaney et al., 2008) Levosimendan has also been shown to have additional beneficial effects on inflammation markers and
apoptosis (Adamopoulos et al., 2006b, Parissis et al., 2004b, Trikas et al., 2006), but the true clinical relevance of these findings is a subject for future research.

2.3.3.4 Clinical studies on oral levosimendan

Data on oral use of levosimendan remain limited. Oral levosimendan has been shown to improve cardiac function in dogs with heart failure and to prevent mortality in rats with coronary occlusion (Masutani et al., 2008, Lepran and Papp, 2003). In patients with heart failure oral levosimendan has been shown to induce a similar inotropic effect as the intravenously administered form (Poder et al., 2003). A very recent study of oral levosimendan in patients with severe chronic heart failure patients (PERSIST) failed to show a significant improvement in cardiac functions. However, patients in PERSIST did have a reduction in BNP values and improved self-reported quality of life. (Nieminen et al., 2008)

2.3.3.5 Experimental studies on levosimendan

Experimental studies on different animal models, including the rat (Boost et al., 2008, Louhelainen et al., 2007), sheep (Dubin et al., 2006, Dubin et al., 2007), guinea pig (Brendt et al., 2008), dog (Papp et al., 2006) and pig (Cunha-Goncalves et al., 2007, Koudouna et al., 2007, Kerbaul et al., 2007), have revealed a marked role of levosimendan in treating various diseases models. Levosimendan has been shown to reduce inflammatory response and mortality after experimental lung injury and chronic hypertension (Boost et al., 2008, Louhelainen et al., 2007), to improve outcome of cardiopulmonary resuscitation after cardiac arrest (Koudouna et al., 2007) and to restore right ventricular failure due to pulmonary embolism (Kerbaul et al., 2007). Reports on levosimendan in management of endotoxaemia are somewhat conflicting, and beneficial effects, such as improved renal function or prevention of intramucosal acidosis, might be overshadowed by episodes of systemic hypotension or ischaemia (Dubin et al., 2006, Cunha-Goncalves et al., 2007, Zager et al., 2006). In cells exposed to inflammatory stimuli, levosimendan decreases nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-mediated transcription, inducible nitric oxide synthase (iNOS) expression and NO production (Sareila et al., 2008).

Levosimendan provides an interesting option for treatment of nerve injuries. In models of spinal cord damage by aorta clamping, administration of levosimendan has reduced ischaemic damage and provided a better neurological outcome (Katircioğlu et al., 2008, Lafci et al., 2008).

Ischaemic preconditioning prior to ischaemia-reperfusion injury is known to protect cardiac muscle and reduce size of infarction. Levosimendan is, besides its mainly recognized role as a calcium sensitizer, also an opener of both sarcolemmal and mitochondrial KATP channels without elevation of Ca\(^{2+}\) currents in both humans and experimental animal models (Kopustinskiene et al., 2004, Yokoshiki et al., 1997b, Hohn et al., 2004, Kaheinen et al., 2001, Kopustinskiene et al., 2001, Usta et
al., 2006, Yokoshiki et al., 1997a). Pretreatment with levosimendan and ischaemic preconditioning improve survival and offer cardioprotection in a similar manner (Kersten et al., 2000, Das and Sarkar, 2007). These cardioprotective effects are speculated to be mediated via both opening of mitochondrial KATP channels and NO –dependent pathways since vasodilatory and preconditioning effects can be abolished with potassium channel antagonists (Kopustinskiene et al., 2001, Das and Sarkar, 2007). Anti-apoptotic effects of levosimendan, discovered in a hypertensive rat model (Dahl/Rapp SS rat), are also speculated to be mediated via its blood pressure-independent potassium channel opening properties (Louhelainen et al., 2007).

2.3.4 The active metabolite OR-1896

Levosimendan has two active circulating metabolites, OR-1896 and OR-1855, both found in humans and rats (Koskinen et al., 2008). The active metabolite OR-1896 has similar haemodynamic functions as the parent drug, while OR-1855 unable to produce any haemodynamic effects (Segreti et al., 2008, Takahashi et al., 2000b, Takahashi et al., 2000a, Banfor et al., 2008). OR-1896 has a considerably longer half-life than levosimendan, approximately 80 h vs. 1 h in humans and 6.5 h vs. 0.76 h in rats (Orion Pharma, unpublished data), making it responsible for long-term - up to one week - effects of a single levosimendan infusion (Kivikko et al., 2003, Lilleberg et al., 2007, Puttonen et al., 2008). OR-1896 is also less bound to plasma proteins than levosimendan, approximately 40% vs 97%, which could explain its greater effects (Antila et al., 2004a).

The steady-state equilibrium of metabolites of levosimendan differs based on the genetic polymorphism of the metabolizing enzyme, N-acetyltransferase. In slow acetylators, the dominant metabolite is OR-1855, whereas rapid acetylators have more OR-1896 in their system. Asian patients tend to be fast acetylators (70-80%) and Caucasians slow acetylators (40-70%). (Parissis et al., 2008) Rapid acetylators show 3.5 times higher plasma OR-1896 concentration than slow acetylators with similar levosimendan dosing. However, this polymorphism does not seem to impair haemodynamic responses in the therapeutic dose range. (Antila et al., 2004b)

2.3.4.1 Mechanism of action of OR-1896

Although studied to a lesser extent than levosimendan, OR-1896 has been shown to have similar pharmacological actions. As a parent compound, OR-1896 also exhibits calcium-sensitizing properties, and its effects are mediated via increased contractility without elevated levels of intracellular calcium or impaired relaxation. (Takahashi et al., 2000b, Takahashi et al., 2000a, Papp et al., 2004, Szilagyi et al., 2004, Takahashi and Endoh, 2002) OR-1896 also induces arterial vasodilatation by activating potassium channels (Segreti et al., 2008, Banfor et al., 2008, Erdei et al., 2006). Despite OR-1896 having weak PDE inhibiting-properties, it is believed to function through the mechanisms of calcium sensitization and potassium channel opening (Szilagyi et al., 2004).
3 Aims of the study

The aim of this study was to investigate the cardiovascular effects of oral calcium sensitizers levosimendan and OR-1896 in two different experimental models of heart failure, namely hypertension-induced heart failure with preserved systolic function and experimental myocardial infarction induced by coronary ligation leading to post-infarct heart failure with decreased systolic function. Furthermore, the molecular mechanisms of cardiac remodelling associated with heart failure were investigated.

Specific objectives were the following:

I. To investigate whether oral treatment with calcium sensitizer levosimendan could prevent excess cardiovascular mortality and hypertension-induced cardiac remodelling in Dahl/Rapp SS rats on a high-salt diet. The possible mechanisms of action mediating the beneficial effects of oral levosimendan were also examined.

II. To evaluate whether OR-1896, the active metabolite of levosimendan, could also prevent excess cardiovascular mortality and hypertension-induced cardiac damage in Dahl/Rapp SS rats on a high-salt diet.

III. To assess whether oral levosimendan treatment could prevent post-infarct heart failure and cardiac remodelling in spontaneously diabetic Goto-Kakizaki rats as well as in non-diabetic Wistar rats. The influence of oral levosimendan treatment on cardiomyocyte hypertrophy, cardiomyocyte apoptotic signalling, inflammatory response, cardiac oxidative stress and fibrosis and myocardial expression of calcium-handling proteins was also examined.

IV. To investigate the cardiovascular effects of oral OR-1896 treatment on post-infarct heart failure and cardiac remodelling in spontaneously diabetic Goto-Kakizaki rats.
4 Materials and methods

4.1 Experimental models and animal welfare

4.1.1 Dahl/Rapp salt sensitive (SS) rat

The inbred salt-sensitive Dahl/Rapp salt sensitive (SS) rat is a model of salt-induced hypertension with severe target organ damage, including cardiac hypertrophy leading to congestive heart failure, vascular damage and renal failure with relatively preserved ejection fraction (Klotz et al., 2006, Rapp and Dene, 1985, Walder et al., 1996). The model mimics well the hypertension-induced cardiac hypertrophy and heart failure found in humans.

When kept on high-salt (6%) diet, Dahl/Rapp SS rats rapidly develop fulminant hypertension with increased mortality visible already 3 weeks into the diet. The overall mortality is almost 100% after 8 weeks on the high-salt diet.

Development of hypertensive heart disease in Dahl/Rapp SS rats has been shown to involve several different mechanisms. Alterations in neurohumoral and natriuretic activation and increased apoptosis all play major roles in the development of heart failure. Dahl/Rapp SS rats exhibit salt induced abnormal expression of calcium-handling proteins, especially a decrease in SERCA2 expression and an imbalance in the SERCA2 to NCX ratio or the SERCA2 to PLN ratio. Desensitization to calcium has been demonstrated to be related to transition to heart failure; Dahl/Rapp SS rats have a defect in the thin filament system making them susceptible to calcium insensitivity (Yoneda et al., 2001, Seki et al., 2003b, Kihara and Sasayama, 1997, Noguchi et al., 2003). Moreover, the inability to increase production of vasorelaxant NO, with its need increasing with elevating blood pressure, damages the heart muscle similarly as seen in hypertensive nephrosclerosis (Chen et al., 1993).

4.1.2 Diabetic Goto Kakizaki (GK) rat

The Goto Kakizaki (GK) rat, developed by selective breeding of glucose-intolerant Wistar rats, is a spontaneous, non-obese and normotensive model of type 2 diabetes exhibiting very similar metabolic, hormonal and vascular features to type 2 diabetes found in humans (Goto et al., 1976). GK rats develop mild hyperglycaemia (1.5 to 2 times normal) and hyperinsulinaemia, glucose intolerance with impaired glucose-induced insulin secretion and peripheral insulin resistance. GK rats also exhibit slight differences in blood lipids, renal damage, cardiac hypertrophy and hypoxia-inducible cardiac dysfunction (Schrijvers et al., 2004, Bisbis et al., 1993, Gauguier et al., 1996, Kristiansen et al., 2004, El-Omar et al., 2004). Hypertension, albuminuria, cardiac hypertrophy and impaired endothelin-dependent relaxation can be further induced on Goto-Kakizaki rat by adding salt (6%) to the diet. Increased functions of the RAS system plays a major role in development of diabetes induced tissue damage in the GK rat since treatment with drugs affecting to RAS system is able to prevent or alleviate end-organ damage. (Cheng et al., 2001) Heart failure can be induced to GK rat with
experimental infarction. The Wistar rat, genetic basis for the GK rat, is used as normoglycemic control for the GK rat.

4.1.3 Animal welfare

This investigation conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The protocols were approved by the Animal Experimentation Committee of the University of Helsinki, Finland, and the Provincial State Office of Southern Finland.

Animals were purchased from M&B, Denmark (GK rat) or Harlan, USA (Dahl/Rapp SS rat and Wistar rat). The rats had free access to standard chow (Harlan, Eystrup, Germany) and drinking water. High salt diet was produced by adding NaCl (Honeywell Riedel-de-Haen, Hanover, Germany) to commercial low salt diet (Na 0.3 %, K 0.8 %, Mg 0.2 %; Harlan).

4.2 Study design

The study design, including animal model, experimental procedure, treatment and dosing, and follow-up time are seen in tables 4-6.

Table 4 Study designs for Studies I and II. FU denotes follow-up.

<table>
<thead>
<tr>
<th>Study I</th>
<th>Groups</th>
<th>Rat strain</th>
<th>Salt in food</th>
<th>Oral levosimendan dosage</th>
<th>FU, weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAHL HS</td>
<td>Dahl/Rapp SS</td>
<td>High, 6 %</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>DAHL HS + levo high</td>
<td>Dahl/Rapp SS</td>
<td>High, 6 %</td>
<td>3 mg/kg</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>DAHL HS + levo low</td>
<td>Dahl/Rapp SS</td>
<td>High, 6 %</td>
<td>0.3 mg/kg</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>DAHL LS</td>
<td>Dahl/Rapp SS</td>
<td>Normal</td>
<td>-</td>
<td>7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study II</th>
<th>Groups</th>
<th>Rat strain</th>
<th>Salt in food</th>
<th>Oral OR-1896 dosage</th>
<th>FU, weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAHL HS</td>
<td>Dahl/Rapp SS</td>
<td>High, 6 %</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>DAHL HS + OR-1896 high</td>
<td>Dahl/Rapp SS</td>
<td>High, 6 %</td>
<td>0.2 mg/kg</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>DAHL HS + OR-1896 low</td>
<td>Dahl/Rapp SS</td>
<td>High, 6 %</td>
<td>0.02 mg/kg</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>DAHL LS</td>
<td>Dahl/Rapp SS</td>
<td>Normal</td>
<td>-</td>
<td>7</td>
</tr>
</tbody>
</table>
4.3 Experimental myocardial infarction

Experimental myocardial infarction was induced the left anterior descending coronary artery (LAD) ligation in male eight-week-old spontaneously diabetic GK rat and non-diabetic Wistar rats as described previously (Palojoki et al., 2001) under ketamine (65 mg/kg, i.p.) and medetomidine (0.5 mg/kg, i.p.) anaesthesia. Long-acting insulin (1 IU/rat) was given two hours before anaesthesia to GK rats to prevent hyperglycemia. Buprenorphine (0.02 mg/kg s.c.), given twice a day for two consecutive days, was used as post-operative analgesia. 24 hours after surgery surviving rats were randomized into study groups (tables 4 and 5).

4.4 Levosimendan and OR-1896 dosage

Levosimendan (10 mg/l) and OR-1896 (0.5 mg/l) (Orion Pharma, Espoo, Finland) were given orally via drinking fluid to produce a daily dose of approximately 0.3-3 mg/kg and 0.02-0.4 mg/kg, respectively. The levosimendan and OR-1896 dosages were chosen based on our previous experiments (Louhelainen et al., 2007, Levijoki et al., 2001, Louhelainen et al., 2009). In these

<table>
<thead>
<tr>
<th>Study III</th>
<th>Rat strain</th>
<th>MI</th>
<th>Oral levosimendan dosage</th>
<th>FU, weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>GK MI</td>
<td>Goto Kakizaki</td>
<td>X</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>GK MI + levo</td>
<td>Goto Kakizaki</td>
<td>X</td>
<td>1 mg/kg</td>
<td>12</td>
</tr>
<tr>
<td>GK SHAM</td>
<td>Goto Kakizaki</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>GK SHAM + levo</td>
<td>Goto Kakizaki</td>
<td>-</td>
<td>1 mg/kg</td>
<td>12</td>
</tr>
<tr>
<td>WIS MI</td>
<td>Wistar</td>
<td>X</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>WIS MI + levo</td>
<td>Wistar</td>
<td>X</td>
<td>1 mg/kg</td>
<td>12</td>
</tr>
<tr>
<td>WIS SHAM</td>
<td>Wistar</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>WIS SHAM + levo</td>
<td>Wistar</td>
<td>-</td>
<td>1 mg/kg</td>
<td>12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study IV</th>
<th>Rat strain</th>
<th>MI</th>
<th>Oral OR-1896 dosage</th>
<th>FU, weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>GK MI, 1w</td>
<td>Goto Kakizaki</td>
<td>X</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>GK SHAM, 1w</td>
<td>Goto Kakizaki</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>GK MI, 4w</td>
<td>Goto Kakizaki</td>
<td>X</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>GK MI + OR-1896, 4w</td>
<td>Goto Kakizaki</td>
<td>X</td>
<td>0.4 mg/kg</td>
<td>4</td>
</tr>
<tr>
<td>GK SHAM, 4w</td>
<td>Goto Kakizaki</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>GK SHAM + OR-1896, 4w</td>
<td>Goto Kakizaki</td>
<td>-</td>
<td>0.4 mg/kg</td>
<td>4</td>
</tr>
<tr>
<td>GK MI, 12w</td>
<td>Goto Kakizaki</td>
<td>X</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>GK MI + OR-1896, 12w</td>
<td>Goto Kakizaki</td>
<td>X</td>
<td>0.4 mg/kg</td>
<td>12</td>
</tr>
<tr>
<td>GK SHAM, 12w</td>
<td>Goto Kakizaki</td>
<td>-</td>
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<td>12</td>
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<tr>
<td>GK SHAM + OR-1896, 12w</td>
<td>Goto Kakizaki</td>
<td>-</td>
<td>0.4 mg/kg</td>
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studies, the levosimendan and OR-1896 dosage used here exerted a significant therapeutic effect and produced a clinically relevant plasma drug concentration (Kivikko et al., 2002b). Fresh levosimendan and OR-1896 solutions were prepared daily.

4.5 Blood pressure measurement and sample preparation

Systolic blood pressure was measured using a tail-cuff blood pressure analyzer (Apollo-2AB Blood Pressure Analyzer, Model 179-2AB, IITC Life Science, Woodland Hills, CA, USA). Urine samples were collected in one or two consecutive 24-h periods in metabolic cages. Rats were anaesthetized with CO$_2$/O$_2$ (AGA, Riihimäki, Finland) and decapitated. Blood samples were collected for using ethylenediaminetetra-acetic acid (EDTA) as an anticoagulant. The hearts were excised, washed with ice-cold saline, blotted dry, weighed and snap-frozen in liquid nitrogen or isopentane (-35°C). All samples were stored at -80°C until assayed.

4.6 Echocardiography

Transthoracic echocardiography (Toshiba Ultrasound, Tokyo, Japan) was performed on all rats under isoflurane anaesthesia (AGA, Riihimäki, Finland) in a blinded fashion. Parameters needed for the calculation of cardiac function and cardiac dimensions were measured from three systole-diastole cycles. A short-axis view of the left ventricle at the level of the papillary muscles was obtained by a two-dimensional imaging method (Gibson method), using a 15-MHz linear transducer. Two-dimensionally guided M-mode recording through the anterior and posterior walls of the left ventricle was used to measure the left ventricle (LV) end-systolic (LVESD), and end-diastolic (LVEDD) dimensions. Also interventricular septum (IVS) and posterior wall (PW) thickness were measured. LV fractional shortening (FS) and ejection fraction (EF), were calculated from the M-mode LV dimensions using the following equations:

\[
\text{FS} \text{ (%) } = \frac{(\text{LVEDD} - \text{LVESD})}{\text{LVEDD}} \times 100
\]
\[
\text{EF} = \frac{\text{SV}}{\text{EDV}}
\]
\[
\text{SV} = \text{EDV} - \text{ESV}
\]
\[
\text{EDV} = 0.52 \times (0.98 \times (\text{LVIDD}/10) + 5.90) \times (\text{LVIDD}/10)^2
\]
\[
\text{ESV} = 0.52 \times (1.14 \times (\text{LVIDS}/10) + 4.18) \times (\text{LVIDS}/10)^2
\]

LVIDD = Diameter of the short-axis left ventricle in end diastole. LVIDS = Diameter of the short-axis left ventricle in end systole. Cardiac output (CO) was calculated from heart rate (HR) and stroke volume (SV=LVEDV-LVESV), CO = HR x SV.

4.7 Infarct size, collagen volume fraction and cardiomyocyte cross-sectional area

The hearts were fixed in 10% buffered formalin solution for histological analysis. Transversal 5-µm-thick sections of the paraffin-embedded left ventricle were cut and stained with Masson’s trichrome or
picrosirius red. The infarct sizes were determined planimetrically from the trichrome-stained histological sections as described elsewhere (Palojoki et al., 2001, Pfeffer et al., 1979). Remote area interstitial collagen volume fraction was measured from picrosirius red-stained histological sections by light microscopy (x200) using computerized software (ImageJ, NIH). Cardiomyocyte cross-sectional area was measured by conventional light microscopy at x400 magnification as described previously (Vahtola et al., 2008).

4.8 Myocardial morphology

Histology of the myocardial samples was evaluated with conventional light microscopy. The severity of observed lesions was graded with numerical values characterizing the degree of damage on a whole tissue level as follows: “0, no abnormalities detected; 1, minimal; 2, mild; 3, moderate; 4, marked; or 5, severe”. Severity grading was performed on the coronary arteries and the myocardium. (Herbert et al., 2002)

4.9 Immunohistochemistry

Immunoperoxidase staining for p16\textsuperscript{INK4a} was performed using 5-µm frozen sections with 1:100 diluted primary monoclonal p16\textsuperscript{INK4a} antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and peroxidase-conjugated rabbit anti-mouse secondary antibody (DAKO A/S, Glostrup, Denmark). 3-amino-9-ethylcarbazole, was added to yield a red reaction product, and finally, the sections were slightly counterstained with Mayer’s hemalaun (Merck, Whitehouse, NJ, USA), blued in tap water. Perivascular monocyte/macrophage infiltration was examined by immunohistochemistry using rat ED-1 (Serotec Ltd., Oslo, Norway) as a primary antibody. Cardiac nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression was assessed using p47phox (BD Biosciences, Pharmingen, USA) as primary a antibody. Heart paraffin sections were deparaffinized and nonspecific antibody binding was blocked with 1% bovine serum albumin and 10% goat serum in phosphate buffered saline. The sections were incubated with polyclonal C-kit antibody (Santa Cruz Biotechnologies inc., Santa Cruz, CA, USA) at dilution 1:100 for overnight at +4°C. After washing, the sections were incubated with secondary biotin-conjugated antibody for 1 h at room temperature, and avidin biotin enzyme reagent (Vector Laboratories, Burlingame, CA, USA) was used. The sections were counterstained with hematoxylin before being examined under a light microscope.

4.10 TUNEL apoptosis assay

Cardiomyocyte apoptosis was assessed by the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) assay. Nuclear DNA strand breaks were end-labelled with digoxigenin-conjugated dideoxy-UTP by terminal transferase and visualized immunohistochemically with alkaline
phosphatate-conjugated digoxigenin antibody. Adjacent tissue sections treated with DNase I to induce DNA fragmentation were used as a positive control for apoptosis. The percentages of TUNEL-positive cardiomyocytes were calculated using light microscopy (X 200 magnification) with an ocular grid. (Palojoki et al., 2001, Kyto et al., 2004)

4.11 Apoptosis microarray

RNA from hearts (n=4-5 in each group) was extracted with Trizol (Gibco, Invitrogen Co., Carlsbad, CA, USA) and purified with RNeasy mini kit (Qiagen N.V., Venlo, The Netherlands). RNA was reverse-transcribed to cDNA and back to cRNA for microarray analysis using the Oligo GEArray for Rat Apoptosis (Superarray, Frederick, MD, USA) containing 114 known, apoptosis-related genes. Samples were hybridized to separate GEArray plates overnight in a hybridization oven (Stratagene Personal Hyb, La Jolla, CA, USA) and scanned (Packard Biosciences ScanArray 4000, Boston, MA, USA). Data extraction and analysis were performed with the GEArray Expression Analysis Suite software (Superarray, Bethesda, MD, USA). Significant differences in apoptotic microarrays were tested with Student’s t-test.

4.12 Western blotting

Samples from left ventricle were homogenized and the proteins were isolated with Micro BCA Protein Assay kit (Thermo scientific, Rockford, IL, USA). Myocardial samples were electrophoretically separated by 8% SDS-PAGE (15 µg total protein per lane). Proteins were transferred to a PVDF membrane (Immobilon-P®, Millipore, Bedford, MA, USA), and the membranes were probed with the following dilutions of primary antibodies; rabbit anti-NCX, 1:5000 (Alpha Diagnostics, San Antonio, TX, USA), rabbit anti-Serca2, 1:5000 (Abcam, Cambridge, England). Tubulin was used as a loading control (Anti-alpha tubulin, Abcam). Horseradish peroxidase-conjugated anti-rabbit secondary antibody (Chemicon, Temecula, CA, USA) was subjected to enhanced chemiluminescence solution (GE Healthcare, Uppsala, Sweden) and exposed to x-ray film (Hyperfilm-ECL, GE Healthcare). The relative protein expression was quantified from the x-ray film by densitometry (Genesnap, Synoptics, Cambridge, UK).

4.13 Electrophoretic mobility shift assay of nuclear FOXO3a transcription factor

Electrophoretic mobility shift assay (EMSA) was used to determine the quantity of DNA-binding FOXO3a transcription factor from cardiac left ventricular nuclear extracts. Nuclear proteins were extracted by grinding in liquid nitrogen and incubating in a buffer with protease inhibitor, membrane proteins were solubilized with Nonidet P-40 (Roche Diagnostics GmbH, Mannheim, Germany). 5 µg of nuclear extracts were incubated with the following digoxigenin-tagged FOXO3a oligonucleotides: 5’-DIG-ATT GCT AGC AAG CAA AAC AAA CCG CTA GCT TA-3’ and 5’-DIG-TAA GCT AGC
GGT TTG TTT TGC TTG CTA GCA AT-3′ (Oligomer, Helsinki, Finland). Nuclear proteins were separated by 5% SDS-PAGE, blotted to a nylon membrane (Hoefer semiPhor, TE77, Amersham Biosciences, Buckinghamshire, UK) and cross-linked to the membrane. The membrane was probed with rabbit anti-digoxigenin-AP-conjugate (Roche Diagnostics GmbH), incubated in detection reagent (CDP Star, Roche Diagnostics GmbH) and exposed to an x-ray film (Kodak Inc., Japan). The indication reaction was done by incubating the samples with tagged and untagged oligonucleotides and probing with rabbit anti-forkhead (Drosophila) homolog (rhabdomyosarcoma) like 1 (FKHRL1) (sc-11351, Santa Cruz, CA, USA). Proteins extracted from HeLa-cells was used as positive and water as negative control.

4.14 Quantitative real-time RT-PCR

Total RNA from rat hearts was collected with Trizol® (Gibco, Invitrogen, Carlsbad, CA, USA). 1 µg of isolated RNA was treated with DNase 1 (Deoxyribonuclease 1, Sigma Chemicals Co., St Louis, MO, USA) and reverse transcribed to cDNA by RT enzyme (Enhanced avian Hot Start RT-PCR kit, Sigma Chemicals Co or Im-Prom-II RT system, Promega, Madison, WI, USA). One µl of cDNA was subjected to quantitative real-time RT-PCR using the Lightcycler® instrument (Roche Diagnostics, Neuilly sur Seine, France) for detection of ANP, BNP, connective tissue growth factor (CTGF), interleukin-6 (IL-6), atrogin-1, Bim (BCL2L11), tumour necrosis factor receptor superfamily member 12a (Tnfrsf12a), p16INK4A, p19ARF, vascular endothelial growth factor (VEGF), nuclear receptor 4a3 (NR4a3), monocyte chemoattractant protein 1 (MCP-1), transcription factor 5 (E2F-5), PPARγ coactivator-1α (PCG-1α), transcription factor A, mitochondria (TFAM), nuclear respiratory factor 1 (NRF-1), SERCA2, NCX, osteopontin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal 18S mRNA. Primer sequences are presented in table 7. The samples were amplified using FastStart DNA Master SYBR Green 1 (Roche Diagnostics) according manufacturer’s protocol. The quantities of the PCR products were quantified with an external standard curve amplified from purified PCR product.
<table>
<thead>
<tr>
<th>Table 7. RT-PCR primer sequences.</th>
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<td><strong>Forward primer</strong></td>
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<tr>
<td>18S</td>
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<td>ANP</td>
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<td>Tnfrsf12a</td>
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<tr>
<td>Bim</td>
</tr>
<tr>
<td>p16(^{INK4a})</td>
</tr>
<tr>
<td>p19(^{ARF})</td>
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<tr>
<td>E2F-5</td>
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<tr>
<td>VEGF</td>
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<td>MCP-1</td>
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<tr>
<td>SERCA-2</td>
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<tr>
<td>NCX</td>
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<td>Osteopontin</td>
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4.15 Biochemical analyses

Blood glucose was determined with a handheld test meter (Contour, Bayer Diabetes Care, Basel, Switzerland), and plasma BNP (BNP-45, Peninsula Laboratories, Belmont, CA, USA), plasma renin activity (Angiotensin I RIA kit, Diasorin, Italy), serum aldosterone (Coat-a-Count Aldosterone RIA kit, DPC Biermann, Bad Nauheim, Germany) and serum insulin (Rat insulin RIA kit, Linco, St. Charles, Missouri, USA) were determined by radioimmunoassay according to the manufacturers’ instructions. Urinary noradrenalin and dopamine were analysed using the isocratic ion-pair reversed-phase high-performance liquid chromatography method with electrochemical detection. (Helkamaa et al., 2003) Urinary albumin was measured by ELISA using rat albumin as a standard (Celltrend, Luckenwalde, Germany). Plasma samples were analysed for levosimendan and OR-1896 by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Kivikko et al., 2003).

4.16 Statistical analyses

Data are presented as means ± standard error of mean. Statistically significant differences in mean values were tested by analysis of variance (ANOVA) and Tukey’s and Bonferroni’s post-hoc test for comparisons of multiple groups. The Kaplan-Meier test was used for survival analysis.
5 Results

5.1 Survival (All studies)

When kept on high-salt diet, Dahl/Rapp SS rats develop fulminant hypertension and severe end-organ damage, with a mortality of almost 100% after 8 weeks. During 7-week follow-up period Dahl/Rapp SS rats on a high-salt diet exhibited total mortality of 62%, significantly more than mortality rate of 0% in similar rats on a low-salt diet. Both levosimendan and OR-1896 were able to significantly improve survival. Of levosimendan treated groups, higher dose yielded a survival rate of 75% and a lower dose 100%. Both OR-1896 doses were able to improve survival in a similar manner; 75% of the Dahl/Rapp SS rats on a high-salt diet survived (Figure 6). Interestingly, also late treatment, started at midpoint of the 7-week follow-up prevented mortality in both levosimendan, and OR-1896-treated rats (survival rates of 80% and 58% vs 38% in untreated Dahl/Rapp rats on high-salt diet).

![Figure 6. Survival curves of Dahl/Rapp SS rats on high salt diet treated with levosimendan (0.3 (low) and 3 (high) mg/kg) and OR-1896 (0.02 (low) and 0.2 (high) mg/kg) compared to untreated Dahl/Rapp SS rats on high salt diet and on low salt diets. * denotes p<0.05 compared to Dahl/Rapp SS rats on high salt diet.](image)

Survival after MI is impaired in the presence of diabetes. In diabetic GK rats, survival rate after LAD-induced MI was only 35%, while the survival rate of non-diabetic Wistar rats was 58% (p<0.05). Neither levosimendan nor OR-1896 had effect on mortality.

5.2 Cardiac functions and blood pressure (All studies)

A high-salt diet induced moderate deterioration of cardiac functions and left ventricular hypertrophy in Dahl/Rapp SS rats when assessed by echocardiography. The ejection fraction and fractional shortening decreased slightly in Dahl/Rapp SS rats on a high-salt diet. Both levosimendan doses and the higher dose of OR-1896 significantly improved contractility and normalized ejection fraction and fractional shortening, even over the levels found in Dahl/Rapp SS rats on a low-salt diet.
Echocardiography revealed post-infarct heart failure in GK and Wistar rats with an ejection fraction 20-40% (p<0.05) at different time points. In GK rats, levosimendan increased ejection fraction, fractional shortening and cardiac output to levels found in sham-operated GK rats. However, administered levosimendan in non-diabetic Wistar rats was clearly less beneficial. In sham-operated GK or Wistar rats, levosimendan did not significantly influence cardiac function. In post-infarct GK rats, OR-1896 also increased ejection fraction and fractional shortening at all time-points but significant increase in cardiac output was only seen in the later stages of remodelling. A high-salt diet induced fulminant hypertension in Dahl/Rapp SS rats (215 mmHg vs. 148 mmHg, p<0.05). Both levosimendan doses and the higher dose of OR-1896 produced a slight decrease in blood pressure in the middle of 7-week follow-up period but at the end of experiment all groups had similar blood pressure values.

In diabetic GK rats, MI resulted in approximately 30 mmHg lower blood pressure (p<0.05) than in SHAM operated rats. Levosimendan or OR-1896 had no effect on blood pressure in Wistar rats or MI operated GK rats, but in SHAM operated GK rats both levosimendan and OR-1896 treatments resulted in an approximately 30 mmHg reduction in blood pressure compared to non-medicated SHAM-operated rats.

5.3 Hypertrophy (All studies), cardiomyocyte cross-sectional area (Study III and IV)

Dahl/Rapp SS rats on a high-salt diet showed a markedly elevated heart weight to body weight ratio compared with low-salt controls (5.0 ± 0.15 mg/g vs. 3.0 ± 0.07 mg/g, p<0.001). Treatment with levosimendan or OR-1896 was able to partially prevent cardiac hypertrophy measured by the heart weight/body weight ratio in hypertensive Dahl/Rapp SS rat. MI induced pronounced cardiac hypertrophy measured as heart-weight-to-body weight ratio 1, 4 and 12 weeks post-MI (increase of 61%, 62% and 71%, respectively, p<0.01, when compared with age-matched SHAM and MI-operated GK rats).

MI induced 55% increase in cardiomyocyte cross-sectional area in the diabetic GK rat (Figure 7), which was preventable with both treatments. Levosimendan and OR-1896 also lowered diabetes-induced elevation of cardiomyocyte cross-sectional area in SHAM-operated GK rats by 7%. In Wistar rats MI induced an increase in cardiomyocyte cross-sectional area relative to SHAM-operated rats (372.3 ± 7.8 µm² vs. 271.6 ± 5.4µm², p<0.001). Levosimendan further elevated cardiomyocyte cross-sectional area by roughly 45 µm².
Figure 7 MI induced a clear hypertrophic response, measured as cardiomyocyte cross-sectional area, 12 weeks post-MI in GK and Wistar rats. Oral levosimendan and OR-1896 treatments were able to prevent hypertrophic activation and to reduce cross-sectional area even in SHAM-operated GK rats. Levosimendan had no effect on cross-sectional area in Wistar rats. * p<0.05 compared with GK or WIS MI, # p<0.05 compared with GK or WIS SHAM

5.3.1 Cardiomyocyte morphology (Studies I and II), infarct size, fibrosis (Studies III and IV)

Dahl/Rapp SS rats on a high-salt diet developed clear coronary damage and to lesser extent myocardial damage. Animals showed intimal hyperplasia, fibrinoid necrosis of the arteries with marked medial thickening and adventitial scarring, myocardial infarcts with inflammation, perivascular monocyte/macrophage infiltration and p47phox (subunit of ROS-generating NAPDH oxidase) expression by immunohistochemistry. Neither levosimendan nor OR-1896 treatment had an effect on the coronary or myocardial damage score.

LAD ligation resulted in an infarction size of approximately 37% in diabetic GK rats and 32% in Wistar rats. Levosimendan or OR-1896 treatments had no effect on infarct size.

Interstitial collagen volume fraction tended to be increased after MI in GK and Wistar rats, but this change did not quite reach statistical significance. Levosimendan treatment tended to decrease remote area collagen volume fraction in MI-operated rats, while interstitial collagen volume fraction in sham-operated GK rats was unaffected (p=0.34).

In both GK and Wistar rats, MI also induced mRNA expression of CTGF, a potent inductor of fibrosis, throughout the 12-week follow-up with the most prominent induction one week after infarction. Both treatment with levosimendan and treatment with OR-1896 were able to prevent this fibrotic signalling.
5.4 Calcium handling proteins (Studies I and III)

Development of heart failure is associated with disturbances in calcium handling. In hypertension and diabetic MI, myocardial SERCA2 expression was decreased by 40% and 30%, respectively. Hypertension in Dahl/Rapp SS rats had no effect on myocardial NXC expression, but MI induced a 74% increase in NCX protein expression in GK rats. MI had no impact on expression of calcium handling proteins in Wistar rats. Levosimendan was able to prevent abnormalities in expression of calcium handling proteins in both Dahl/Rapp SS rats and GK rats.

5.5 Expression of natriuretic peptides (All studies)

Hypertension induced a 5-fold expression of atrial natriuretic peptide (ANP) in Dahl/Rapp SS rats and a 50% non-significant induction of brain natriuretic peptide (BNP) accompanied by increased BNP plasma levels. A higher dose of levosimendan was able to reduce myocardial ANP-mRNA expression to a level similar to that found in Dahl/Rapp SS low-salt controls, and both doses were able to normalize BNP in both mRNA level and plasma.

In GK rats, MI induced a significant increase in ANP mRNA (Figure 8) as well as a slight, non-significant increase in plasma BNP throughout the 12-week follow-up. Post-infarct Wistar rats also showed increased expression of ANP mRNA. Levosimendan and OR-1896 were able to normalize expressions of myocardial natriuretic peptides.

**Figure 8** Expression of ANP mRNA during post-infarct remodelling in GK and Wistar rats 1, 4 and 12 weeks after MI. Levosimendan and OR-1896 normalized ANP mRNA expression in GK rats, while levosimendan had no effect on ANP mRNA expression in Wistar rats. * p<0.05 compared with GK MI, # p<0.05 compared with GK SHAM.
5.6 Apoptosis (Studies I and III)

Cardiomyocyte apoptosis was assessed by TUNEL staining (Figure 9) and apoptosis-specific microarray. In hypertensive Dahl/Rapp SS rats on a high-salt diet cardiomyocyte apoptosis was increased by 6-fold compared with low-salt controls. Levosimendan dose-dependently decreased the number of apoptotic cardiomyocytes in Dahl/Rapp SS rats on a high-salt diet.

Both GK and Wistar rats exhibited increased cardiomyocyte apoptosis after MI, but the degree of apoptosis was higher in GK rats. Cardiomyocyte apoptosis was more prominent in the border than in the remote area (Figure 8). Based on microarray experiments, a >1.8 fold increase in mRNA expression was found in 90% (103/114) of selected apoptotic genes in GK rat hearts after MI. Wistar rats also showed induction of apoptosis, but based on microarray analysis the profile of apoptotic expression was different from that in GK rats. Levosimendan significantly reduced sustained cardiomyocyte apoptosis in both border and remote areas and prevented induction of apoptotic genes in the heart after MI. Levosimendan suppressed mRNA expression of apoptosis-related genes on a broad range, several member of apoptosis-related caspases (including caspase 9), tumour necrosis superfamily and BCL-pathway.

MI induced a 2-fold increase in proapoptotic DNA-bound, nuclear FOXO3a, followed by a 100% increase in FOXO3a controlled atrogin-1 mRNA expression in the GK heart. Levosimendan prevented MI-induced activation of transcription factor FOXO3a and atrogin-1. Furthermore, apoptotic microarray results revealed that FOXO3a-associated proapoptotic genes, such as Bcl2l11, Bad and Fadd, were induced in GK rats with MI compared with SHAM controls. Levosimendan was able to suppress apoptotic signalling caused by myocardial infarction.
5.7 Premature cardiomyocyte senescence (Studies II and IV)

A high-salt diet induced a 4-fold change in p16\textsuperscript{INK4a} mRNA expression in Dahl/Rapp SS rats, while expression of tumour suppressor p19\textsuperscript{ARF} mRNA remained unaltered (Figure 10). OR-1896 treatment was able to reduce p16\textsuperscript{INK4a} mRNA expression and induce p19\textsuperscript{ARF} mRNA expression. The p16\textsuperscript{INK4A} positive staining in cardiac samples was located in the nuclei of myocytes, arterial smooth muscle cells and to a lesser extent in vascular endothelial cells. The perivascularly located leucocytes also showed positive staining.

MI induced a clear, 5-fold induction in the cardiomyocyte senescence marker p16\textsuperscript{INK4a} at later stages of post-infarct remodelling in GK rats, while induction of p19\textsuperscript{ARF} was most prominent at the early stages of remodelling (Figure 10). MI had no effect on p16\textsuperscript{INK4a} mRNA expression in Wistar rats.

Transcription factor, cell cycle suppressor E2F-5 mRNA was induced parallel to p16\textsuperscript{INK4a} expression in post-infarct GK rats. Oral OR-1896 treatment was able to completely normalize myocardial p19\textsuperscript{ARF} mRNA expression and to prevent upregulation of myocardial p16\textsuperscript{INK4A} and E2F-5 mRNA expressions.
Figure 10. Induction of senescence markers \(p16^{INK4A}\) and \(p19^{ARF}\) mRNA during hypertensive remodelling in Dahl/Rapp SS rats (A and B) and after myocardial infarction in spontaneously diabetic GK rat (C and D). Oral OR-1896 was able to prevent upregulation of \(p16^{INK4A}\) mRNA in both rat strains and induce potentially protective \(p19^{ARF}\) mRNA in Dahl/Rapp SS rats. * \(p<0.05\) compared with Dahl HS or GK MI, # \(p<0.05\) compared with Dahl LS.

5.8 VEGF, NR4a3 and MCP-1 expression (Study IV)

VEGF, NR4a3 and MCP-1 mRNA expression were measured as markers of inflammation and oxidative stress. VEGF mRNA expression was transiently reduced 1 week after MI in GK rats, with induction of expression occurring later in remodelling (unpublished data). A VEGF-induced increase in nuclear factor NR4a3 mRNA expression was seen at 12 weeks post-MI, while a smaller increase was also seen earlier in post-MI remodelling. MCP-1 mRNA expression was measured as a marker of VEGF- and NR4a3-induced oxidative stress. (Martinez-Gonzalez and Badimon, 2005, Liu et al., 2003, Pols et al., 2007) MCP-1 mRNA expression was upregulated during post-MI cardiac remodelling in a similar manner as VEGF mRNA expression, resulting in a >3.5-fold induction 12 weeks after MI. OR-1896 was able to prevent the late induction of VEGF, even to lower levels than observed in SHAM-operated animals and to reduce NR4a3 mRNA expression throughout the study.
OR-1896 treatment completely prevented induction MCP-1 mRNA, indicating reduced oxidative stress.

5.9 Expression of markers of mitochondrial biogenesis (Study IV)

To study the mitochondrial biogenesis, mRNA expression of typical markers of mitochondrial biogenesis, NRF-1, TFAM and PGC-1α, were measured. MI induced a significant increase in myocardial mRNA expression of NRF-1, while expressions of PGC-1α and TFAM showed only a positive trend towards increased expression. Oral OR-1896 was able to reduce NRF-1 expression to levels found in SHAM-operated rats, with similar trend in PGC-1 and TFAM mRNA expressions.

5.10 Biochemical and hormonal analyses (All studies)

A high-salt diet induced clear renal damage, measured by urinary albumin (113.3 ±44.2 vs 52.4 ±36.4, p<0.05) but no differences in plasma renin activity, serum aldosterone or urinary noradrenaline in Dahl/Rapp SS rats when compared to low salt controls. Levosimendan had no effect on urinary albumin, plasma renin activity or serum aldosterone, but OR-1896 was able to reduce serum aldosterone by 50% (p<0.05) compared with both high- and low-salt controls.

In GK and Wistar rats, neither MI nor levosimendan treatment influenced plasma renin activity, plasma glucose, serum insulin, serum aldosterone or urinary albumin.

5.11 Plasma drug concentrations (All studies)

The mean levosimendan daily dosages of low- and high-dose levosimendan in Dahl/Rapp SS rats were 0.36 ± 0.01 and 3.6±0.1 mg/kg, respectively. The mean plasma concentrations of levosimendan and OR-1896 in the low-dose levosimendan group were 6.4 ± 1.3 and 4.5 ± 0.2 ng/ml and in the high dose levosimendan group 79 ± 19 and 42 ± 15 ng/ml. Plasma levosimendan concentration showed typical diurnal variation with the highest plasma concentrations occurring during the night (Figure 11).

The respective average daily dosages for OR-1896 in Dahl/Rapp SS rats were 192 ± 24 µg/kg for the high-dose OR-1896 group, and 19 ± 4 µg/kg for the low-dose OR-1896 group, resulting in steady diurnal blood drug concentrations (Figure 11). The mean 24-h plasma concentrations of OR-1896 in the high- and low-dose groups were 49.1 ± 10.6 and 4.1 ± 0.43 ng/ml, respectively.

At the end of the study in GK rats treated with levosimendan, the plasma concentrations of OR-1896 were 20.4 ± 10.5 ng/ml. In GK rats treated with OR-1896, the mean plasma concentration of OR-1896 was 20.1 ± 2.0 ng/ml.
Figure 11. Mean plasma concentrations of levosimendan (A) and OR-1896 (B) after oral levosimendan or OR-1896 treatments (C) in hypertensive Dahl/Rapp SS rat on a high-salt diet.
6 Discussion

Despite of marked advancements in understanding the molecular mechanisms behind development of heart failure and in the treatment options available, a great deal remains to be elucidated. Current standard treatment with drugs affecting to the renin-angiotensin-aldosterone and sympathoadrenergic systems have strong preventive effect on mortality, but is not able to improve already lost cardiac capacity. Moreover the ageing population and unfavourable lifestyle choices, including tobacco, alcohol, unhealthy nutritional composition and lack of physical activity increase the prevalence of the heart failure and add strain to the public health care systems. Thus, demand for new, effective ways to prevent harmful cardiac remodelling and improve cardiac pumping capacity are being sought. (Kupari and Lommi, 2004)

Our study demonstrated that chronic oral treatment with the calcium sensitizers levosimendan and its active metabolite OR-1896 provides a safe and efficient means of preventing mortality and end-organ damage in hypertensive heart disease, and improve cardiac functions and preventing deleterious cardiac remodelling in a post-infarct model complicated with type II diabetes.

6.1 Methodological aspects

6.1.1 Validation of the models

Hypertension and associated left ventricular hypertrophy are powerful risk factors for severe cardiac conditions. Left ventricular hypertrophy reduces diastolic relaxation, renders heart susceptible to ischaemia and increases the risk of cardiac arrhythmias and sudden death. (for review see (Artham et al., 2009)) To study the heart failure with preserved systolic function, the type of heart failure often related to hypertension and left ventricular hypertrophy in humans, hypertensive Dahl/Rapp SS rat was used. The Dahl/Rapp SS rat is a model of salt-sensitive hypertension. It represents the early phase in the progression of hypertensive heart failure, with increased left ventricular hypertrophy and impaired diastolic functions. When fed a diet containing 6% NaCl, the rats develop severe hypertension and end-organ damage with markedly increased cardiac hypertrophy, myocardial infarctions and severely impaired renal function. Mortality approaches 100% after 7 weeks on the high-salt diet. Surprisingly though, cardiac functions of Dahl/Rapp SS hearts remain at relative high level (Klotz et al., 2006). In our studies, the average decrease in ejection fraction was roughly 5%, which is not enough to explain the increased mortality. However, hypertensive Dahl/Rapp SS exhibit clear cardiac hypertrophy with increased left ventricular wall thickness, indicating the presence of heart failure with preserved systolic function. Increased left ventricular hypertrophy is associated with hypoxia and cardiac arrhythmia, and regression of the left ventricular hypertrophy by angiotensin II blockade prevents ischaemia-induced lethal arrhythmias. (Kohya et al., 1995) Indeed, the actual cause of death in
Dahl/Rapp SS rats seems to be sudden death, due to hypertrophy related cardiac arrhythmias, and strokes rather than heart failure (Qu et al., 2000). Diabetes is a known risk factor for cardiovascular diseases, and diabetes increases the risk of first time myocardial infarction by 5-fold (Bauters et al., 2003). As 15-20% of heart failure patients are diabetics, we wanted to investigate the post-infarct remodelling in a situation complicated with diabetes. Heart failure was produced by ligation of the left ascending coronary artery (LAD), widely used experimental heart failure model, in diabetic GK rats. Wistar rats were used as non-diabetic controls. GK rats have heterogenic, spontaneous type II diabetes, which is not associated with obesity, providing a good model for type II diabetes found in humans. We noted that survival of myocardial infarction was impaired in diabetic GK rats compared with non-diabetic Wistar rats, leading to a higher number of animals needed for the studies. To improve survival of GK rats, 1 IU of long-acting insulin was used to reduce the ketamin-induced increase in blood glucose values, but the difference in survival remained significantly different. The level of cardiomyocyte hypertrophy, inflammation status and apoptosis were also affected by diabetes, with GK rats exhibiting larger cardiomyocytes, greater activation of inflammation and more cardiomyocyte apoptosis than non-diabetic Wistar rats.

6.2 Cardiovascular effects of oral levosimendan and OR-1896

6.2.1 Mortality

Clinical data on levosimendan’s effects on mortality remain conflicting. Earlier reports showed beneficial, long-lasting effect on improved survival (Follath et al., 2002, Moiseyev et al., 2002, Zairis et al., 2004), which were not confirmed by later, larger studies on both iv and oral levosimendan (Packer, 2005, Nieminen et al., 2008). In present study hypertensive Dahl/Rapp SS rats exhibited increased mortality when compared to normotensive ones. Especially interesting is that both oral levosimendan and OR-1896, also started after development of hypertension, were able to significantly reduce or totally prevent mortality without changes in systolic blood pressure or renal functions. Due to the relatively preserved systolic function in hypertensive Dahl/Rapp SS rats it seems reasonable to assume despite that minor significant improvement in cardiac functions by levosimendan or OR-1896 other mechanisms than pure inotrophy are also associated. Interestingly, levosimendan has been shown to prevent ventricular tachyarrhythmias in ischaemia-reperfusion models (Papp et al., 2006, Du Toit et al., 1999), which could at least partly explain improved survival in levosimendan medicated Dahl/Rapp SS rats, found in a present study. Levosimendan and OR-1896 could also have mediated improved survival via reduction in left ventricular hypertrophy as regression of left ventricular hypertrophy has also been shown to improve diastolic functions and decrease cardiac arrhythmias and sudden cardiac death in hypertensive patients, even without reduction of blood pressure (Okin et al., 2006, Wachtell et al., 2002).
Diabetes is linked to increased mortality after MI (Laakso, 2001). In good accordance with previous knowledge, acute mortality from MI was higher in diabetic GK rats compared to non-diabetic controls despite of peri-operative glucose control and similar size of experimental infarctions (Goyal et al., 2009). The underlying molecular mechanisms behind increased mortality are not clearly defined, but it is evident that present diabetic cardiomyopathy with compensatory cardiac hypertrophy, increased apoptotic signalling and vulnerability to ischaemia-induced arrhythmias play a major role in it (Marwick, 2008).

In GK rats with MI most of the mortality took place during first 24 h after MI, before the study treatments were started. It has been shown that cardiomyocyte damage and necrosis is evident 6-hours after MI (Vivaldi et al., 1985), well before treatments were started in a present study. This 24 h delay before beginning of the treatments was done to select those individuals with none-fatal MI in order to study the effects of levosimendan or OR-1896 on existing, chronic heart failure. However, this approach limits the correlation to clinical situation where treatment would be started as sooner. During follow up, there were only few occasional deaths in GK rats and due to relatively small sample size and short follow up neither levosimendan nor OR-1896 had effect on long-term mortality in a present study.

### 6.2.2 Cardiac functions and remodelling

Transition from the left ventricular hypertrophy to the heart failure is associated with complicated network of molecular mechanisms, which remodel cardiac structure and functions (Wright et al., 2008). In this study both hypertension and myocardial infarction resulted in signs of cardiac remodeling, namely clear cardiac hypertrophy, measured by heart weight to body weight ratio or cardiomyocyte cross sectional area, and increased fibrotic cardiac remodeling with compromised cardiac contractility.

In this study hypertensive Dahl/Rapp SS rats had severe hypertension, clear cardiac hypertrophy and, as described previously, only modest impairment in systolic functions (Klotz et al., 2006). Oral levosimendan and OR-1896 were both able to improve cardiac function even above levels found in low salt controls and reduce cardiac hypertrophy. As reduction of left ventricular hypertrophy by antihypertensive agents has been shown to be beneficial in hypertensive patients (Gupta et al., 2007, Lorell and Carabello, 2000), temporary reduction in blood pressure could have been partially responsible with reduced left ventricular mass and improved contractility discovered in a present study in treatment groups. However, it should be noted that levosimendan or OR-1896 had no effect terminal blood pressure indicating improvement in cardiac hypertrophy by another mechanisms, such as improved microcirculation or reduced inflammation or apoptosis, which are discussed further. Levosimendan and OR-1896 were able to reduce plasma BNP concentration and myocardial mRNA expression of ANP in hypertensive Dahl/Rapp SS rats, indicating a decrease in myocardial tension
and cardiac overload (Haug et al., 1993). This could have been lead to improved cardiac microcirculation, improved oxygen delivery and improved cardiac performance.

Besides cardiac functions, it should also be noted, that Dahl/Rapp SS rats on a high-salt diet exhibited marker albuminuria and renal damage. Levosimendan or OR-1896 was unable to alleviate renal damage caused by severe hypertension indicating that beneficial effects discovered in a present study were not mediated either by renal protection. Interestingly though, in humans levosimendan has been shown to improve renal function (Yilmaz et al., 2007, Zemljic et al., 2007).

Development of post-infarct heart failure was assessed 1, 4 and 12 weeks after MI in diabetic GK rats. MI resulted severe pumping failure, which, like in humans (Sezer et al., 2009), was moderately compensated over time. Oral levosimendan and OR-1896 were able to totally normalize cardiac function without any effects on infarct size, cardiac morphology, blood pressure or diabetic status. Since cardiac output is directly related to heart rate and stroke volume (Heikkilä et al., 2008), levosimendan and OR-1896-induced normalization of cardiac output in GK rats, could have been partially due to drug-induced increase in heart rate. It is possible that the increased in heart rate found in the present study in levosimendan-treated GK rats are rather due to PDE III inhibition (Kaheinen et al., 2006, Ajiro et al., 2002, Szilagyi et al., 2005). However, it should be noted that in Wistar rats with MI levosimendan was able to improve cardiac output without elevation in heart rate.

Oxidative stress plays a major role in post-infarct cardiac remodelling and suppression of ROS protects the heart (Sun, 2007). In GK rats MI induced clear hypertrophic response, measured by increased cardiomyocyte cross sectional area, profibrotic CTGF mRNA expression and interstitial fibrosis, associated with increased diabetic oxidative stress (Vahtola et al., 2008). Interestingly, in GK rats oral levosimendan or OR-1896 were able to suppress oxidative stress and inflammation, measured by several different markers, which could explain the observed reduction in the cardiac hypertrophy and alleviation of the fibrosis.

Interestingly, effects of levosimendan were more beneficial in diabetic GK rats than in normoglycemic Wistar rats. Mechanisms behind this are not clearly defined yet, but it could be argued that alleviating effects of levosimendan and OR-1896, on diabetes induced inflammation, fibrotic response, imbalance in expression of calcium handling proteins and apoptosis are involved (Adamopoulos et al., 2006b, Parissis et al., 2004b, Trikas et al., 2006). However, further clinical studies on diabetic subjects are warranted to clarify whether levosimendan greater efficacy it seen also in humans.

### 6.2.3 Calcium handling

Altered calcium handling has been shown to play a major role in development of heart failure (Bers, 2002, Hasenfuss and Pieske, 2002). Both experimental studies and failing human hearts show reduced SERCA2 protein level and activity, which leads to reduced uptake of calcium to SR, impairment of relaxation and longer duration of calcium transients (Zarain-Herzberg et al., 1996, Seki et al., 2003a,
Schmidt et al., 1998, Okayama et al., 1997, Meyer et al., 1995, Aoyagi et al., 1999). Interestingly, altered insulin signalling has also been linked to reduced myocardial expression of SERCA2 after MI (Sena et al., 2009). Consistent with the previous results (Seki et al., 2003a, Yoneda et al., 2001, Sena et al., 2009), both hypertensive Dahl/Rapp SS rats and diabetic GK rats revealed hindered expression of myocardial calcium handling proteins, namely SERCA2 and NCX, leading to impaired calcium handling, while non-diabetic Wistar rats showed no post-MI changes in calcium handling protein expressions. Oral treatment with levosimendan normalized the SERCA2 to NCX ratio in Dahl/Rapp SS and diabetic GK rats. In fact, levosimendan-corrected impaired calcium handling in GK rats could explain the beneficial effects of levosimendan treatment in the presence of diabetes as increased expression SERCA2 in diabetic SERCA2-transgenic mice has been previously shown to improve cardiac contractility (Vetter et al., 2002).

Reactive oxygen species (ROS) have been noted to have a detrimental effect on calcium handling proteins, such as SERCA2 and NCX (Guerra et al., 1996, Goldhaber, 1996, Kaplan et al., 2003). In addition to disturbances in calcium handling protein expression, hypertensive Dahl/Rapp SS and diabetic GK rats showed activation of several markers of inflammation, namely p47phox, MCP-1, IL-6 and osteopontin. In addition to its effects on calcium handling proteins, levosimendan was able to reduce inflammatory status in both heart failure models used. Terentyev et al. recently showed, that antioxidant treatment is improves function of another calcium handling protein, ryanodine receptor, indicating that reduced inflammatory status might explain improved calcium handling found in this study (Terentyev et al., 2008).

6.2.4 Apoptosis

Increased apoptosis has been linked to development and progression of heart failure in both clinical and experimental setting (Narula et al., 1996, Olivetti et al., 1997a, Kang and Izumo, 2000). In good agreement with previous knowledge, the present study showed elevated levels of cardiomyocyte apoptosis associated with hypertension and post-infarct cardiac remodelling (Narula et al., 1996, Olivetti et al., 1997a, Kang and Izumo, 2000). The level of post-infarct apoptosis was more elevated in the presence of diabetes, and diabetic animals had different profiles in apoptotic microarray analyses examining expression of genes involved in apoptotic events than their normoglycaemic controls. In diabetic GK rats, oral levosimendan reduced the number of apoptotic cardiomyocytes to a level similar to that found in SHAM-operated GK rats.

The exact mechanisms related to reduced cardiomyocyte apoptosis remain under investigation, but ROS and ATP-sensitive potassium channels apparently play a major role in apoptosis (for reviews see (Giordano, 2005, Ardehali and O’Rourke, 2005)). Levosimendan is a known opener of sarcolemmal and mitochondrial ATP-sensitive potassium channels (Kopustinskiene et al., 2004, Kopustinskiene et al., 2001), and it has previously been shown to reduce plasma markers of apoptosis in human plasma and cell cultures (Adamopoulos et al., 2006b, Parissis et al., 2004b, Maytin and Colucci, 2005). The
present study revealed for the first time the effectiveness of chronic levosimendan treatment in preventing heart failure-related cardiomyocyte apoptosis in two different experimental models. Microarray analysis also showed that levosimendan is able to suppress all apoptotic signalling at the mRNA level. Levosimendan also reduced activity of proapoptotic transcription factor FOXO3A and downregulated its downstream signalling. Based on this finding, PDE III inhibiting properties of levosimendan appear not to be clinically relevant, as traditional pharmacological PDE III inhibitors have been linked to increased apoptosis in patients with heart failure (Ding et al., 2005). Whether levosimendan is also able to suppress apoptosis in an actual clinical setting remains a subject for further clinical studies.

ROS play a major role in several physiological processes, such as control of apoptosis. However, an elevated level of oxidative stress, as often found in heart failure, can lead to several types of tissue damage, including activated apoptosis (for review see (Giordano, 2005)). In the present study, levosimendan reduced markers of systemic inflammation in both animal models used, indicating that besides KATP opening properties antiapoptotic effects of levosimendan could be, at least partially, mediated via reduction of oxidative stress. Indeed, improved oxidative status prevents apoptosis in different experimental approaches (Dekkers et al., 2008, Zhao et al., 2009, Looi et al., 2008). Especially reduction of VEGF-controlled NR4a3 mRNA expression indicates the beneficial role in suppression of ROS-induced apoptosis, as OR-1896 was able to significantly reduce MI-induced mRNA expressions of VEGF, NR4a3 and MCP-1 mRNA expressions (Martinez-Gonzalez and Badimon, 2005, Liu et al., 2003, Pols et al., 2007).

### 6.2.5 Cardiomyocyte senescence

Activation of premature senescence has recently been linked to several pathological conditions such as premature ageing, hypertension and heart failure (Westhoff et al., 2008, Sharpless and DePinho, 2007, Melzer, 2008). Human hearts with premature cardiac ageing and heart failure show an accumulation of p16INK4a positive primitive cells and myocytes combined with shortened telomeres, representing the imbalance between cellular growth and death (Chimenti et al., 2003). The INK4a/ARF locus codes the cell cycle inhibitor p16INK4A and the p53 activator p19ARF, which have been related to premature senescence (For review see (Sharpless, 2004, Sharpless and DePinho, 2007, Melzer, 2008). In our study, hypertensive Dahl/Rapp SS rats presented along with fulminant hypertension and severe tissue damage also activation of cardiomyocyte senescence, measured as 4-fold increase in p16INK4a mRNA. These results are in accordance with previous findings by Westhoff et al, who showed hypertension-induced cardiac senescence, mechanistically linked to increased renin-angiotensin activity. (Westhoff et al., 2008) Ang II has been demonstrated to induce cellular senescence via NADPH oxidase-derived oxidative stress and DNA damage. (Herbert et al., 2008) As increased ROS formation plays a major role in development of target-organ damage in Dahl/Rapp SS rats (Tojo et al., 2002), it seems reasonable to assume that discovered activation of cardiomyocyte senescence is
truly mediated via increased oxidative stress. Treatment with chronic OR-1896 administration was able to completely prevent induction in expression of $p16^{INK4A}$ mRNA independent of blood pressure status or renin-angiotensin system activity. However, both levsimendan and OR-1896 have been shown by us and others to reduce markers of inflammation, which provide a possible mechanistic explanation for discovered prevention of senescence (Parissis et al., 2007, Louhelainen et al., 2007, Adamopoulos et al., 2006a, Parissis et al., 2004a).

Myocardial infarction induced a clear senescent response in diabetic GK rats. Myocardial mRNA expression of $p19^{ARF}$ was induced immediately after MI, with the most prominent expression occurring during the early weeks of remodelling. However, $p16^{INK4a}$ mRNA was expressed several-fold only at later stages of remodelling. Thus, the present findings support the results of Baker et al, who reported that $p19^{ARF}$ functions as a protective factor to alleviate MI-induced trauma. (Baker et al., 2008a) By contrast, induction of $p16^{INK4A}$ in late remodelling seems to be linked more to ageing and diabetes-induced tissue damage than plain MI. (Rota et al., 2006) Further induction of senescent cascades was examined by mRNA expression of transcription factor E2F-5, which has been shown to be a key downstream mediator for $p16^{INK4A}$-induced G1 arrest (Ohtani et al., 2003, Gaubatz et al., 2000). E2F-5 mRNA expression was induced along with $p16^{INK4A}$ mRNA expression, indicating a true senescent activation.

In GK rats, post-infarct chronic treatment with OR-1896 was able to completely prevent upregulation of senescent signalling, measured by $p19^{ARF}$, $p16^{INK4a}$ and E2F-5 mRNA expressions. Mechanisms for this prevention of senescence remain unclear, but involvement of reduced oxidative stress via treatment is likely to play a major role.

Taken together, this novel senescence preventing effect of calcium sensitizers levsimendan and OR-1896 provides intriguing possibilities for future treatment and prevention of heart failure. However, studies with human subjects are warranted to clarify the effectiveness in clinical use.

6.2.6 Mitochondrial biogenesis

Failure in normal mitochondrial functions has been shown to play a major role in energetics of failing heart (Rimbaud et al., 2009), with special emphasis with type 2 diabetes (Sack, 2009). In the present study, markers of mitochondrial biogenesis, namely TFAM, PGC-1α and NRF-1, were evaluated in the hearts of spontaneously diabetic GK rat after MI. NRF-1 mRNA expression was induced after MI as compared to SHAM-operated animals. NRF-1 regulates a majority of the genes involved in mitochondrial respiratory function (for review see (Scarpulla, 2008)). NRF-1 offers a link between mitochondrial biogenesis and oxidative stress, as it is induced via NRF-2 –dependent manner in response to CO-induced oxidative stress (Piantadosi et al., 2008). Sano and Fukuda recently speculated that mitochondrial biogenesis can in fact be triggered by low-dose formation of ROS (Sano and Fukuda, 2008). In the present study mRNA expression of NRF-1 was significantly induced with
similar trend towards increased mRNA expressions on TFAM and PGC-1α. Interestingly, OR-1896 treatment prevented the MI-induced increase in mitochondrial biogenesis markers, thus supporting further OR-1896’s anti-inflammatory effects.

6.3 Mechanisms of action of levosimendan and OR-1896

The main mechanism of action of both levosimendan and OR-1896 is considered to be calcium sensitization, but additional mechanisms of actions, such as KATP-channel opening and PDE-inhibiting properties are also involved (Kopustinskiene et al., 2004, Yokoshiki et al., 1997b, Yokoshiki et al., 1997a, Takahashi et al., 2000b, Takahashi et al., 2000a, Banfor et al., 2008, Erdei et al., 2006). Although this study was not aimed at describing the mechanism of actions for discovered findings, based on these it could be argued that calcium sensitization provides only part of the beneficial effects during oral levosimendan and OR-1896 treatments. Especially the fact that levosimendan was able to significantly suppress apoptosis, which is generally considered to be mediated via opening of mitochondrial KATP channels, in both experimental models supports this notion. Levosimendan and to a weaker extent OR-1896 are inhibitors of PDE III, but these inhibiting properties seem to have only minor significant relevance at clinical doses (Kaheinen et al., 2006, Ajiro et al., 2002, Szilagyi et al., 2005, Szilagyi et al., 2004). The irrelevance of PDE inhibition is further supported by our study, which showed antiapoptotic effects of levosimendan, while traditional PDE inhibitors have been found to have a detrimental effect when given to patients with heart failure via an increased level of apoptosis (Ding et al., 2005). Based on our results, further investigations into the roles of calcium sensitization, KATP channel opening and PDE inhibition are required.

6.4 Clinical implications and future aspects

The role of OR-1896 in beneficial effects associated with levosimendan treatment remains unknown. OR-1896 has a considerably longer half time (75-78 h in humans, 6.5 h in rats) than levosimendan (1 h in humans, 0.6 h in rats (Orion Pharma, unpublished information)), indicating that it is responsible for long-term, up to 7 days, effects seen after single levosimendan infusion (Kivikko et al., 2003, Sandell et al., 1995, Kivikko et al., 2002a). In clinical use, the infusion time of levosimendan has been limited to 24 h due to possible cumulation OR-1896. However, previous studies by others and us indicate that oral, continuous administration of levosimendan is a safe and effective treatment option (Nieminen et al., 2008, Louhelainen et al., 2007). The doses of levosimendan and OR-1896 used, yielded plasma concentrations of approximately 6-80 ng/ml for levosimendan and 4-50 ng/ml for OR-1896, which are at relevant clinical level (Kivikko et al., 2002b). We were unable to detect any adverse effects with chronic oral levosimendan or OR-1896 in either of the experimental heart failure models used. It could also be argued, that despite mother compound has some additional benefits in prevention of mortality, OR-1896 is as effective in treatment of heart failure, at least in rats.
Beneficial effects could be mediated via steady plasma level of OR-1896, based on its considerably longer half-life. In our study, the plasma concentration of OR-1896 during chronic OR-1896 administration showed no diurnal fluctuation as was seen during levosimendan treatment. Prolonged administration of levosimendan has been associated with side effects, namely hypotonic events and atrial fibrillation, most likely due to accumulation of the long-lived active metabolite OR-1896. Therefore, current clinical recommendations limit the maximal infusion time of levosimendan to 24 h. These adverse reactions could be avoided by repeated administrations of levosimendan infusion, as Parissis et al. in a small-scale study reported the safety and beneficial effects of serial levosimendan infusions on patients with heart failure (Parissis et al., 2006a). However, the costs and inconvenience of this time-consuming invasive method of intravenous administration restricts its widespread use. Neither levosimendan nor OR-1896, while administered orally in a chronic manner showed any signs of adverse reactions or cumulative toxicity. However, the transient decrease in blood pressure and the slight increase in heart rate discovered in here could potentially explain side effects, such as hypotension and arrhythmias, in human populations.

Data on oral use of levosimendan remain limited. In the only large-scale study of oral levosimendan in humans to date, the PERSIST study, levosimendan failed to show improvement in the novel main end-point, Patient journey (Nieminen et al., 2008). However, the PERSIST patients had very severe heart failure with recent hospitalizations, very high NT-proBNP values and symptoms at rest or very mild exercise, which could, explain the discovered ineffectiveness of levosimendan treatment. The PERSIST study also had some problems in baseline imbalance and in patient randomization, pro-BNP levels in the lower levosimendan dose group being 40% greater than in controls. Taken together, improvements in the quality of life score and in renal function and a reduction in NT-proBNP provide encouragement for further studies.

Even though studies in rats cannot be extrapolated to humans, results of the present study indicate that oral levosimendan and OR-1896 provide interesting possibilities in prevention of experimental hypertensive heart disease and post-infarct cardiac remodelling as well as other low cardiac output conditions such as shock and pre-, peri- and post-operative cardiac interventions. Indeed, levosimendan is currently under evaluation in several clinical studies ranging from septic shock to weaning from cardio-pulmonary by-pass (ClinicalTrials.gov, 2009).

Especially the safety of continuous oral administration discovered in this study gives encouragement for future development of a suitable oral drug form for man, even though preliminary findings of oral levosimendan in humans have been disappointing. Therefore, further studies of the beneficial role of oral calcium sensitization in human heart failure of different origins are warranted.
7 Conclusions

The aim of this study was to investigate the cardiovascular effects of oral calcium sensitizers levosimendan and OR-1896, an active metabolite, in two different models of experimental heart failure, namely an experimental model of myocardial infarction induced by coronary artery ligation and salt-sensitive model of essential hypertension. These models represent post-infarct heart failure and hypertension-induced heart failure with preserved systolic function, respectively. The main findings of these non-clinical cardiovascular studies were as follows:

I. Oral treatment with calcium sensitizer levsimendan and OR-1896 improved survival, produced a transient decrease in systolic blood pressure, and prevented hypertension-induced cardiac remodelling, apoptosis and neurohumoral activation in hypertensive Dahl/Rapp SS rats on a high-salt diet. Our findings indicate that the beneficial effects of oral levsimendan treatment were mediated, to a great extent, by the active and long-lasting metabolite OR-1896.

II. Post-infarct cardiac remodelling in spontaneously diabetic GK rat was associated with increased inflammation response, oxidative stress, neurohumoral activation, sustained cardiomyocyte apoptosis, increased cellular senescence and disturbances in the expression of calcium-handling proteins when compared to post-infarct cardiac remodelling in non-diabetic Wistar rats.

III. Oral levsimendan prevented post-infarct cardiac remodelling and restored systolic function in both spontaneously diabetic GK rats and non-diabetic Wistar rats. Levsimendan did not influence systolic blood pressure, blood glucose level, myocardial infarct size or cardiovascular mortality. The cardiovascular effects of levsimendan were more pronounced in diabetic rats than in the non-diabetic controls.

IV. In spontaneously diabetic GK rats with MI, oral OR-1896 restored cardiac functions at systole, ameliorated post-infarct cardiac hypertrophy and prevented MI-induced increases in cardiac atrial natriuretic peptide (ANP), monocyte chemoattractant protein 1 (MCP-1) and connective tissue growth factor (CTGF) mRNA expressions, markers of pressure/volume overload, inflammation and fibrosis, respectively. OR-1896 suppressed MI-induced cardiomyocyte senescence, but did not influence systolic blood pressure, blood glucose level, myocardial infarct size or cardiovascular mortality.

V. Our results suggest a therapeutic role for oral calcium sensitizers in the prevention of hypertension-induced heart failure with preserved systolic function as well as in the prevention of post-infarct heart failure with decreased systolic function.
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