Levosimendan: Studies on its mechanisms of action and beyond

Petri Kaheinen

Institute of Biomedicine
Pharmacology
University of Helsinki

and

Division of Pharmacology and Toxicology
Faculty of Pharmacy
University of Helsinki

Academic Dissertation

To be presented, with the permission of the Faculty of Medicine, University of Helsinki, for public examination in the lecture hall 2 of the Biomedicum, Helsinki on 13 November, 2009, at 12 noon

Supervisors

Professor Eero Mervaala, MD, PhD
Institute of Biomedicine, Pharmacology
University of Helsinki
Helsinki, Finland

Docent Piero Pollesello, PhD
Orion Pharma
Espoo, Finland

Reviewers

Senior Lecturer Ewen Macdonald, PhD
Department of Pharmacology and Toxicology
University of Kuopio
Kuopio, Finland

Docent Veli-Pekka Harjola, MD, PhD
Department of Medicine Division of
Emergency Care
Helsinki University Central Hospital
Helsinki, Finland

Opponent

Professor Markku Koulu, MD, PhD
Health Biosciences
University of Turku
Turku, Finland

ISBN 978-952-92-6140-6 (paperback)
ISBN 978-952-10-5735-9 (PDF)
Helsinki 2009
Helsinki University Print

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to the text by the Roman numerals I-V, and some unpublished data:

I Haikala H, Kaheinen P, Levijoki J, Linden IB. The role of cAMP- and cGMP-dependent protein kinases in the cardiac actions of the new calcium sensitizer, levosimendan. *Cardiovasc Res* 1997;34:536-46.

II Szilagyi S, Pollesello P, Levijoki J, Kaheinen P, Haikala H, Edes I, et al. The effects of levosimendan and OR-1896 on isolated hearts, myocyte-sized preparations and phosphodiesterase enzymes of the guinea pig. *Eur J Pharmacol* 2004;486:67-74.

III Kaheinen P, Pollesello P, Hertelendi Z, Borbely A, Szilagyi S, Nissinen E, et al. Positive inotropic effect of levosimendan is correlated to its stereoselective Ca^{2+}-sensitizing effect but not to stereoselective phosphodiesterase inhibition. *Basic Clin Pharmacol Toxicol* 2006;98:74-8.

IV Kaheinen P, Pollesello P, Levijoki J, Haikala H. Levosimendan increases diastolic coronary flow in isolated guinea-pig heart by opening ATP-sensitive potassium channels. *J Cardiovasc Pharmacol* 2001;37:367-74.

V Kaheinen P, Pollesello P, Levijoki J, Haikala H. Effects of levosimendan and milrinone on oxygen consumption in isolated guinea-pig heart. *J Cardiovasc Pharmacol* 2004;43:555-61.

ABBREVIATIONS

4-AP	4-aminopyridine
α_1-AR	adrenergic alpha-1 receptor
AMI	acute myocardial infarction
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AC	adenylate cyclase
ACE	angiotensin converting enzyme
ACEI	angiotensin converting enzyme inhibitors
ADHF	acutely decompensated heart failure
ADP	adenosine diphosphate
AE	adverse effect
AF	atrial fibrillation
AHF	acute heart failure
ARB	angiotensin receptor antagonist
ATP	adenosine triphosphate
β_1-AR	adrenergic beta-1 receptor
β_2-AR	adrenergic beta-2 receptor
BK_{Ca}	calcium-activated potassium channel
BIM	bisindolylmaleimide
BP	blood pressure
CABG	coronary artery bypass graft
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
$[Ca^{2+}]_i$	intracellular calcium concentration
CAD	coronary artery disease
CBP	cardiopulmonary bypass
CCB	calcium channel blockers
CF	coronary flow
CHD	coronary heart disease
CHF	congestive heart failure
CI	cardiac index
CIP	Cahn Ingold Prelog priority rules

CMK	calmodulin-kinase
CN	calcineurin
CP	cardiac power
CRT	cardiac resynchronization therapy
CS	cardiogenic shock
cTn	cardiac troponin complex
DCFV	diastolic coronary flow velocity
DCM	dilated cardiomyopathy
DHF	diastolic heart failure
ECG	electrocardiogram
EF	ejection fraction
ESC	European Society of Cardiology
ET_B	endothelin-receptor type-B
GC	guanylate cyclase
hBNP	human B-type natriuretic peptide
HF	heart failure
HR	heart rate
IBTX	iberiotoxin
IC_{50}	50% inhibitory concentration
IL	interleukin
IP3	inositol triphosphate
IRAG	cGMP kinase substrate
IRK	inwardly rectifying potassium channels
K_{ATP}	ATP-sensitive potassium channels
KO	knockout
KV	voltage-gated potassium channels
LV $+dP/dt_{max}$	left ventricular maximal positive pressure derivative
LV $-dP/dt_{max}$	left ventricular maximal negative pressure derivative
MRI	Magnetic Resonance Imaging
M1	muscarinic type-1 receptor
M3	muscarinic type-3 receptor
MI	myocardial infarction
mitoK_{ATP}	mitochondrial K_{ATP} channels
MOF	multiorgan organ failure

MVO_2	oxygen consumption
Na^+-K^+ pump	sodium–potassium ATPase
NCX	sodium-calcium exchanger
NO	nitric oxide
NOS	nitric oxide synthase
NRP	natriuretic peptide receptor
PCWP	pulmonary capillary wedge pressure
PDE	phosphodiesterase
PDEI	phosphodiesterase inhibitor
PCI	percutaneous coronary intervention
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
PKC	protein kinase C
RAAS	renin angitensin aldosterone system
S1	subfragment 1
sarcK_{ATP}	sarcolemmal K_{ATP} channels
SBP	systolic blood pressure
SERCA-2	sarcoplasmic reticulum calcium ATPase isoform 2
SNS	sympathetic nervous system
SR	sarcoplasmic reticulum
SVT	supraventricular tachycardia
SUR	sulfonylurea receptor
SVR	systemic vascular resistance
Tm	tropomyosin
Tn	troponin complex
TnC	troponin C
TNF- α	tumor necrosis factor-α
TnI	troponin I
TnT	troponin T
VT	ventricular tachycardia

ABSTRACT

Acute heart failure syndrome represents a prominent and growing health problem all around the world. Ideally, medical treatment for patients admitted to hospital because of this syndrome, in addition to alleviating the acute symptoms, should also prevent myocardial damage, modulate neurohumoral and inflammatory activation, and preserve or even improve renal function. Levosimendan is a cardiac enhancer having both inotropic and vasodilatory effects. It is approved for the short-term treatment of acutely decompensated chronic heart failure, but it has been shown to have beneficial clinical effects also in ischemic heart disease and septic shock as well as in perioperative cardiac support.

In the present study, the mechanisms of action of levosimendan were studied in isolated guinea-pig heart preparations: Langendorff-perfused heart, papillary muscle and permeabilized cardiomyocytes as well as in purified phosphodiesterase isoenzyme preparations. Levosimendan was shown to be a potent inotropic agent in isolated Langendorff-perfused heart and right ventricle papillary muscle. In permeabilized cardiomyocytes, it was demonstrated to be a potent calcium sensitizer in contrast to its enantiomer, dextrosimendan. It was additionally shown to be a very selective phosphodiesterase (PDE) type-3 inhibitor, the selectivity factor for PDE3 over PDE4 being 10000 for levosimendan. Irrespective of this very selective PDE3 inhibitory property in purified enzyme preparations, the inotropic effect of levosimendan was demonstrated to be mediated mainly through calcium sensitization in the isolated heart as well as the papillary muscle preparations at clinically relevant concentrations. In the isolated Lagendorff-perfused heart, glibenclamide antagonized the levosimendan-induced increase in coronary flow (CF). Therefore, the main vasodilatory mechanism in coronary veins is believed to be the opening of the ATP-sensitive potassium (K_{ATP}) channels. In the paced hearts, CF did not increase in parallel with oxygen consumption (MVO_2), thus indicating that levosimendan had a direct vasodilatory effect on coronary veins. The pharmacology of levosimendan was clearly different from that of milrinone, which induced an increase in CF in parallel with MVO_2.

In conclusion, levosimendan was demonstrated to increase cardiac contractility by binding to cardiac troponin C and sensitizing the myofilament contractile proteins to calcium, and further to induce coronary vasodilatation by opening K_{ATP} channels in vascular smooth muscle. In addition, the efficiency of the cardiac contraction was shown to be more advantageous when the heart was perfused with levosimendan in comparison to milrinone perfusion.

1 INTRODUCTION

Acute heart failure syndromes are a most common cause of hospitalization all over the world with a dismal long-term prognosis (Goldberg et al. 2007). According to the European Society of Cardiology (ESC) guidelines for the diagnosis and treatment of acute and chronic heart failure (HF) issued in 2008, acute heart failure is defined as 'rapid onset or change in the signs and symptoms of HF, resulting in the need for urgent therapy' (Dickstein et al. 2008). HF is defined as a syndrome in which patients should have the following features: Typical symptons like breathlessness at rest or on exercise, fatigue, tiredness and ankle swelling; typical signs like tachycardia, tachypnoea, pulmonary rales, pleural effusion, raised jugular venous pressure, peripheral oedema and hepatomegaly and; objective evidence of a structural or functional abnormality in the heart at rest like cardiomegaly, third heart sound, cardiac murmurs, abnormality on the echocardiogram and raised natriuretic peptide concentration (Dickstein et al. 2008). Acute heart failure (AHF) may be either new HF or worsening of pre-existing chronic HF. Patients differ in terms of their clinical presentation, pathophysiology, prognosis, and therapeutic options (Flaherty et al. 2009). Primarily the symptoms of AHF are the result of severe pulmonary congestion due to elevated left ventricular filling pressure and/or diastolic dysfunction, which can be related to low cardiac output. AHF patients can have both preserved and reduced ejection fraction (EF) and be suffering from a variety of cardiovascular conditions such as coronary heart disease (CHD), hypertension, valvular heart disease and atrial arrhythmias (Gheorghiade et al. 2005c). In addition, noncardiac conditions like renal dysfunction, diabetes and anaemia are often present (Gheorghiade et al. 2005c).

AHF can be categorized into 6 clinical distinct entities, which can overlap with each other: worsening or decompensated chronic HF, pulmonary oedema, hypertensive HF, cardiogenic shock, isolated right HF and, acute coronary syndrome (ACS) and HF (Dickstein et al. 2008) (Fig. 1.). The majority of patients presenting with AHF have coronary artery disease (CAD) (Flaherty et al. 2009). These patients can be divided into two groups, those with acute CAD and those with underlying or chronic CAD. Thus, therapeutic strategies can be designated according to appearance of CAD.

The most common entities of symptoms of AHF patients admitted to the intensive care unit are acutely decompensated chronic heart failure and pulmonary edema with elevated systemic blood pressure (Gheorghiade et al. 2005c). Conventionally, the primary therapeutic management for acute HF deterioration are reduction of PCWP and/or increase of cardiac output, but blood pressure control, myocardial protection, neurohormonal modulation, and preservation of renal function may also be needed. According to Finnish Acute Heart Failure Study (FINN-AKVA), acute congestion

(63.5%) was the most common manifestation of AHF and over one quarter of the patients (26.3%) displayed pulmonary oedema. The rest had either cardiogenic shock (2.3%) or hypertensive crisis (3.1%) or right ventricular failure (4.8%). Furthermore, half of the patients had a history of HF (Siirila-Waris et al. 2006).

According to the ESC Guidelines (Dickstein et al. 2008), inotropic agents should be considered in patients with low output state, in the presence of signs of hypoperfusion or congestion despite the use of vasodilators and/or diuretics to alleviate the symptoms. Traditionally inotropes are categorised into three classes: cardiac glycosides (e.g. digitalis); sympathomimetic amines having β-adrenergic activity (e.g. dopamine, dobutamine, norepinephrine and epinephrine) and; phosphodiesterase inhibitors (PDEIs) (e.g. milrinone and enoximone) (Braunwald 1992). These inotropic agents generally improve the hemodynamic status of the AHF patients, but on the other hand, have not been shown to improve survival, in fact may even worsen survival (Amidon and Parmley 1994; Thackray et al. 2002; Felker et al. 2003) probably due to elevating intracellular calcium levels, an action which is crucial to their mechanism of inotropic action (Papp et al. 2005). Therefore, these drugs should be withdrawn as soon as the desired hemodynamic effects are reached (e.g. adequate organ perfusion or reduced congestion) (Dickstein et al. 2008).

One of the latest entries in the family of cardiac enhancers is levosimendan, a novel compound having both inotropic and vasodilatory effects (Parissis et al. 2008). The present studies were designed to characterize the mechanisms of action of levosimendan and to compare its inodilatory effects to those of phosphodiesterase inhibitors, acting via a different mechanism of action. The *ex-vivo* experiments were done in isolated heart preparations, a technique which allows untangling the inotropic and vasodilatory effects of the drugs studied. Levosimendan increases cardiac contractility by enhancing the sensitivity of the contractile proteins to calcium ions (Pollesello et al. 1994), and exerts a vasodilatory effect by opening adenosine triphosphate ATP-sensitive potassium (K_{ATP}) channels in vascular smooth muscle (Yokoshiki et al. 1997). Due to its novel mechanism of action, which is not dependent upon an increase in the intracellular level of calcium, it is believed to be more beneficial than the other inodilators in being able to reduce both morbidity and mortality.

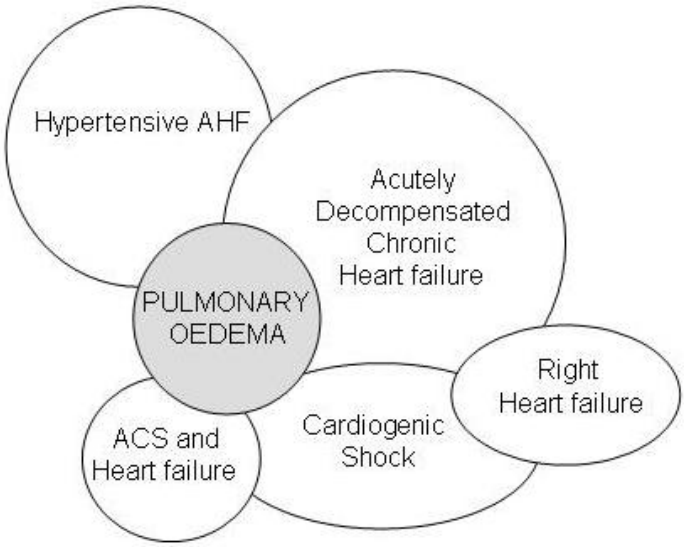

Figure 1. Clinical classification of AHF. Acute heart failure (AHF); Acute coronary syndrome (ACS). Modified from (Dickstein et al. 2008) *Eur Heart J* 29(19): 2388-442.

2 REVIEW OF THE LITERATURE

2.1 Regulation of the cardiac contraction

Two characteristic intrinsic mechanisms, independent of neural and humoral influences, determine the contractile regulation of cardiac myocytes; the Frank- Starling mechanism and the positive force-frequency relationship. The Frank-Starling mechanism is defined as the ability of the heart to change its force of contraction and therefore the stroke volume in response to changes in venous return, i.e. an increase in ventricular wall stretch, leading to an increase in contractile force. The positive force-frequency relationship, i.e. increased heart rate which induces an increase in contractile force, also leads to an increase in stroke volume. The immediate response to the stretch (Frank- Starling mechanism) does not increase the Ca^{2+} transient, but is followed by slowly developing increase in intracellular calcium concentration $[Ca^{2+}]_i$, probably due to the increase in Ca^{2+} binding affinity to troponin C induced by the force generation (Endoh 2008). The force-frequency relationship is, in turn, associated with the mobilization of $[Ca^{2+}]_i$. Furthermore, cardiac muscle contracts phasically, so that the difference between systolic and diastolic $[Ca^{2+}]_i$, determine the effect on the left ventricular function. Thus, heart muscle contraction can be enhanced by increasing the $[Ca^{2+}]_i$ in the cardiomyocytes. Relaxation, in turn, depends on how fast Ca^{2+} is extruded or taken back into the sarcoplasmic reticulum (SR). Myocyte $[Ca^{2+}]_i$ can be increased by increasing intracellular levels of cyclic adenylate monophosphate (cAMP) or by affecting the sodium-calcium exchanger (NCX). The increased amount of Ca^{2+} is accumulated into the cytosol and stored into the SR. This Ca^{2+} is then released from SR by each heart-beat-cycle, which leads to increased contractility of the heart (Braunwald 1992).

Contraction of the cardiac cell

Skeletal and cardiac muscles are both composed of striated muscle cells and the sarcomere structure and the general mechanism involved in the muscle contraction is the same. The striated muscle cell contains myofibrils formed by repeating units of sarcomeres. They are arranged in series, which are composed of two type parallel filaments, a thin filament and a thick filament. The thick filament is a polymer, composed of myosin molecules which in turn consists of two heavy and four light chains. A bundle of myosin heavy chain coiled tails forms the backbone of the thick filament. The globular heads of the heavy chain N-terminus named as subfragment 1 (S1) are located in the thick filament at regular intervals and interact with the thin filaments to form strong crossbridges. The thin filament, in turn, has a two-stranded helical structure. The backbone of the thin filament is

composed of polymerized globular actin monomers (G-actin). Actin monomers consist of two equal-sized domains which are available for myosin interaction or interact with the corresponding sub-domains of the adjacent strand (Holmes et al. 1990).

A series of regulatory proteins modulate the interaction between the thick and thin filaments. The most elongated of them, tropomyosin (Tm) overlaps with the neighboring tropomyosins in a head-to-tail configuration. The overlapping regions of adjacent tropomyosins are mainly responsible for the affinity of Tm for actin. It binds to the actin filament by electrostatic interaction (Lorenz et al. 1995). Instead of being fixed in one position, Tm rolls over the surface of the thin filament depending on the phase of the contraction cycle. This movement is influenced by Ca^{2+} and it affects myosin S1 binding to actin (Gordon et al. 2000; Gordon et al. 2001; Sorsa et al. 2004). Every tropomyosin is spatially coupled to a troponin complex (Tn), and together they form the calcium dependent trigger of the contractile apparatus. Tn consists of a Tm binding unit troponin T (TnT), an actomyosin ATPase inhibitory unit troponin I (TnI), and a calcium-binding unit troponin C (TnC). Striated-muscle troponin C is expressed in two isoforms in vertebrates, in fast skeletal muscle there is skeletal troponin C (sTnC) and in slow skeletal and cardiac muscles it is cardiac troponin C (cTnC). TnT is an asymmetric protein that attaches the Tn complex to a defined position on the thin filament. It is needed for full, Ca^{2+}-dependent activity of the thin filament. The interaction of TnT with Tm is calcium sensitive. Calcium-binding to TnC initiates the cascade of events leading to muscle contraction. The interaction between TnC and TnI is essential for further transmission of the contraction signal to the other components of the thin filament (Sorsa et al. 2004).

Mechanism of action of cardiotonic agents

The adrenergic beta1-receptor (β_1-AR) is the main adrenergic receptor type in heart cells. It is stimulated by the endogenous norepinephrine (NE), released from the sympathetic nerve-endings. Drugs that enhance NE release can be inotropic, but mainly the receptor is stimulated by the beta-adrenergic agonists. Stimulation of β_1-AR activates adenylyl cyclase (AC) due to the G-stimulatory protein (G_s). The activation of AC increases the intracellular levels of cAMP, which activates cAMP dependent protein kinase (PKA). PKA, in turn, phosphorylates L-type calcium channels leading to their opening and increase in $[Ca^{2+}]_i$. Increased $[Ca^{2+}]_i$ triggers the release of Ca^{2+} from SR via ryanodine receptors and furthermore, there is increased force generation by the actin-myosin apparatus when Ca^{2+} binds to the cTnC (Sorsa et al. 2004).

PDE inhibitors increase the amount of intracellular cAMP by inhibiting its breakdown. Thus, this mechanism is downstream to β_1-AR stimulation and acts in a parallel manner with it. In all, 11

families of PDE enzymes have been identified, 5 of them in heart tissue. They differ in terms of their affinity for cAMP and cGMP, cellular expression, intracellular localization, and mechanisms of regulation. Their function is dependent on their compartmentation with protein kinases and other proteins related to signal transduction cascades (Fischmeister et al. 2006; Vandecasteele et al. 2006). Two families, PDE3 and PDE4, are the most important in the regulation of the cardiac contractility. PDE4 is cAMP specific, but PDE3 has a very high affinity for both cAMP and cGMP. However, its turnover rate for cGMP is much lower than its turnover rate for cAMP (Shimizu et al. 2002). Thereby, PDE3 may function primarily as a cGMP-inhibited cAMP phosphodiesterase. Inhibition of the cAMP-hydrolytic activity of PDE3 by cGMP contributes to the potentiation of delayed rectifier K^+ currents and L-type Ca^{2+} currents in cardiac myocytes (Shimizu et al. 2002; Movsesian et al. 2008).

Cardiac glycosides, like digoxin, bind to the α-subunit of the sodium/potassium ATPase (Na^+/K^+ pump) in the extracellular membranes of the myocytes and inhibit its function. The inhibition of the Na^+/K^+ pump causes an increase in the intracellular sodium concentration $[Na^+]_i$ in the myocytes, which leads further to an elevation in $[Ca^{2+}]_i$ by the NCX. Basically, NCX that normally extrudes Ca^{2+} from the cell (forward mode), also brings Ca^{2+} into the cell (reverse mode) during membrane depolarization in the resting phase of the cardiac action potential (Iwamoto et al. 2007). NCX can run in the reverse mode also when Na^+ accumulates into the cytosol. As a result of the inhibition of the Na^+/K^+ pump, Ca^{2+} accumulates in the cytosol and is then stored in the SR. In principle, the inhibition of the forward mode of NCX can also cause an inotropic effect.

The above-mentioned mechanisms increase the cytosolic calcium concentration $[Ca^{2+}]_i$ and thus, result in higher demands for oxygen and elevated risk of arrhythmias, cell injury, apoptosis or necrosis due to Ca^{2+} overload. An alternative idea is to enhance the response of myofilaments to calcium without an increase in the $[Ca^{2+}]_i$, and thus, have a positive inotropic effect on cardiac contractility. Calcium sensitizers can increase the Ca^{2+} affinity to troponin, like pimopendan. They can stabilize the Ca^{2+} bound conformation of troponin C, like levosimendan or furthermore, they can have a direct effect on the cycling rate of actinmyosin cross-bridges, lowering the threshold of $[Ca^{2+}]_i$ needed for actin sliding, like MCI-154 (Kass and Solaro 2006).

Role of stereoisomerism on the pharmacodynamic action

The symmetry of a molecule determines its chirality. A molecule is chiral when it is non-superposable on its mirror image. Enantiomers are two stereoisomers that are mirror images of each other. However, a chiral molecule is not necessarily asymmetric. Different enantiomers of a compound have the same physical properties, but they rotate polarized light in different directions and interact differently with optical isomers of other compounds. Therefore, the enantiomers of a compound may have substantially different biological effects. A mixture of equal parts of the enantiomers is called racemic.

Many drugs are chiral and only one of the enantiomers are biologically active or more effective than the other. A good example is adrenaline of which the natural form is the (R)-(−)-L-adrenaline. An enantiomer can be named by the direction in which it rotates the plane of polarized light. If it rotates the light clockwise the enantiomer is labeled (+), if counter-clockwise it is labeled (−). An optical isomer can be named by the D/L system, a spatial configuration of its atoms relating the molecule to glyceraldehyde. The R/S system is the most important nomenclature system for symbolizing enantiomers, which does not involve a reference molecule such as glyceraldehyde. It labels each chiral center R or S according to the CIP system(Cahn Ingold Prelog priority rules) (Cahn et al. 1966). The R/S system has no fixed relation to the (+)/(-) or the D/L systems.

Classical examples of cardiotonic drugs, which have two enantiomers are propranolol and carvedilol. Only L-propranolol is a β_1-AR antagonist, whereas D-propranolol has little binding affinity. However, both isomers possess a local anesthetic effect. Furthermore, S(-) isomer of carvedilol is 100 times more potent as β_1-AR antagonist than R(+) isomer. However, both of the isomers are approximately equipotent as adrenergic α_1-receptor (α_1-AR) antagonists.

2.2 Regulation of the vascular resistance

Vascular resistance is dependent on contraction and relaxation of smooth muscle myocytes. The molecular mechanisms involved in the contraction-relaxation process in the smooth muscle cells and its modulation can be manipulated pharmacologically in many ways. Smooth muscle contracts tonically, thereby the vascular tone is in direct proportion to a function of $[Ca^{2+}]_i$ itself, not to the difference between systolic and diastolic $[Ca^{2+}]_i$. Agents that increase $[Ca^{2+}]_i$ in vascular smooth muscle cells are vasoconstrictors, while agents that decrease $[Ca^{2+}]_i$ in vascular smooth muscle cells are vasodilators.

Mechanism of action of vasodilatory agents

The inhibition of L-type calcium channels leads to a decrease in vascular myocyte $[Ca^{2+}]_i$. Less Ca^{2+} is bound to calmodulin, which decreases the activation of myosin kinase and further reduces the phosphorylation of myosin thick filament. L-type calcium channels are voltage-dependent and are inhibited by three type of chemical substances, the phenylalkylamines (like verapamil), the dihydropyridines (like nifedipide) and by benzothiazephines (like diltiazem). They are vasodilators, but verapamil is used also as an antiarrhythmic agent.

Calcium release via inositol trisphosphate (IP3)-sensitive Ca^{2+} channels from SR is another source of $[Ca^{2+}]_i$ in vascular myocytes. IP3 is generated from phosphatidylinositol when phospholipase C is activated by $G_{\alpha q}$-coupled receptors i.e. α_1-adrenergic, angiotensin, and endothelin receptors. M1/M3 muscarinic receptors and ET_B endothelin-receptor are also coupled to inositol triphosphate (IP_3) generation, but in endothelial cells. Increased endothelial $[Ca^{2+}]_i$ leads to vasodilatation contrary to the situation in vascular smooth muscle cells (see below, cGMP-dependent vasodilatation). The main drug categories acting ultimately through IP_3 generation are angiotensin converting enzyme inhibitors (ACEIs), angiotensin II receptor antagonists (ARBs), endothelin receptor antagonists and α_1-AR antagonists.

The formation of cGMP from GTP by guanylate cyclase (GC) is analogous to the formation of cAMP from ATP by AC. Two forms of GC exist, a soluble enzyme and a membrane-bound enzyme. GC activates cGMP-dependent protein kinase (PKG), which phosphorylates the IP_3 receptor-associated cGMP kinase substrate (IRAG). Phosphorylation of IRAG further reduces IP_3-induced Ca^{2+} release, leading to relaxation of vascular myocytes. PKG also phosphorylates K^+ channels and thereby, hyperpolarizes the cell membrane and decreases Ca^{2+} influx through L-type channels. In addition, PKC phosphorylates myosin light chain phosphatase and so, dephosphorylates the myosin ATPase and reduces the contraction of vascular myocytes. Furthermore, it phosphorylates $G_{\alpha q}$-protein and thus, inhibits IP3 formation. Soluble GC is a target for nitric oxide (NO), synthesised endogenously from arginine and oxygen by nitric oxide synthase (NOS) enzymes or formed by reduction from organic nitrates and then released from endothelial cells. Nitroglycerin (or glyceryl trinitrate) and sodium nitroprusside, are both sources of NO and thus, common vasodilatory agents. The natriuretic peptides (ANP, BNP and CNP), instead, bind to the specific NP receptors (NPRs). Three subtypes of NPRs have been characterized, namely NPR1, NPR2 and NPR3. Two of them, NPR1 and NPR2, contain the GC catalytic domain (membrane bound GC). ANP and BNP selectively stimulate NPR1, whereas CNP activates primarily NPR2. All three NPs

bind to NPR3, which clears them from the circulation through receptor-mediated internalization and degradation (Potter et al. 2006; Potter et al. 2009). Moreover, PDE5 is essentially the most cGMP specific of the cardiac phosphodiesterases. The benefits of PDE5 inhibition in heart failure originate from a decrease of the right ventricle afterload resulting from pulmonary vasodilatation (Movsesian et al. 2008).

The increase in the cAMP content in smooth muscle cells evokes vasodilatation. The cAMP level increases when vascular β_2-adrenergic receptors are stimulated. Instead of PKA, cAMP activates PKG in vascular myocytes. PKG has a much lower affinity for cAMP than for cGMP, but cAMP levels can rise high enough to activate PKG in smooth muscle cells, despite its relatively low affinity for cAMP. In this manner, vasodilatation occurs when PDE3 is inhibited and cAMP hydrolysis is restricted.

Activation of potassium channels in vascular smooth muscle cells results in membrane hyperpolarization and thus, a decrease Ca^{2+} influx through L-type channels and further vasodilatation (Brayden 2002). Four major classes of potassium channels have been classified: calcium-activated potassium channel (BK_{Ca}), inwardly rectifying potassium channels (IRK), two-pore-domain potassium channels and voltage-gated potassium channels (KV). K_{ATP} channels belong to the IRK channels, containing four pore-forming Kir6.x subunits and a large regulatory sulfonylurea receptor (SUR) (Aguilar-Bryan et al. 1998; Brayden 2002). They appear to be tonically active in some vascular beds, e.g. in the coronary veins, and contribute to the physiological regulation of vascular tone and blood flow. K_{ATP} channels are expressed also in the vascular endothelium (Yoshida et al. 2004) and may modulate vasodilatation in response to shear stress and pathophysiological conditions, such as hypoxia, ischemia, acidosis and septic shock as well as some vasodilators, e.g. adenosine (Yoshida et al. 2004; Adebiyi et al. 2008).

2.3 Acute Heart Failure Syndromes

2.3.1 Pathophysiology and risk markers of AHF

Multiple reasons are involved in the pathogenesis of AHF, starting from nonadherence to diet, pharmacologic therapy or fluid restriction and ending in cardiogenic shock and multiorgan failure. However, fluid accumulation is the most important factor causing hospitalization of patients with AHF and traditionally it has been considered simply as the major cause of the syndromes (Metra et al. 2008a). This opinion has now been challenged. Most patients with AHF can be divided into two main types according to their symptoms, especially according to their blood pressure (Gheorghiade

et al. 2005a). Furthermore, Cotter et al (Cotter et al. 2008) proposed that the mechanisms behind AHF are more complex than simply fluid accumulation. They distinguished two categories of symptoms of ADHF. Usually ADHF is the end result of a relatively slow (days to weeks) deterioration of severe chronic HF. However, it can be a rapidly progressive disorder of high blood pressure (BP) seen in the emergency rooms, accompanied by severe acute dyspnoea. Moreover, ADHF may be the result of the decrease in myocardial contractility due to ischemia. On the other hand, it can be caused by a combination of increased vascular resistance with decreased cardiac contractility, even though EF is relatively well preserved. This leads to severe hypertension with increased diastolic left ventricular failure (Cotter et al. 2008).

Volume overload is typical for AHF and is traditionally believed to be the main cause of congestion. Thus, treatment with diuretics results in recovery of the fluid homeostasis. However, recent studies do not necessarily support this concept. The symptoms of HF e.g. fatigue and increased shortness of breath as well as an increase in pulmonary pressure have been shown to occur days to weeks before any weight gain was first observed (Cotter et al. 2008). Furthermore, there is evidence that even a dramatic rise in cardiac filling pressure (preceding an AHF hospitalization) often occurs without any significant change in weight gain in patients with chronical hemodynamic monitoring devices (Cotter et al. 2008). In addition, even treatment with a high dose diuretic has not been associated with improved outcome (Cotter et al. 1997). In fact, a high dose of loop diuretics has been associated with a higher risk of renal failure (Butler et al. 2004).

Myocardial ischaemia is considered as an important trigger of AHF and more than a half of the patients hospitalized with AHF have a history of chronic ischemic heart disease in the USA, in Europe as well as in Finland (Siirila-Waris et al. 2006; Alla et al. 2007). Furthermore, Gheorghiade et al (2005) in PRESERVD-HF study (Gheorghiade et al. 2005b) studied 51 patients with AHF who were admitted with worsening HF and a history of CAD but not with an acute coronary event. Most of the patients had detectable levels of cardiac troponin (cTn) already at the initial measurement, and some patients displayed cTn release during hospitalization. The release of cTn is thought to be a marker for myocardial injury, and therefore the study raised the possibility that injury occurred in most patients admitted with AHF.

According to FINN-AKVA, elevated cTn levels are common in AHF patients without ACS, cTnI being more often elevated than cTnT. Increase in both cTnI and cTnT correlated with increased mortality. However, they did not act as independent risk markers (Ilva et al. 2008). Cystatin C is a novel marker of renal function, which has been discovered as a prognostic marker in AHF.

According to FINN-AKVA, cystatin C was a strong and independent predictor of outcome at 12 months in AHF. In spite of normal plasma creatinine, cystatin C identifies patients with poor prognosis (Lassus et al. 2007).

Arrhythmias are common in chronic heart failure. In congestive heart failure (CHF), atrial fibrillation (AF) is accompanied by a marked attenuation of the cardiac sympathetic response to acute hemodynamic stress in isometric exercise (Gould et al. 2008). Previously, impairment of the baroreceptor responses has been associated with abnormal hemodynamic responses to isometric exercise (Fukuma et al. 2004). This indicates that AF could be associated with the impairment of the baroreceptor response in CHF. According to the EuroHeart Failure Survey II (EHFS II) (Nieminen et al. 2006), 38.7% of the patients had AF. However, the role of new arrhythmias in acute heart failure is largely unknown. Nevertheless, AF has been shown to be a strong predictor of the reappearance of symptoms and death in patients admitted for AHF (Benza et al. 2004).

Rupture of chordae tendineae or a papillary muscle can lead to acute mitral regurgitation, which in turn, may be a cause of acute pulmonary edema and a rapid decrease in myocardial contractility, leading further to AHF. Instead, the role of chronic ischemic mitral regurgitation as a cause for AHF is less clear. However, major increases in mitral regurgitation and systolic pulmonary-artery pressure during exercise have been observed a few days after acute pulmonary edema (Pierard and Lancellotti 2004). This suggests that acute pulmonary edema is associated with the dynamic changes in ischemic mitral regurgitation and the further development of AHF.

Recent studies have revealed that elevated BP plays a central role in the pathogenesis of AHF in most patients. Initial BP measurements in the emergency room before any treatment has shown that BP is elevated prominently at the onset of AHF (Milo-Cotter et al. 2007). A marked increase in systemic vascular resistance (SVR) may also be an important trigger for AHF. The extreme vasoconstriction combined with impaired cardiac power (CP), defined as the product of CO and systemic arterial pressure, induces a vicious cycle of afterload mismatch leading to a reduction of CO and elevated left ventricular end-diastolic pressure. This affects the pulmonary capillaries resulting in pulmonary edema. However, the basic reason for the increase in SVR is still unknown. Increased arterial stiffness could be one reason for the increased SVR in AHF, since the incidence of both AHF and arterial stiffness increases with age (Redfield et al. 2005).

Diastolic heart failure (DHF) is defined as heart failure with preserved systolic function accompanied with other signs of heart failure. DHF is diagnosed by echocardiography as the normal ejection fraction (EF >40-45%) combined with diastolic dysfunction. Preserved EF is detected in

24% to 55% of HF patients depending on the study population (Gorelik et al. 2009). DHF patients are usually older females. They are more often hypertensive, obese, and suffering from chronic obstructive pulmonary disease or atrial fibrillation, but not from coronary artery disease. They may well receive calcium antagonists, but are less likely to be treated with ACEIs or digoxin (Gorelik et al. 2009). A study on patients with hypertensive pulmonary edema has shown that EF during an episode of acute hypertensive pulmonary edema is similar to that measured after the treatment of the hypertension. (Gandhi et al. 2001). Thus, a normal EF after the BP has been controlled, indicates with high probability that the pulmonary congestion was due diastolic dysfunction. However, in patients with AHF, echocardiographic EF may correlate poorly with hemodynamic measures of the left ventricular contractility and outcome (Uriel et al. 2005). One possibility is that heart failure in patients with diastolic dysfunction might be paroxysmal rather than chronic (Banerjee et al. 2004). Thus, an acute worsening of diastolic function may be one of the main echocardiographic findings in patients with AHF, present predominantly in emergency units.

Chronic renal dysfunction is one of the strongest risk factors for mortality in patients with chronic HF. In this respect, renal function is considered as useful indicator for heart failure prognosis. Worsening of renal function has been diagnosed also in patients hospitalised for AHF (Metra et al. 2008b) and it may be a marker of the disease severity. The best single predictor for mortality in AHF patients admitted to hospital according to ADHERE registry in the United States was the presence of concomitant high admission levels of blood urea nitrogen followed by low admission systolic blood pressure and then by high levels of serum creatinine (Fonarow et al. 2005).

Activation of neurohormonal pathways and inflammatory cascades has been known to contribute to the chronic HF for a long time. The participation of neurohormones and inflammation in AHF is more complex and has been less extensively studied. However, it has been shown that the levels of both neurohormones and inflammatory markers increase during the acute phase of AHF (Milo et al. 2003). In addition, a high lymphocyte ratio has been observed to be related to high BP (Cotter et al. 2008). A low lymphocyte ratio, on the other hand, was related to higher troponin and more severe recurrent HF and death (Cotter et al. 2008). The parallel increase in systolic BP and lymphocytes supports the idea that there is an interaction between inflammatory activation and increased arterial stiffness, further evidence for a link between inflammatory and hemodynamic events in AHF (Vlachopoulos et al. 2005).

The Cardiac Resynchronization-Heart Failure (CARE-HF) trial (Cleland et al. 2001) showed that patients with chronic heart failure and cardiac dyssynchrony benefitted from cardiac resynchronization therapy (CRT). Not only were the cardiac function and symptoms improved and

the hospitalization and complications reduced, but also the mortality was reduced (Cleland et al. 2005). In addition, a recent study utilizing exercise Doppler echocardiography, showed that the response to CRT largely depended on the presence of contractile reserve collaterally with the extent of LV dyssynchrony and the severity of mitral regurgitation (Moonen et al. 2008). However, the importance of dyssynchrony in AHF is obscure. The only study on dyssynchrony in AHF, showed that the intraventricular conduction delay was associated with more adverse outcome in patients admitted with AHF (Brophy et al. 1994). Subsequently, a multicenter, international, observational study, Karolinska-Rennes (KaRen), has been designed to characterize electrical and mechanical dyssynchrony and to assess its prognostic impact in patients presenting acute heart failure with preserved ejection fraction (Donal et al. 2009).

2.3.2 Current pharmacological management of AHF

Diverse pharmacological agents are used in the treatment of AHF. Nevertheless, there is sparse data from clinical trials, mostly done with heterogenous patient populations without appropriate randomization. These trials have shown improvements in hemodynamics, but none of the agents used have reduced mortality. The multiple mechanism of action of the drugs in use are listed in table 1.

Opiates

Traditionally *morphine* has been used in the treatment of acute pulmonary oedema and it is still in practise for relieving the symptoms of AHF. ESC guidelines states that morphine should be considered in the early stage of the treatment of patients with severe AHF, particularly in patients with restlessness, anxiety, or chest pain (Dickstein et al. 2008). However, morphine can cause many adverse side-effects, including depressed respiration, which need to be monitored.

Diuretics

Administration of diuretics is an effective treatment for symptoms secondary to congestion and volume overload and is recommended for the sodium and water retention in AHF. The loop diuretics are the most effective and most frequently used diuretics in treating of AHF (Somberg and Molnar 2009) and are therefore recommended either alone or in combination with a vasodilator as initial therapy in patients with volume overload (Amin 2008). *Furosemide,* in turn, is the most often used loop diuretic. Before the increase in urinary sodium and water output, *i.v.* furosemide provides rapid symptomatic relief. This beneficial effect is due to the dilatation of peripheral capacitance vessels, consequently there is a decrease in left ventricular filling pressure and immediate relief of

the symptoms of pulmonary congestion (Jhund et al. 2000; Somberg and Molnar 2009). Plasma vasopressin concentrations are elevated in patients with worsening heart failure due to fluid overload and LV systolic dysfunction. This may lead to fluid retention and hemodynamic abnormalities. Vasopressin antagonists, which are able to treat congestion without causing electrolyte abnormalities or worsening renal function, can relieve the symptoms in AHF. *Tolvaptan* is one of the most extensively investigated oral vasopressin V_2-receptor antagonist that decreases body weight and increases urine volume without inducing renal dysfunction or hypokalemia (Gheorghiade et al. 2005c; Udelson et al. 2008).

Vasodilators

Intravenous vasodilators (nitrates, sodium nitroprusside and nesiritide) are used as a continuous infusion in the treatment of hospitalized patients during the early stages of AHF and ESC guidelines recommend their use for patients without symptomatic hypotension (SBP <90mmHg) or serious obstructive valvular disease (Dickstein et al. 2008). *Nitroglycerin* is most commonly used intravenous vasodilator in ADHF. It is predominantly a venous vasodilator compared to nesiritine, which dilates both venous and arterial blood vessels and has a combined diuretic and natriuretic effect. The most common adverse effect of nitroglycerin treatment is headache, which occurred in 20% of the patients with ADHF in the VMAC study (VMAC 2002). This was followed by asymptomatic hypotension (8%) and nausea (6%) and there were also some cases of symptomatic hypotension (Elkayam et al. 2008). *Nesiritide* is a recombinant 32 amino acid human B-type natriuretic peptide (hBNP) and therefore chemically and structurally identical to the endogenous hormone. It was introduced into clinical practice in the USA in 2001. Nesiritide increases cytosolic cGMP levels by binding to NP receptors. It has venous and arterial vasodilatory effects that reduce preload and afterload, and induces coronary vasodilatation. In addition, nesiritide increases cardiac output without having any direct inotropic effects. The use of nesiritide is normally restricted to patients hospitalized with acutely decompensated heart failure and who have dyspnea at rest (Colucci 2001; Maisel et al. 2008).

Inotropic agents

The infusion rate of the inotropic drugs needs to be modified according to symptoms. Also blood pressure should be continuously monitored. Furthermore, weaning patients from the drug infusion needs to be done carefully. *Dobutamine* is a positive inotropic agent, acting via stimulation of the β_1-ARs. It causes dose-dependent positive inotropic and chronotropic effects, starting rapidly after the beginning of the *i.v.* infusion. *Dopamine* also stimulates β_1-ARs, both directly and indirectly and

is used as an additional inotropic agent. PDEIs inhibit the breakdown of cyclic AMP causing inotropic and peripheral vasodilating effects. *Milrinone* and *enoximone* are two PDEIs widely used in clinical practice. They are administered by continuous infusion, evoking an increase in cardiac output and stroke volume, and a simultaneous decrease in pulmonary artery and wedge pressures as well as pulmonary vascular resistance (Dickstein et al. 2008). Compared to beta-agonists, PDEIs preserve hemodynamic effects fully during complete beta-blockade, since their site of action is beyond the beta-adrenergic receptor. Thus, PDEIs have been recommended for inotrope-requiring patients with decompensated heart failure who are undergoing long-term beta-blocking therapy, where a beta-agonist such as dobutamine would be less effective (Bristow et al. 2001). Cardiac glycosides produce a small increase in CO and a reduction of filling pressure. According to ESC guidelines, in AHF, cardiac glycosides like *digitalis*, may be useful in slowing the vetricular rate in rapid AF (Dickstein et al. 2008). However, a meta-analysis of digitalis studies has revealed that there is no evidence for any benefits in terms of mortality between treatment and control groups. Nonetheless, digitalis therapy lowered the rate of both hospitalization and clinical decline (Hood et al. 2004). *Levosimendan* is an inodilator compound, having both inotropic and vasodilatory effects. It belongs to the group of calcium sensitizers and its clinical use is discussed in detail in chapter 2.4.

Vasopressors

Norepinephrine, the recommended vasopressor in cardiogenic shock, is not a first-line agent and it should be used only if other inotropic agents and fluids do not restore the systolic blood pressure. In addition, sepsis patients with AHF may need vasopressor therapy. Norepinephrine can be used with other inotropic agents, but must be withdrawn as soon as possible. *Epinephrine* is not recommended as vasopressor or inotropic agent in cardiogenic shock. Its use should be limited to rescue therapy in cardiac arrest (Dickstein et al. 2008).

Renin angiotensin aldosterone system (RAAS) inhibitors and β-blockers

Beta-blockers, ACEIs, ARBs as well as aldosterone antagonists have been success stories in drug development for chronic HF. However, there is no consensus about the advantages of ACEI/ARB therapy in AHF. In general, the ESC guideline recommends that treatment with these agents should be initiated before discharge from hospital (Dickstein et al. 2008). In particular, in patients at high risk for development chronic HF, ACEIs and ARBs have an important role in the early management of AHF and acute MI (Dickstein et al. 2008). These agents attenuate remodelling and reduce morbidity and mortality. Patients on ACEI/ARB therapy admitted with worsening HF should have treatment continued with the highest tolerated dose whenever possible (Dickstein et al. 2008).

In patients admitted with acutely decompensated HF, the dose of β-blockers may need to be reduced temporarily. In general, treatment should not be stopped, unless the patient is clinically unstable with signs of low output. In patients admitted with de novo AHF, β-blockers should be considered when the patient has become stabilized on an ACEI or ARB and treatment with the β-blocker preferably initiated before hospital discharge (Dickstein et al. 2008). Data from the Italian survey on acute heart failure, established that in patients hospitalized for worsening HF, non-use or discontinuation of β-blockers was associated with a significant higher mortality (Orso et al. 2009). Furthermore, intravenous β-blockers are not contraindicated in patients with AHF who present with hypertension and/or atrial fibrillation with a rapid ventricular response (Khan et al. 2008). Cardioselective (β_1-selective) β-blockers are usually used in HF patients, because many of the side-effects of the non-selective blockers i.e. bronchospasm, peripheral vasoconstriction, alteration of glucose and lipid metabolism of these drugs are attributable to their blockade of β_2-receptors. Therefore, β-blockers that selectively block β_1-receptors are thought to produce fewer adverse side-effects than those drugs that non-selectively block both β_1- and β_2-receptors.

Table 1. Pharmacological management of Acute Heart Failure Syndromes

Category	Agents	Mechanism of action	Indication	Major adverse side-effecs
Morphine and its analogues	Morphine	μ_1 and μ_2 opioid receptor agonism	Restlessness, anxiety, chest pain	Depression of respiration, nausea
Loop diuretics	Furosemide Bumetadide Torasemide	Inhibition of the Na-K-Cl cotransporter-2 (NKCC2)	Volume overload, congestion	Hypovolemia, hypokalemia, hyponatremia, neurohumoral activation, hypotension following initiation of ACEI/ARB therapy
Vasopressin antagonists	Tolvaptan	Arginine vasopressin receptor 2 (V_2) antagonism	Volume overload, congestion	
	Conivaptan	V_1- and V_2- receptor antagonism		
Vasodilators	Nitroglycerin	Formation of NO (effect via soluble GC)	Pulmonary congestion, edema	Hypotension, headache
	Sodium nitroprusside			Hypotension, cyanide toxicity
Natriuretic peptides	Nesiritide	NPR1 (membrane-bound GC) and NPR3 receptor agonism	Pulmonary congestion, edema	Hypotension
Beta-agonists	Dobutamine	Adrenergic β_1-receptor agonism	Need of inotropic support, low CO, hypotension	Tachycardia, arrhythmias
	Dopamine	Dopamine D_1- and adrenergic β_1- and α_1-receptor agonism		Tachycardia, arrhythmias, vasoconstriction
Phosphodiesterase inhibitors	Milrinone Enoximone	Inhibition of the PDE3 and PDE4	Need for inotropic support, low CO	Arrhythmias, increase in mortality
Vasopressors	Noradrenaline	Adrenergic β_1-, α_1- and α_2-receptor agonism	Cardiogenic shock	Increase in systemic vascular resistance
	Adrenaline	Adrenergic β_1-, β_2-, α_1- and α_2-receptor agonism	Rescue therapy in cardiac arrest	
Cardiac glycosides	Digoxin	Inhibition of the Na^+/K^+ -ATPase	Rapid AF	Arrhythmias, paroxysmal atrial tachycardia with A-V block (digoxin toxicity)
Calcium sensitizers	Levosimendan	Improvement of Ca^{2+} binding to troponin C, opening of the K_{ATP} channels and inhibition of PDE3	Need for inotropic support, low CO, signs of organ hypoperfusion	Hypotension
Chronical treatment			*Objective*	
Beta blockers	Nebivolol Bisoprolol Metoprolol	Adrenergic β_1-receptor antagonism	Attenuation of remodelling and reduction of morbidity and mortality	Bradycardia, hypotension, worsening of asthma
	Carvedilol	Adrenergic β_1- and α_1-receptor antagonism		Orthostatic hypotension, edema
Angiotensin converting enzyme inhibitors	Captopril Enalapril Lisinopril Ramipril Trandolapril	Inhibition of the conversion of Ang I to Ang II	Attenuation of remodelling and reduction of morbidity and mortality	Cough, hyperkalemia, hypotension, dizziness
Angiotensin receptor antagonists	Candesartan Valsartan	Ang II receptor type 1 (AT_1) antagonism	Attenuation of remodelling and reduction of morbidity and mortality	Headache, dizziness
Aldosterone antagonists	Eplerenone Spironolactone	Mineralocorticoid receptor antagonism	Attenuation of remodelling and reduction of morbidity and mortality	Hyperkalemia, hypotension, dizziness

2.3.3 Unmet needs for the treatment of AHF

Undoubtedly, therapy for AHF should improve symptoms and hemodynamics of acutely hospitalized patients without adverse effects and the risk of mortality. However, patients with AHF have usually been diagnosed with other cardiac and also noncardiac contributing disorders, such as coronary artery disease, chronic ischemic heart disease, hypertension, atrial fibrillation, type 2 diabetes mellitus. In severe types of the syndrome, there may be cardiorenal or multiorgan failure which needs to be taken into account in the choice of appropriate medication. Ideal medical treatment should prevent myocardial damage, modulate neurohumoral and inflammatory activation, and preserve or even improve renal function (Figure 2.). Moreover, costs of index hospitalization increase depending on the severity of the different classes AHF (Harjola et al. 2009). This has created a growing need for effective treatment for the most severe classes of the syndrome.

There is a vigorous debate of the role of inotropic therapy in the management of AHF and counterarguments have been postulated (Petersen and Felker 2008). Nevertheless, the use of conventional inotropic agents has been shown to have favourable haemodynamic effects in patients with decreased cardiac contractility. According to Acute Decompensated Heart Failure National (ADHERE) Registry, 9.6% of patients hospitalized for AHF in the United States receive intravenous inotrope therapy, either PDEIs or beta1-adrenergic agonists (β_1-AR agonists) (Abraham et al. 2005). These patients tended to have a clinical profile associated with more severe disease, including lower blood pressure, lower ejection fraction, and higher blood urea nitrogen. The use of PDEIs and β_1-AR agonists in heart failure has consistently been associated with increased myocardial oxygen demand, more cardiac arrhythmias, and worsened mortality in many clinical trials. This unsatisfactory outcome is believed to be related to the fact that these agents increase myocardial concentrations of cAMP, leading to an increase in the level of intracellular calcium which in turn can trigger myocardial cell death and provoke lethal arrhythmias. Furthermore, according to FINN-AKVA, the mortality increased in proportion to the number of inotropes used (Rossinen et al. 2008). The administration of inotropes was related to low BP, low ejection fraction, elevated C-reactive protein and cardiac markers and was a marker of increased mortality in patients with AHF. However, the use of a single inotrope during hospitalization appeared to be rather safe (Rossinen et al. 2008).

For these reasons, there is a clear need for improvements in therapy. Some new drugs have been recently introduced for the treatment of AHF. One of them is istaroxime, a new agent with inotropic and especially powerful lusitropic properties, that is improvement in relaxation. It inhibits the sodium–potassium ATPase (Na^+-K^+ pump) and stimulates the sarcoplasmic reticulum calcium

ATPase isoform 2 (SERCA-2) and is consequently believed to improve contractility as well as causing diastolic relaxation (Khan et al. 2009). Data from human phase II study (HORIZON-HF) indicated that istaroxime decreases PCWP and possibly improves diastolic function without evoking any significant change in heart rate (HR), blood pressure, ischemic or arrhythmic events (Blair et al. 2008; Gheorghiade et al. 2008). However, the mechanism of action of istaroxime means that it can contribute to an increase in cytosolic calcium $[Ca^{2+}]_i$ in a manner similar to the cardiac glycosides, and to this extent, harmful effects attributable to increased $[Ca^{2+}]_i$ cannot be excluded. In addition, the enhanced calcium uptake in the SR causes a greater amount of calcium release in every diastole-systole cycle. Cardiac-specific myosin ATPase activators are another novel class of agents designed to improve myocardial contractility. They accelerate the productive phosphate-release step of the crossbridge cycle (Bragadeesh et al. 2007). CK-1827452 is a new agent that directly activates myosin, prolonging the duration of time that myosin remains in a force-generating reaction with actin and enhancing the extent of myocyte shortening, with no effect on the Ca^{2+} transient (Solaro 2009).

Calcium sensitization is a mechanism that is thought to resolve many of the disadvantages of the therapies that increase the cytosolic calcium concentration $[Ca^{2+}]_i$. Calcium sensitizers do not increase the energy required for Ca^{2+} handling and are able to reverse contractile dysfunction under pathophysiological conditions. However, calcium sensitizers carry a potential risk of the diastolic dysfunction due to the impaired relaxation, which will reduce the rate of ventricular filling (Endoh 2008). A set of compounds has been under development as calcium sensitizers during the past two decades. Most of them have a combination of mechanism of action of calcium sensitization and PDE3 inhibition. Some of them have only weak PDE3 inhibitory effect like EMD 57033, MCI-154, Org 30029 and SCH00013, but also pure calcium sensitizers, like CGP 48506, have been developed (Endoh 2002; Kass and Solaro 2006). However, none of them have been a success story in the clinic. Pimobendan is a calcium sensitizer with potent PDE3 inhibitory properties in the same concentration range (Endoh 2002). It is approved in HF patients in Japan only (Kass and Solaro 2006). However, pimobendan has been used in the clinical management of CHF secondary to both dilated cardiomyopathy (DCM) and chronic degenerative valvular disease in dogs (Gordon et al. 2006).

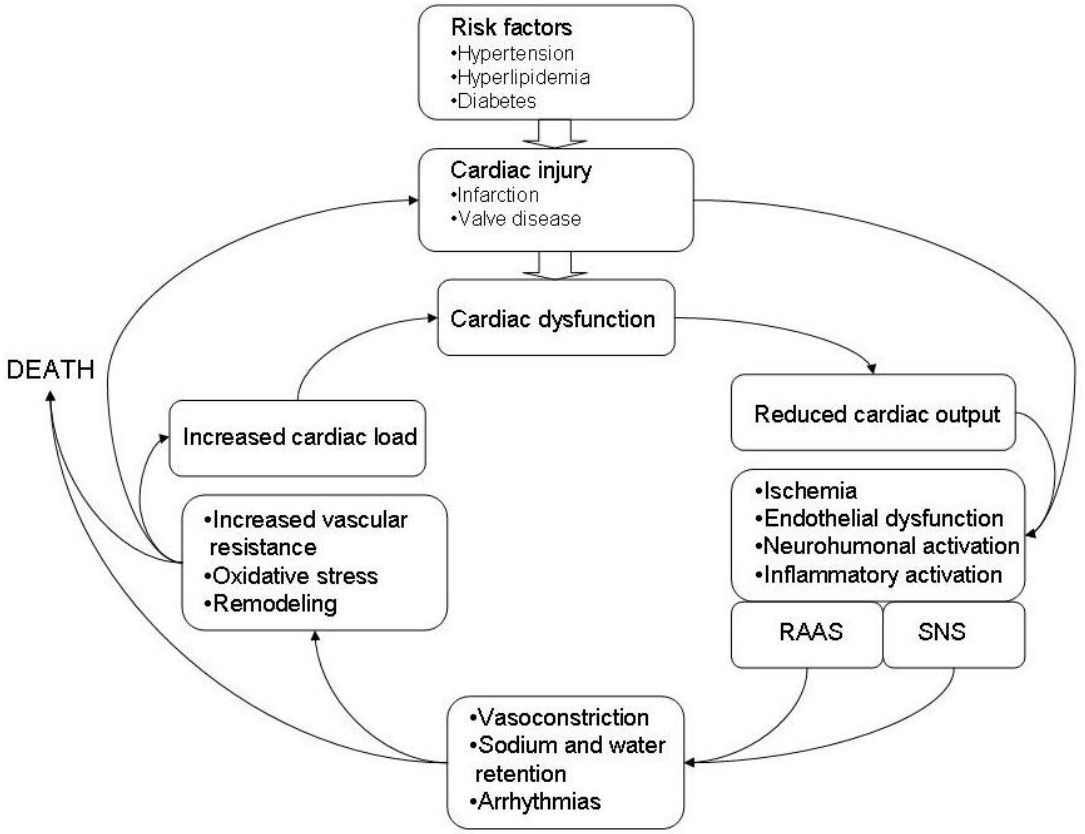

Figure 2. The vicious cycle of heart failure. Renin angiotensin aldosterone system (RAAS); Sympathetic nervous system (SNS).

2.4 Levosimendan

2.4.1 Physicochemical properties

Levosimendan is commercially available as a powder, to be dissolved in a solution of glucose 5% in water and administered as an intravenous infusion. The chemical name of levosimendan is (-) (R)-[[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3- pyridazinyl) phenyl] hydrazono] propanedinitrile (WHO Drug Information Vol. 7, No. 3, 1993). Its molecular weight is 280.291 and the molecular formula $C_{14}H_{12}N_6O$ (Figure 4). Levosimendan, is a weak acid and a moderately lipophilic compound with a pKa value of 6.2.

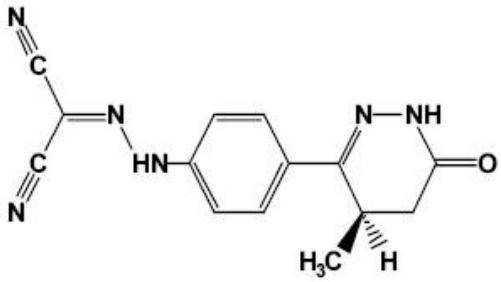

Figure 4. **The molecular formula of levosimendan.**

2.4.2 Pharmacokinetics

The molecule has a short elimination half-life of approximately 1 h, and is 95– 98% bound to plasma proteins. The drug is mostly metabolized through glutathione conjugation at one of its nitrile groups followed by amino acid cleavage and cyclization or acetylation (Lehtonen et al. 2004; Koskinen et al. 2008). Even though it is administered intravenously, levosimendan is excreted into the small intestine and reduced by intestinal bacteria to an amino phenolpyridazinone metabolite (OR-1855), which is further metabolised by acetylation to N-acetylated conjugate (OR-1896). The circulating metabolites OR-1855 and OR-1896 are formed slowly, and their maximum concentrations are seen on average 2 days after cessation of a 24 h infusion. The haemodynamic effects after levosimendan infusion appear to be similar between fast and slow acetylators. OR-1896, an active metabolite, is approximately 40% bound to plasma proteins. It has a longer half life (75 to 80 h) than levosimendan and it is responsible for the prolonged hemodynamic effects, which can be detected for 7–9 days after a 24 h continuous infusion of levosimendan (Antila et al. 2007; Puttonen et al. 2007). The hemodynamic effects after levosimendan infusion are thus, combination of the acute effects by levosimendan itself and the long lasting effects by OR-1896.

2.4.3 Mechanism of action

2.4.3.1 **Positive inotropic effect**

Since it is a calcium sensitizer, levosimendan does not increase $[Ca^{2+}]_i$. It rather increases myofilament calcium sensitivity by binding to cTnC in a calcium-dependent manner. Levosimendan binds to the calcium-saturated N-terminal domain of troponin C and stabilizes the troponin molecule with subsequent prolongation of its effect on the contractile proteins (Pollesello et al. 1994). The heart contracts when calcium binds to cTnC and it relaxes when calcium dissociates from cTnC. Therefore, the calcium saturated form of cTnC is an ideal target for calcium sensitisers (Haikala and Linden 1995). Furthermore, a desirable feature of a calcium sensitizer is that it should not impair the relaxation of cardiac muscle, i.e. it must detach from cTnC when Ca^{2+} dissociates. In fact,

levosimendan behaves in this way (Figure 5.) (Pollesello et al. 1994; Levijoki et al. 2000; Sorsa et al. 2004; Antoniades et al. 2007). In addition, levosimendan has been shown to actually enhance diastolic function in pacing induced heart failure dogs during exercise (Tachibana et al. 2005). It is also essential that an efficacious calcium sensitizer must not prevent or inhibit any of the protein–protein interactions required for muscle contraction and relaxation, and furthermore, it should not exert any detrimental actions on troponin's functions, levosimendan does not (Sorsa et al. 2004).

2.4.3.2 **Stereoselective interaction with cardiac troponin C**

Levosimendan has a chiral carbon atom in its pyridazine ring (Figure 4.). Therefore, it has an optical stereoisomer, dextrosimendan . Levosimendan and dextrosimendan are the (R)-(-) and (S)-(+) enantiomers of simendan. The interaction of simendan stereoisomers on cTnC has been shown to be different in the absence of cardiac troponin I (cTnI) (Sorsa et al. 2004). Both stereoisomers interacted with both, C- and N-domains of the isolated cTnC. However, levosimendan has been shown to have an order of magnitude higher affinity for the N-domain than dextrosimendan (Sorsa et al. 2004). The binding of calcium ion to the N-domain of cTnC appropriately initiates the contraction of the cardiac muscle, and thus, explains the stereospecific mode of action of the simendans in cardiac preparations.

The positive inotropic effect of levosimendan has been demonstrated in many *in vitro* experiments. Levosimendan has been shown to increase cardiac contractility in isolated cardiomyocytes, papillary muscles, atrial muscle strips, ventricular muscle strips, and in isolated hearts (Haikala et al. 1995; Lancaster and Cook 1997; Hasenfuss et al. 1998; Janssen et al. 2000). In *in-vivo* experimental models, levosimendan has shown positive inotropic effects in normal rats, dogs, cats and rabbits. In addition, levosimendan has been proven to be even more potent in animal model of heart failure (Levijoki et al. 2001; Louhelainen et al. 2007; Masutani et al. 2008).

One plasma metabolite of levosimendan which possesses biological activity, OR-1896, is also a potent positive inotropic compound. In anaesthetised dogs, the overall cardiovascular pharmacodynamic profile of OR-1896 was shown to be rather similar to that of levosimendan (Banfor et al. 2008). Levosimendan and OR-1896 were intravenously infused at 30 min intervals in cumulatively increasing doses. They were hemodynamically active in anaesthetised dogs and elicited equivalent cardiovascular, whereas another metabolite, OR-1855 was inactive (Banfor et al. 2008). In anesthetized rats, levosimendan and OR-1896 have also been shown to have similar hemodynamic effects (Banfor et al. 2008; Segreti et al. 2008). The relatively long-lasting duration

of levosimendan's positive inotropic effect and the possible accumulation of OR-1896 after prolonged administration of levosimendan may modify the effects of levosimendan.

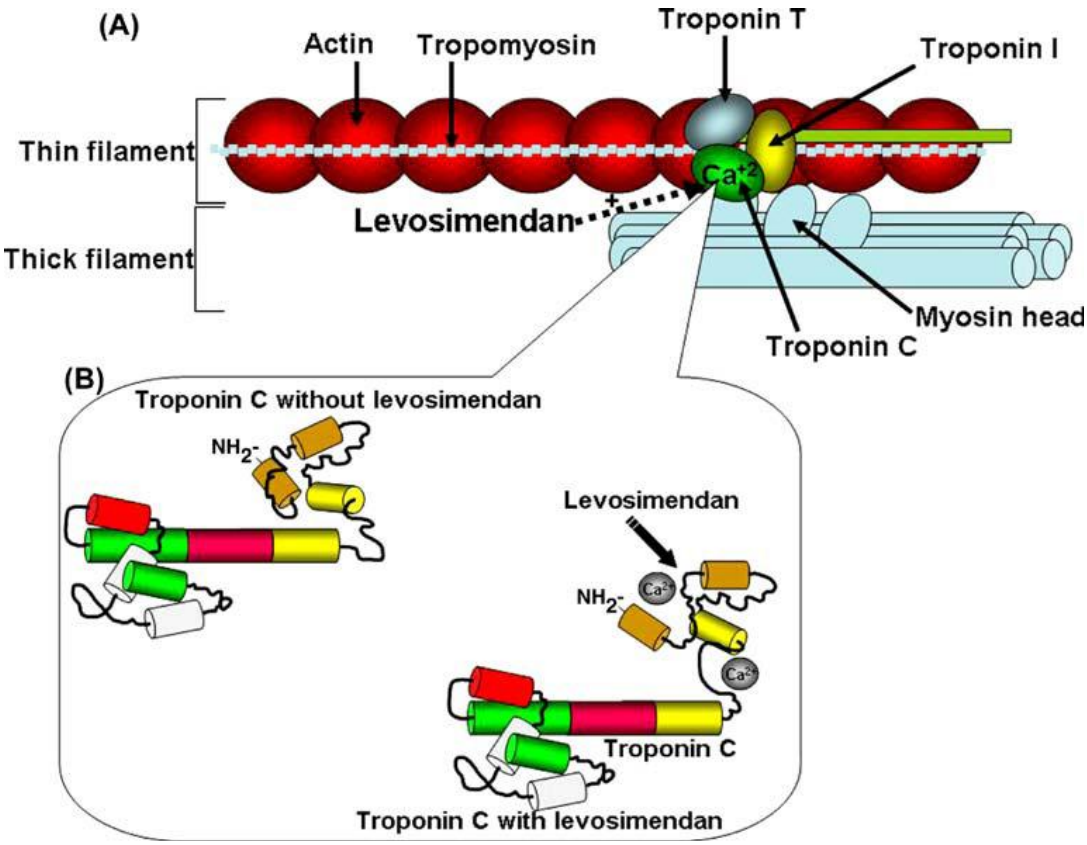

Figure 5. Mechanism of levosimendan as an inotropic agent. (A) Levosimendan binds to troponin C during systole, increasing the sensitivity of myofilaments to Ca2+ levels. This phenomenon increases the contractility of myocardium during systole, but it does not affect diastolic function. **(B)** The presence of levosimendan leads to an opening of the active sites of troponin C, increasing in this way its sensitivity to Ca^{2+}. Reprinted from *Pharmacol Ther*, **114** (2), Antoniades, C., D. Tousoulis, N. Koumallos, K. Marinou and C. Stefanadis, "Levosimendan: beyond its simple inotropic effect in heart failure", Pages 184-97, Copyright (2007), with permission from Elsevier.

2.4.3.3 **Vasodilatory effect**

Levosimendan has a pronounced vasodilatory effect. The opening of the K_{ATP} channels is believed to be the main mechanism of the vasodilatory effect of levosimendan. This has been shown in arterial and venous preparations as well as in coronary arteries. In rat mesenteric arterial myocytes, levosimendan hyperpolarized the cells, probably through the activation of K_{ATP} channels, i.e. an effect which is known to have a vasodilating action (Yokoshiki et al. 1997). Furthermore, in the isolated rat small mesenteric artery preparation, levosimendan had a relaxant response in KCl precontracted arteries. Glibenclamide, a K_{ATP} channel blocker, inhibited the response, supporting

the theory that the opening of K_{ATP} channels was the important vasodilatory mechanism. The response did not differ significantly between endothelium-intact and endothelium-denuded preparations (Ozdem et al. 2006). In isolated human portal vein, levosimendan was found to be about 16-fold more potent as a relaxing agent than cromakalim in noradrenaline-precontracted portal venous preparations. Glibenclamide totally inhibited the cromakalim-induced relaxation of the portal vein. However, the effect of levosimendan was only partially prevented (Pataricza et al. 2000), indicating that levosimendan might also have some other vasodilatory effects in addition to opening of the K_{ATP} channels.

Subsequently, in isolated porcine epicardial coronary arteries, it has been shown that the specific BK_{Ca} channel blocker, iberiotoxin (IBTX), could suppress the maximum effect of levosimendan, and the KV channel blocker, 4-aminopyridine (4-AP) significantly shifted the concentration-response curve to the right. This indicated that the vasorelaxing mechanism of levosimendan involves the activation of voltage-sensitive and, at large concentrations, calcium-activated potassium channels (Pataricza et al. 2003). However, IBTX and 4-AP were not able to suppress the vasodilatory effect of levosimendan in isolated rat small mesenteric arteries (Ozdem et al. 2006). In addition, studies with porcine coronary arteries suggest that levosimendan may also interact with smooth muscle EF-hand proteins, such as, calmodulin, the regulatory myosin light chains, or S100 proteins and thus, relax coronary smooth muscle through calcium desensitization (Bowman et al. 1999). Furthermore, the role of PDE3 inhibition cannot be excluded, since levosimendan has been shown to potentiate the relaxant effect of a cAMP-stimulating drug, isoprenaline in isolated porcine arteries (Gruhn et al. 1998).

The vasodilatory effect of OR-1896 has been shown to be comparable to that of levosimendan in isolated, pressurized rat coronary and skeletal muscle arterioles. The vasodilatation was proposed to be mediated primarily by activation of BK_{Ca} and K_{ATP} channels, respectively (Erdei et al. 2006). In anaesthetised rats and dogs, the vasodilatory effect of OR-1896 is rather similar to that of levosimendan (Banfor et al. 2008; Segreti et al. 2008).

Thus, it can be concluded that levosimendan predominantly stimulates K_{ATP} channels in small resistance vessels. In large conductance vessels, the vasodilatation may be mediated mainly through opening of KV as well as BK_{Ca} channels. Moreover, the duration of the vasodilatory effect is proportionately long-lasting, because of the formation of an active metabolite, OR-1896.

2.4.3.4 **Anti-ischemic effect**

Endogenous protective mechanisms in heart cells, so called preconditioning, have been previously claimed to be mediated via K_{ATP} channels. Preconditioning is known to exist in many mammalian species and tissue types. It is described as a cardiac phenomenon in which a short period of ischemia will protect the heart from a subsequent, much longer, ischemic episode (Grover and Garlid 2000). The opening of the sarcolemmal K_{ATP} (sarcK_{ATP}) channels in vascular smooth muscle cell, resulting in membrane hyperpolarization and vasodilatation, was previously believed to be behind this protective mechanism. Subsequently, mitochondria K_{ATP} (mitoK_{ATP}) channels have been described (Liu et al. 1999; O'Rourke 2004) and the mitochondrial hypothesis has become more generally accepted. Furthermore, it has been shown that K_{ATP} channel openers can induce vasodilatation by activating two different signaling mechanisms, one pathway that is mitochondrial and another pathway that involves sarcK_{ATP} channel activation (Adebiyi et al. 2008). That study indicated that the K_{ATP} channel openers, pinacidil and diazoxide, could induce vasodilatation by two distinct mechanisms. The action of pinacidil required the sulfonylurea receptor (SUR 2B) whereas diazoxide targeted the mitochondrial electron transport chain (ETC).

K_{ATP} openers have been shown to mimic preconditioning and the results from several studies with a variety of K^+ channel openers have demonstrated their effects on mitochondrial function (O'Rourke 2004). This is evidence for a link between the mitoK_{ATP} channels and protection against ischemic injury in intact hearts (Garlid et al. 1997) and isolated myocytes (Liu et al. 1998). The mechanisms believed to be behind this protective effect are prevention of mitochondrial calcium overload, preservation of high energy phosphates and regulation of mitochondrial volume (O'Rourke 2004). Mitochondrial Ca^{2+}-activated K^+ (mitoK_{Ca}) channels also exist in cardiac myocytes, and are known to play a key role in cardioprotection. Potassium influx through mitoK_{ATP} or mitoK_{Ca} channels occurs independently of each other and induces cardioprotection in a similar manner (Nishida et al. 2009). Activation of mitoK_{ATP} channel is enhanced by protein kinase C (PKC), whereas mitoK_{Ca} channel is activated by protein kinase A (PKA) (Nishida et al. 2009).

Levosimendan has been demonstrated to open mitoK_{ATP} channels in liver, but also in cardiac preparations, indicating that it may have a cardioprotective effect. In the rat liver mitochondria, levosimendan at a concentration of 0.7-2.6 μM could open the mitoK_{ATP} channels (Kopustinskiene et al. 2001). The effect was abolished by the selective mitochondrial K_{ATP} channel blocker 5-hydroxydecanoate. In addition, levosimendan up to 2.2 μM had no effect on the respiration rate of rat liver mitochondria. In the rat cardiac cells, levosimendan activated potassium flux to the mitochondrial matrix (median effective concentration, EC_{50}, 0.83 +/- 0.24 μM) (Kopustinskiene et

al. 2004). The anti-ischemic effect has been seen also in a dog-infarction model, where levosimendan (24 μg/kg bolus followed by an infusion of 0.4 μg/kg/min), when given before ischemia, reduced the infarct size by 50%. This reduction of infarct size was abolished by treatment with the K_{ATP} channel blocker glibenclamide, indicating that levosimendan had an anti-ischemic effect, which was mediated via opening of the K_{ATP} channels (Kersten et al. 2000).

In addition, oral simendan (the racemate of levosimendan) has been shown to improve survival in rats with healed myocardial infarction during the follow-up period of 312 days. The incidences of mortality in control, simendan, enalapril and milrinone groups were 81%, 53%, 56% and 63%, respectively. The calculated mean daily dose of simendan was 2-2.5 μg/kg from the 10th week onwards. The mean plasma concentration of simendan was 97 ng/ml at the end of the follow-up period. The hemodynamic responses to simendan, measured prior to sacrifice, had been well preserved i.e. no tolerance developed to the hemodynamic effects of simendan (Levijoki et al. 2001).

2.4.3.5 **Antiaggregatory, anti-inflammatory and antiapoptotic effects**

Levosimendan has been shown to inhibit concentration-dependently the platelet aggregation induced by ADP (5 and 10 μM), and collagen (2 and 5 μg/mL) in human platelet-rich plasma. Levosimendan inhibited also the secondary wave of platelet aggregation induced by ADP, indicating that it may inhibit platelet signal transduction, e.g. thromboxane A2 formation (Kaptan et al. 2008). In vitro levosimendan was able to reverse interleukin (IL)-5-related survival of human eosinophils i.e. it increased the number of apoptotic eosinophils in the presence of 10 pM IL-5 with an EC50 value of 6.5 ± 0.7 μM. The increase in the number of apoptotic cells was confirmed by the percentage of annexin V-positive cells, which were in the absence or presence of levosimendan (10 μM) 7 ± 1 and 86 ± 4%, respectively (Kankaanranta et al. 2007). Moreover, levosimendan and dextrosimendan decreased NO production in a dose-dependent manner in macrophages and in fibroblasts exposed to inflammatory stimuli. They reduced IL-6 production slightly but they had no effect on TNF-α synthesis (Sareila et al. 2008).

2.4.4 Effects in combined heart and renal failure model

When administered via the drinking water, levosimendan dose-dependently prevented cardiomyocyte apoptosis and normalized salt-induced increased expression of natriuretic peptide, and decreased urinary noradrenaline excretion in salt-sensitive hypertensive Dahl/Rapp rats (Louhelainen et al. 2007). Preventative treatment with levosimendan for seven weeks in rats fed a high-salt diet, improved also survival, increased cardiac function, and ameliorated cardiac

hypertrophy. In addition, levosimendan also corrected salt-induced declines in myocardial SERCA2a protein expression and myocardial SERCA2a/NCX-ratio. In conclusion, the modulation of pro-inflammatory and pro-apoptotic pathways by levosimendan may be part of its beneficial effects in the progression of decompensated heart failure.

2.4.5 Effects in stroke models

In addition to the above mentioned studies, there is strong experimental evidence that levosimendan may have preventive effects on stroke. Recent unpublished studies in experimental models of primary and secondary prevention of stroke, confirmed by using MRI and/or histology, have demonstrated this effect. Oral treatment with levosimendan prevented strokes and mortality in Dahl salt-sensitive and stroke-prone spontaneously hypertensive rats. The effects were seen at low daily doses of levosimendan alone and in combination with the angiotensin II receptor blocker, valsartan. An international patent for a combination treatment with levosimendan and ACE inhibitor or AngII receptor blocker has been accepted and published (Sallinen et al. 2009).

2.5 Clinical use of levosimendan

Levosimendan is the only calcium sensitizer, which is widely used in the clinical management in humans. Today it has an established place in the treatment of AHF. Levosimendan increases myofilament calcium sensitivity and has a highly selective PDE3 inhibitory action. It has been shown to enhance cardiac contraction without increasing oxygen consumption, and thus, having an energetically advantageous effect on the contraction (Ukkonen et al. 1997; Ukkonen et al. 2000). Levosimendan induces vasodilatation by opening the sarcolemmal K_{ATP} channels in the resistance arteries. There is some evidence that it may be also a venodilator and therefore, reduce the central venous pressure and improve the pulmonary congestion, and this may be the mechanism behind the observed improvement in renal function (Yilmaz et al. 2007). Moreover, levosimendan-evoked mitochondrial K_{ATP} channel opening may have cardioprotective effects due to the ATP synthesis during ischemia. In summary, the positive effects on the energy balance could be of major significance in the use of levosimendan in the treatment of AHF (Nieminen et al. 2009).

2.5.1 Use in acute heart failure

To date, levosimendan has been approved in 47 countries worldwide for short-term treatment of AHF. According to ESC guidelines, levosimendan infusion is recommended in acutely decompensated heart failure and may be effective in patients with decompensated chronic HF, too (Dickstein et al. 2008). The effects of levosimendan on the symptoms of AHF have been evaluated

in several clinical trials. The effects on overall hemodynamics in different trials are presented in table 3. Some of these trials have suggested that levosimendan might improve prognosis and mortality in patients with decompensated heart failure. There are three trials, LIDO, RUSSLAN and CASINO, which were carried out in patients with high filling pressures, where intravenous levosimendan was compared with placebo or dobutamine. The mortality rate of levosimendan was statistically significantly lower in the levosimendan group than in the dobutamine group (LIDO) or placebo group (RUSSLAN) or in both (CASINO) (Follath et al. 2002; 2002; Cleland et al. 2004; Lehtonen 2004; Lehtonen and Poder 2007). However, two larger trials (SURVIVE and REVIVE II) in patients who were hospitalized for worsening of heart failure, but in whom it was not essential to measure the filling pressure, indicated that though levosimendan did improve the symptoms of HF, it did not improve survival (Cleland et al. 2006; Lehtonen and Poder 2007; Mebazaa et al. 2007) (Table 2.). Nevertheless, levosimendan proved beneficial compared to dobutamine in specific subgroups of patients in the SURVIVE trial. Levosimendan treatment lowered all-cause mortality during the first weeks following treatment, in patients admitted with acute decompensated heart failure and with a known history of CHF and/or currently treated with oral β-blockers (Mebazaa et al. 2009).

Table 2. Major comparative studies (over 100 patients including per study) with levosimendan.

Study	Design	Number of patients (total /LS)	Type of patients	Levosimendan dose	Primary end point	Secondary end points	Prespecified follow-up time	Effects on primary end point	Main outcome on mortality
European dose finding study [1]	Randomized, placebo controlled, parallel group (fixed-dose)	151/95	NYHA III–IV CHF [a] with ischemic etiology (EF <40%)	Bolus 3–36 µg/kg + infusion of 0.05, 0.1, 0.2, 0.4 or 0.6 µg/kg/min for 24 hrs	Hemodynamic improvement (response criteria based on SV and PCWP)	Individual hemodynamic parameters	7 days	Levosimendan was well tolerated and leads to favorable hemodynamic effects	No difference compared to placebo and dobutamine
US dose duration study [2]	Randomized, placebo controlled, parallel group (forced uptitration + withdrawal)	146/98	Decompensated CHF (PCWP ≥15 mmHg, CI ≤2.5 L/min/kg)	Repeated bolus of 6 µg/kg and infusion of 0.1– 0.4 µg/kg/min for 24 or 48 hrs	Hemodynamic improvement (response criteria based on SV and PCWP)	Individual hemodynamic parameters, symptoms of heart failure, 14-day outcome	14 days	Levosimendan was well tolerated and leads to favorable hemodynamic effects	No difference compared to placebo
LIDO [3]	Randomized, double blind (double dummy) comparison between levosimendan and dobutamine	203/103	Severe low-output heart failure requiring inotropic support	Levosimendan bolus of 24 µg/kg and infusion of 0.1–0.2 µg/kg/min versus dobutamine 6-12 µg/kg/min for 24 hrs	Hemodynamic improvement (response criteria based on CO and PCWP)	Symptoms of heart failure, 31-day outcome	31 days [a]	Levosimendan improved haemodynamic performance more effectively than dobutamine	Levosimendan was superior compared to dobutamine
RUSSLAN [4]	Randomized, placebo controlled, parallel group	504/402	Heart failure after acute myocardial infarction and clinical need for inotropic support	Levosimendan bolus of 6, 12 or 24 µg/kg and infusion of 0.1, 0.2 or 0.4 µg/kg/min for 6 hrs	Safety (clinically significant ischemia or hypotension)	Symptoms of heart failure, 14-day mortality	14 days [a]	Levosimendan did not induce hypotension or ischaemia.	Levosimendan was superior compared to placebo
CASINO [5]	Randomized, comparison between levosimendan, dobutamine and placebo	299/100	Patients hospitalized with NYHA class IV HF, LVEF ≤35%	Levosimendan bolus of 16 µg/kg and infusion of 0.2 µg/kg/min versus placebo / dobutamine 10 µg/kg/min for 24 hrs	Mortality at 1 month, 6 months and 1 year	Death or rehospitalization due to worsening heart failure	1 year (the study was prematurely halted by the data safety monitoring board, citing the superior results of levosimendan)		Levosimendan was superior compared to placebo and dobutamine

Table 2. (continued)

Study	Design	Number of patients (total /LS)	Type of patients	Levosimendan dose	Primary end point	Secondary end points	Prespecified follow-up time	Effects on primary end point	Main outcome on mortality
REVIVE-2 [6]	Randomized, placebo controlled, parallel group (fixed dose)	600/299	Primary or secondary heart failure, LVEF <35%, breathlessness at rest despite diuretic and vasodilator therapy	Levosimendan bolus of 6–12 µg/kg infusion of 0.1–0.2 µg/kg/min versus placebo for 24 hrs	Death or worsening heart failure over 5 days with a composite end point consisting of the patient's selfassessment of symptoms together with a physician's assessment of the occurrence of clinical deterioration	Mortality at 90 days, duration of hospital stay, change in BNP at 24 hours, patient global assessment and dyspnea at 6 hours	90 days	Levosimendan was superior on the composite primary outcome compared to placebo	No difference compared to placebo
SURVIVE [7]	Randomized, double blind (double dummy) comparison between levosimendan and dobutamine	1327/664	Heart failure with features of low cardiac output (oliguria), LVEF ≤30%), breathlessness at rest despite diuretic and vasodilator therapy	Levosimendan bolus of 12 µg/kg and infusion of 0.1–0.2 µg/kg/min versus dobutamine 5 µg/kg/min for 24 hrs	180-day all cause mortality	31-day all-cause mortality, 180-day cardiovascular mortality, days alive and out of hospital for 180 days, change in BNP at 24 hours, symptoms of heart failure	180 days	Levosimendan did not significantly reduce all-cause mortality at 180 days	No overall difference compared to dobutamine, however, levosimendan was superior in patients on β-blockers compared to dobutamine

[1] (Nieminen et al. 2000), [2] (Slawsky et al. 2000; Kivikko et al. 2003), [3] (Follath et al. 2002),[4] (Moiseyev et al. 2002), [5] (Cleland et al. 2004; Lehtonen 2004), [6] (Cleland et al. 2006), [7] (Mebazaa et al. 2007); [a] Six-month follow-up data has been collected for both LIDO and RUSSLAN as a part of the drug approval process.

BNP = b-type natriuretic peptide; CHF = congestive heart failure; CI = cardiac index; CO = cardiac output; EF = ejection fraction; HF = heart failure; LVEF = left ventricular ejection fraction; NYHA = New York Heart Association; PCWP = pulmonary capillary wedge pressure; SV = stroke volume.

Modified from (Lehtonen and Poder 2007) *Ann Med* **39**(1): 2-17

2.5.2 Additional clinical use

In addition to its use in ADHF, as recommended according to ESC guidelines, levosimendan has found increasing off-label use also for other cardiovascular symptoms in acute cardiovascular events and in intensive care units. Ischemic heart disease and septic shock as well as perioperative cardiac support are conditions where the use of levosimendan has been shown to have beneficial clinical effects.

2.5.2.1 **Ischemic heart disease and cardiogenic shock**

Cardiogenic shock (CS) is the leading cause of death in patients hospitalized for acute myocardial infarction (AMI) (Hochman et al. 2000). The syndrome has been defined as the inability of the heart, with its impairment of pumping function, to deliver sufficient blood flow to the tissues to meet resting metabolic demands (Califf and Bengtson 1994). The symptoms of CS are diagnosed as the combination of low mean arterial blood pressure, low cardiac index (CI), elevated PCWP, and an increase in systemic vascular resistance index (Hochman et al. 2000). Moreover, there is believed to be a systemic inflammatory response owing to the release of inflammatory cytokines and the expression of inducible nitric oxide synthase and extraneous vasodilatation may also play an important role (Kohsaka et al. 2005). Furthermore, patients with large MIs often have signs of inflammation like elevation of body temperature, white blood cell count, complement, interleukins, C-reactive protein, and other inflammatory markers (Hochman 2003) (Figure 3.). Early revascularization, either by percutaneous coronary intervention (PCI) or coronary artery bypass graft (CABG) surgery, has been shown to increase the long-term survival (Hochman et al. 2006).

Levosimendan infusion was shown to improve hemodynamics in critically ill patients with CS requiring catecholamine therapy (Delle Karth et al. 2003). In patients with AMI complicated by severe and refractory CS, therapy with levosimendan added to current therapy resulted in a better outcome and may have contributed to improved survival compared with phosphodiesterase inhibitor, enoximone (Fuhrmann et al. 2008). Particular, in the levosimendan-treated patients, no deaths because of multiorgan failure (MOF) were observed compared to mortality of 25% in the enoximone group. It is worth noting that in the multicenter trial SURVIVE, where levosimendan failed to improve mortality in acute heart failure compared with dobutamine, all patients with CS were excluded (Mebazaa et al. 2007). In addition, clinical data demonstrate that levosimendan does not increase markers of oxidative and nitrosative stress, in contrast to placebo treatment, in patients with advanced chronic heart failure (Parissis et al. 2008). Thus, the severity of heart failure may be

quite different in different patient populations and the occurrence of MOF and the systemic inflammatory response can be variable. Furthermore, in patients having ADHF with ischemic cardiomyopathy and LV ejection fraction (LVEF) <40% , left atrial functions, calculated from left atrial volume i.e. the active emptying fraction, the passive emptying fraction, and the reservoir fraction were respond better to levosimendan than to dobutamine (Duygu et al. 2008). In conclusion, pharmacological modulation of mitochondrial K_{ATP} channels may be beneficial in patients at risk of myocardial ischemia, particularly those requiring inotropic support.

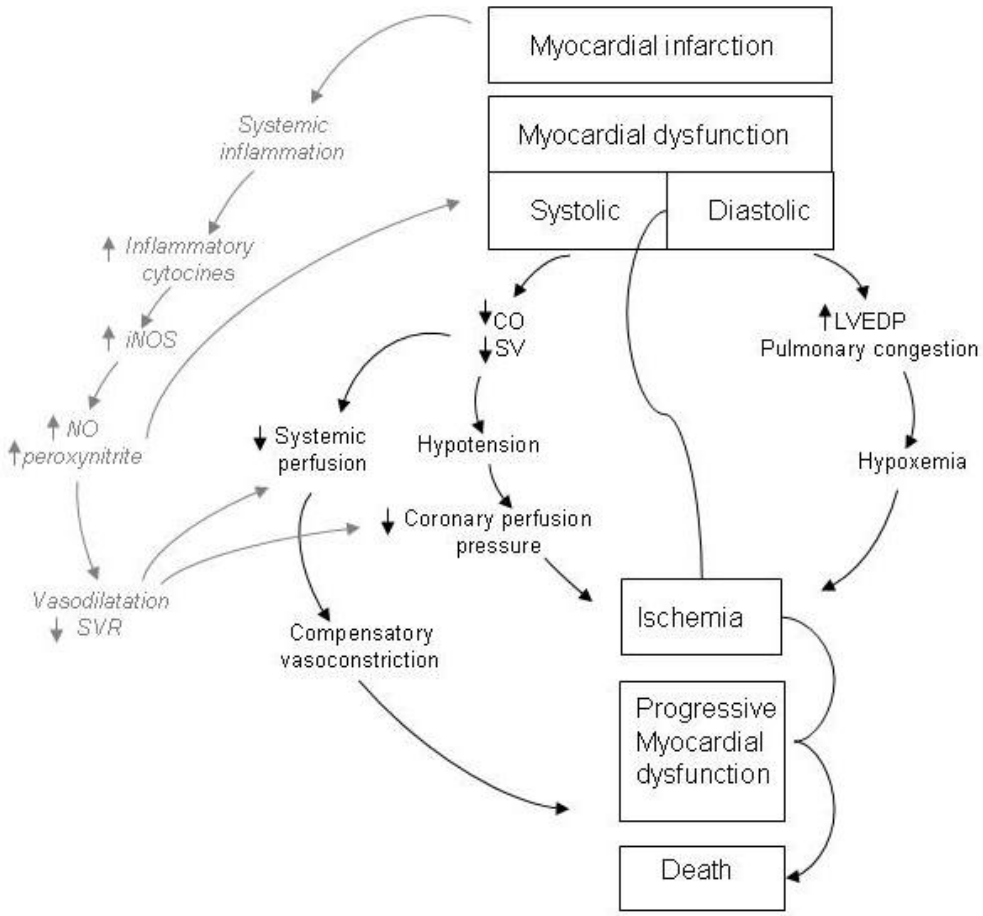

Figure 3. Shock paradigms. Classic shock paradigm is shown in black. The influence of the inflammatory response syndrome initiated by a large MI is illustrated in gray. Left ventricular end diastolic pressure (LVEDP); Cardiac output (CO); Stroke volume (SV); Systemic vascular resistance (SVR); Inducible nitric oxide synthase (iNOS): Nitric oxide (NO). Modified from (Hochman 2003) *Circulation* **107**(24): 2998-3002.

2.5.2.2 **Sepsis and septic shock**

Sepsis is referred to as a systemic disease caused by microbial invasion of normally sterile parts of the body. More accurately defined, sepsis is a set of non-specific inflammatory responses with evidence, or suspicion, of a microbial origin (Lever and Mackenzie 2007). Severe sepsis is accompanied by evidence of hypoperfusion or dysfunction of at least one organ system. Septic shock, in turn, is sepsis accompanied by hypotension or the need for vasopressors, despite adequate fluid resuscitation. Severe sepsis and septic shock together are some of the leading causes of death in noncoronary intensive care units. Increasing severity of sepsis correlates with increasing mortality, i.e. 25-30% for severe sepsis up to 40-70% for septic shock (Lever and Mackenzie 2007).

Myocardial depression is related to symptoms of sepsis, although cardiac output (CO) may be preserved or even increased. This is explained by a maintained stroke volume due to a dilated ventricle and an increased heart rate (Rabuel and Mebazaa 2006). Sepsis can induce myocardial dysfunction which is seen in both ventricles. Echocardiography studies have noted that 40% to 50% of patients with prolonged septic shock have reduced ejection fraction as a sign of develop myocardial depression (Rudiger and Singer 2007). Alterations in sympathetic beta-adrenoceptor signalling are considered to impair the myocardial response to endogenous and exogenous catecholamines. Moreover, sepsis-induced cardiomyopathy can affect calcium homeostasis in the cardiomyocytes. The calcium current can be disturbed and myofilament calcium sensitivity be decreased (Rudiger and Singer 2007). Calcium is also related to mitochondrial dysfunction, which is believed to be important in the pathophysiology of sepsis, and seems to correlate to the severity of the disease. In brief, calcineurin (*CN*), activated by calmodulin-kinase (*CMK*), interacts with the mitochondrial permeability transition pore, triggering mitochondria-related death pathways (Rudiger and Singer 2007). Also circulatory abnormalities related to vasodilatation and vascular leakage are often encountered in sepsis.

The use of levosimendan in patients with septic shock has been described in a few case reports. The drug was reported to improve hemodynamics, global oxygen transport, pulmonary circulation, metabolism and vasopressor requirements, mostly in patients with long-lasting refractory septic shock (Pinto et al. 2008; Salmenpera and Eriksson 2009). Levosimendan can exert beneficial effects in both the left and right ventricles. As a calcium sensitizer, it does not influence the beta-adrenoceptor signalling or evoke changes in the intracellular Ca^{2+} concentration. Therefore, it was postulated that levosimendan could theoretically be an ideal therapy for sepsis-induced myocardial dysfunction (Pinto et al. 2008). In addition, levosimendan enhances pulmonary and intestinal microcirculation a priori due to its vasodilatory actions and therefore, prevents multi-organ failure

(Figure 4.). Moreover, some recent experimental studies have shown that levosimendan has a positive effects in the rat sepsis model of cecal ligation and puncture. Treatment with levosimendan had a marked effect on attenuating or decreasing apoptosis and inflammation in the lung (Erbuyun et al. 2009). In other recent study, levosimendan equally attenuated arterial hypotension, metabolic acidosis and prolonged survival on mortality (Scheiermann et al. 2009).

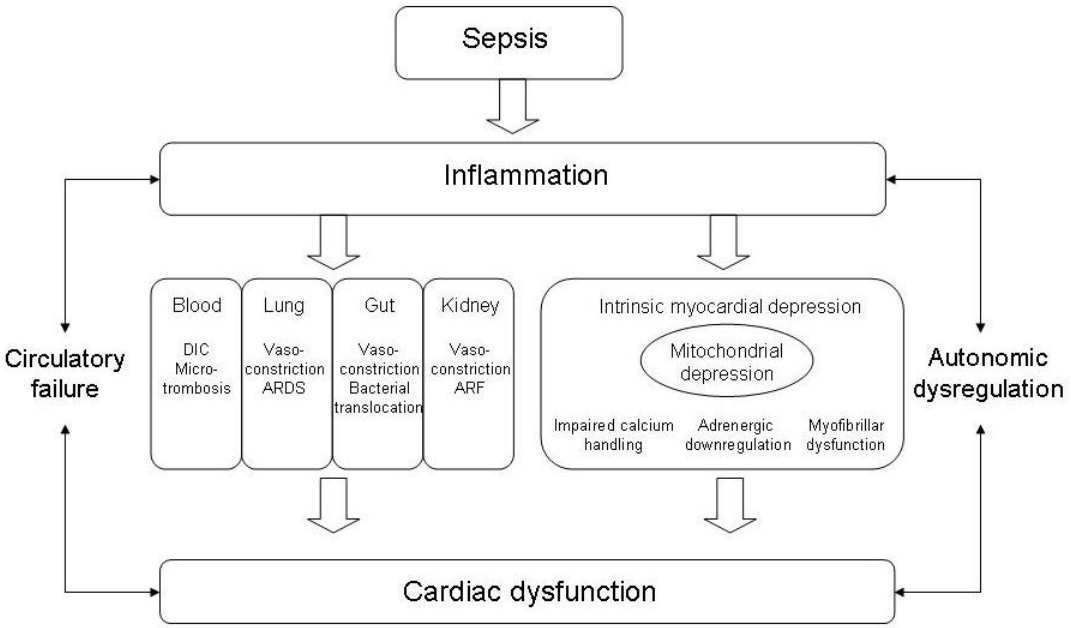

Figure 4. Sepsis-induced organ dysfunction. Acute respiratory distress syndrome (ARDS); Acute renal failure (ARF); Disseminated intravascular coagulation (DIC). Modified from (Pinto et al. 2008) *Curr Opin Anaesthesiol* **21**(2): 168-77.

2.5.2.3 **Perioperative cardiac support**

Inotropic support is frequently required to wean patients from cardiopulmonary bypass (CBP). In particular, in patients with preoperative impaired ventricular function, weaning failure without medical or mechanical support is a common occurrence occuring in as many as 70% to 80% of cases (Eriksson et al. 2009). Moreover, postoperative renal dysfunction and acute renal failure are frequent and serious complications of cardiac surgery and these are associated with prolonged intensive care unit stay and hospitalization, as well as significant increases in mortality (Mangano et al. 1998).

Levosimendan has been demonstrated to have beneficial effects on postoperative conditions and it has been postulated as a bridge therapy for the perioperative phase of cardiac surgery (Nieminen et al. 2009). Levosimendan has been shown to increase cardiac output without increasing myocardial

oxygen consumption in patients early after weaning from CPB following coronary artery bypass grafting (CABG) (Lilleberg et al. 1998; Nijhawan et al. 1999). Moreover, the effect of prophylactic levosimendan administration on weaning from and early recovery after CPB in patients with preoperatively impaired left ventricular function undergoing CABG was studied recently. Levosimendan showed a positive outcome and significantly facilitated the primary weaning from CPB compared with placebo (Eriksson et al. 2009). Furthermore, a comprehensive meta-analysis of existing trials to determine the impact of levosimendan on cardiac troponin release in patients undergoing cardiac surgery was done by the senior training project in Center for Overview, Meta-analysis, and Evidence-based Medicine Training (COMET) in Milano (Zangrillo et al. 2009). The results suggested that the use of levosimendan was associated with a significant reduction in cardiac troponin I (cTnI) release in patients undergoing cardiac surgery. The authors hypothesized that the better preservation of early cardiac function with levosimendan may have been the result of the improved global tissue perfusion leading to better recovery from surgery (Zangrillo et al. 2009).

2.5.2.4 **Effects in calcium channel poisoning**

Poisoning with calcium channel blockers (CCB), accidental or intentional, has become an increasing caseload in the poisoning centers in recent years. This is associated with the more frequent use of these drugs. CCB and beta-blockers account for approximately 40% of the cardiovascular drug exposures reported to the American Association of Poison Centers (DeWitt and Waksman 2004). Verapamil causes the most severe cases and tends to produce the most hypotension, bradycardia, and conduction disturbances and deaths from the calcium channel blockers seen in emergency rooms. The adverse effects of verapamil consist of its negative effects on the sino-atrial and atrio-ventricular nodes reducing heart rate and atrioventricular conduction, reduction of myocardial contractility by antagonism of myocardial L-type calcium channels, and antagonism of L-type calcium channels in vascular smooth muscle, resulting in peripheral vasodilatation. The toxicity of calcium channel blockers can lead also to a wide variety of manifestations in the central nervous system, gastrointestinal system, endocrine-metabolic, hematologic and respiratory systems.

Levosimendan is believed to improve the cardiac depression and prognosis of the patients. There is some experimental evidence which supports these claims. In anaesthetized guinea-pigs, levosimendan markedly decreased mortality in the diltiazem intoxicated group (12% versus 100%) and in the verapamil-intoxicated group (0% versus 89%) ((Levijoki, unpublished data). However, in a rat model, levosimendan failed to cause any improvement in the survival time (Abraham et al. 2009). In one other rat study, levosimendan increased CO to a similar degree as $CaCl_2$ alone, but it

did not improve BP from the time of maximal toxicity. The addition of $CaCl_2$ to levosimendan did not confer any further improvement in CO and BP compared to $CaCl_2$ alone. There was either no mortality benefit observed with levosimendan compared to $CaCl_2$ treated animals. However, a recent case report did reveal a beneficial hemodynamic effect of levosimendan in CCB overdose patients (Varpula et al. 2009). Clearly, more studies are needed to evaluate the place of levosimendan in the treatment of verapamil poisoning in humans.

2.5.3 Other clinical effects

Short-term levosimendan therapy of heart failure has been shown to have no tendency to increase the incidence of cardiac arrhythmias. A meta-analysis of cardiac arrhythmias by analysing ECG recordings from clinical studies on intravenously administered levosimendan in heart failure patients showed no difference between levosimendan and control groups in the occurrence of atrial fibrillation (AF), supraventricular tachycardia (SVT), or ventricular tachycardia (VT). In addition, the frequency of VT was similar and no torsade de pointes or sustained VT occurred (Lilleberg et al. 2004). In a recent open-label study in 50 patients with heart failure, given a 24-hour intravenous protocol once monthly for 6 months, no increase was seen in the incidence of supraventricular or ventricular beats or SVT and VT episodes in levosimendan group, compared with controls (Mavrogeni et al. 2007). However, in REVIVE II and SURVIVE trials, ventricular tachycardia as an adverse effect (AE) was statistically significantly more frequent with levosimendan than with placebo (Cleland et al. 2006), or occurred at a similar frequency with both levosimendan and dobutamine (Mebazaa et al. 2007). To sum up all of the i.v. studies, levosimendan seems to increase the occurrence of atrial fibrillation according to AEs (Levijoki, unpublished data). However, with respect to ventricular tachycardia, contradictory results have been reported in clinical trials.

Levosimendan (0.5 mg four times daily) orally for 9 days in healthy subjects had no effects on blood coagulation (Antila et al. 2000). In addition, levosimendan has been shown to possess some anti-inflammatory and antiapoptotic properties. In patients with decompensated advanced heart failure after 48 hours compared with placebo, levosimendan induced a significant reduction in the concentration of interleukin-6 and a slight decrease in the level of tumor necrosis factor-α (TNF-α). It also reduced soluble apoptosis mediators, such as soluble Fas and Fas ligand (Parissis et al. 2004; Paraskevaidis et al. 2005).

2.5.4 Drug interactions

Levosimendan seems to have no serious interactions with most of the drugs used in the treatment of CHF. The combination of levosimendan and dobutamine has been shown to be relatively safe and

effective in patients with severe heart failure (Nanas et al. 2004; Nanas et al. 2005). In cardiogenic shock patients requiring catecholamines, additive levosimendan infusion was shown to improve hemodynamics (Delle Karth et al. 2003). No pharmacodynamic interactions between levosimendan and warfarin, were seen in healthy volunteers (Antila et al. 2000). Levosimendan in combination with isosorbide-5 mononitrate was shown to have no major additive haemodynamic effects compared with each drug alone at rest. However, the response to an orthostatic heart rate test was potentiated with the combination of levosimendan and isosorbide-5-mononitrate in healthy subjects (Sundberg and Lehtonen 2000). When levosimendan was given in addition to carvedilol in healthy subjects, no additive positive inotropic, positive chronotropic, or effects on diastolic blood pressure were seen though the effect on systolic blood pressure was attenuated (Lehtonen and Sundberg 2002). No major haemodynamic interactions between levosimendan and captopril, an ACE inhibitor, (Antila et al. 1996) or between levosimendan and felodipine, a calcium channel blocker, (Poder et al. 2003) have been observed in humans. Moreover, no pharmacodynamic interactions between levosimendan and ethanol, used as a diluent in the intravenous formulation, were found (Antila et al. 1997). Furthermore, levosimendan did not demonstrate any interaction with itraconazole (Antila et al. 1998), a potent inhibitor of CYP3A4 isoenzyme and an antifungal agent

2.6 Summary of the effects of levosimendan on the cardiovascular system

Levosimendan is defined as a inodilator agent based on its combined positive inotropic and vasodilator effects related to calcium sensitization and potassium channel opening. In addition to these properties it can evoke mitochondrial K_{ATP} channel opening, a unique assortment of mechanisms, which may be responsible for a positive synergistic effects on the whole cardiovascular system (Figure 6.). The abilities of levosimendan to improve myocardial function without increasing oxygen consumption practically at all and its property to enhance the perfusion of peripheral organs generates energetically favourable conditions for the restoration of impaired cardiac performance in addition to AHF also in ischemic heart disease, sepsis and perioperative cardiac support.

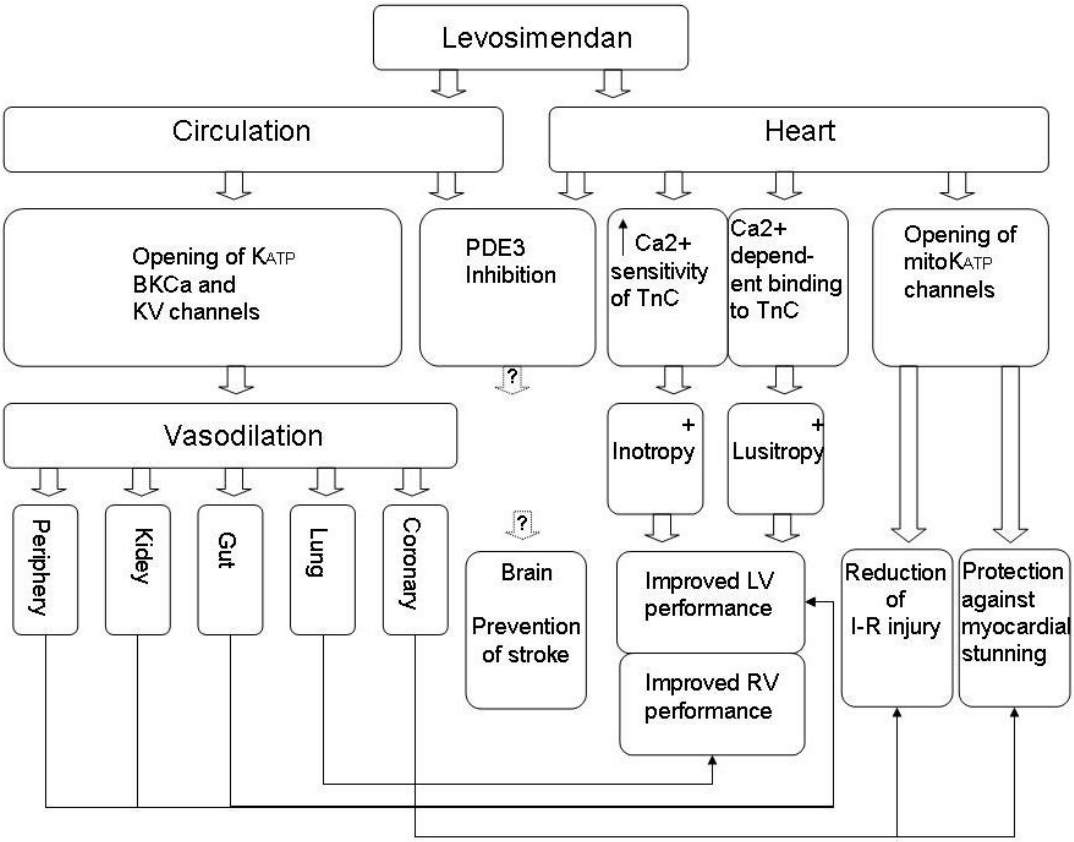

Figure 6. Summary of the effects of levosimendan on cardiovascular functions. ATP-dependent potassium channels (K_{ATP}); Large conductance calcium-activated potassium channels (BK_{Ca}); Voltage gated potassium channels (KV); Phosphodiesterase type-3 (PDE3); Troponin C (TnC); Ischemia-reperfusion (I-R); Left ventricle (LV); Right ventricle (RV). Modified from (Pinto et al. 2008) *Curr Opin Anaesthesiol* **21**(2): 168-77.

3 AIMS OF THE STUDY

The inodilatory effects of levosimendan have been described in many of the above-mentioned *in vitro* and *in vivo* studies. It is a calcium sensitizer and an opener of ATP-dependent potassium channels and exerts its primary pharmacological effects via this unique double mechanism of action. There is another family of drugs with an inodilatory effect, the PDEIs, of which milrinone is a classical example. However, these drugs exert their inotropic and vasodilatory effects by a mechanism of action based on the increase of intracellular calcium, which has been associated with undesirable effects such as increased myocardial oxygen demand, cardiac arrhythmias, and mortality. However, it has been shown that levosimendan is an effective and potent PDE3 inhibitor, *in vitro*. The consequence of this PDE3 inhibition, *in vivo* is obscure, since levosimendan enhances cardiac contraction without increasing oxygen consumption, in contrast to the PDEIs.

The present *ex-vivo* experiments were designed to further characterize the mechanisms of action of levosimendan and to differentiate it from the inodilatory effects of the PDEIs. The model of isolated heart preparation was selected in order to study the dual effects of levosimendan in the target organ, possibly untangling the inotropic and vasodilatory effects. The experiments were conducted in guinea-pig isolated hearts because the cardiomyocytes of this species have a calcium handling similar to human myocytes.

Five different study settings were designed in order to answer five main questions about the mechanisms of action of levosimendan:

1. What is the role of cAMP- and cGMP-dependent protein kinases in the effects of levosimendan on cardiac contraction and coronary vasodilatation compared to the effects of the PDE inhibitor milrinone?

2. How potent a inotropic agent and Ca^{2+}-sensitizer is the active plasma metabolite of levosimendan, OR-1896, compared to levosimendan, and how selective inhibitors are these two agents on phosphodiesterase isoforms (PDE3 and PDE4)?

3. Is the difference between the positive inotropic effects of levosimendan and its stereoisomer, dextrosimendan, correlated primarily to their stereoselective Ca^{2+}-sensitizing effect or to their stereoselective phosphodiesterase inhibory functions?

4. What is the main vasodilatory mechanism of levosimendan in the coronary arteries?

5. Is the inotropic mechanism of levosimendan energetically more advantageous than the mechanism of the PDE inhibitor, milrinone?

4 MATERIALS AND METHODS

4.1 Experimental animals

Adult guinea pigs of either sex (MOL-DUHA in studies I and III-V and Duntley Hartley in study II, purchased from Mollegaard Breeding Center, Denmark), weighing 300–400 g were used. The guinea-pigs were housed in polycarbonate cages (Macrolone IV, Scanbur A/S, Denmark), in a thermostatically controlled room at 20 ± 1°C at a relative humidity of 50 ± 10%. The room was artificially illuminated from 06.00 to 20.00 hours. The guinea pigs received commercial, pelleted, guinea-pig feed (Altromin 3120, Chr.Petersen A/S, Denmark) autoclaved at 120°C and sterile, filtered tap water ad libitum. The study was conducted with the permission of the Animal Ethic Committee of Orion Pharma, in accordance with Finnish law and government regulations complying with the European Community guidelines for the use of experimental animals.

4.2 Preparations

4.2.1 Isolated heart

The guinea-pig isolated heart was the most common preparation used in this study. Langendorff-perfused heart is described in four studies, I, II, IV and V. Briefly, the guinea-pig was sacrificed and the heart was rapidly excised and rinsed in ice-cold oxygenated perfusion buffer. A cannula was inserted into the aorta and retrograde perfusion of the heart began as soon as the heart was placed in a thermostatically controlled moist chamber of the Langendorff apparatus. Modified Tyrode solution was used as a perfusion buffer. Most of the experiments were carried out under constant pressure conditions (50 mmHg). A latex balloon, attached to a stainless-steel cannula coupled to a pressure transducer, was placed in the left ventricle. The isovolumetric left ventricular pressure was recorded with a pressure transducer. Coronary flow was continuously recorded by an electromagnetic flow meter with a flow probe inserted above the aortic cannula. The HR and the left ventricular systolic and end-diastolic pressure of the heart were obtained from the digitised pressure signals. The left ventricular pressure signals were used to calculate the maximal positive and negative pressure derivatives (LV + dP/dt_{max} and LV − dP/dt_{max}, respectively). In studies II and V, the hearts were overpaced at 5 Hz. Furthermore, in study II, the experiment was carried out in the presence of constant coronary flow. Under these conditions, myocardial contractility could be increased either by an increase in the Ca^{2+}-sensitivity of the contractile system and/or by an increase in the amplitude of the Ca^{2+} transient due to phosphodiesterase inhibition.

4.2.2 Papillary muscle

The papillary muscle preparation is described in studies I and III. Briefly, the right ventricular papillary muscle of the guinea-pig heart was mounted in an organ bath containing modified Tyrode solution for measurement of isometric tension. For pacing of the papillary muscle, the electrical pulses were conducted through platinum wire electrodes inserted on both sides of the muscle. The field stimulation occurred at 20% above threshold or at double the threshold voltage. A force-displacement transducer was connected to a driver amplifier and a programmable scanner. The amplified signal was digitised at 1 kHz frequency by a programmable digitizer and the twitch tension was measured.

4.2.3 Permeabilized cardiomyocytes

Force measurements in permeabilized myocyte-sized preparations (skinned fibres) are described in studies I, II and III. Briefly, the calcium-sensitizing effect of levosimendan was investigated in two types of skinned fibers obtained either from unpretreated or isoprenaline-pretreated isolated, spontaneously beating guinea-pig hearts. Fast and complete perforation of the cell membranes was achieved by retrogradely conducting saponin solution through the aorta into the coronary arteries of the heart. The fibers dissected from the papillary muscles were further mildly sonicated and treated with saponin. A slightly acidic pH of 6.7 was chosen in order to mimic the pH in the ischemic myocardium in which the calcium sensitivity is decreased and in which the benefits of levosimendan should be most prominent. The fibers were induced to contract in the calculated desired free pCa (Fabiato and Fabiato 1979). The absolute stability constants used were as reported by Fabiato (Fabiato 1981). At the beginning of the experiment, the fiber was stretched in 'relaxing' calcium free solution until the resting tension amounted to approximately 2% of the maximum force able to produce by the fiber. Then, the 'relaxing' solution was replaced with the 'activating' pCa 5.8. solution which roughly corresponds to the intracellular cytoplasmic calcium during muscle contraction. When the tension produced by the fiber had reached a steady state, various concentrations of levosimendan were successively added to the solution. The maximum tension which was produced by the fiber at pCa 4.8 was determined after washing out the test compound.

4.2.4 Inhibition of phosphodiesterase isoenzymes

Inhibition of PDE is described in studies II and III. PDE III and IV were isolated from guinea pig hearts and from a human myeloid leukemia promonocytic cell line (U-937), respectively, to achieve high level PDE purity. The isolation procedure of PDE and the determination of their activities have been described earlier (Alajoutsijarvi and Nissinen 1987; Frodsham and Jones 1992; Torphy et al.

1992). Briefly, tissues were homogenized and the homogenate was centrifuged and further applied to DEAE cellulose columns and subsequently eluted with a linear gradient of sodium buffers. The collected fractions were subsequently assayed for cAMP and cGMP phosphodiesterase activity. The fractions containing phosphodiesterase activity were pooled to form isoenzyme specific pools. The effects of levosimendan, dextrosimendan, milrinone and OR-1896 on PDE activity were measured by using a liquid chromatograph system for on-line radiochemical detection of the formation of [^{3}H]cAMP. PDE inhibitory potential determinations were performed in duplicate and the results are given as the means of the data from the individual test runs. The IC_{50}-values for PDE inhibition were calculated.

4.3 Statistical analysis

In study I, ANCOVA (repeated measures) was used in order to verify that the selected concentration range for levosimendan and milrinone produced positive inotropy to the same extent in the isolated heart. The same statistical test was used when the hearts treated with the protein kinase inhibitors were compared with those exposed to levosimendan or milrinone alone. In all studies ANOVA (repeated measures), followed by Dunnett's *t*-test was used, when the measured parameters were compared to the initial values. Statistical significance was accepted at $P<0.05$. The EC_{50} values from the sigmoidal dose-response data were calculated by using the curve-fitting algorithm of Microsoft® Office Excel program.

4.4 Ethical statement

The study was conducted with the permission of the Animal Ethic Committee of Orion Pharma, in accordance with Finnish law and government regulations complying with the European Community guidelines for the use of experimental animals.

5 RESULTS

The main results are summarized in table 3.

5.1 Brief summary of the main effects in the separate studies

Study I

In the isolated Langendorff-perfused spontaneously beating guinea-pig heart, levosimendan and milrinone increased the left ventricular systolic peak pressure to almost the same extent. The inotropic and lusitropic effects of milrinone were mediated via PKA mediated phosphorylation, while the effects of levosimendan were not mediated via PKA up to its EC_{50}. Furthermore, the stimulation of AC potentiated the positive inotropic effect of milrinone but not that of levosimendan in the papillary muscle.

Study II

In the isolated Langendorff-perfused paced guinea-pig heart, in the presence of constant coronary flow, levosimendan and OR-1896 were equally potent as positive inotropes. In the permeabilized cardiomyocytes, levosimendan and OR-1896 both increased isometric force production similarly. Furthermore, levosimendan was 40-fold more potent and a 3-fold more selective PDE3 inhibitor than OR-1896.

Study III

In the isolated guinea-pig papillary muscle preparation, levosimendan increased the twitch tension at 47 times lower EC_{50} value than dextrosimendan and in the permeabilized cardiomyocytes, levosimendan and dextrosimendan increased isometric force production due to Ca^{2+}-sensitization with a similar relative potency difference of 76. However, levosimendan was 427 times more potent PDE3 inhibitor than dextrosimendan.

Study IV

In the isolated Langendorff-perfused spontaneously beating guinea-pig heart, levosimendan increased diastolic coronary flow velocity (DCFV) concentration-dependently, and this effect was noncompetitively antagonized by glibenclamide, whereas the effects of pinacidil were antagonized competitively by glibenclamide. In the presence of glibenclamide, the positive inotropic and

chronotropic effects of levosimendan were unaltered. The effect of a PKC inhibitor, BIM and levosimendan on DCFV was additive.

Study V

In the isolated Langendorff-perfused paced guinea-pig heart, perfusion with levosimendan consumed proportionally less oxygen than perfusion with milrinone, while the compounds increased the mechanical performance of the heart equally.

5.2 Isolated Langendorff-perfused heart

The inotropic and vasodilatory effects of levosimendan and its active metabolite, OR-1896, were studied in retrogradely perfused isolated guinea-pig hearts and compared to the effects of milrinone and pinacidil. In the paced hearts, levosimendan and OR-1896 were equally potent positive inotropes with very similar concentration dependences with a slightly lower EC_{50} value for levosimendan than that for OR-1896 (15 ± 2 nM vs 25 ± 1 nM, $P < 0.05$). They were also equal efficacious, increasing the left ventricular + dP/dt_{max} by 26 ± 4% and 25 ± 3% (mean ± S.E.M.), respectively (study II). Compared to milrinone, levosimendan was 10 to 30 times more potent than milrinone as a positive inotropic and lusitropic agent. The maximum increase in left ventricular systolic pressure (LVSP) was 24 ± 10% in the levosimendan-treated group and 20 ± 10% in the milrinone-treated group. The maximum increase in contractility (+dP/dt max) was 30 ± 10% and 28 ± 11% and the maximum increase in relaxation (−dP/dt max) was 36 ± 17% and 37 ± 14% in the levosimendan and milrinone groups, respectively (study V). In the spontaneously beating hearts, levosimendan was 10 to 20 times more potent as an inotropic agent than milrinone. The EC_{50} concentration for the elevation in LVSP by levosimendan was about 0.03 μM and that for milrinone was about 0.6 μM. However, levosimendan and milrinone increased the left LVSP almost equally, levosimendan maximally by 17 mmHg (+19%) at 0.3 mM and milrinone maximally by 14 mmHg (+15%) at 3 mM (study I). The PKA inhibitor, KT5720 (1 μM) inhibited the contraction (LVSP and +dP/dt_{max}) of levosimendan at the highest studied concentrations (≥ 0.1 μM). Instead the inotropic effects of milrinone, were inhibited totally by KT5720. The effects of KT5720 on relaxation (-dP/dt_{max}) of levosimendan and milrinone were similar to those on contraction. The PKG inhibitor, KT5823 (1 μM), did not affect statistically significantly the increasing effect of levosimendan or milrinone on the contraction or relaxation (study I). The PKC inhibitor, BIM, did not have any effects either on the contraction or relaxation in the levosimendan group (study IV).

In spontaneously beating guinea-pig hearts, levosimendan concentration-dependently increased the heart rate (HR) to the same extent in the absence and presence of KT5720 or KT5823. In all groups, a maximum increase of 27–30% was seen at 1 μM levosimendan. The magnitude of the positive chronotropic effect of milrinone (0.1–10 mM) was comparable to that of levosimendan (0.01–1 mM), but unlike the situation with levosimendan, the maximum effect was not reached at the concentrations used (study I). Moreover, the PKC inhibitor, BIM, did not have any effects on the HR in the levosimendan group (study IV).

Levosimendan alone increased the coronary flow concentration-dependently and maximally by 64% at 1 μM. The increase in coronary flow by milrinone alone did not differ from that induced by levosimendan except that the maximum effect was not reached at the milrinone concentrations used (study I). Also the increase in the DCFV by levosimendan reached a maximum effect at 1 μM. The effect of pinasidil on DCFV was equal with levosimendan (study IV). The total change in the oxygen consumption was significantly lower during levosimendan than the corresponding value during milrinone perfusion. Furthermore, the maximum increase in oxygen consumption was 10 ± 4% in the levosimendan group compared with 38 ± 15% in the milrinone group ($P = 0.031$ between the concentration-dependent effects of the two drugs on VO_2) (study V). The response to levosimendan or milrinone was slightly and to the same extent decreased in the presence of KT5720 (1 μM). In contrast, the levosimendan-induced increase in coronary flow was almost doubled in the presence of KT5823 (1 μM), whereas KT5823 did not alter the milrinone-induced increase in coronary flow (study I). The effect of levosimendan on DCFV was noncompetitively (nonparallel shift of the concentration-response curve to the right) antagonized by glibenclamide on the contrary to pinacidil, which was antagonized competitively (parallel shift of the concentration-response curve to the right) by glibenclamide. Furthermore, the concentration-response curve of levosimendan in the presence of the BIM, was markedly potentiated (study IV). In addition, it should be mentioned that glibenclamide alone decreased the DCFV and BIM increased it, while KT5720 and KT5823 had no effects on the baseline DCFV.

5.3 Papillary muscle

The concentration–response curve of levosimendan on twitch tension displayed a bell-shaped form and was markedly dependent on the stimulus strength used in the pacing of the papillary muscle. No such similar phenomenon was seen with milrinone (study I). The EC_{50} concentrations for levosimendan were 0.1 and 0.3 μM when the papillary muscles were paced at 100% or at 20% above threshold voltage, respectively. The maximum increases of 330 mg (+127%) and 90 mg

(+45%) in twitch tension were reached at 1 and 3 μM, respectively. The EC_{50} value for milrinone was about 2 μM independently of the stimulus strength used. The maximum increase of 240 mg in twitch tension induced by milrinone was also not affected by the stimulus strength (study I). Moreover, twitch tension increased with EC_{50} values of 60 nM for levosimendan compared to 2.8 μM for dextrosimendan. Hence, the two enantiomers exhibited a 47 times potency difference in their positive inotropic effects (study III).

In guinea-pig papillary muscles paced at twofold threshold voltage, forskolin (0.1 μM) increased twitch tension due to stimulation of the cAMP synthesis. The levosimendan-induced increase in twitch tension was not markedly changed in the presence of forskolin, while the positive inotropic effect of milrinone was almost doubled when it was combined with forskolin (study I). The initial twitch tension changed less than 10% by treatment with the β_1-AR antagonist, atenolol (1 μM). However, atenolol abolished the stimulus sensitivity of the twitch tension and thereby eliminated the stimulus strength dependency of the inotropic response to levosimendan. Furthermore, levosimendan at 1 μM and milrinone at 10 μM increased twitch to the same extent (+180% to +190%) when these concentrations were tested before isoprenaline. After wash-out of isoprenaline, in percentage terms, milrinone was as efficacious as before isoprenaline, but the levosimendan-induced increase in twitch tension was reduced to 78% of pre-isoprenaline values (study I).

5.4 Permeabilized cardiomyocytes

In permeabilized guinea-pig cardiomyocytes, levosimendan (0.3–10 μM) concentration-dependently increased the calcium-induced tension at pCa 5.8 from 7% to 26% of the maximum tension produced by the skinned fibers at pCa 4.8 (study I). In the comparison of levosimendan with OR-1896, both compounds increased isometric force production at pCa 6.2 by 51% ± 7% and 52% ± 6%, with EC_{50} values of 8 ± 1 nM and 36 ±7 nM, respectively (study II). In addition, levosimendan and dextrosimendan increased isometric force production (at pCa 6.2) with EC_{50} values of 8.4 nM and 0.64 μM, respectively, i.e. a relative potency difference of 76 (study III). However, when the permeabilized cardiomyocytes were obtained from isoprenaline-pretreated (0.1 μM, for 10 min) hearts, the levosimendan-induced increase in tension of the skinned fibres was only from 6% to 16% of the maximum tension (study I).

5.5 Purified PDE enzymes

Increasing concentrations of levosimendan or OR-1896 decreased the PDE3 activity in a dose-dependent manner. The half-maximal inhibition of PDE3 was achieved at a concentration (IC_{50}) of

2.5 nM by levosimendan, while in the case of OR-1896, the IC_{50} was 94 nM. Similarly to the results with PDE3, increasing concentrations of levosimendan or OR-1896 also progressively decreased the PDE4 activity. However, to achieve half maximal inhibition of PDE4, significantly higher drug concentrations were required. The IC_{50} value for levosimendan was 25 μM, comparison to 286 μM for OR-1896 (study II). Furthermore, levosimendan was a 427 times more potent PDE3 inhibitor than dextrosimendan, with IC_{50} values of 7.5 nM, and 3.2 μM, respectively (study III).

Table 3. Summary table of the main results

Preparation	Study conditions	Studied compound	Modulatory agent	Inotropic effect	Vasodilatory effect	Oxygen consumption	PDE inhibition
isolated Langendorff-perfused guinea-pig heart [1, 2, 4, 5]	constant pressure (50mmHg), spontaneously beating heart [1, 4]	levosimendan milrinone pinacidil	KT5720 KT5823 BIM glibenclamide	levosimendan > milrinone KT5720 + milrinone ↓	levosimendan > milrinone KT5823 + levosimendan ↑ BIM + levosimendan ↑ glibenclamide + pinacidil ↓ glibenclamide + levosimendan ↓		
	constant pressure (50mmHg), paced heart at 5 Hz [5]	levosimendan milrinone		levosimendan > milrinone	levosimendan > milrinone	levosimendan < milrinone	
	constant flow, paced heart at 5 Hz [2]	levosimendan OR-1896		levosimendan ≈ OR-1896			
guinea-pig papillary muscle [1, 3]	paced at 20% above threshold [1]	levosimendan milrinone	atenolol	levosimendan < milrinone atenolol + levosimendan ↑			
	paced at twofold of threshold [1, 3]	levosimendan milrinone dextrosimendan	forskolin isoprenaline atenolol	levosimendan > milrinone levosimendan > dextrosimendan forskolin + milrinone ↑ isoprenaline + levosimendan ↓ atenolol + levosimendan ↓			
permeabilized cardiomyocytes [1, 2, 3]		levosimendan milrinone OR-1896, dextrosimendan		levosimendan ≈ OR-1896 levosimendan > dextrosimendan			
	isoprenaline-pretreatment [1]	levosimendan		levosimendan ↓			

Table 3. (continued)

Preparation	Study conditions	Studied compound	Modulatory agent	Inotropic effect	Vasodilatory effect	Oxygen consumption	PDE inhibition
PDE3 enzyme from guinea pig hearts [2, 3]		levosimendan OR-1896 dextrosimendan					levosimendan > OR-1896 levosimendan > > dextrosimendan
PDE4 enzyme from a human myeloid leukemia promonocytic cell line (U-937) [2]		levosimendan OR-1896					levosimendan > OR-1896

[1] study I, [2] study II, [3] study III, [4] study IV, [5] study V

pinacidil = K_{ATP} channel opener, KT5720 = PKA inhibitor, KT5823 = PKG inhibitor, BIM (bisindolylmaleimide) = PKC inhibitor , glibenclamide = K_{ATP} channel antagonist, atenolol = β_1-AR antagonist, forskolin = adenylate cyclase activator, isoprenaline = β_1-AR agonist

↑ = additive effect, ↓ = antagonistic effect

6 DISCUSSION

The main conclusion emerging from the present study is the concept that levosimendan possesses of two main mechanisms of action, an inotropic effect via calcium sensitization and a vasodilatory effect mediated through the opening of the K_{ATP} channels. However, levosimendan was found to be a potent PDE3 inhibitor, a finding which is at odds with the fact that levosimendan does not increase intracellular calcium concentration $[Ca^{2+}]_i$ and oxygen consumption in proportion to its inotropic effect. This dilemma is discussed in the following chapters.

6.1 Inotropic effects

In the present study, the economy of the contraction was shown to be more advantageous in levosimendan-perfused hearts than in milrinone-perfused hearts (study V). Previously, Lancaster and Cook have shown that levosimendan produced an increase in cell shortening in guinea-pig cardiomyocytes without affecting the $[Ca^{2+}]_i$ transient or the Ca^{2+} content of the sarcoplasmic reticulum at clinically relevant concentrations ≤ 0.1 μM (Lancaster and Cook 1997). However, other studies have revealed that levosimendan can increase the calcium transient already at low inotropic concentrations and emphasized its dual mechanism of action in its positive inotropic effects (Sato et al. 1998; Takahashi and Endoh 2005). In canine ventricular trabeculae loaded with aequorin, the positive inotropic effect of levosimendan up to 10 μM was associated with an increase in Ca^{2+} transients (Takahashi and Endoh 2005). On the other hand, in isolated failing human myocardium, levosimendan induced moderate inotropic effects without any increased intracellular calcium transients (Hasenfuss et al. 1998). Levosimendan increased twitch tension statistically significantly and aequorin light emission statistically nonsignificantly at a concentrations of 0.3 and 1μM. In comparison, at concentrations of 10 μM and 100 μM, milrinone increased both twitch tension and aequorin light emission statistically significantly. Therefore, these findings support the concept that the positive inotropic effect originates predominantly from myofilament calcium sensitization.

In the present study, levosimendan was found to be 10–20 times more potent as a positive inotropic compound than the PDE inhibitor, milrinone, in isolated guinea-pig heart. Previously, the positive inotropic effect of levosimendan has been shown to be sensitive to carbachol (Sato et al. 1998). The inhibitory action of carbachol may be exerted selectively on the cAMP mediated positive inotropic effect, because it is virtually unaffected by the inotropic effect of other drugs e.g. α-adrenoceptor agonists and cardiac glycosides, that do not involve the generation of cAMP (Endoh 1995).

However, the PKA inhibitor, KT5720, did not antagonise the increase in LVSP produced by levosimendan at concentrations up to its EC_{50}. This effect of levosimendan contrasts with that of milrinone, which was devoid of the positive inotropic effect in the presence of KT5720, i.e. evidence that PDE inhibition is the major mechanism of action of milrinone. In heart failure patients, 95–98% of levosimendan is bound to plasma proteins and therefore its therapeutically active free plasma concentration is less than 0.02 μM (Sandell et al. 1995). Additionally, during the treatment of acutely decompensated heart failure, a maximal free concentration of 3–6 nM of levosimendan has been measured (Kivikko et al. 2002). Therefore, the concentration range used in the present study was at the clinically relevant level. In guinea-pig papillary muscle, the EC_{50} value of levosimendan for the inotropic effect was about 3 times higher than in isolated heart, being comparable to the effects in isolated human cardiac muscle strips. In canine ventricular trabeculae, the EC_{50} value of levosimendan to promote contraction was even higher.

Compartmentation of cAMP is thought to generate the specificity of G_s-coupled receptor activation in cardiac myocytes. The PDEs have a major role in this signal cascade by preventing cAMP diffusion. For example, selective inhibition of PDE3 by cilostamide and of PDE4 by Ro 20-1724 have both been shown to potentiate β_1-AR cAMP signals, whereas the Glu-R activated cAMP level was increased only by PDE4 inhibition. Furthermore, cAMP levels increased by PGE1-R and β_2-AR stimulation only when PDE3 and PDE4 were blocked (Rochais et al. 2006). In the recent study with cilostamide and Ro 20-1724 in adult rat ventricular myocytes, Leroy et al (Leroy et al. 2008) revealed that PDE3 inhibition has a direct inotropic effect on basal contractility and PDE4 inhibition causes inotropic responses in the presence of β-AR stimulation. Thus, they hypothetized that PDE3 can regulate a "constitutive" cAMP pool linked to contractility, whereas PDE4 regulates the cAMP microdomains mobilized by β-AR stimulation.

In study II, the selectivity factors for levosimendan and OR-1896 to inhibit PDE3 in preference to PDE4 were 10000 and 3000, respectively. The corresponding value for milrinone has been found to be only 14 (de Cheffoy de Courcelles et al. 1992). Leroy et al observed that when PDE4 was inhibited, PDE3 became the predominant enzyme to hydrolyze membrane and cytosolic cAMP, as well as regulating $I_{Ca,L}$ recovery (Leroy et al. 2008). Therefore, PDE4 seems to be able to compensate for PDE3 inhibition, at least in the cytosol and in sites near the L-type Ca^{2+} channels. Moreover, PDE4D subtype is related to the A kinase anchoring protein-organized signaling complexes (Dodge-Kafka et al. 2006), thus activation of PDE4 can be regulated by PKA. This could explain why KT5720 did not antagonize the effects of levosimendan, a very selective PDE3

inhibitor, and therefore, the role of PDE3 inhibition in levosimendan induced inotropy seems to be modest.

Thus, one could speculate that when only one phosphodiesterase isoenzyme is inhibited and one signal cascade is blunted, cAMP is presumably metabolized by the other phosphodiesterase isoenzymes and another inotropic signal cascade is active. In addition, PDE inhibitors with poor PDE selectivity have been shown to be more potent elevators of intracellular cAMP in cardiomyocytes, supporting the concept of additive effect on simultaneous activation of parallel inotropic signal cascades (Cone et al. 1999; Shakur et al. 2002). It is notable that in rapid pacing-induced heart failure in dog, PDE3 mRNA and activity had decreased, whereas PDE4D was unchanged, another indication that the role of PDE3 inhibition is not very significant in the effects of levosimendan (Smith et al. 1997). Moreover, dextrosimendan, the stereoisomer of levosimendan, had 47 times less positive inotropic effect in papillary muscle preparations and 76 times less effect on the contractile apparatus, the EC_{50}-values being comparable in permeabilized cardiomyocytes. However, the PDE3 inhibitory activities of the two stereoisomers differed by 427 fold (study III).

Furthermore, the positive inotropy by levosimendan was found to depend on the strength of electrical stimuli. This phenomenon was not seen with milrinone, indicating that it was not due to PDE inhibition and cAMP. However, the β_1-AR antagonist, atenolol, decreased the sensitivity of twitch tension to the stimulus strength. Thus, the high stimulus strength may have enhanced the release of noradrenaline from adrenergic nerve terminals, leading to activation of β_1-ARs and to the direct G_s-protein-mediated opening of calcium channels (Rochais et al. 2006). In this respect, a contributory role for PDE3 inhibition cannot be excluded. Otherwise, the high stimulus strength finally increases intracellular calcium transient, which in turn increases the number of activated cTnC molecules. Since the binding of levosimendan to cTnC is calcium-dependent, there will be an increased number of binding sites when more cTnC molecules become activated. Therefore, the calcium sensitization and subsequently the positive inotropy caused by levosimendan can be potentiated when a high stimulus strength is used. Moreover, forskolin activates AC and further increases cAMP and the intracellular calcium transient. Theoretically, selective inhibition of PDE3 should potentiate the stimulatory effect of forskolin on β_1-AR associated AC. However, milrinone, but not levosimendan, was able to potentiate the effect of forskolin. Alternatively, the increased intracellular calcium content may activate more cTnC molecules and this could then potentiate calcium sensitization evoked by levosimendan.

Isoprenaline, in turn, induces PKA-mediated phosphorylation of cTnI which leads to an alteration in the conformation of cTnC (Rapundalo et al. 1989; Wattanapermpool et al. 1995). This may inhibit the binding of levosimendan to cTnC and thereby antagonize its positive inotropy. The present study with isoprenaline showed that the positive inotropy induced by levosimendan at the concentration giving a maximum effect was decreased by 60–70% in isoprenaline-pretreated muscles. In contrast, the inotropic response to milrinone was not altered by similar isoprenaline pretreatment. Thus, cTnC seems to be the target protein for levosimendan also in the intact muscle and most of its positive inotropic properties may then be due to calcium sensitization. Furthermore, when levosimendan was added to the buffer, the positive inotropy evoked by isoprenaline had completely expired due to its wash-out. Therefore, the effect of levosimendan was affected only by the altered phosphorylation of contractile proteins and no longer by the increased intracellular calcium transient. When forskolin and levosimendan were investigated simultaneously, also the increased calcium transient induced by forskolin influenced the response to levosimendan. These differential experimental results explain why the action of levosimendan was altered in different ways in the forskolin and isoprenaline studies.

Acceleration of the relaxation induced by levosimendan could be due to cAMP accumulation induced by PDE3 inhibition, because like the contraction, it is inhibited by carbachol (Boknik et al. 1997; Sato et al. 1998). However, the present study revealed that in the presence of the PKA inhibitor, KT5720, levosimendan did not impair relaxation but increased the negative dP/dt_{max}. The milrinone-induced increase in the negative dP/dt_{max} was instead markedly antagonised by KT5720. However, the effect was not completely abolished probably because of the increase in HR, which was not abolished by KT5720 (study I). Moreover, levosimendan does not impair relaxation either under normal conditions or during acidosis (Takahashi and Endoh 2005). In the failing heart, cAMP-mediated signaling is weakened (Feldman et al. 1987), and therefore, it is probable that the increase in Ca^{2+}-sensitivity could be more pronounced than any PDE3 inhibitory action in failing ventricular myocardium (Hasenfuss et al. 1998). Furthermore, during acidosis, where cAMP loses its effectiveness, lack of impairment of relaxation due to the Ca^{2+}-dependent nature of the binding of levosimendan to troponin C is even more pronounced than in normal conditions (Haikala et al. 1995).

6.2 Vasodilatory effects

In the present study, levosimendan increased coronary flow both in the spontaneously beating heart (study I) and in the overpaced heart (study V). In their classical work, Braunwald et al (Braunwald

et al. 1958) demonstrated that oxygen consumption (MVO_2) is the major determinant of coronary blood flow. Heart rate (HR), in turn, is one of the main determinants of MVO_2. Therefore, any increase in HR is in proportion to the increase in CF and MVO_2. Even though levosimendan increased HR and CF in a parallel manner in the spontaneous beating hearts, MVO_2 was not increased in parallel with CF after levosimendan administration in the paced hearts. Thus, levosimendan had a direct vasodilatory effect on coronary veins. Moreover, the PKG inhibitor, KT5823 potentiated the levosimendan- but not the milrinone-induced increase in CF i.e. the coronary dilatory mechanism of action of levosimendan differs from that of milrinone and it does not seem to be mediated through the activation of PKG. The K^+ current through the delayed rectifier K^+ channels should be limited during PKG inhibition since these channels are normally activated by PKG (Sperelakis et al. 1994; Ko et al. 2008). In this situation, the efflux of K^+ ions may occur more through the K_{ATP} channels, which may potentiate any vasodilatory effect mediated through these channels.

Patch clamp studies in rat mesenteric arterial cells have revealed that levosimendan can open K_{ATP} channels (Yokoshiki et al. 1997). In study IV, it was shown that glibenclamide antagonized the increase in DCFV induced by levosimendan, demonstrating that the opening of the K_{ATP} channels was the mechanism of action of the observed vasodilatory effect. Pinacidil was three times more efficacious and ten times less potent than levosimendan at increasing DCFV. Moreover, glibenclamide antagonized the coronary dilation induced by levosimendan and pinacidil in two different ways, suggesting that these two drugs may have different binding sites on the K_{ATP} channels. The effect of pinacidil was antagonized competitively by glibenclamide, as expected (Nielsen-Kudsk et al. 1996), whereas levosimendan was inhibited in a non-competitive manner. One possible explanation for this result is that levosimendan opens the channel indirectly or, alternatively, interacts with different subtypes of glibenclamide-sensitive channels (Quast and Cook 1989; Quast et al. 1994; Adebiyi et al. 2008). However, the effect of levosimendan on K_{ATP} channel was also revealed previously in single-channel recordings (Yokoshiki et al. 1997), which suggests that it does have a direct effect. An alternative explanation is that levosimendan has another vasodilatory mechanism.

As previously mentioned, the specific BK_{Ca} channel blocker, IBTX suppressed the maximum effect of levosimendan, and the KV channel blocker, 4-AP significantly shifted the concentration-response curve to the right in isolated porcine epicardial coronary arteries, indicating that the vasorelaxing mechanism of levosimendan involves the activation of voltage-sensitive and, at large concentrations, of calcium-activated potassium channels (Pataricza et al. 2003). In addition,

levosimendan is believed to be able to relax porcine coronary arteries through calcium desensitization, interacting with smooth muscle EF-hand proteins, such as, calmodulin, the regulatory myosin light chains, or S100 proteins (Bowman et al. 1999). This effect was seen at ten times higher concentrations (0.1–10 μM) than those used in the present study (0.01–1 μM). However, this could explain the non-competitive inhibitory mode of action of glibenclamide on the increase in DCFV by levosimendan.

The role of PDE3 inhibition cannot be totally excluded, since levosimendan has been shown to potentiate the relaxant effect of cAMP-stimulation evoked by isoprenaline (Gruhn et al. 1998). On the other hand, the PKA inhibitor, KT5720 did not antagonize the effect of levosimendan on CF (study I). Nevertheless, the effect of milrinone on CF was also not antagonized by KT5720. This can be explained by the differences in the MVO_2 of the contraction-relaxation cycle between levosimendan and milrinone. In isolated heart, milrinone increased CF in parallel with MVO_2, in contrast to levosimendan, which increased CF independently of MVO_2 (study V). In addition, in patch clamp studies, a non-specific protein kinase inhibitor, H-7 did not prevent the hyperpolarisation and stimulation of K^+ current by levosimendan in mesenteric arterial myocytes (Yokoshiki et al. 1997). Thus, it seems unlikely that PDE3 inhibition plays any major role in the vasodilatory effects of levosimendan.

The concentration response of levosimendan was markedly potentiated in the presence of the PKC inhibitor, bisindolylmaleimide (BIM). PKC is a mediator of the effects of several vasoconstrictors on K^+ channels (Ko et al. 2008) and it is known that inhibition of the PKC-mediated phosphorylation of the K_{ATP} channels in the isolated rabbit heart can evoke a vasodilatory effect (Pongo et al. 2001). Therefore, it could be hypothesized that the potentiation of the increase in DCFV was due to a specific binding and action of levosimendan on the non-phosphorylated form of the K_{ATP} channels. In the present study, glibenclamide did not antagonize the effect of levosimendan on the contractile parameters of the heart, indicating that its action on the K_{ATP} channels was not indirectly mediated through the changes in the contraction parameters. Rather it is directly mediated via the coronary vasculature smooth muscle relaxation, since glibenclamide markedly antagonized the levosimendan-induced increase in DCFV. On the contrary, the negative inotropic responses of pinacidil were shown to competitively antagonized by glibenclamide (Lau 1992).

In addition, K_{ATP} channel activation is an important mediator of ischemic preconditioning (Gross and Auchampach 1992; O'Rourke 2004). The stimulation of K_{ATP} channels has been shown to

decrease myocardial infarct size (Gross and Auchampach 1992) and it can also enhance recovery of the stunned myocardium (Yao and Gross 1994). Moreover, the studies of Kersten et al. (Kersten et al. 2000) showed that levosimendan decreased the myocardial infarct size in coronary-ligated dogs and that the effect was mediated through K_{ATP} channels. Although, vasodilatation may be beneficial in ischemia, it seems that mitoK_{ATP} channels are especially linked to the cardioprotective effects (Nishida et al. 2009). The effects of levosimendan on ischemia-reperfusion were not studied in the present experiments. However, we have noticed in guinea-pigs, that levosimendan could prevent the myocardial stunning in the isolated, Langendorff-perfused heart after global ischemia (Kaheinen, unpublished data).

6.3 Limitations of the methods and proposal for future studies

Isolated Langendorff-perfused heart was the most widely used preparation used in this study. It was also the most complex method and has a number of limitations, which have been previously reviewed extensively (Sutherland and Hearse 2000). Briefly, the lack of any oxygen carrier in the salt solution requires the use of high oxygen partial pressure to saturate the perfusion solvent and this causes considerably higher basal coronary flow than that normally present *in vivo*. A consequence of this unnatural coronary perfusion is a deterioration of the contractile function during the experiment. In study IV, the incomplete oxygen supply was seen as a decrease in basal CF in the presence of glibenclamide, indicating at least partially open K_{ATP} channels. Moreover, use of salt-base buffer lacking plasma proteins can cause edema, which could induce a decrease in coronary flow. It took at least 1-2 h to reach a stable function of the heart and achieve a balance between oxygen supply and need (seen as stable coronary flow, oxygen consumption, and contractility parameters) in the present study. Subsequently, it was found that addition of pyruvate as well as glucose in the perfusate could shorten the stabilization period, but pyruvate was not used in the present study. Furthermore, in constant flow mode, unlike constant pressure perfusion, autoregulatory mechanisms are bypassed, which does not automatically ensure that the perfusate would be delivered uniformly to the whole heart as it responds to changes in heart rate or work. In addition, in the intact heart, the atria and sino-atrial node are not perfused by coronary vessels but by extracardiac vessels, which are damaged when the heart is excised for perfusion. This makes it difficult to maintain essential oxygen supply for the sino-atrial node and a stable heart rate. However, this problem can be avoided by pacing the heart, which was done in the part of the study (studies II and V).

Although, the stature of PDE3 inhibition on the inotropic and vasodilatory effects of levosimendan was shown to be slight in the experimental settings used in the present study, its final status still remains unclear. In addition, the intracellular calcium content was not measured. Simultaneous measurement of Ca^{2+} transient with alteration of contractile function in the isolated heart perhaps would have made it possible to elucidate the mode of action of levosimendan, especially the role of PDE inhibition. Interestingly, Sun et al (Sun et al. 2007) have recently published a study using PDE3A and PDE3B gene knockout (KO) mice. They proposed that PDE3A is the main subtype of PDE3 expressed in cardiac ventricular myocytes, and that it is responsible for the functional changes caused by PDE3 inhibition. Thus, further studies are warranted to examine the effects of levosimendan in these mice, though the species differences may complicate comparisons with the present studies. In addition, studies examining the mechanism of action behind the stroke-preventive effects of levosimendan would be scientifically interesting, in addition to being beneficial for future drug design.

6.4 Overall clinical aspects

In drug development, two characteristics come before all others, efficacy and safety in patients. In clinical use, the originally designed indication may be changed and/or new off-label indications stand out. That is what has happened to levosimendan. As mentioned above, it is approved for short-term treatment of acutely decompensated chronic heart failure. However, in the clinic, levosimendan has been demonstrated to have beneficial effects in ischemic heart disease and also against septic shock. It has been also demonstrated that levosimendan can be used as a bridge therapy for the perioperative cardiac support e.g. it facilitates weaning of patients from cardiopulmonary bypass (Eriksson et al. 2009). The last-mentioned indication is possible because of the ability of levosimendan to improve myocardial function without substantially increasing oxygen consumption.

7 SUMMARY AND CONCLUSIONS

The mechanisms of action of an inodilator, levosimendan, were studied in isolated guinea-pig heart preparations. Levosimendan was shown to be a potent inotropic agent in isolated Langendorff-perfused heart and right ventricle papillary muscle. In permeabilized cardiomyocytes, it was proved to be a potent calcium sensitizer in contrast to its positive enantiomer, dextrosimendan. Despite its very selective PDE3 inhibitory properties in purified enzyme preparations, the inotropic effect of levosimendan was observed to be mainly mediated through calcium sensitization also in the isolated heart as well as the papillary muscle preparations at clinically relevant concentrations. The effects of levosimendan differed considerably from the effects of milrinone. In particular, the energy efficiency of the contraction was shown to be advantageous in levosimendan-perfused hearts compared to milrinone-perfused hearts. Levosimendan binds to cardiac troponin C in a calcium-dependent manner and thus, does not impair the relaxation, as confirmed in the present study. Furthermore, the vasodilatory effect of levosimendan was shown to be mediated mostly through the opening of the K_{ATP} channels in the isolated heart.

The main findings and conclusions of the present study are as follows:

1. The main inotropic and vasodilatory mechanisms of action of levosimendan are not related to the PKA mediated phosphorylation.

2. Levosimendan and OR-1896 are equally potent as positive inotropes and Ca^{2+}-sensitizers and reveal highly selective PDE3 inhibitory properties.

3. Levosimendan and its positive stereoisomer, dextrosimendan seem to exert their positive inotropic effects via a stereoselective Ca^{2+}-sensitizing mechanism and not via stereoselective inhibition of PDE3.

4. The main vasodilatory mechanism in coronary veins is the opening of the K_{ATP} channels in the vascular smooth muscle.

5. The inotropic mechanism of levosimendan is energetically more advantageous than the mechanism of the PDE inhibitor milrinone, this being seen as low oxygen consumption in relation to the produced force of contraction.

8 ACKNOWLEDGEMENTS

This study was carried out in the Nonclinical Cardiovascular Research in Orion Pharma.

First, I wish to express my warm gratitude to my supervisor, Professor Eero Mervaala. His support and inspiration helped encourage me to complete my doctoral studies. He is a font of knowledge in the field of cardiovascular research.

My warmest thanks are expressed also to my other supervisor, Docent Piero Pollesello. His belief that publication is necessary also in R&D in the pharmaceutical industry and that peer review guarantee the quality of the research were essential for this academic dissertation.

I owe my respectful acknowledgement to Professor Raimo Tuominen who was the originator of the topic investigated in this thesis and whose appreciation of the sub-studies convinced me that they fulfil the academic requirements.

I would like to express my sincere gratitude to Senior Lecturer, Doctor Ewen MacDonald and Docent Veli-Pekka Harjola, the reviewers appointed by the Medical Faculty of the University of Helsinki, for their valuable criticism and comments.

I would like to express my heartfelt gratitude to Doctor Heimo Haikala, previous Director of the Nonclinical Cardiovascular Research of Orion Pharma and the “father of levosimendan”. His innovative way of thinking and refusal to bow to anything other than the truth, created a scientific atmosphere in the laboratory.

I am grateful to my colleague and later Head of the Cardiovascular Research, Master of Pharmacology Jouko Levijoki. His inspiring attitude and tolerant way of management, led to a good spirit in the laboratory.

I would also like to express my gratitude to Docent Pentti Pohto, previous Research Director, to Docent Inge-Britt Lindén, previous Registration Director and to Docent Lasse Lehtonen, previous Director of the levosimendan project for their visionary leadership and their strong faith in levosimendan.

Particularly, I would like to thank Ms Ritva Huuhilo, Mr Heikki Olkkonen and Mr Timo Oksanen for their skilful technical assistance. In addition to their accurate work, their professional criticism and the methodological advances which they made have guaranteed the reliability of the results.

My special thanks goes to everyone who participated in the levosimendan and other cardiovascular projects in Orion Pharma, especially to Mr Juha Kaivola, a dyed-in-the-wool biochemist, to Docent Erkki Nissinen, leading biochemist, to Doctor Kristiina Haasio, responsible veterinarian, to Doctor Jukka Tenhunen, expert in genetics, to Doctor Piet Finckenberg, expert in pathology, to Docent Carola Tilgmann, expert in molecular biology, to Mrs Ulla Kotro-Kervinen, previous Head of the laboratory animal unit, to Doctor Matti Kivikko, physican and the previous leader of the clinical research of levosimendan and to numerous other individuals in Orion Pharma.

I would also like to thank all of my friends and colleagues in the former Nonclinical Cardiovascular Research for inspiring conversations: Mrs Maria Arraño-Marchant, Docent Mikko Ares, Mrs Minja Hyttilä-Hopponen, Mrs Reetta Junnila, Ms Sanna Kärkkäinen, Mrs Ulla-Riikka Laitinen, Mrs Päivi Leinikka, Docent Ken Lindstedt, Ms Tiina Oksala, Mrs Saara-Elisa Peltokorpi, Mr Miikka Tarkia and Mrs Mari Virtanen.

I wish also to express my warm gratitude to Mrs Eeva Harju for the practical arrangements.

Finally, I would like to give my loving thanks to my family, my wife Hanna and my children Kaisla and Juho for their patience, especially during the hectic writing period over the past months.

9 REFERENCES

Abraham, M., S. Scott, A. Meltzer and F. Barrueto (2009). Levosimendan does not improve survival time in a rat model of verapamil toxicity. *J Med Toxicol* **5**(1): 3-7.

Abraham, W. T., K. F. Adams, G. C. Fonarow, M. R. Costanzo, R. L. Berkowitz, T. H. LeJemtel, M. L. Cheng and J. Wynne (2005). In-hospital mortality in patients with acute decompensated heart failure requiring intravenous vasoactive medications: an analysis from the Acute Decompensated Heart Failure National Registry (ADHERE). *J Am Coll Cardiol* **46**(1): 57-64.

Adebiyi, A., E. M. McNally and J. H. Jaggar (2008). Sulfonylurea receptor-dependent and -independent pathways mediate vasodilation induced by ATP-sensitive K+ channel openers. *Mol Pharmacol* **74**(3): 736-43.

Aguilar-Bryan, L., J. P. t. Clement, G. Gonzalez, K. Kunjilwar, A. Babenko and J. Bryan (1998). Toward understanding the assembly and structure of KATP channels. *Physiol Rev* **78**(1): 227-45.

Alajoutsijarvi, A. and E. Nissinen (1987). Determination of cyclic nucleotide phosphodiesterase activity by high-performance liquid chromatography. *Anal Biochem* **165**(1): 128-32.

Alla, F., F. Zannad and G. Filippatos (2007). Epidemiology of acute heart failure syndromes. *Heart Fail Rev* **12**(2): 91-5.

Amidon, T. M. and W. W. Parmley (1994). Is there a role for positive inotropic agents in congestive heart failure: focus on mortality. *Clin Cardiol* **17**(12): 641-7.

Amin, A. (2008). Hospitalized patients with acute decompensated heart failure: recognition, risk stratification, and treatment review. *J Hosp Med* **3**(6 Suppl): S16-24.

Antila, S., J. Eha, M. Heinpalu, L. Lehtonen, I. Loogna, A. Mesikepp, U. Planken and E. P. Sandell (1996). Haemodynamic interactions of a new calcium sensitizing drug levosimendan and captopril. *Eur J Clin Pharmacol* **49**(6): 451-8.

Antila, S., T. Honkanen, L. Lehtonen and P. J. Neuvonen (1998). The CYP3A4 inhibitor intraconazole does not affect the pharmacokinetics of a new calcium-sensitizing drug levosimendan. *Int J Clin Pharmacol Ther* **36**(8): 446-9.

Antila, S., A. Jarvinen, J. Akkila, T. Honkanen, M. Karlsson and L. Lehtonen (1997). Studies on psychomotoric effects and pharmacokinetic interactions of the new calcium sensitizing drug levosimendan and ethanol. *Arzneimittelforschung* **47**(7): 816-20.

Antila, S., A. Jarvinen, T. Honkanen and L. Lehtonen (2000). Pharmacokinetic and pharmacodynamic interactions between the novel calcium sensitiser levosimendan and warfarin. *Eur J Clin Pharmacol* **56**(9-10): 705-10.

Antila, S., S. Sundberg and L. A. Lehtonen (2007). Clinical pharmacology of levosimendan. *Clin Pharmacokinet* **46**(7): 535-52.

Antoniades, C., D. Tousoulis, N. Koumallos, K. Marinou and C. Stefanadis (2007). Levosimendan: beyond its simple inotropic effect in heart failure. *Pharmacol Ther* **114**(2): 184-97.

Banerjee, P., A. L. Clark, N. Nikitin and J. G. Cleland (2004). Diastolic heart failure. Paroxysmal or chronic? *Eur J Heart Fail* **6**(4): 427-31.

Banfor, P. N., L. C. Preusser, T. J. Campbell, K. C. Marsh, J. S. Polakowski, G. A. Reinhart, B. F. Cox and R. M. Fryer (2008). Comparative effects of levosimendan, OR-1896, OR-1855, dobutamine, and milrinone on vascular resistance, indexes of cardiac function, and O2 consumption in dogs. *Am J Physiol Heart Circ Physiol* **294**(1): H238-48.

Benza, R. L., J. A. Tallaj, G. M. Felker, K. M. Zabel, W. Kao, R. C. Bourge, D. Pearce, J. D. Leimberger, S. Borzak, M. O'Connor C and M. Gheorghiade (2004). The impact of arrhythmias in acute heart failure. *J Card Fail* **10**(4): 279-84.

Blair, J. E., C. Macarie, W. Ruzyllo, A. Bacchieri, G. Valentini, M. Bianchetti, P. S. Pang, M. E. Harinstein, H. N. Sabbah, G. S. Filippatos and M. Gheorghiade (2008). Rationale and design of the hemodynamic, echocardiographic and neurohormonal effects of istaroxime, a novel intravenous inotropic and lusitropic agent: a randomized controlled trial in patients hospitalized with heart failure (HORIZON-HF) trial. *Am J Ther* **15**(3): 231-40.

Boknik, P., J. Neumann, G. Kaspareit, W. Schmitz, H. Scholz, U. Vahlensieck and N. Zimmermann (1997). Mechanisms of the contractile effects of levosimendan in the mammalian heart. *J Pharmacol Exp Ther* **280**(1): 277-83.

Bowman, P., H. Haikala and R. J. Paul (1999). Levosimendan, a calcium sensitizer in cardiac muscle, induces relaxation in coronary smooth muscle through calcium desensitization. *J Pharmacol Exp Ther* **288**(1): 316-25.

Bragadeesh, T. K., G. Mathur, A. L. Clark and J. G. Cleland (2007). Novel cardiac myosin activators for acute heart failure. *Expert Opin Investig Drugs* **16**(10): 1541-8.

Braunwald, E. (1992). HEART DISEASE, A Textbook of Cardiovascular Medicide. Philadelphia, W.B. Saunders Company.

Braunwald, E., S. J. Sarnoff, R. B. Case, W. N. Stainsby and G. H. Welch, Jr. (1958). Hemodynamic determinants of coronary flow: effect of changes in aortic pressure and cardiac output on the relationship between myocardial oxygen consumption and coronary flow. *Am J Physiol* **192**(1): 157-63.

Brayden, J. E. (2002). Functional roles of KATP channels in vascular smooth muscle. *Clin Exp Pharmacol Physiol* **29**(4): 312-6.

Bristow, M. R., S. F. Shakar, J. V. Linseman and B. D. Lowes (2001). Inotropes and beta-blockers: is there a need for new guidelines? *J Card Fail* **7**(2 Suppl 1): 8-12.

Brophy, J. M., G. Deslauriers and J. L. Rouleau (1994). Long-term prognosis of patients presenting to the emergency room with decompensated congestive heart failure. *Can J Cardiol* **10**(5): 543-7.

Butler, J., D. E. Forman, W. T. Abraham, S. S. Gottlieb, E. Loh, B. M. Massie, C. M. O'Connor, M. W. Rich, L. W. Stevenson, Y. Wang, J. B. Young and H. M. Krumholz (2004). Relationship between heart failure treatment and development of worsening renal function among hospitalized patients. *Am Heart J* **147**(2): 331-8.

Cahn, R. S., C. Ingold and V. Prelog (1966). Specification of Molecular Chirality. *Angew. Chem. internat. Edit* **5**(4): 385-415.

Califf, R. M. and J. R. Bengtson (1994). Cardiogenic shock. *N Engl J Med* **330**(24): 1724-30.

Cleland, J. G., J. C. Daubert, E. Erdmann, N. Freemantle, D. Gras, L. Kappenberger, W. Klein and L. Tavazzi (2001). The CARE-HF study (CArdiac REsynchronisation in Heart Failure study): rationale, design and end-points. *Eur J Heart Fail* **3**(4): 481-9.

Cleland, J. G., J. C. Daubert, E. Erdmann, N. Freemantle, D. Gras, L. Kappenberger and L. Tavazzi (2005). The effect of cardiac resynchronization on morbidity and mortality in heart failure. *N Engl J Med* **352**(15): 1539-49.

Cleland, J. G., N. Freemantle, A. P. Coletta and A. L. Clark (2006). Clinical trials update from the American Heart Association: REPAIR-AMI, ASTAMI, JELIS, MEGA, REVIVE-II, SURVIVE, and PROACTIVE. *Eur J Heart Fail* **8**(1): 105-10.

Cleland, J. G., J. Ghosh, N. Freemantle, G. C. Kaye, M. Nasir, A. L. Clark and A. P. Coletta (2004). Clinical trials update and cumulative meta-analyses from the American College of Cardiology: WATCH, SCD-HeFT, DINAMIT, CASINO, INSPIRE, STRATUS-US, RIO-Lipids and cardiac resynchronisation therapy in heart failure. *Eur J Heart Fail* **6**(4): 501-8.

Colucci, W. S. (2001). Nesiritide for the treatment of decompensated heart failure. *J Card Fail* **7**(1): 92-100.

Cone, J., S. Wang, N. Tandon, M. Fong, B. Sun, K. Sakurai, M. Yoshitake, J. Kambayashi and Y. Liu (1999). Comparison of the effects of cilostazol and milrinone on intracellular cAMP levels and cellular function in platelets and cardiac cells. *J Cardiovasc Pharmacol* **34**(4): 497-504.

Cotter, G., G. M. Felker, K. F. Adams, O. Milo-Cotter and C. M. O'Connor (2008). The pathophysiology of acute heart failure--is it all about fluid accumulation? *Am Heart J* **155**(1): 9-18.

Cotter, G., J. Weissgarten, E. Metzkor, Y. Moshkovitz, I. Litinski, U. Tavori, C. Perry, R. Zaidenstein and A. Golik (1997). Increased toxicity of high-dose furosemide versus low-dose dopamine in the treatment of refractory congestive heart failure. *Clin Pharmacol Ther* **62**(2): 187-93.

de Cheffoy de Courcelles, D., K. de Loore, E. Freyne and P. A. Janssen (1992). Inhibition of human cardiac cyclic AMP-phosphodiesterases by R 80122, a new selective cyclic AMP-phosphodiesterase III inhibitor: a comparison with other cardiotonic compounds. *J Pharmacol Exp Ther* **263**(1): 6-14.

Delle Karth, G., A. Buberl, A. Geppert, T. Neunteufl, M. Huelsmann, C. Kopp, M. Nikfardjam, R. Berger and G. Heinz (2003). Hemodynamic effects of a continuous infusion of levosimendan in critically ill patients with cardiogenic shock requiring catecholamines. *Acta Anaesthesiol Scand* **47**(10): 1251-6.

DeWitt, C. R. and J. C. Waksman (2004). Pharmacology, pathophysiology and management of calcium channel blocker and beta-blocker toxicity. *Toxicol Rev* **23**(4): 223-38.

Dickstein, K., A. Cohen-Solal, G. Filippatos, J. J. McMurray, P. Ponikowski, P. A. Poole-Wilson, A. Stromberg, D. J. van Veldhuisen, D. Atar, A. W. Hoes, A. Keren, A. Mebazaa, M. Nieminen, S. G. Priori, K. Swedberg, A. Vahanian, J. Camm, R. De Caterina, V. Dean, C. Funck-Brentano, I. Hellemans, S. D. Kristensen, K. McGregor, U. Sechtem, S. Silber, M. Tendera, P. Widimsky and J. L. Zamorano (2008). ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). *Eur Heart J* **29**(19): 2388-442.

Dodge-Kafka, K. L., L. Langeberg and J. D. Scott (2006). Compartmentation of cyclic nucleotide signaling in the heart: the role of A-kinase anchoring proteins. *Circ Res* **98**(8): 993-1001.

Donal, E., L. H. Lund, C. Linde, M. Edner, S. Lafitte, H. Persson, F. Bauer, J. Ohrvik, P. V. Ennezat, C. Hage, I. Lofman, Y. Juilliere, D. Logeart, G. Derumeaux, P. Gueret and J. C. Daubert (2009).

Rationale and design of the Karolinska-Rennes (KaRen) prospective study of dyssynchrony in heart failure with preserved ejection fraction. *Eur J Heart Fail* **11**(2): 198-204.

Duygu, H., S. Nalbantgil, F. Ozerkan, M. Zoghi, A. Akilli, U. Erturk, M. Akin, C. Nazli and O. Ergene (2008). Effects of levosimendan on left atrial functions in patients with ischemic heart failure. *Clin Cardiol* **31**(12): 607-13.

Elkayam, U., M. Janmohamed, M. Habib and P. Hatamizadeh (2008). Vasodilators in the management of acute heart failure. *Crit Care Med* **36**(1 Suppl): S95-105.

Endoh, M. (1995). The effects of various drugs on the myocardial inotropic response. *Gen Pharmacol* **26**(1): 1-31.

Endoh, M. (2002). Mechanisms of action of novel cardiotonic agents. *J Cardiovasc Pharmacol* **40**(3): 323-38.

Endoh, M. (2008). Cardiac Ca2+ signaling and Ca2+ sensitizers. *Circ J* **72**(12): 1915-25.

Erbuyun, K., S. Vatansever, D. Tok, G. Ok, E. Turkoz, H. Aydede, Y. Erhan and I. Tekin (2009). Effects of levosimendan and dobutamine on experimental acute lung injury in rats. *Acta Histochem* **111**(5): 404-14.

Erdei, N., Z. Papp, P. Pollesello, I. Edes and Z. Bagi (2006). The levosimendan metabolite OR-1896 elicits vasodilation by activating the K(ATP) and BK(Ca) channels in rat isolated arterioles. *Br J Pharmacol* **148**(5): 696-702.

Eriksson, H. I., J. R. Jalonen, L. O. Heikkinen, M. Kivikko, M. Laine, K. A. Leino, A. H. Kuitunen, K. T. Kuttila, T. K. Perakyla, T. Sarapohja, R. T. Suojaranta-Ylinen, M. Valtonen and M. T. Salmenpera (2009). Levosimendan facilitates weaning from cardiopulmonary bypass in patients undergoing coronary artery bypass grafting with impaired left ventricular function. *Ann Thorac Surg* **87**(2): 448-54.

Fabiato, A. (1981). Myoplasmic free calcium concentration reached during the twitch of an intact isolated cardiac cell and during calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned cardiac cell from the adult rat or rabbit ventricle. *J Gen Physiol* **78**(5): 457-97.

Fabiato, A. and F. Fabiato (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J Physiol (Paris)* **75**(5): 463-505.

Feldman, M. D., L. Copelas, J. K. Gwathmey, P. Phillips, S. E. Warren, F. J. Schoen, W. Grossman and J. P. Morgan (1987). Deficient production of cyclic AMP: pharmacologic evidence of an important cause of contractile dysfunction in patients with end-stage heart failure. *Circulation* **75**(2): 331-9.

Felker, G. M., W. A. Gattis, J. D. Leimberger, K. F. Adams, M. S. Cuffe, M. Gheorghiade and C. M. O'Connor (2003). Usefulness of anemia as a predictor of death and rehospitalization in patients with decompensated heart failure. *Am J Cardiol* **92**(5): 625-8.

Fischmeister, R., L. R. Castro, A. Abi-Gerges, F. Rochais, J. Jurevicius, J. Leroy and G. Vandecasteele (2006). Compartmentation of cyclic nucleotide signaling in the heart: the role of cyclic nucleotide phosphodiesterases. *Circ Res* **99**(8): 816-28.

Flaherty, J. D., J. J. Bax, L. De Luca, J. S. Rossi, C. J. Davidson, G. Filippatos, P. P. Liu, M. A. Konstam, B. Greenberg, M. R. Mehra, G. Breithardt, P. S. Pang, J. B. Young, G. C. Fonarow, R. O. Bonow and M. Gheorghiade (2009). Acute heart failure syndromes in patients with coronary artery disease early assessment and treatment. *J Am Coll Cardiol* **53**(3): 254-63.

Follath, F., J. G. Cleland, H. Just, J. G. Papp, H. Scholz, K. Peuhkurinen, V. P. Harjola, V. Mitrovic, M. Abdalla, E. P. Sandell and L. Lehtonen (2002). Efficacy and safety of intravenous levosimendan compared with dobutamine in severe low-output heart failure (the LIDO study): a randomised double-blind trial. *Lancet* **360**(9328): 196-202.

Fonarow, G. C., K. F. Adams, Jr., W. T. Abraham, C. W. Yancy and W. J. Boscardin (2005). Risk stratification for in-hospital mortality in acutely decompensated heart failure: classification and regression tree analysis. *Jama* **293**(5): 572-80.

Frodsham, G. and R. B. Jones (1992). Effect of flosequinan upon isoenzymes of phosphodiesterase from guinea-pig cardiac and vascular smooth muscle. *Eur J Pharmacol* **211**(3): 383-91.

Fuhrmann, J. T., A. Schmeisser, M. R. Schulze, C. Wunderlich, S. P. Schoen, T. Rauwolf, C. Weinbrenner and R. H. Strasser (2008). Levosimendan is superior to enoximone in refractory cardiogenic shock complicating acute myocardial infarction. *Crit Care Med* **36**(8): 2257-66.

Fukuma, N., K. Oikawa, N. Aisu, K. Kato, Y. K. Kimura-Kato, T. Tuchida, K. Mabuchi and T. Takano (2004). Impaired baroreflex as a cause of chronotropic incompetence during exercise via autonomic mechanism in patients with heart disease. *Int J Cardiol* **97**(3): 503-8.

Gandhi, S. K., J. C. Powers, A. M. Nomeir, K. Fowle, D. W. Kitzman, K. M. Rankin and W. C. Little (2001). The pathogenesis of acute pulmonary edema associated with hypertension. *N Engl J Med* **344**(1): 17-22.

Garlid, K. D., P. Paucek, V. Yarov-Yarovoy, H. N. Murray, R. B. Darbenzio, A. J. D'Alonzo, N. J. Lodge, M. A. Smith and G. J. Grover (1997). Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K+ channels. Possible mechanism of cardioprotection. *Circ Res* **81**(6): 1072-82.

Gheorghiade, M., J. E. Blair, G. S. Filippatos, C. Macarie, W. Ruzyllo, J. Korewicki, S. I. Bubenek-Turconi, M. Ceracchi, M. Bianchetti, P. Carminati, D. Kremastinos, G. Valentini and H. N. Sabbah (2008). Hemodynamic, echocardiographic, and neurohormonal effects of istaroxime, a novel intravenous inotropic and lusitropic agent: a randomized controlled trial in patients hospitalized with heart failure. *J Am Coll Cardiol* **51**(23): 2276-85.

Gheorghiade, M., L. De Luca, G. C. Fonarow, G. Filippatos, M. Metra and G. S. Francis (2005a). Pathophysiologic targets in the early phase of acute heart failure syndromes. *Am J Cardiol* **96**(6A): 11G-17G.

Gheorghiade, M., W. Gattis Stough, K. F. Adams, Jr., A. S. Jaffe, V. Hasselblad and C. M. O'Connor (2005b). The Pilot Randomized Study of Nesiritide Versus Dobutamine in Heart Failure (PRESERVD-HF). *Am J Cardiol* **96**(6A): 18G-25G.

Gheorghiade, M., C. Orlandi, J. C. Burnett, D. Demets, L. Grinfeld, A. Maggioni, K. Swedberg, J. E. Udelson, F. Zannad, C. Zimmer and M. A. Konstam (2005c). Rationale and design of the multicenter, randomized, double-blind, placebo-controlled study to evaluate the Efficacy of Vasopressin antagonism in Heart Failure: Outcome Study with Tolvaptan (EVEREST). *J Card Fail* **11**(4): 260-9.

Goldberg, R. J., J. Ciampa, D. Lessard, T. E. Meyer and F. A. Spencer (2007). Long-term survival after heart failure: a contemporary population-based perspective. *Arch Intern Med* **167**(5): 490-6.

Gordon, A. M., E. Homsher and M. Regnier (2000). Regulation of contraction in striated muscle. *Physiol Rev* **80**(2): 853-924.

Gordon, A. M., M. Regnier and E. Homsher (2001). Skeletal and cardiac muscle contractile activation: tropomyosin "rocks and rolls". *News Physiol Sci* **16**: 49-55.

Gordon, S. G., M. W. Miller and A. B. Saunders (2006). Pimobendan in heart failure therapy--a silver bullet? *J Am Anim Hosp Assoc* **42**(2): 90-3.

Gorelik, O., D. Almoznino-Sarafian, M. Shteinshnaider, I. Alon, I. Tzur, I. Sokolsky, S. Efrati, Z. Babakin, D. Modai and N. Cohen (2009). Clinical variables affecting survival in patients with decompensated diastolic versus systolic heart failure. *Clin Res Cardiol*.

Gould, P. A., M. D. Esler and D. M. Kaye (2008). Atrial fibrillation is associated with decreased cardiac sympathetic response to isometric exercise in CHF in comparison to sinus rhythm. *Pacing Clin Electrophysiol* **31**(9): 1125-9.

Gross, G. J. and J. A. Auchampach (1992). Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res* **70**(2): 223-33.

Grover, G. J. and K. D. Garlid (2000). ATP-Sensitive potassium channels: a review of their cardioprotective pharmacology. *J Mol Cell Cardiol* **32**(4): 677-95.

Gruhn, N., J. E. Nielsen-Kudsk, S. Theilgaard, L. Bang, S. P. Olesen and J. Aldershvile (1998). Coronary vasorelaxant effect of levosimendan, a new inodilator with calcium-sensitizing properties. *J Cardiovasc Pharmacol* **31**(5): 741-9.

Haikala, H. and I. B. Linden (1995). Mechanisms of action of calcium-sensitizing drugs. *J Cardiovasc Pharmacol* **26 Suppl 1**: S10-9.

Haikala, H., E. Nissinen, E. Etemadzadeh, J. Levijoki and I. B. Linden (1995). Troponin C-mediated calcium sensitization induced by levosimendan does not impair relaxation. *J Cardiovasc Pharmacol* **25**(5): 794-801.

Harjola, V. P., S. Costa, R. Sund, S. Ylikangas, K. Siirila-Waris, J. Melin, K. Peuhkurinen and M. S. Nieminen (2009). The type of acute heart failure and the costs of hospitalization. *Int J Cardiol*.

Hasenfuss, G., B. Pieske, M. Castell, B. Kretschmann, L. S. Maier and H. Just (1998). Influence of the novel inotropic agent levosimendan on isometric tension and calcium cycling in failing human myocardium. *Circulation* **98**(20): 2141-7.

Hochman, J. S. (2003). Cardiogenic shock complicating acute myocardial infarction: expanding the paradigm. *Circulation* **107**(24): 2998-3002.

Hochman, J. S., C. E. Buller, L. A. Sleeper, J. Boland, V. Dzavik, T. A. Sanborn, E. Godfrey, H. D. White, J. Lim and T. LeJemtel (2000). Cardiogenic shock complicating acute myocardial infarction--etiologies, management and outcome: a report from the SHOCK Trial Registry. SHould we emergently revascularize Occluded Coronaries for cardiogenic shocK? *J Am Coll Cardiol* **36**(3 Suppl A): 1063-70.

Hochman, J. S., L. A. Sleeper, J. G. Webb, V. Dzavik, C. E. Buller, P. Aylward, J. Col and H. D. White (2006). Early revascularization and long-term survival in cardiogenic shock complicating acute myocardial infarction. *Jama* **295**(21): 2511-5.

Holmes, K. C., D. Popp, W. Gebhard and W. Kabsch (1990). Atomic model of the actin filament. *Nature* **347**(6288): 44-9.

Hood, W. B., Jr., A. L. Dans, G. H. Guyatt, R. Jaeschke and J. J. McMurray (2004). Digitalis for treatment of congestive heart failure in patients in sinus rhythm. *Cochrane Database Syst Rev*(2): CD002901.

Ilva, T., J. Lassus, K. Siirila-Waris, J. Melin, K. Peuhkurinen, K. Pulkki, M. S. Nieminen, H. Mustonen, P. Porela and V. P. Harjola (2008). Clinical significance of cardiac troponins I and T in acute heart failure. *Eur J Heart Fail* **10**(8): 772-9.

Iwamoto, T., Y. Watanabe, S. Kita and M. P. Blaustein (2007). Na+/Ca2+ exchange inhibitors: a new class of calcium regulators. *Cardiovasc Hematol Disord Drug Targets* **7**(3): 188-98.

Janssen, P. M., N. Datz, O. Zeitz and G. Hasenfuss (2000). Levosimendan improves diastolic and systolic function in failing human myocardium. *Eur J Pharmacol* **404**(1-2): 191-9.

Jhund, P. S., J. J. McMurray and A. P. Davie (2000). The acute vascular effects of frusemide in heart failure. *Br J Clin Pharmacol* **50**(1): 9-13.

Kankaanranta, H., X. Zhang, R. Tumelius, M. Ruotsalainen, H. Haikala, E. Nissinen and E. Moilanen (2007). Antieosinophilic activity of simendans. *J Pharmacol Exp Ther* **323**(1): 31-8.

Kaptan, K., K. Erinc, A. Ifran, V. Yildirim, M. Uzun, C. Beyan and E. Isik (2008). Levosimendan has an inhibitory effect on platelet function. *Am J Hematol* **83**(1): 46-9.

Kass, D. A. and R. J. Solaro (2006). Mechanisms and use of calcium-sensitizing agents in the failing heart. *Circulation* **113**(2): 305-15.

Kersten, J. R., M. W. Montgomery, P. S. Pagel and D. C. Warltier (2000). Levosimendan, a new positive inotropic drug, decreases myocardial infarct size via activation of K(ATP) channels. *Anesth Analg* **90**(1): 5-11.

Khan, H., M. Metra, J. E. Blair, M. Vogel, M. E. Harinstein, G. S. Filippatos, H. N. Sabbah, H. Porchet, G. Valentini and M. Gheorghiade (2009). Istaroxime, a first in class new chemical entity exhibiting SERCA-2 activation and Na-K-ATPase inhibition: a new promising treatment for acute heart failure syndromes? *Heart Fail Rev*.

Khan, S. S., M. Gheorghiade, J. D. Dunn, E. Pezalla and G. C. Fonarow (2008). Managed care interventions for improving outcomes in acute heart failure syndromes. *Am J Manag Care* **14**(12 Suppl Managed): S273-86; quiz S287-91.

Kivikko, M., S. Antila, J. Eha, L. Lehtonen and P. J. Pentikainen (2002). Pharmacokinetics of levosimendan and its metabolites during and after a 24-hour continuous infusion in patients with severe heart failure. *Int J Clin Pharmacol Ther* **40**(10): 465-71.

Kivikko, M., L. Lehtonen and W. S. Colucci (2003). Sustained hemodynamic effects of intravenous levosimendan. *Circulation* **107**(1): 81-6.

Ko, E. A., J. Han, I. D. Jung and W. S. Park (2008). Physiological roles of K+ channels in vascular smooth muscle cells. *J Smooth Muscle Res* **44**(2): 65-81.

Kohsaka, S., V. Menon, A. M. Lowe, M. Lange, V. Dzavik, L. A. Sleeper and J. S. Hochman (2005). Systemic inflammatory response syndrome after acute myocardial infarction complicated by cardiogenic shock. *Arch Intern Med* **165**(14): 1643-50.

Kopustinskiene, D. M., P. Pollesello and N. E. Saris (2001). Levosimendan is a mitochondrial K(ATP) channel opener. *Eur J Pharmacol* **428**(3): 311-4.

Kopustinskiene, D. M., P. Pollesello and N. E. Saris (2004). Potassium-specific effects of levosimendan on heart mitochondria. *Biochem Pharmacol* **68**(5): 807-12.

Koskinen, M., J. Puttonen, M. Pykalainen, A. Vuorela and T. Lotta (2008). Metabolism of OR-1896, a metabolite of levosimendan, in rats and humans. *Xenobiotica* **38**(2): 156-70.

Lancaster, M. K. and S. J. Cook (1997). The effects of levosimendan on [Ca2+]i in guinea-pig isolated ventricular myocytes. *Eur J Pharmacol* **339**(1): 97-100.

Lassus, J., V. P. Harjola, R. Sund, K. Siirila-Waris, J. Melin, K. Peuhkurinen, K. Pulkki and M. S. Nieminen (2007). Prognostic value of cystatin C in acute heart failure in relation to other markers of renal function and NT-proBNP. *Eur Heart J* **28**(15): 1841-7.

Lau, W. M. (1992). Effects of potassium channel blockers on the negative inotropic responses induced by cromakalim and pinacidil in guinea pig atrium. *Pharmacology* **45**(1): 9-16.

Lehtonen, L. (2004). Levosimendan: a calcium-sensitizing agent for the treatment of patients with decompensated heart failure. *Curr Heart Fail Rep* **1**(3): 136-44.

Lehtonen, L. and P. Poder (2007). The utility of levosimendan in the treatment of heart failure. *Ann Med* **39**(1): 2-17.

Lehtonen, L. and S. Sundberg (2002). The contractility enhancing effect of the calcium sensitiser levosimendan is not attenuated by carvedilol in healthy subjects. *Eur J Clin Pharmacol* **58**(7): 449-52.

Lehtonen, L. A., S. Antila and P. J. Pentikainen (2004). Pharmacokinetics and pharmacodynamics of intravenous inotropic agents. *Clin Pharmacokinet* **43**(3): 187-203.

Leroy, J., A. Abi-Gerges, V. O. Nikolaev, W. Richter, P. Lechene, J. L. Mazet, M. Conti, R. Fischmeister and G. Vandecasteele (2008). Spatiotemporal dynamics of beta-adrenergic cAMP signals and L-type Ca2+ channel regulation in adult rat ventricular myocytes: role of phosphodiesterases. *Circ Res* **102**(9): 1091-100.

Lever, A. and I. Mackenzie (2007). Sepsis: definition, epidemiology, and diagnosis. *Bmj* **335**(7625): 879-83.

Levijoki, J., P. Pollesello, P. Kaheinen and H. Haikala (2001). Improved survival with simendan after experimental myocardial infarction in rats. *Eur J Pharmacol* **419**(2-3): 243-8.

Levijoki, J., P. Pollesello, J. Kaivola, C. Tilgmann, T. Sorsa, A. Annila, I. Kilpelainen and H. Haikala (2000). Further evidence for the cardiac troponin C mediated calcium sensitization by levosimendan: structure-response and binding analysis with analogs of levosimendan. *J Mol Cell Cardiol* **32**(3): 479-91.

Lilleberg, J., M. S. Nieminen, J. Akkila, L. Heikkila, A. Kuitunen, L. Lehtonen, K. Verkkala, S. Mattila and M. Salmenpera (1998). Effects of a new calcium sensitizer, levosimendan, on haemodynamics, coronary blood flow and myocardial substrate utilization early after coronary artery bypass grafting. *Eur Heart J* **19**(4): 660-8.

Lilleberg, J., V. Ylonen, L. Lehtonen and L. Toivonen (2004). The calcium sensitizer levosimendan and cardiac arrhythmias: an analysis of the safety database of heart failure treatment studies. *Scand Cardiovasc J* **38**(2): 80-4.

Liu, Y., T. Sato, B. O'Rourke and E. Marban (1998). Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation* **97**(24): 2463-9.

Liu, Y., T. Sato, J. Seharaseyon, A. Szewczyk, B. O'Rourke and E. Marban (1999). Mitochondrial ATP-dependent potassium channels. Viable candidate effectors of ischemic preconditioning. *Ann N Y Acad Sci* **874**: 27-37.

Lorenz, M., K. J. Poole, D. Popp, G. Rosenbaum and K. C. Holmes (1995). An atomic model of the unregulated thin filament obtained by X-ray fiber diffraction on oriented actin-tropomyosin gels. *J Mol Biol* **246**(1): 108-19.

Louhelainen, M., E. Vahtola, P. Kaheinen, H. Leskinen, S. Merasto, V. Kyto, P. Finckenberg, W. S. Colucci, J. Levijoki, P. Pollesello, H. Haikala and E. M. Mervaala (2007). Effects of levosimendan on cardiac remodeling and cardiomyocyte apoptosis in hypertensive Dahl/Rapp rats. *Br J Pharmacol* **150**(7): 851-61.

Maisel, A., C. Mueller, K. Adams, Jr., S. D. Anker, N. Aspromonte, J. G. Cleland, A. Cohen-Solal, U. Dahlstrom, A. DeMaria, S. Di Somma, G. S. Filippatos, G. C. Fonarow, P. Jourdain, M. Komajda, P. P. Liu, T. McDonagh, K. McDonald, A. Mebazaa, M. S. Nieminen, W. F. Peacock, M. Tubaro, R. Valle, M. Vanderhyden, C. W. Yancy, F. Zannad and E. Braunwald (2008). State of the art: using natriuretic peptide levels in clinical practice. *Eur J Heart Fail* **10**(9): 824-39.

Mangano, C. M., L. S. Diamondstone, J. G. Ramsay, A. Aggarwal, A. Herskowitz and D. T. Mangano (1998). Renal dysfunction after myocardial revascularization: risk factors, adverse outcomes, and hospital resource utilization. The Multicenter Study of Perioperative Ischemia Research Group. *Ann Intern Med* **128**(3): 194-203.

Masutani, S., H. J. Cheng, M. Hyttila-Hopponen, J. Levijoki, A. Heikkila, A. Vuorela, W. C. Little and C. P. Cheng (2008). Orally available levosimendan dose-related positive inotropic and lusitropic effect in conscious chronically instrumented normal and heart failure dogs. *J Pharmacol Exp Ther* **325**(1): 236-47.

Mavrogeni, S., G. Giamouzis, E. Papadopoulou, S. Thomopoulou, A. Dritsas, G. Athanasopoulos, E. Adreanides, I. Vassiliadis, K. Spargias, D. Panagiotakos and D. V. Cokkinos (2007). A 6-month follow-up of intermittent levosimendan administration effect on systolic function, specific activity questionnaire, and arrhythmia in advanced heart failure. *J Card Fail* **13**(7): 556-9.

Mebazaa, A., M. S. Nieminen, G. S. Filippatos, J. G. Cleland, J. E. Salon, R. Thakkar, R. J. Padley, B. Huang and A. Cohen-Solal (2009). Levosimendan vs. dobutamine: outcomes for acute heart failure patients on {beta}-blockers in SURVIVE. *Eur J Heart Fail* **11**(3): 304-11.

Mebazaa, A., M. S. Nieminen, M. Packer, A. Cohen-Solal, F. X. Kleber, S. J. Pocock, R. Thakkar, R. J. Padley, P. Poder and M. Kivikko (2007). Levosimendan vs dobutamine for patients with acute decompensated heart failure: the SURVIVE Randomized Trial. *Jama* **297**(17): 1883-91.

Metra, M., L. Dei Cas and M. R. Bristow (2008a). The pathophysiology of acute heart failure--it is a lot about fluid accumulation. *Am Heart J* **155**(1): 1-5.

Metra, M., S. Nodari, G. Parrinello, T. Bordonali, S. Bugatti, R. Danesi, B. Fontanella, C. Lombardi, P. Milani, G. Verzura, G. Cotter, H. Dittrich, B. M. Massie and L. Dei Cas (2008b). Worsening renal function in patients hospitalised for acute heart failure: clinical implications and prognostic significance. *Eur J Heart Fail* **10**(2): 188-95.

Milo, O., G. Cotter, E. Kaluski, A. Brill, A. Blatt, R. Krakover, Z. Vered and R. Hershkoviz (2003). Comparison of inflammatory and neurohormonal activation in cardiogenic pulmonary edema secondary to ischemic versus nonischemic causes. *Am J Cardiol* **92**(2): 222-6.

Milo-Cotter, O., K. F. Adams, C. M. O'Connor, N. Uriel, E. Kaluski, G. M. Felker, B. Weatherley, Z. Vered and G. Cotter (2007). Acute heart failure associated with high admission blood pressure--a distinct vascular disorder? *Eur J Heart Fail* **9**(2): 178-83.

Moiseyev, V. S., P. Poder, N. Andrejevs, M. Y. Ruda, A. P. Golikov, L. B. Lazebnik, Z. D. Kobalava, L. A. Lehtonen, T. Laine, M. S. Nieminen and K. I. Lie (2002). 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 (RUSSLAN). *Eur Heart J* **23**(18): 1422-32.

Moonen, M., M. Senechal, B. Cosyns, P. Melon, E. Nellessen, L. Pierard and P. Lancellotti (2008). Impact of contractile reserve on acute response to cardiac resynchronization therapy. *Cardiovasc Ultrasound* **6**: 65.

Movsesian, M., J. Stehlik, F. Vandeput and M. R. Bristow (2008). Phosphodiesterase inhibition in heart failure. *Heart Fail Rev*.

Nanas, J. N., P. Papazoglou, E. P. Tsagalou, A. Ntalianis, E. Tsolakis, J. V. Terrovitis, J. Kanakakis, S. N. Nanas, G. P. Alexopoulos and M. I. Anastasiou-Nana (2005). Efficacy and safety of intermittent, long-term, concomitant dobutamine and levosimendan infusions in severe heart failure refractory to dobutamine alone. *Am J Cardiol* **95**(6): 768-71.

Nanas, J. N., P. P. Papazoglou, J. V. Terrovitis, J. Kanakakis, A. Dalianis, E. Tsolakis, E. P. Tsagalou, N. Agrios, K. Christodoulou and M. I. Anastasiou-Nana (2004). Hemodynamic effects of levosimendan added to dobutamine in patients with decompensated advanced heart failure refractory to dobutamine alone. *Am J Cardiol* **94**(10): 1329-32.

Nielsen-Kudsk, J. E., S. Boesgaard and J. Aldershvile (1996). K+ channel opening: a new drug principle in cardiovascular medicine. *Heart* **76**(2): 109-16.

Nieminen, M. S., J. Akkila, G. Hasenfuss, F. X. Kleber, L. A. Lehtonen, V. Mitrovic, O. Nyquist and W. J. Remme (2000). Hemodynamic and neurohumoral effects of continuous infusion of levosimendan in patients with congestive heart failure. *J Am Coll Cardiol* **36**(6): 1903-12.

Nieminen, M. S., D. Brutsaert, K. Dickstein, H. Drexler, F. Follath, V. P. Harjola, M. Hochadel, M. Komajda, J. Lassus, J. L. Lopez-Sendon, P. Ponikowski and L. Tavazzi (2006). EuroHeart Failure Survey II (EHFS II): a survey on hospitalized acute heart failure patients: description of population. *Eur Heart J* **27**(22): 2725-36.

Nieminen, M. S., P. Pollesello, G. Vajda and Z. Papp (2009). Effects of Levosimendan on the Energy Balance: Preclinical and Clinical Evidence. *J Cardiovasc Pharmacol*.

Nijhawan, N., A. C. Nicolosi, M. W. Montgomery, A. Aggarwal, P. S. Pagel and D. C. Warltier (1999). Levosimendan enhances cardiac performance after cardiopulmonary bypass: a prospective, randomized placebo-controlled trial. *J Cardiovasc Pharmacol* **34**(2): 219-28.

Nishida, H., T. Sato, T. Ogura and H. Nakaya (2009). New aspects for the treatment of cardiac diseases based on the diversity of functional controls on cardiac muscles: mitochondrial ion channels and cardioprotection. *J Pharmacol Sci* **109**(3): 341-7.

O'Rourke, B. (2004). Evidence for mitochondrial K+ channels and their role in cardioprotection. *Circ Res* **94**(4): 420-32.

Orso, F., S. Baldasseroni, G. Fabbri, L. Gonzini, D. Lucci, C. D'Ambrosi, M. Gobbi, G. Lecchi, S. Randazzo, G. Masotti, L. Tavazzi and A. P. Maggioni (2009). Role of beta-blockers in patients admitted for worsening heart failure in a real world setting: data from the Italian Survey on Acute Heart Failure. *Eur J Heart Fail* **11**(1): 77-84.

Ozdem, S. S., O. Yalcin, H. J. Meiselman, O. K. Baskurt and C. Usta (2006). The role of potassium channels in relaxant effect of levosimendan in rat small mesenteric arteries. *Cardiovasc Drugs Ther* **20**(2): 123-7.

Papp, Z., K. Csapo, P. Pollesello, H. Haikala and I. Edes (2005). Pharmacological mechanisms contributing to the clinical efficacy of levosimendan. *Cardiovasc Drug Rev* **23**(1): 71-98.

Paraskevaidis, I. A., J. T. Parissis and D. Th Kremastinos (2005). Anti-inflammatory and anti-apoptotic effects of levosimendan in decompensated heart failure: a novel mechanism of drug-induced improvement in contractile performance of the failing heart. *Curr Med Chem Cardiovasc Hematol Agents* **3**(3): 243-7.

Parissis, J. T., S. Adamopoulos, C. Antoniades, G. Kostakis, A. Rigas, S. Kyrzopoulos, E. Iliodromitis and D. Kremastinos (2004). Effects of levosimendan on circulating pro-inflammatory cytokines and soluble apoptosis mediators in patients with decompensated advanced heart failure. *Am J Cardiol* **93**(10): 1309-12.

Parissis, J. T., I. Andreadou, V. Bistola, I. Paraskevaidis, G. Filippatos and D. T. Kremastinos (2008). Novel biologic mechanisms of levosimendan and its effect on the failing heart. *Expert Opin Investig Drugs* **17**(8): 1143-50.

Pataricza, J., J. Hohn, A. Petri, A. Balogh and J. G. Papp (2000). Comparison of the vasorelaxing effect of cromakalim and the new inodilator, levosimendan, in human isolated portal vein. *J Pharm Pharmacol* **52**(2): 213-7.

Pataricza, J., I. Krassoi, J. Hohn, A. Kun and J. G. Papp (2003). Functional role of potassium channels in the vasodilating mechanism of levosimendan in porcine isolated coronary artery. *Cardiovasc Drugs Ther* **17**(2): 115-21.

Petersen, J. W. and G. M. Felker (2008). Inotropes in the management of acute heart failure. *Crit Care Med* **36**(1 Suppl): S106-11.

Pierard, L. A. and P. Lancellotti (2004). The role of ischemic mitral regurgitation in the pathogenesis of acute pulmonary edema. *N Engl J Med* **351**(16): 1627-34.

Pinto, B. B., S. Rehberg, C. Ertmer and M. Westphal (2008). Role of levosimendan in sepsis and septic shock. *Curr Opin Anaesthesiol* **21**(2): 168-77.

Poder, P., J. Eha, S. Antila, M. Heinpalu, U. Planken, I. Loogna, A. Mesikepp, J. Akkila and L. Lehtonen (2003). Pharmacodynamic interactions of levosimendan and felodipine in patients with coronary heart disease. *Cardiovasc Drugs Ther* **17**(5-6): 451-8.

Pollesello, P., M. Ovaska, J. Kaivola, C. Tilgmann, K. Lundstrom, N. Kalkkinen, I. Ulmanen, E. Nissinen and J. Taskinen (1994). Binding of a new Ca2+ sensitizer, levosimendan, to recombinant human cardiac troponin C. A molecular modelling, fluorescence probe, and proton nuclear magnetic resonance study. *J Biol Chem* **269**(46): 28584-90.

Pongo, E., Z. Balla, K. Mubagwa, W. Flameng, I. Edes, Z. Szilvassy and P. Ferdinandy (2001). Deterioration of the protein kinase C-K(ATP) channel pathway in regulation of coronary flow in hypercholesterolaemic rabbits. *Eur J Pharmacol* **418**(3): 217-23.

Potter, L. R., S. Abbey-Hosch and D. M. Dickey (2006). Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocr Rev* **27**(1): 47-72.

Potter, L. R., A. R. Yoder, D. R. Flora, L. K. Antos and D. M. Dickey (2009). Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications. *Handb Exp Pharmacol*(191): 341-66.

Puttonen, J., T. Laine, M. Ramela, S. Hakkinen, W. Zhang, R. Pradhan, P. Pentikainen and M. Koskinen (2007). Pharmacokinetics and excretion balance of OR-1896, a pharmacologically active metabolite of levosimendan, in healthy men. *Eur J Pharm Sci* **32**(4-5): 271-7.

Quast, U. and N. S. Cook (1989). Moving together: K+ channel openers and ATP-sensitive K+ channels. *Trends Pharmacol Sci* **10**(11): 431-5.

Quast, U., J. M. Guillon and I. Cavero (1994). Cellular pharmacology of potassium channel openers in vascular smooth muscle. *Cardiovasc Res* **28**(6): 805-10.

Rabuel, C. and A. Mebazaa (2006). Septic shock: a heart story since the 1960s. *Intensive Care Med* **32**(6): 799-807.

Rapundalo, S. T., R. J. Solaro and E. G. Kranias (1989). Inotropic responses to isoproterenol and phosphodiesterase inhibitors in intact guinea pig hearts: comparison of cyclic AMP levels and phosphorylation of sarcoplasmic reticulum and myofibrillar proteins. *Circ Res* **64**(1): 104-11.

Redfield, M. M., S. J. Jacobsen, B. A. Borlaug, R. J. Rodeheffer and D. A. Kass (2005). Age- and gender-related ventricular-vascular stiffening: a community-based study. *Circulation* **112**(15): 2254-62.

Rochais, F., A. Abi-Gerges, K. Horner, F. Lefebvre, D. M. Cooper, M. Conti, R. Fischmeister and G. Vandecasteele (2006). A specific pattern of phosphodiesterases controls the cAMP signals generated by different Gs-coupled receptors in adult rat ventricular myocytes. *Circ Res* **98**(8): 1081-8.

Rossinen, J., V. P. Harjola, K. Siirila-Waris, J. Lassus, J. Melin, K. Peuhkurinen and M. S. Nieminen (2008). The use of more than one inotrope in acute heart failure is associated with increased mortality: a multi-centre observational study. *Acute Card Care* **10**(4): 209-13.

Rudiger, A. and M. Singer (2007). Mechanisms of sepsis-induced cardiac dysfunction. *Crit Care Med* **35**(6): 1599-608.

Sallinen, J., M. Kuoppamäki and J. Levijoki (2009). A Combination Treatment. *World Intellectual Property Organisation* **WO 2009**(027577).

Salmenpera, M. and H. Eriksson (2009). Levosimendan in perioperative and critical care patients. *Curr Opin Anaesthesiol* **22**(4): 496-501.

Sandell, E. P., M. Hayha, S. Antila, P. Heikkinen, P. Ottoila, L. A. Lehtonen and P. J. Pentikainen (1995). Pharmacokinetics of levosimendan in healthy volunteers and patients with congestive heart failure. *J Cardiovasc Pharmacol* **26 Suppl 1**: S57-62.

Sareila, O., R. Korhonen, H. Auvinen, M. Hamalainen, H. Kankaanranta, E. Nissinen and E. Moilanen (2008). Effects of levo- and dextrosimendan on NF-kappaB-mediated transcription, iNOS expression and NO production in response to inflammatory stimuli. *Br J Pharmacol* **155**(6): 884-95.

Sato, S., M. A. Talukder, H. Sugawara, H. Sawada and M. Endoh (1998). Effects of levosimendan on myocardial contractility and Ca2+ transients in aequorin-loaded right-ventricular papillary muscles and indo-1-loaded single ventricular cardiomyocytes of the rabbit. *J Mol Cell Cardiol* **30**(6): 1115-28.

Scheiermann, P., D. Ahluwalia, S. Hoegl, A. Dolfen, M. Revermann, B. Zwissler, H. Muhl, K. A. Boost and C. Hofstetter (2009). Effects of intravenous and inhaled levosimendan in severe rodent sepsis. *Intensive Care Med* **35**(8): 1412-9.

Segreti, J. A., K. C. Marsh, J. S. Polakowski and R. M. Fryer (2008). Evoked changes in cardiovascular function in rats by infusion of levosimendan, OR-1896 [(R)-N-(4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenyl)acetamid e], OR-1855 [(R)-6-(4-aminophenyl)-5-methyl-4,5-dihydropyridazin-3(2H)-one], dobutamine, and milrinone: comparative effects on peripheral resistance, cardiac output, dP/dt, pulse rate, and blood pressure. *J Pharmacol Exp Ther* **325**(1): 331-40.

Shakur, Y., M. Fong, J. Hensley, J. Cone, M. A. Movsesian, J. Kambayashi, M. Yoshitake and Y. Liu (2002). Comparison of the effects of cilostazol and milrinone on cAMP-PDE activity, intracellular cAMP and calcium in the heart. *Cardiovasc Drugs Ther* **16**(5): 417-27.

Shimizu, K., Y. Shintani, W. G. Ding, H. Matsuura and T. Bamba (2002). Potentiation of slow component of delayed rectifier K(+) current by cGMP via two distinct mechanisms: inhibition of phosphodiesterase 3 and activation of protein kinase G. *Br J Pharmacol* **137**(1): 127-37.

Siirila-Waris, K., J. Lassus, J. Melin, K. Peuhkurinen, M. S. Nieminen and V. P. Harjola (2006). Characteristics, outcomes, and predictors of 1-year mortality in patients hospitalized for acute heart failure. *Eur Heart J* **27**(24): 3011-7.

Slawsky, M. T., W. S. Colucci, S. S. Gottlieb, B. H. Greenberg, E. Haeusslein, J. Hare, S. Hutchins, C. V. Leier, T. H. LeJemtel, E. Loh, J. Nicklas, D. Ogilby, B. N. Singh and W. Smith (2000). Acute hemodynamic and clinical effects of levosimendan in patients with severe heart failure. Study Investigators. *Circulation* **102**(18): 2222-7.

Smith, C. J., R. Huang, D. Sun, S. Ricketts, C. Hoegler, J. Z. Ding, R. A. Moggio and T. H. Hintze (1997). Development of decompensated dilated cardiomyopathy is associated with decreased gene expression and activity of the milrinone-sensitive cAMP phosphodiesterase PDE3A. *Circulation* **96**(9): 3116-23.

Solaro, R. J. (2009). CK-1827452, a sarcomere-directed cardiac myosin activator for acute and chronic heart disease. *IDrugs* **12**(4): 243-51.

Somberg, J. C. and J. Molnar (2009). The Management of Acute Heart Failure and Diuretic Therapy. *Am J Ther*.

Sorsa, T., P. Pollesello and R. J. Solaro (2004). The contractile apparatus as a target for drugs against heart failure: interaction of levosimendan, a calcium sensitiser, with cardiac troponin c. *Mol Cell Biochem* **266**(1-2): 87-107.

Sperelakis, N., N. Tohse, Y. Ohya and H. Masuda (1994). Cyclic GMP regulation of calcium slow channels in cardiac muscle and vascular smooth muscle cells. *Adv Pharmacol* **26**: 217-52.

Sun, B., H. Li, Y. Shakur, J. Hensley, S. Hockman, J. Kambayashi, V. C. Manganiello and Y. Liu (2007). Role of phosphodiesterase type 3A and 3B in regulating platelet and cardiac function using subtype-selective knockout mice. *Cell Signal* **19**(8): 1765-71.

Sundberg, S. and L. Lehtonen (2000). Haemodynamic interactions between the novel calcium sensitiser levosimendan and isosorbide-5-mononitrate in healthy subjects. *Eur J Clin Pharmacol* **55**(11-12): 793-9.

Sutherland, F. J. and D. J. Hearse (2000). The isolated blood and perfusion fluid perfused heart. *Pharmacol Res* **41**(6): 613-27.

Tachibana, H., H. J. Cheng, T. Ukai, A. Igawa, Z. S. Zhang, W. C. Little and C. P. Cheng (2005). Levosimendan improves LV systolic and diastolic performance at rest and during exercise after heart failure. *Am J Physiol Heart Circ Physiol* **288**(2): H914-22.

Takahashi, R. and M. Endoh (2005). Dual regulation of myofilament Ca2+ sensitivity by levosimendan in normal and acidotic conditions in aequorin-loaded canine ventricular myocardium. *Br J Pharmacol* **145**(8): 1143-52.

Thackray, S., J. Easthaugh, N. Freemantle and J. G. Cleland (2002). The effectiveness and relative effectiveness of intravenous inotropic drugs acting through the adrenergic pathway in patients with heart failure-a meta-regression analysis. *Eur J Heart Fail* **4**(4): 515-29.

Torphy, T. J., H. L. Zhou and L. B. Cieslinski (1992). Stimulation of beta adrenoceptors in a human monocyte cell line (U937) up-regulates cyclic AMP-specific phosphodiesterase activity. *J Pharmacol Exp Ther* **263**(3): 1195-205.

Udelson, J. E., C. Orlandi, J. Ouyang, H. Krasa, C. A. Zimmer, G. Frivold, W. H. Haught, S. Meymandi, C. Macarie, D. Raef, P. Wedge, M. A. Konstam and M. Gheorghiade (2008). Acute hemodynamic effects of tolvaptan, a vasopressin V2 receptor blocker, in patients with symptomatic heart failure and systolic dysfunction: an international, multicenter, randomized, placebo-controlled trial. *J Am Coll Cardiol* **52**(19): 1540-5.

Ukkonen, H., M. Saraste, J. Akkila, J. Knuuti, M. Karanko, H. Iida, P. Lehikoinen, K. Nagren, L. Lehtonen and L. M. Voipio-Pulkki (2000). Myocardial efficiency during levosimendan infusion in congestive heart failure. *Clin Pharmacol Ther* **68**(5): 522-31.

Ukkonen, H., M. Saraste, J. Akkila, M. J. Knuuti, P. Lehikoinen, K. Nagren, L. Lehtonen and L. M. Voipio-Pulkki (1997). Myocardial efficiency during calcium sensitization with levosimendan: a noninvasive study with positron emission tomography and echocardiography in healthy volunteers. *Clin Pharmacol Ther* **61**(5): 596-607.

Uriel, N., G. Torre-Amione, O. Milo, E. Kaluski, L. Perchenet, A. Blatt, I. Kobrin, A. Turnovski, S. Kaplan, M. Rainisio, A. Frey, Z. Vered and G. Cotter (2005). Echocardiographic ejection fraction in patients with acute heart failure: correlations with hemodynamic, clinical, and neurohormonal measures and short-term outcome. *Eur J Heart Fail* **7**(5): 815-9.

Vandecasteele, G., F. Rochais, A. Abi-Gerges and R. Fischmeister (2006). Functional localization of cAMP signalling in cardiac myocytes. *Biochem Soc Trans* **34**(Pt 4): 484-8.

Varpula, T., J. Rapola, M. Sallisalmi and J. Kurola (2009). Treatment of serious calcium channel blocker overdose with levosimendan, a calcium sensitizer. *Anesth Analg* **108**(3): 790-2.

Wattanapermpool, J., X. Guo and R. J. Solaro (1995). The unique amino-terminal peptide of cardiac troponin I regulates myofibrillar activity only when it is phosphorylated. *J Mol Cell Cardiol* **27**(7): 1383-91.

Vlachopoulos, C., I. Dima, K. Aznaouridis, C. Vasiliadou, N. Ioakeimidis, C. Aggeli, M. Toutouza and C. Stefanadis (2005). Acute systemic inflammation increases arterial stiffness and decreases wave reflections in healthy individuals. *Circulation* **112**(14): 2193-200.

VMAC, P. C. f. t. (2002). Intravenous nesiritide vs nitroglycerin for treatment of decompensated congestive heart failure: a randomized controlled trial. *Jama* **287**(12): 1531-40.

Yao, Z. and G. J. Gross (1994). Effects of the KATP channel opener bimakalim on coronary blood flow, monophasic action potential duration, and infarct size in dogs. *Circulation* **89**(4): 1769-75.

Yilmaz, M. B., K. Yalta, C. Yontar, F. Karadas, A. Erdem, O. O. Turgut, A. Yilmaz and I. Tandogan (2007). Levosimendan improves renal function in patients with acute decompensated heart failure: comparison with dobutamine. *Cardiovasc Drugs Ther* **21**(6): 431-5.

Yokoshiki, H., Y. Katsube, M. Sunagawa and N. Sperelakis (1997). Levosimendan, a novel Ca2+ sensitizer, activates the glibenclamide-sensitive K+ channel in rat arterial myocytes. *Eur J Pharmacol* **333**(2-3): 249-59.

Yoshida, H., J. E. Feig, A. Morrissey, I. A. Ghiu, M. Artman and W. A. Coetzee (2004). K ATP channels of primary human coronary artery endothelial cells consist of a heteromultimeric complex of Kir6.1, Kir6.2, and SUR2B subunits. *J Mol Cell Cardiol* **37**(4): 857-69.

Zangrillo, A., G. Biondi-Zoccai, A. Mizzi, G. Bruno, E. Bignami, C. Gerli, V. De Santis, L. Tritapepe and G. Landoni (2009). Levosimendan Reduces Cardiac Troponin Release After Cardiac Surgery: A Meta-analysis of Randomized Controlled Studies. *J Cardiothorac Vasc Anesth*.