Diabetic cardiomyopathy and post-infarct ventricular remodelling

Effects of levosimendan in a rodent model of type II diabetes

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ACADEMIC DISSERTATION

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To my family
Sofia, Benjamin, Fredrik and Viktor

“Solve mentem et cor sequetur”
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List of original publications

The thesis is based on the following original publications (studies I-IV):


*Shared first authorship*
Main abbreviations

ACS  Acute coronary syndromes
Agt1r Angiotensin II type 1 receptor
Akt  Akt kinase
ANP  A-type natriuretic peptide
Bax  Bcl2-associated X protein
Bcl2l11 Bcl2-like 11 apoptosis facilitator
BIM  Bcl2-like 11 apoptosis facilitator
BNP  B-type natriuretic peptide
Caspase-3 Apoptosis related cysteine peptidase 3
CBP  Calcium binding protein
CHF  Congestive heart failure
CTGF Connective tissue growth factor
DD  Diastolic dysfunction
E/A  Early/Atrial(late) filling of left ventricle
EF  Ejection fraction
FOXO3a  Forkhead class O 3a
GADD45 Growth arrest and DNA damage-inducible protein 45
GK  Goto-Kakizaki
HbA1c  Glycosylated haemoglobin A1c
HDAC Histone deacetylase
IL-6  Interleukin 6
IR  Insulin receptor
LV  Left ventricle
LVEDP  Left ventricular end-diastolic pressure
MI  Myocardial infarction
mTOR Mammalian target of rapamycin
NA  Noradrenaline
NAD+/NADH Nicotinamide dinucleotide/reduced
NCX Sodium-Calcium exchanger
p300 E1 binding protein transcriptional coactivator
p38 MAPK Mitogen activated protein kinase 38
p53 Tumour suppressor protein 53
pI3K Phosphatidylinositol 3-kinase
PIP3 Phosphatidylinositol (3,4,5)-triphosphate
PPAR Peroxisome proliferator –activated receptor
PRA Plasma renin activity
RA(A)S Renin-angiotensin (-aldosterone) system
ROS Reactive oxygen species
RTK Receptor tyrosine kinase
RT-PCR Reverse transcriptase polymerase chain reaction
SERCA2 Sarco-Endoplasmic Reticulum Ca2+-ATPase
Sirt1 Sirtuin 1 (silent mating type information regulation 2 homolog), a NAD –dependent class III HDAC
Sod2 Superoxide dismutase 2
TNFalpha Tumour necrosis factor alpha
Abstract

Type 2 diabetes is a risk factor for the development of cardiovascular disease. Recently, the term diabetic cardiomyopathy has been proposed to describe the changes in the heart that occur in response to chronic hyperglycemia and insulin resistance. Ventricular remodelling in diabetic cardiomyopathy includes left ventricular hypertrophy, increased interstitial fibrosis, apoptosis and diastolic dysfunction. Mechanisms behind these changes are increased oxidative stress and renin-angiotensin system activation. The diabetic Goto-Kakizaki rat is a non-obese model of type 2 diabetes that exhibits defective insulin signalling. Recently two interconnected stress response pathways have been discovered that link insulin signalling, longevity, apoptosis and cardiomyocyte hypertrophy. The insulin-receptor – PI3K/Akt –pathway inhibits proapoptotic FOXO3a in response to insulin signalling and the nuclear Sirt1 –deacetylase inhibits proapoptotic p53 and modulates FOXO3a in favour of survival and growth.

Levosimendan is a calcium sensitizing agent used for the management of acute decompensated heart failure. Levosimendan acts as a positive inotrope by sensitizing cardiac troponin C to calcium and exerts vasodilation by opening mitochondrial and sarcolemmal ATP-sensitive potassium channels. Levosimendan has been described to have beneficial effects in ventricular remodelling after myocardial infarction.

The aims of the study were to characterize whether diabetic cardiomyopathy associates with cardiac dysfunction, cardiomyocyte apoptosis, hypertrophy and fibrosis in spontaneously diabetic Goto-Kakizaki (GK) rats, which were used to model type 2 diabetes. Protein expression and activation of the Akt – FOXO3a and Sirt1 – p53 pathways were examined in the development of ventricular remodelling in GK rats with and without myocardial infarction (MI). The third and fourth studies examined the effects of levosimendan on ventricular remodelling and gene expression in post-MI GK rats.
The results demonstrated that diabetic GK rats develop both modest hypertension and features similar to diabetic cardiomyopathy including cardiac dysfunction, LV hypertrophy and fibrosis and increased apoptotic signalling. MI induced a sustained increase in cardiomyocyte apoptosis in GK rats together with aggravated LV hypertrophy and fibrosis. The GK rat myocardium exhibited decreased Akt- FOXO3a phosphorylation and increased nuclear translocation of FOXO3a and overproduction of the Sirt1 protein. Treatment with levosimendan decreased cardiomyocyte apoptosis, senescence and LV hypertrophy and altered the gene expression profile in GK rat myocardium.

The findings indicate that impaired cardioprotection via Akt – FOXO3a and p38 MAPK is associated with increased apoptosis, whereas Sirt1 functions in counteracting apoptosis and the development of LV hypertrophy in the GK rat myocardium. Overall, levosimendan treatment protects against post-MI ventricular remodelling and alters the gene expression profile in the GK rat myocardium.
1 Introduction

Diabetes is an increasing health concern in most developed countries with prevalence reaching an estimated 10% of the population. During the last few decades the increase in the number of patients with diabetes has accelerated and the latest estimates depict alarming numbers for the future: within the next 10-15 years the number of patients with diabetes may be twice of that today. Diabetes is a burden to society and it has been calculated that up to 15% of healthcare costs in Finland may be attributed to the care of patients with diabetes (Current Care guideline for diabetes, Duodecim 2009). The majority of these costs arise from the care for diabetes related complications. Diabetes is associated with several complications but the main cause of death for type 2 diabetic subjects is of cardiovascular origin.

Type 2 diabetes is often associated with risk factors for cardiovascular disease; these so called co-morbid states include increased blood pressure (hypertension), dysregulation of lipids in the blood (dyslipidemia) and coronary artery disease (CAD). Over the last decades the concept of diabetic cardiomyopathy has been introduced (Rubler et al. 1972; Regan et al. 1997). This describes the independent adverse effect diabetes has on the myocardium (i.e. the heart as a muscle). The rationale for introducing this term was based upon findings showing that, when adjusted for the co-morbid states mentioned above, diabetes is a positive predictor for cardiac disease. Diabetic cardiomyopathy is associated with various changes in the heart including increased growth, an increase in the formation of connective tissue and an increase in the rate of cell death. These changes alter the structure of the heart and therefore, its function as a pump is compromised. Diabetic cardiomyopathy is associated with diastolic dysfunction owing to the increased stiffness due to hypertrophy and connective tissue build-up (Regan et al. 1972). In diastolic dysfunction the heart is unable to relax and blood flow into the ventricles is decreased at diastole. Later, systolic dysfunction may develop. At present several mechanisms involved in the development of diabetic
cardiomyopathy are known. Changes in energy source utilization, generation of free oxygen radicals, activation of the renin-angiotensin system (RAS), crosslinking of excess glucose to structural and functional proteins such as collagen (glycosylation) and ryanodine receptors (RyR) and mitochondrial dysfunction are all known to enhance the development of diabetic cardiomyopathy (for a review, see Dobrin et al. 2010).

Lifestyle changes provide the cornerstone in both the prevention and treatment of type 2 diabetes. However there is also a need for new and effective pharmacological treatment for the disease and the arising complications. When treating the complications related to diabetic cardiomyopathy there are strikingly few drugs available. The calcium sensitizer Levosimendan is a novel inodilator used for the short-term treatment of acute heart failure (for a review, see Milligan et al. 2010). The mechanism by which levosimendan acts is unique from the traditional positive inotropes, such as dobutamine and dopamine. Levosimendan has vasodilatory effects in addition to its positive inotropic effects, and hence the term inodilator is used. At present, levosimendan is used clinically for acute decompensated heart failure. So far there have been few studies concerning its use in diabetes-related cardiomyopathy.
2 Review of the literature

2.1 Type 2 diabetes

2.1.1 Definition and etiology

The trigger for type 1 diabetes is a rapid loss of insulin–producing cells from the islets of Langerhans in the pancreas. The disease is presented when endogenous insulin production has decreased significantly. Typically type 2 diabetes is characterised by an increase in blood glucose with sustained endogenous production of insulin. The onset of the disease is slower in type 2 diabetes. However, the division between type 1 and 2 diabetes is artificial and is useful only at the extreme ends of the spectrum as most patients develop aspects from both types. For simplicity and lack of better option, it is customary to use the abovementioned division. Type 2 diabetes is a heterogenous disease with elusive diagnostic criteria. A typical subject may be middle aged, obese, hypertensive and dyslipidemic. However the increased occurrence is rapidly broadening the spectrum of afflicted people. Etiology is multifactorial with both genetic and environmental factors playing a role in the pathogenesis. Obesity is a known risk factor for insulin resistance and at present patients who are prediabetic (impaired fasting glucose or impaired glucose tolerance or both) are commonly overweight. Insulin resistance and a relative insulin deficiency are common in newly diagnosed type 2 diabetic patients. Relative insulin deficiency indicates the inability of tissues to utilize insulin at normal concentrations due to insulin resistance which results in a subsequent increase in insulin production. In the long-term the compensatory increase in insulin secretion may be followed by a decrease in insulin production leading to overt insulin deficiency. Hence the older term non-insulin dependent diabetes mellitus (NIDDM) is an unsuitable description for type 2 diabetes.
2.1.2 Diagnostics

Although precise diagnosis of type 2 diabetes is difficult, there are some established laboratory procedures that are commonly used. At present, established criteria for diagnosis in a symptom-free individual are a fasting plasma glucose concentration of >7 mmol/l or failure of plasma glucose to decrease under 11 mmol/L 2h after an oral glucose tolerance test. In an individual with classic symptoms for diabetes, such as polyuria, increased thirst and/or unexpected weight loss, a non-fasting plasma glucose concentration of >11 mmol/L is diagnostic. Individuals with impaired fasting glucose (fP-Gluc 6.1-6.9 mmol/L) and/or with impaired glucose tolerance (P-Gluc 7.8-11 mmol/L 2h after oral glucose test) are considered to be in a prediabetic state. Measurement of glycated haemoglobin (HbA1c) is used to follow response to therapy in diabetic patients. For diabetic animal models there are no established diagnostic criteria and thus when evaluating diabetes in experimental rats the corresponding human threshold values are widely used.

2.1.3 Prevention and treatment

Type 2 diabetes is foremost a disease of lifestyle. In order to slow down the number of incidences of diabetes today there are several programmes for the primary prevention of type 2 diabetes. These share the aim of increasing awareness of the beneficial effects of a healthy diet and exercise. Patients with the metabolic syndrome have an accumulation of risk factors such as dyslipidemia, obesity, hypertension and insulin resistance, and should be among the primary targets for prevention of type 2 diabetes (for a review, see Tuomilehto 2005). Treatment options for type 2 diabetes include changes in lifestyle, oral anti-diabetic pharmacotherapy and sometimes insulin therapy. It is recommended that at time of diagnosis, the oral antidiabetic drug metformin is started. As add-on therapy there are several other oral anti-diabetic drugs (Table 1). Insulin treatment is indicated for insulin deficient type 2 diabetic patients and/or for those whose hyperglycaemia is unresponsive to oral anti-diabetic drugs. In some cases insulin
Table 1. Pharmacological management of type 2 diabetes

<table>
<thead>
<tr>
<th>Class</th>
<th>Drug</th>
<th>Effects on S-insulin</th>
<th>Indication</th>
<th>Mechanism in brief</th>
<th>Adverse effects in brief</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biguanidines</td>
<td>Metformin</td>
<td>No</td>
<td>First-line at presentation of diagnosis</td>
<td>Activation of AMPK suppresses hepatic gluconeogenesis</td>
<td>Well tolerated in general, lactic acidosis</td>
</tr>
<tr>
<td>Thiazolidinediones</td>
<td>Pioglitazone</td>
<td>No</td>
<td>Second-line</td>
<td>PPAR-gamma activation</td>
<td>Worsening of heart failure, AMI, fluid retention</td>
</tr>
<tr>
<td></td>
<td>Rosiglitazone*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gliptins</td>
<td>Sitagliptine</td>
<td>Glucose – independent increase</td>
<td>Second-line</td>
<td>DPP-4 inhibitor, GLP-1 increase</td>
<td>Long-term effects unknown</td>
</tr>
<tr>
<td></td>
<td>Vildagliptine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saxagliptine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meglitinides</td>
<td>Repaglinide</td>
<td>Glucose – independent increase</td>
<td>Second-line</td>
<td>Blocking K⁺ -channels in pancreatic beta cells</td>
<td>Hypoglycaemia</td>
</tr>
<tr>
<td></td>
<td>Nateglinide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphonylureas</td>
<td>Glibenclamide</td>
<td>Glucose – independent increase</td>
<td>Second-line</td>
<td>Blocking K⁺ -channels in pancreatic beta cells</td>
<td>Hypoglycaemia</td>
</tr>
<tr>
<td></td>
<td>Glimepiride</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Glipizide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incretin mimetics</td>
<td>Exenatide</td>
<td>Glucose – dependent increase</td>
<td>Second-line</td>
<td>GLP-1 mimetic</td>
<td>Nausea and other GI adverse effects, long-term effects unknown</td>
</tr>
<tr>
<td></td>
<td>Liraglutide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class</td>
<td>Drug</td>
<td>Effects on S-insulin</td>
<td>Indication</td>
<td>Mechanism in brief</td>
<td>Adverse effects in brief</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------</td>
<td>----------------------</td>
<td>------------------------------------------------------</td>
<td>--------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Long/intermediate-</td>
<td>Glargin</td>
<td></td>
<td></td>
<td>20-30h duration</td>
<td>Hypoglycaemia</td>
</tr>
<tr>
<td>acting insulin</td>
<td>Detemir</td>
<td></td>
<td>Adjunctive for treatment -unresponsive hyperglycaemia and/or insulin deficient type 2 diabetes</td>
<td>12-24h duration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NPH</td>
<td></td>
<td></td>
<td>12-20h duration</td>
<td></td>
</tr>
<tr>
<td>Short-acting insulin</td>
<td>Humulin</td>
<td></td>
<td></td>
<td>2-4h duration</td>
<td>Hypoglycaemia</td>
</tr>
<tr>
<td>Rapid-acting</td>
<td>Aspart</td>
<td></td>
<td></td>
<td>10-20 min. to start</td>
<td>Hypoglycaemia</td>
</tr>
<tr>
<td></td>
<td>Lispro</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Withdrawn

(Modified from Goodman & Gilman’s the pharmacological basis of therapeutics, 12th ed. 2010).
treatment may be first-line therapy in type 2 diabetes, e.g. when plasma glucose concentration exceeds 15 mmol/L, \( \text{HbA}_{1c} \) is over 10% and/or patients show unintentional weight loss. The common co-morbid states in type 2 diabetes are commonly treated with statins (dyslipidemia), ACE inhibitors/ARB:s (hypertension) and mini-acetylsalicylic acid (anti-thrombotic). The following laboratory values are aims for subjects with diabetes (Current Care guideline for diabetes, Duodecim 2009): \( \text{HbA}_{1c} \) (%): <6.0-7.0, fasting glucose: 4-6 mmol/L, postprandial glucose (ca. 2h after last meal): <8 mmol/L, LDL cholesterol: <2.5 mmol/L, <1.8 mmol/L if known arterial disease, blood pressure: <130/80.

### 2.1.4 Insulin resistance

Insulin is a hormone secreted from the pancreas and its main actions are anabolic, i.e. it builds up energy stores. In carbohydrate metabolism its main role is to increase glucose uptake from blood to tissues. Insulin resistance is one of the hallmarks of type 2 diabetes and at present it is assumed that insulin resistance develops years before hyperglycemia and the onset of clinical symptoms. In short, insulin resistance is a state where the glucose lowering effect of insulin is reduced. This occurs due to the organism’s inability to utilize glucose in an insulin dependent manner in organs such as skeletal muscle and adipose tissue (Becker 2001). In the insulin resistant liver, the normal increase in glycogen synthesis and storage and the suppression of glucose production is absent. The insulin resistant state triggers a compensatory increase in insulin production in pancreatic beta cells. After a period of hyperinsulinemia, a characteristic reduction in insulin production slowly occurs, due to beta cell failure, at which time insulin substitution therapy may be needed.

A high degree of visceral fat, independent of age, is associated with an increased risk of developing the metabolic syndrome including insulin resistance, hypertension and dyslipidemia (Abrams et al. 2010). It has recently become clear that adipose tissue secretes high numbers of cytokines (adipokines) that are metabolically active in most organs of the body. The adipokines and their roles implicated in the development of insulin resistance include decreased levels of
adiponectin and increased secretion of inflammatory mediators IL-6 and TNF-alpha together with leptin and resistin (for a review see Dobrin et al. 2010). In the liver the accumulation of fat presents itself as non-alcoholic fatty liver disease (NAFLD). In NAFLD an increase in lipolysis leads to the accumulation of fatty acids and their by-products which may further disturb insulin signalling contributing to insulin resistance in the liver. The insulin—regulated Glut4 protein is the most important glucose transporter in striated muscle, cardiac muscle and adipose tissue (Becker 2001) (Figure 1). Studies have shown that accumulation of metabolites of fatty acids impairs Glut4 recruitment to the cell membrane in skeletal muscle and adipose tissue (Kim et al. 2004). Furthermore increased insulin secretion during the hyperinsulinemic state reduces the amount of Glut4 receptors through a negative feedback system.

Figure 1. A simplified scheme showing insulin-mediated glucose uptake into the cell. IR: insulin receptor, IRS-1: insulin receptor substrate 1, PI3K: phosphatidylinositol 3 kinase, PIP2/3: phosphatidylinositol (3),4,5-bis/(tris)phosphate, Akt: Akt serine/threonine kinase, GLUT4: glucose transporter subtype 4.

2.1.5 Diabetic cardiomyopathy and ventricular remodelling

Target organ damage is the major reason for the increased risk of premature death, poor quality of life and the social economic burden that can be attributed to diabetes (Current Care guideline for diabetes, Duodecim 2009). Notorious complications of diabetes include damage to small blood vessels called microangiopathy, with target organs including the retina of the eye (retinopathy),
the kidney (nephropathy) and damage to cells of the peripheral nervous system (neuropathy). On the other hand the macrovascular complications of diabetes include myocardial infarction and stroke. The latest addition to the growing list of target organ damaged by diabetes is diabetic cardiomyopathy, i.e. the direct adverse effects of diabetes on the heart, or more strictly the adverse effects on the myocardium. However, since type 2 diabetes most is commonly accompanied by hypertension and dyslipidemia, it is difficult to discern the precise role of diabetes in the birth of these complications. Cardiovascular causes are the leading complications attributed to the increased mortality and morbidity in type 2 diabetes. This study is focused on the adverse effects of diabetes on the heart. The heart is made up of cardiomyocytes (heart muscle cells), coronary arteries and veins and fibroblasts producing an extracellular matrix and electrical conductance fibres, which are all enveloped in the pericardial sac. The functional part of the myocardial apparatus is made up of cardiomyocytes that contract in unison to produce a rhythmic pumping effect propelling the blood through the arteries. As with all muscles in the body, the heart is able to adapt in the event of an increased perfusion demand in peripheral organs. However, maladaptive changes in the heart may occur in pathologic states such as diabetes, hypertension, ischemic heart disease or valvular disease. Ventricular remodelling is an adaptive process that results in reversible or irreversible changes in heart architecture (for a review, see Opie et al. 2006). The extent of the remodelling is determined by the underlying reason and may range from a physiological increase in stroke volume (e.g. endurance athletes) to a pathological decrease in stroke volume (e.g. after myocardial infarction). The term ventricular remodelling was first introduced in 1985 when Pfeffer and colleagues studied the effects of coronary artery ligation on ventricular topography in the rat myocardium (Pfeffer et al. 1985). Since then the term has commonly been used to describe the post-infarct changes in the myocardium (Pfeffer et al. 1990). Major hallmarks in ventricular remodelling include increased growth of myocytes leading to LV hypertrophy, an increase in programmed cell death i.e. apoptosis and the build -
up of connective tissue, i.e. fibrosis. In LV remodelling an increase in LV hypertrophy is associated with an inability of the heart to produce sufficient blood flow to compensate for the need of increased oxygen demand, i.e. capillary density is decreased. In the following section we shall inspect the major aspects of ventricular remodelling in the diabetic heart.

For decades it was controversial whether diabetic cardiomyopathy existed independently from the common co-morbidities such as hypertension and coronary heart disease. The concept of diabetic cardiomyopathy was introduced in 1972 when diabetes-associated changes in the heart were first suspected (Rubler et al. 1972). Since then it has become increasingly evident that diabetes is an independent risk factor for the development of cardiac disease. In 1998 Haffner and colleagues showed that diabetes constitutes as high a risk of mortality from coronary heart disease as a prior myocardial infarction (Haffner et al. 1998). And more recently diabetes has been shown to act as an independent risk for heart failure when the effects of age, weight, cholesterol, blood pressure and history of coronary artery disease have been considered (Bertoni et al. 2003). Patients with idiopathic dilated cardiomyopathy were found to be 75% more likely to have diabetes than age-matched controls (Poornima et al. 2006). In 2007 a cohort study with 62,000 patients from 55 countries showed that 30 days and 1 year after acute coronary syndromes (ACS: UAP/NSTEMI or STEMI) the mortality risk was significantly higher in diabetic patients than in non-diabetic population when adjusted for hypertension, smoking and any other confounding factor (Donahoe et al. 2007).

Hallmark findings in diabetic cardiomyopathy include increased LV hypertrophy, fibrosis, diastolic and systolic dysfunction, calcium dysregulation and mitochondrial dysfunction together with changes in substrate utilization that lead to increased oxidative stress and lipotoxicity (for a review see Boudina et al. 2010). These alterations are reflected in the multifactorial pathogenesis that constitutes diabetic cardiomyopathy. The following definition of diabetic cardiomyopathy has been proposed recently by Aneja et al. 2008: After excluding
other contributory causes, diabetic cardiomyopathy can be defined as the presence of both of the following: 1) Evidence of cardiac hypertrophy determined by conventional echocardiography or MRI, 2) evidence of LV diastolic dysfunction (with or without LV systolic dysfunction) either clinically by transmitral Doppler or tissue Doppler imaging, or evidence of left atrial enlargement, or subclinically by novel imaging techniques or provocative testing (e.g. strain and strain-rate imaging, or stress-imaging).

2.1.5.1 Functional aspects

The factors that contribute to myocardial dysfunction in diabetic cardiomyopathy have been identified as dysregulation of calcium homeostasis, renin-angiotensin system upregulation, increased oxidative stress, altered substrate metabolism and mitochondrial dysfunction. In type 2 diabetes it is not uncommon to find a mix of systolic and diastolic dysfunction (for a review, see Fang et al. 2004). However, the hallmark functional disturbance in diabetic cardiomyopathy is primarily diastolic. Diastolic dysfunction (DD) occurs when relaxation of the ventricle is impaired due to reduced compliance of the ventricular wall. Usually fibrosis and hypertrophy are underlying causative factors which are in turn caused by hypertension, diabetes, ischemia or a mix of these. In DD, echocardiography show a characteristic increase in LV end-diastolic pressure (LVEDP), increased isovolumetric relaxation time and doppler flow changes including a primary decrease in the ratio of early to late (atrial) filling (E/A) of the LV. It has been shown that the prevalence of DD is increased in both types of diabetes when other confounding factors such as coronary artery disease have been controlled (Shivalkar et al. 2006; Brooks et al. 2008). Systolic dysfunction in type 2 diabetes is normally a later manifestation occurring as a consequence of DD. However, with modern techniques subtle changes in systolic function can be found in diabetic patients with formerly isolated DD (Yu et al. 2002; Fang et al. 2005). Interestingly the Framingham study with 292 diabetics and 4900 non-diabetics showed a gender-dependency of diabetes and congestive heart failure; the incidence of CHF was increased 2.4 -fold in diabetic men in contrast to a 5.1 –
fold increase in diabetic women (Kannel 1976). Animal models of diabetes have shown a reduction in systolic function in STZ-treated rats compared to Wistar (Wold et al. 2001). In one study in diabetic GK rats, diastolic dysfunction was not present at baseline compared to Wistar rats; however, in hypoxic conditions LVEDP increased significantly more in isolated hearts of GK rats compared to Wistar, indicating an increased susceptibility to develop DD (El-Omar et al. 2004). Furthermore, mitral inflow pattern was changed towards DD in a recent study where GK rats were fed a high sodium diet (Grönholm et al. 2005). Altered intracellular calcium regulation, accumulation of intracellular fatty acids and glycation of interstitial collagen and intracellular ryanodine receptors (RyR) have been associated with the development of DD in diabetes (Choi et al. 2002; Dong et al. 2006; Bidasee et al. 2003; Avendano et al. 1999). Furthermore, insulin–receptor knock-out (CIRKO) mice with myocardial infarction showed a reduction in the protein content of the Sarco-Endoplasmic Reticulum Calcium ATPase 2 (SERCA2) (Sena et al. 2009). A decrease in SERCA2 protein impairs relaxation as the protein is largely responsible for the uptake of calcium into the sarcoplasmic reticulum during diastole.

### 2.1.5.2 The renin - angiotensin system

The circulating renin angiotensin system (RAS) is commonly known for its role as a regulator of blood pressure by regulating blood vessel constriction and water and mineral balance. Renin is released from the kidney in response to decreased perfusion (i.e. low blood pressure) of the kidney in the juxtaglomerular apparatus (JGA) of the afferent arteriole. Renin is an enzyme that cleaves circulating angiotensinogen (secreted from the liver) to angiotensin I (ang I). Ang I is further cleaved to Ang II by angiotensin converting enzyme (ACE) mainly on the surface of pulmonary endothelial cells. Ang II increases systemic blood pressure (JGA perfusion) by 1) arteriolar vasoconstriction, 2) increasing sympathetic nervous system activity 3) increasing tubular Na\(^+\) and Cl\(^-\) reabsorption leading to H\(_2\)O retention alone and by stimulating aldosterone secretion from the adrenal cortex.
and 4) by reducing ADH secretion. The effects of RAS activation on organs such as the heart include hypertrophy, proliferation and fibrogenesis.

In the adult cardiovascular system Ang II induces its effects by binding to the AT1-receptor whereas expression of the AT2-receptor primarily declines after birth (for a review, see Lavoie et al. 2003). Recently a third component of the RAS has been identified, namely the Ang (1-7) – Mas receptor axis. Ang (1-7) is formed from Ang I and Ang II by ACE2, neutral endopeptidase (NEP) and ACE and acts by binding to the Mas receptor (Figure 2) (Donoghue et al. 2000). Recent evidence suggests that at least part of the beneficial effects of ACE inhibitors in the heart can be attributed to the shunting of Ang I to the Ang (1-7) – Mas axis from the Ang I – Ang II –AT1 receptor axis (Simões e Silva et al. 2006). In many ways the Ang (1-7) – Mas receptor axis acts on the heart in opposition to the Ang II – AT1R pathway. Ang (1-7) – Mas receptor activation has been shown to be anti-proliferative, anti-hypertrophic and antifibrinogenic (Santos et al. 2004).

2.1.5.2.1 The local renin angiotensin system

The revelation of a local or tissue RAS, independent but interacting with the systemic RAS, was a breakthrough finding in the 1970s (Ganten et al. 1971). At present a local RAS has been found in several organs including the heart (Bader et al. 2001). Additionally, the ACE2 – Ang (1-7) - Mas –receptor axis has been suggested to have an important role in intracardiac RAS (Donoghue et al. 2000). Present data indicate that the local cardiac RAS acts to amplify the effects of the systemic RAS; consequently it has been shown that myocardial infarction (MI) increases the activity in circulating RAS and it is associated with an increase in intracardiac RAS (Sun 2010; Bader et al. 2008). Furthermore, angiotensin II type 1 receptors are overexpressed after MI (Sun et al. 1994) and increased local production of Ang II from Ang I occurs when ACE expression is upregulated in endothelial cells of neovasculature, macrophages and myofibroblasts (Falkenhahn et al. 1995; Sun 1996). Local renin production has been shown to occur at the infarct site, however, there is conflict over the significance of local renin production and it has been proposed that activation of the local RAS starts
with increased uptake of angiotensinogen and renin from the circulation (Passier et al. 1996; Sun et al. 2001). Studies in rats have shown that the regulation of tissue AT1 receptor mRNA expression is independent of the circulating RAS (Sechi et al. 1996).

**Figure 2.** A simplified scheme of the components and actions of the renin-angiotensin system. Black boxes indicate peptides/proteins, gray boxes indicate enzymes, white boxes indicate receptors. Ang=angiotensin, PRR=prorenin receptor, AT=angiotensin receptor, ACE=Angiotensin converting enzyme, AP=aminopeptidase, NEP=neutral endopeptidase, PCP=prolyl carboxypeptidase. (Modified from Goodman & Gilman's the pharmacological basis of therapeutics, 12th ed. 2010).
In diabetes, local RAS activation in the heart is believed to contribute to increased fibrosis, apoptosis and oxidative stress; the hallmarks of diabetic cardiomyopathy (Fang et al. 2004). Activation of the RAS, both local and systemic, is strongly associated with the development of insulin resistance and the onset of type 2 diabetes. Indeed blocking the RAS has been shown to attenuate diabetic cardiomyopathy and increase insulin sensitivity and prevent the onset of type 2 diabetes (Machackova et al. 2004; Niklason et al. 2004; Scheen 2004; Abuissa et al. 2005; Liu et al. 2006; Ribero-Oliveira et al. 2008 and de Kloet et al. 2010). On the other hand, studies with type 1 diabetic subjects have shown a decrease in plasma renin activity (PRA) indicating a suppressed circulating RAS in insulin dependent diabetes (Bojestig et al. 1999).

Animal experiments have shown that Ang II infusion induces insulin resistance (Richey et al. 1999); and Mas receptor knockout mice show characteristic signs of the metabolic syndrome including dyslipidemia, hyperinsulinemia and obesity (Santos et al. 2008). Furthermore, increased levels of tissue angiotensinogen, Ang II and AT1 receptors have been shown in diabetic rat hearts (Khattar et al. 1996; Fiordaliso et al. 2000). Spontaneously diabetic BB rats (insulin dependent DM) treated with the ACE inhibitor captopril showed amelioration of cardiac dysfunction and cardioprotection with decreased fibrosis (Rösen et al. 1995). These results provide evidence for the involvement of local RAS in diabetic cardiomyopathy and break ground for further research in this field.

2.1.5.3 Left ventricular hypertrophy

Left ventricular (LV) hypertrophy is an independent risk factor for heart failure. The rationale is simple: an increase in cardiac mass increases oxygen consumption and thereby presents an increased risk for ischemic attacks. Furthermore, changes in LV architecture may predispose for cardiac dysfunction. Numerous studies have shown that patients with type 2 diabetes have an increase in LV hypertrophy independent of other confounding factors including hypertension (Galderisi et al. 1991; Devereux et al. 2000; Eguchi et al. 2008).
Hypertrophy is a form of growth which is characterized by an increase in average cell size of the constituting organ (in contrast to hyperplasia where the number of cells increases). Although left ventricular hypertrophy is more common, the right ventricle can also become enlarged or both may be affected. The common factor is that in physiological cardiac hypertrophy the intermittent load induces an increase in chamber wall thickness; this is compensated by an increase in ventricular volume, thus balancing wall thickness to chamber volume ratio (Dorn 2007). In pathological hypertrophy the chronic pressure or volume load imposed on the heart due to e.g. a stenotic valve or hypertension promotes the pathological changes in wall structure. The three major patterns of ventricular hypertrophy are divided into the following: 1) concentric left ventricular hypertrophy, 2) eccentric left ventricular hypertrophy and 3) mixed post-infarct hypertrophy (Figure 3). In chronic hypertension, concentric hypertrophy develops in response to increased peripheral resistance, this is known as afterload. Echocardiography suggests that obesity per se and diabetes are associated with mainly concentric but also eccentric LV hypertrophy (Woodwiss et al. 2008; Ojji et al. 2009); since hypertension is a common co-morbidity in diabetes, concentric hypertrophy is expected to be far more common.

In type 2 diabetes LV hypertrophy may not be present in the early stages and instead may manifest after a longer period. The mechanisms that contribute to LV hypertrophy in diabetes are not entirely clear. Recently however, clinical and animal studies have proposed evidence that an increase in circulating leptin and resistin from adipose tissue can induce concentric LV hypertrophy (Barouch et al. 2003; Eguchi et al. 2008). Hyperinsulinaemia in early type 2 diabetes has been linked to LV hypertrophy (Karason et al. 2003) and insulin resistance has been shown to be an independent risk factor for CHF in elderly men (Ingelsson et al. 2005). The obvious question that arises from this notion is whether insulin sensitivity can be preserved in some organs while resistance has developed in others. It has been postulated that the distribution and severity of insulin resistance is not uniform in all tissue types; while skeletal muscle and adipose
tissue are insulin resistant, insulin sensitivity may be preserved in the myocardium. This notion would make a logical explanation for the LV hypertrophy commonly seen in type 2 diabetes (for a review, see Poornima et al. 2006). Acutely, insulin sensitive tissue responds to insulin receptor activation by increasing LV hypertrophy. This is mediated by PI3K/Akt–guided increase in glucose uptake, GSK3-beta–guided increase in transcription of prohypertrophic genes and activation of mTOR–guided increase in protein synthesis (Shioi et al. 2002; Dorn et al. 2004). However, in chronic hyperinsulinemia, desensitization of insulin receptor and mitochondrial Akt sequestration leads to alternative Akt–independent pathways becoming increasingly important. Akt–independent pathways that have been shown to induce hypertrophy in insulin resistant tissue include the ERK1/2 and p38 MAPK pathways (Dobrin et al. 2010).

<table>
<thead>
<tr>
<th><strong>Pressure overload</strong> (hypertension)</th>
<th><strong>Volume overload</strong> (mitral regurgitation)</th>
<th><strong>Mixed overload</strong> (post-myocardial infarction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocyte thickening and collagen deposition</td>
<td>Myocyte lengthening and collagen deposition</td>
<td>Scarification and mixed myocyte effects</td>
</tr>
<tr>
<td>Concentric LV hypertrophy and diastolic dysfunction</td>
<td>Eccentric LV hypertrophy and diastolic dysfunction</td>
<td>Stretched and dilated chamber, systolic and diastolic dysf.</td>
</tr>
</tbody>
</table>

**Figure 3.** Three major patterns of ventricular hypertrophy with examples of clinical situations contributing to each pattern. In all types interstitial fibrosis is accelerated (Opie et al. 2006).
2.1.5.4 Changes in energy metabolism

The normal adult heart can use glucose and/or free fatty acids as sources of energy and the switch occurs smoothly depending on momentary supply and demand (Neely et al. 1972). In diabetes, myocardial tissue switches substrate utilization from a mix of fatty acids and glucose to the exclusive use of fatty acids (for a review, see Feuvray et al. 2008). Dysregulated lipid metabolism in the heart has been implicated as one of the key triggers of diabetic cardiomyopathy (for a review, see Dobrin et al. 2010). Peroxisome proliferator-activated receptors (PPARs) are transcription factors implicated in fatty acid (FA) metabolism. In diabetes the high utilization of FAs increase the expression of PPARs and their role in energy metabolism become more important. The overexpression of the PPAR subtype alpha (PPARalpha), the most abundant isoform of the PPARs, is implicated in the advent of diabetic cardiomyopathy (Finck et al. 2002). Experiments have shown that in the cardiomyocyte, an increase in intracellular fatty acids upregulates the expression of PPARalpha. However, in later stages of diabetes the expression of PPARalpha is downregulated and PPARgamma upregulated (for a review, see Saunders et al. 2008). Nuclear receptor PPAR-gamma increases the intake of glucose into peripheral tissues via the GLUT4 glucose transporter and reduces the free fatty acid concentration. This is an important target of the thiazolidinediones (glitazones), which are medicines used for the management of type 2 diabetes and sensitize liver, fat and skeletal muscle tissue towards the actions of insulin.

2.1.5.5 Insulin PI3K/Akt – FOXO3a pathway

When insulin binds the insulin receptor (IR, a transmembrane receptor tyrosine kinase (RTK)) which initiates a number of intracellular protein activation cascades that have the following effects: 1) glucose transport into the cell by recruitment of the glucose transporter Glut-4 membrane protein 2) glycolysis and following ATP synthesis 3) glycogen synthesis, i.e. storage of glucose 4) fatty acid synthesis from glucose and pyruvate 5) protein synthesis 6) survival by inhibition of
proapoptotic factors. IR-activation phosphorylates PI3 kinase (PI3K) which in turn phosphorylates PIP2 to produce PIP3. This enables PIP3 to bind and activate the serine/threonine kinase Akt (Figure 1). The phosphorylation and activation of the PI3K/Akt pathway is counteracted by the phosphatase and tensin homologue PTEN which dephosphorylates PIP3 which consequently leads to dephosphorylation of Akt (Figure 4).

Insulin is a well known anabolic hormone and the role of Akt as a growth promoting factor has been thoroughly investigated. An increase in insulin signalling in the heart has been shown to increase cardiac mass (Belke et al. 2002). This notion has triggered the theory that onset of LV hypertrophy in type 2 diabetes starts in the hyperinsulinemic state when insulin acts as a growth stimulus in non-insulin resistant tissue through the PI3K/Akt pathway. Recent studies have shown that pathological growth is associated with additional recruitment of signalling pathways that crosstalk with PI3K/Akt such as the Gq/phospholipase C (for a review, see Dorn et al. 2005). Activation of the PI3K - Akt pathway has been shown to increase hypertrophy through activation of the mammalian target of rapamycin (mTOR) (Shioi et al. 2002). Akt mediates its anti-apoptotic effects partly by inhibiting the forkhead class O transcription factors (FOXOs) (Brunet et al. 1999). The FOXO family of transcription factors is an important group that modulates expression of genes involved in apoptosis (Bcl2II11, FasL), repair of damaged DNA (GADD45), arrest of the cell cycle (p27, cyclins), and detoxification of ROS (SOD2) (for a review, see Huang et al. 2007). FOXO proteins are tightly regulated, they are activated by cJun N-terminal kinase (JNK) and Mammalian sterile 20-like kinase 1 (MST1), they undergo inhibitory phosphorylation by the Akt kinase and their actions are counterbalanced by deacetylation by the Sirtuin (silent mating type information regulation 2 homolog) 1 (Sirt1) (Huang et al. 2007). Evidence for Akt-mediated inhibition of FOXO was found when insulin and insulin like growth factor (IGF-1) suppressed FOXO activity (Ogg et al. 1996; Kimura et al. 1997). FOXO3a is a target of the insulin receptor - PI3K/Akt signalling pathway in cardiomyocytes and it mediates the
response to oxidative stress; FOXO3a phosphorylation by IR – Akt/PI3K inactivates expression of proapoptotic target genes such as Bcl-like 11 (Bim) and FasL (Franke et al. 1997). On the other hand, reduced insulin signalling reduces Akt/PI3K mediated phosphorylation and thus inactivation of proapoptotic FOXO3a. In spontaneously diabetic Goto-Kakizaki (GK) rats, a model of type 2 diabetes, IR – PI3K/Akt signalling and insulin dependent glucose uptake is defect partly due to depletion of IR subunit beta, IRS-1 and Glut4 (Galli et al. 1999; Desrios et al. 2004) and therefore disturbance in Akt/PI3K mediated FOXO3a – signalling is likely to be present in the GK rat. However this has neither been investigated nor reported in the literature. Akt –mediated FOXO –phosphorylation translocates the FOXOs to the nucleus where they are sequestered to 14-3-3 chaperones.

**Figure 4.** A simplified scheme showing the major effects of insulin - PI3K/Akt pathway. IR: Insulin receptor, P: phosphate group, PI3K: phosphoinositide 3-kinase, PIP2(3): phosphatidylinositol (3),4,5-bis(tris)phosphate, PTEN: phosphatase and tensin homologue 10, mTOR: mammalian target of rapamycin, p27Kip: p27 cyclin dependent kinase inhibitor, GSK3beta: glycogen synthase kinase 3 beta, FOXO: forkhead class O transcription factor.
2.1.5.6 Sirt1 signalling - FOXO3a and p53

Mammalian Sirtuin1 (Sirt1) is a nuclear class III histone deacetylase enzyme (HDAC) with sequence homology to yeast silent information regulator 2 (Sir2) and it is implicated in increased longevity through suppression of DNA transcription (histone deacetylase effect) and through its negative actions on proapoptotic factors. For an overview of the regulation and organ-specific effects of Sirt1, refer to figure 5. In the heart Sirt1 is associated with increased cellular stress resistance, decreased senescence and increased hypertrophy. Sirt1 is activated by caloric restriction and it is one of the key factors that contribute to the increased life span associated with reduced energy intake (Lin et al. 2000; Howitz et al. 2003). As expected, recent studies have provided a connection between Sirt1 –mediated cell survival and the PI3K/Akt –pathway (for a review see Sundaresan et al. 2011). Phosphorylation of cytoplasmic Sirt1 by the Akt kinase promotes nuclear translocation of Sirt1 (Tanno et al. 2010). In the nucleus Sirt1 inactivates transcription of genes implicated in reduced longevity (maintenance of intact telomeres) and apoptosis such as p53 and FOXO3a. Sirt1 –mediated deacetylation is coupled to nicotinamide adenine dinucleotide (NAD\(^+\)) hydrolysis which yields the deacetylated substrate and nicotinamide (NAM). NAD\(^+\) is a coenzyme implicated in the energy transfer chain by accepting electrons (becoming the reduced form: NADH) and donating them (returing to the oxidized state NAD\(^+\)). Hence, Sirt1 activity is sensitive to the oxidative state of the cell, a high NAD\(^+\)/NADH –ratio increases Sirt1 activity and nicotinamide functions as a feedback inhibitor (for a review, see Shore 2000). Gene expression of Sirt1 is regulated by numerous transcription factors. Negative feedback loops are provided by p53, HIC1 and FOXO3 (Yamakuchi et al. 2009). In the posttranslational setting Sirt1 activity and localisation is modulated not only by NAD and PI3K/Akt, but also by direct protein binding and small molecule activation. The main proteins that modify Sirt1 activity include the activator protein active regulator of Sirt1 (AROS) and the negative regulator deleted in breast cancer 1 (DBC1) (for a review, see Haigis et al. 2010). Exogenous small
molecule modulators of Sirt1 include activators such as the polyphenol resveratrol and inhibitors such as sirtinol. Resveratrol binds Sirt1 at the same N-terminal site as AROS and DBC1.

Figure 5. Sirt1 and its organ-specific effects: Sirt1 increases life span and is a key regulator of cellular energy metabolism. The level of Sirt1 protein, NAD, Sirt1-phosphorylation, AROS and resveratrol are positive modulators of Sirt1; whereas DBC1 and sirtinol are negative regulators of Sirt1. LVH=left ventricular hypertrophy, FA=Fatty acid, NAD=nicotinamide dinucleotide, AROS=active regulator of Sirt1, DBC1=deleted in breast cancer 1. (Modified from Haigis et al. 2010).

Hyperglycemia-induced ROS and RNS build-up has been implicated in an increase in p53 –and Caspase -3 dependent cardiomyocyte apoptosis in human diabetes and in animal models (Frustaci et al. 2000; Fiordaliso et al. 2001). Furthermore, the tumor suppressor and transcription factor p53 is implicated in increased cardiomyocyte apoptosis in response to oxidative stress, hypoxia, stretch and DNA damage (for reviews, see Von Harsdorf et al. 1999 and Giordano 2005). In response to cellular stress, p53 is maintained at a relative high level by post-translational modifications including phosphorylation and acetylation. It has been shown that the lysine acetylation at the site K373/K382 is linked to the ability
of p53 to regulate cell cycle arrest and apoptosis (Zhao et al. 2006). The anti-apoptotic effects of Sirt1 are in part mediated through deacetylation of p53 at K373/382 (Luo et al. 2001). Sirt1 modulates the activity of FOXO3a, a transcriptional regulator and member of the forkhead class O (FOXO) family. Nuclear FOXO3a is subject to acetylation by CBP and p300 and FOXO3a – acetylation is increased in response to oxidative stress (Huang et al. 2007). Accordingly Sirt1 modulates FOXO3a transcriptional activity to favour of cell survival. Deacetylation of FOXO3a inhibits transcription of apoptotic genes, increases transcription of genes implicated in cell cycle arrest and increases transcription of genes associated with resistance to oxidative stress (Brunet et al. 2004). In rat and dog hearts, the Sirt1 protein has been shown to inhibit apoptosis and act as a pro-hypertrophic factor (Alcendor et al. 2004; Alcendor et al. 2007). A role for Sirt1 in alleviating insulin resistance was suggested when Sirt1 activation increased insulin sensitivity through activation of the peroxisome proliferator-activated receptor gamma coactivator-alpha (PGC1alpha) (Lagouge et al. 2006). Sirt1 is a key regulator of energy and metabolic homeostasis, and since diabetes is associated with disturbance in these, the role of Sirt1 in the development of diabetic cardiomyopathy is intriguing and should be further investigated.

2.1.5.7 P38 Mitogen activated protein kinase

In the heart, the mitogen activated protein kinase (MAPK) signalling system represent an important mechanism for cellular stress response (for a review, see Rose et al. 2010). The four most thoroughly investigated MAPK:s include ERK1/2, ERK5, JNK and p38. Recent information provides an important role for p38 MAPK activation in insulin resistance (Henriksen et al. 2010). Mitogen activated protein kinase (MAPK) p38 is activated by angiotensin II, inflammation, oxidative stress and ischemia (Gao et al. 2002; Zhang et al. 2004). Intracellular factors such as DNA damage or extracellular factors such as binding of proapoptotic FasL induce protein kinase cascade activation (MEKK1-4, MLK, MKK) and subsequent p38 MAPK activation. Previous studies suggest that p38 MAPK activation reduces contractility (Liao et al. 2002). In experimental MI, inhibition of p38 MAPK has
been shown to reduce ventricular remodelling (See et al. 2004). Long-term, but not short-term activation of the p38 MAPK is deleterious as it increases fibrosis, hypertrophy and apoptosis (Kompa AR. 2008). Previous studies have shown that p38 induces collagen formation, CTGF mRNA expression and proinflammatory cytokine IL-6 in the heart (Tenhunen et al. 2006). The role of p38 MAPK has not been studied in detail in diabetic GK rat hearts. For a review of p38 and its effects on gene expression in spontaneously hypertensive rats with LVH, see (Rysä 2009).

2.1.5.8 Cardiomyocyte renewal and senescence

The ageing heart evokes the typical clinical symptoms resulting from a decline in cardiac function. Until a decade ago it was thought that hypertrophy is the only means of growth in the heart. However, breakthroughs in imaging and the discovery of markers of cellular proliferation have provided evidence that dividing myocytes are present in the non-diseased heart and that proliferation of cardiomyocytes is accelerated in the infarct border zone (for a review, see Anversa et al. 2002). A recent study showed that annual cardiomyocyte turnover occurs at an age-dependent pace ranging from 1% to 0.5% in the human heart (Bergmann et al. 2009). The exact role of the recently discovered resident cardiac stem and progenitor cells (CSC) is currently unclear (for a review, see Torella et al. 2008). The revelation of resident cardiac stem cells has sparked the design of novel therapies for the treatment of myocardial infarction, including the implantation of progenitor cell sheets in the infarcted area.

When stem cells age they lose their restorative capacity. In an aging organism, cells lose their functional abilities, cease to grow and replicate, and ultimately undergo apoptosis, a process called cellular senescence. Age-dependent increases in specific tumour suppressor proteins have been recently discovered; these markers of senescence have been shown to promote apoptosis by activating key apoptotic factors such as p53 (see section on apoptosis above). Two essential markers of cellular senescence in the cardiomyocyte include p16\textsuperscript{INK4a} and p19\textsuperscript{ARF} (for a review, see Sharpless 2004).
2.1.5.9 Programmed cell death (Apoptosis)

Apoptosis or programmed cell death is an important process during development in all organisms. Apoptosis is a tightly regulated process where cells go through the characteristic steps of DNA fragmentation, chromatin condensation, nuclear shrinking and formation of apoptotic bodies. Later in life, apoptosis is used to dispose of cells that are old or have been injured; however apoptosis can be harmful if it is unintentional or occurs in excess. In the heart, programmed cell death has been recognised as an important process in the progression to heart failure (for a review, see van Empel 2005). A cell can undergo apoptosis via either the extrinsic or intrinsic pathways or via a combination of the two. Activation of the extrinsic pathway is initiated by extracellular stimuli such as toxins, hormones or growth factors and involves the binding of one of the death ligands TNF-alpha, FasL or TRAIL to receptors of the TNFR family. Ligand-receptor binding results in the formation of a death inducing signalling complex (DISC). Intra- or extracellular stress factors such as hypoxia, ischemia/reperfusion, oxidative stress or increased intracellular calcium concentration activates the intrinsic pathway. The intrinsic pathway involves proteins located in the ER and the mitochondria, including Bcl2 and cytochrome C and the effector caspases.

In animal models for obesity and diabetes an increase in apoptosis has been shown to occur in the heart (Barouch et al. 2006). Several mechanisms have been proposed to lead to the increase in apoptotic cell death; these include gluco- and lipotoxicity, mitochondrial dysfunction and activation of the RAS. All of these lead to an increase in the build-up of reactive oxygen species (ROS). The high circulating and intracellular stores of fatty acids leads to alternative non-oxidative forms of energy production that result in the accumulation of toxic by-products. The reactive lipids react with oxygen products to produce toxic reactive lipid species that may lead to increased cell death (Dorn 2009).

Mitochondrial dysfunction in diabetes has been widely recognised as a contributor for diabetic cardiomyopathy and a source for excessive ROS build-up (Boudina et al. 2006). Indeed, diabetes, ROS build-up and hyperglycemia have been
implicated in p53- and caspase 3- dependent increase in cardiomyocyte apoptosis (Cai et al. 2001; Fiordaliso et al. 2001; Giordano et al. 2005). Furthermore, increased RAS activity in the diabetic heart has been associated with increased apoptosis (Frustaci et al. 2000).

2.1.5.10 Fibrosis

In cardiac fibrosis the increased production and deposition of collagen and other extracellular matrix proteins leads to stiffening and reduced relaxation of the ventricles. The echocardiographic features of increased LV fibrosis appear as impaired relaxation and diastolic dysfunction (increased LVEDP and primary decrease in E/A ratio). Typically, fibrosis is a highly regulated protective mechanism that restores the general architecture of the parenchyma. For example, after MI interstitial fibrosis occurs at the infarct site to produce the scar and continues somewhat in the border zone; being non-functional this contributes to myocardial stiffness and development of cardiac dysfunction. In diabetic cardiomyopathy increased collagen deposition and fibrosis are considered hallmark histological features (Regan et al. 1977; Frustaci et al. 2000). Consistent with this, it has been proposed that a modest increase in interstitial fibrosis is the initial cue for diabetic cardiomyopathy (Shimizu et al. 1993). In a study with prediabetic OLETF rats there was a correlation between increased extracellular collagen content and a decrease in early mitral peak flow (decreased E/A ratio) (Mizushige et al. 2000). The cause for increased collagen accumulation in diabetes is believed to result from 1) reduced degradation of glycosylated collagen and 2) increased production due to increased RAS activation (for a review, see Fang et al. 2004).

2.1.6 Ischemic heart disease and heart failure

Subjects with type 2 diabetes are at a higher risk for developing atherosclerosis and ischemic heart disease including fatal myocardial infarction (Haffner et al. 1998). Recent evidence has shown that diabetes and prior MI are equal risk factors for mortality following acute coronary syndromes (including unstable
angina pectoris (UAP), non-ST elevation myocardial infarct (NSTEMI) and ST-elevation myocardial infarct (STEMI)) (Donahoe et al. 2007). Several mechanisms have been proposed that contribute to the increased atherogenesis including increased production of reactive oxygen species (ROS), altered substrate metabolism and dyslipidaemia (for a review, see Fang et al. 2004). In the coronary arteries, diabetes is associated with endothelial dysfunction, whereas the myocardium is afflicted by a mixture of insulin resistance and increased RAS activation, which further accelerates the atherogenesis. Hypertension is a risk factor for ischemic heart disease and type 2 diabetic patients' blood pressure values should be kept at below 130/80 mmHg due to the aggregate effect of diabetes and hypertension on the development of coronary heart disease.

Atherogenesis in the medial layer of the coronary arteries may result in the formation of a fibroatheroma. Fibroatheromas can be classified into lipid-rich plaques that risk rupture and embolisation or fibrotic ones that are more likely to undergo thrombosis, both of which are risks for myocardial infarction (MI). MI in the left ventricle results from occlusion somewhere in the left coronary artery (LCA). Occlusion in the left anterior descending (LAD) artery results in cessation of blood flow to the anterior wall and septum of left ventricle. An established method for inducing experimental MI with following post-MI heart failure in experimental animals is to ligate the LAD coronary artery. Complications of myocardial infarction include sudden cardiac death, hypertrophy, fibrosis, rupture of the chordae, and ischemia of the papillary muscle, valvular disease, ventricular aneurysms, septum perforation, pericardial tamponade, and post-MI arrhythmia. If reperfusion does not occur within hours, the hypoxic area undergoes inflammation, apoptosis, necrosis and fibrosis. The non-infarct area undergoes architectural changes to compensate for the loss in function. In the long-term the non-functional scar tissue, the change in architecture of viable tissue together with potential post-MI complications together contribute to the development of heart failure.
2.1.7 Rodent models of diabetes

Type 2 diabetes is a multifactorial disease and both environmental and congenital factors are involved in the pathogenesis; hence there are no animal models that specifically mimic human type 2 diabetes. Although inadequate to fully replicate the clinical situation, the following rodent models have proven useful for studying hyperglycaemia, insulin resistance and the development of cardiomyopathy (for a review, see Bugger et al. 2009) (table 2).

2.1.7.1 The Goto-Kakizaki rat

The Goto-Kakizaki (GK) rat was developed by selective inbreeding of glucose intolerant Wistar rats (Goto et al. 1976). Chromosomal mapping of the genetic defects in the GK rats has revealed 6 independent loci involving mutations (Gaugier et al. 1996). Already at a few weeks of age the GK rats exhibit mild hyperglycemia, hyperinsulinaemia, glucose intolerance and peripheral insulin resistance (Bisbis et al. 1993). The GK rat heart has been shown to be partly insulin resistant. Insulin resistance in the GK rat heart is associated with a 31% reduction in insulin receptor protein expression and 38% reduction in IRS-1 protein expression. This is associated with 37% and 45% decrease in insulin – stimulated phosphorylation of these proteins, respectively (Desrois et al. 2004). An increased susceptibility to oxidative stress has been shown in various studies. ROS build-up due to hyperglycemia and increased FFA, mitochondrial dysfunction and reduced levels of antioxidant has been proposed to play a crucial role in the pathogenesis in GK rats (Santos et al. 2003; Bitar et al. 2005). Our studies have shown that GK rats develop endothelial dysfunction, left ventricular hypertrophy, moderate hypertension and are salt-sensitive. The involvement of RAS activation in the pathogenesis is supported by the beneficial effects of ang II inhibition in GK rats fed with a high salt diet (Cheng et al. 2004).
Table 2. Rodent models of diabetes and obesity

<table>
<thead>
<tr>
<th>Model</th>
<th>Type 1 diabetes</th>
<th>Obesity/Type 2 diabetes</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Mechanism in brief</strong></td>
<td>STZ</td>
<td>OEV26 mouse</td>
</tr>
<tr>
<td></td>
<td>Streptozotocin induced beta cell loss</td>
<td>Calmodulin overexpression induced beta cell loss</td>
</tr>
<tr>
<td><strong>Background strain</strong></td>
<td>Any</td>
<td>FVB</td>
</tr>
<tr>
<td><strong>Latency to diabetes</strong></td>
<td>1-2 weeks after injection</td>
<td>1 week postpartum</td>
</tr>
<tr>
<td><strong>Obese/non-obese</strong></td>
<td>Non-obese</td>
<td>Non-obese</td>
</tr>
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<td><strong>LV hypertrophy</strong></td>
<td>↑</td>
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</tr>
<tr>
<td><strong>Cardiac function</strong></td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

STZ: Streptozotocin, ZDF: Zucker diabetic fatty, ZFR: Zucker fatty rat, GK: Goto-Kakizaki
2.2 Levosimendan

2.2.1 Pharmacological properties and dosing

Levosimendan, or (-) (R)-[[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono]-propanedinitrile has a molecular weight of 280.291 g/mol and the molecular formula is C_{14}H_{12}N_{6}O. Levosimendan is commercially available as 1 ml, 5 ml and 10 ml ampoules with 2.5 mg/ml concentrate for intravenous infusion. The dose and duration of infusion varies on an individual basis and with the dose being based on the clinical situation and response to treatment. Usually infusion in a peripheral or central vein starts with a 10 minute bolus of 6-12 µg/kg and the treatment continues at a rate of 0.1 µg/kg/min. The recommended total infusion duration is 24 hours for patients with severe decompensated congestive heart failure.

![Figure 6. Chemical structure of levosimendan](image)

2.2.2 Pharmacokinetics

Approximately 97% of levosimendan is bound to plasma proteins, mainly albumin. Levosimendan is metabolized by conjugation to cyclic or acetylated cystein (Lehtonen et al 2004). Levosimendan is excreted into the intestine where bacterial metabolism yields the active circulating metabolites OR-1855 and OR-1896. The half-life of elimination for levosimendan is one hour. The metabolites appear slowly in the circulation, ca. 2 days after cessation of infusion. The half lives of OR-1855 and OR-1896 are longer, between 75 to 80 hours. The haemodynamic
effects of levosimendan infusion are a combination of the acute effects (levosimendan) and long-lasting effects (OR-1896). When levosimendan was given to rats in drinking fluid at a concentration of 10 mg/l, the average daily dose calculated from weekly plasma samples over a 7-week period averaged 3.6 mg/kg (Louhelainen et al. 2007). In the same set-up, the mean 24–hour plasma concentrations of levosimendan and its metabolite OR-1896 were 79 ± 19 ng/ml and 42 ± 15 ng/ml respectively.

2.2.3 Pharmacodynamics and mechanisms of action

The myocardial contractile apparatus includes the myofilaments actin and myosin and a complex of interacting regulatory proteins namely tropomyosin and the troponins T (TnT), I (TnI) and C (TnC). When calcium attaches to troponin C it causes a conformational change in the complex allowing the formation of cross bridges between actin and myosin, i.e. contraction occurs. Levosimendan increases calcium sensitivity of contractile proteins in the cardiomyocyte by binding to TnC. Levosimendan stabilizes TnC and does not increase intracellular calcium concentration (Pollesello et al. 1994). In the failing heart levosimendan acts as a positive inotrope, i.e. it increases the strength of contraction. Diastolic function is preserved due to the rapid detachment of levosimendan from TnC during relaxation (Levijoki et al. 2000; Antoniades et al. 2007); additionally levosimendan has been shown to enhance diastolic function in paced dogs (Tachibana et al. 2005).

In addition to acting as a positive inotrope, levosimendan exerts vasodilatory effects. This is mediated mainly through opening of ATP–dependent potassium (K\text{ATP}) –channels in small resistance vessels of arterial vasculature leading to outflow of potassium and subsequent hyperpolarization of the membrane potential (Yokoshiki et al. 1997). Levosimendan has also been shown to activate voltage–sensitive and calcium–activated potassium channels (BK\text{Ca}) (Pataricza et al. 2003). Furthermore, levosimendan has been shown to act as a phosphodiesterase 3–inhibitor (Gruhn et al. 1998). Inhibition of PDE3 increases the amount of second messenger cAMP which on the one hand activates calcium
influx in the cardiomyocyte and thus increase force of contraction and on the other hand induce vasodilation by outflux of calcium. However, the clinical relevance of levosimendan’s PDE3 inhibitory effect remains unclear. The active metabolite OR-1896 activates $K_{\text{ATP}}$ and $BK_{\text{Ca}}$ –channels and its vasodilatory effects are comparable to levosimendan (Erdei et al. 2006).

Prior studies suggest that levosimendan has beneficial effects on myocardial ischemia, apoptosis and inflammation that are independent of its inodilatory effects (Parissis et al. 2004; Adamopoulos et al. 2006). It has been postulated that by opening mitochondrial ATP –dependent potassium channels ($mK_{\text{ATP}}$), levosimendan may exert anti-ischemic effects that mimic preconditioning (Kersten et al. 2000; Kopustinskiene et al. 2004). In the energy deprivation state such as ischemia, opening of the mito$K_{\text{ATP}}$ –channels in the cardiomyocyte has been suggested to maintain mitochondrial matrix volume and preserve sufficient energy transfer upon reperfusion (for a review, see Garlid et al. 2003). The anti-apoptotic effects have been speculated to be due to the potassium channel opening properties (Louhelainen et al. 2007). The clinical relevance of the anti-apoptotic and anti-inflammatory properties are still unclear. The beneficial effects on ventricular remodelling described here provided a basis to study the effects of levosimendan in a rodent model of diabetic cardiomyopathy with post-MI heart failure.

2.2.4 Experimental studies

Levosimendan has been investigated in several animal models. A recent study examined levosimendan, milrinone and dobutamine in anaesthetized dogs and confirmed that levosimendan acts as an inodilator and does not increase myocardial oxygen consumption in contrast to the other compounds (Banfor et al. 2008). In isolated rabbit hearts, pre-treatment with levosimendan prior to ischemia-reperfusion was shown to have a positive effect on recovery (Leprán et al. 2006). In porcine models levosimendan has been shown to increase success rate after cardiopulmonary resuscitation (Koudona et al. 2007) and to partly restore right ventricular function after pulmonary embolism (Kerbaul et al. 2007).
compared with placebo treatments. The effects of levosimendan on endotoxaemia has been studied in sheep (Dubin et al. 2006), and further studies have been performed in the guinea pigs (Kaheinen et al. 2004), rats (Louhelainen et al. 2007; Boost et al. 2008) and dogs (Banfor et al. 2008).

2.2.5 Clinical use

Levosimendan is the only calcium sensitizer presently used and it is prescribed for the management of acute decompensated heart failure (ADHF). Levosimendan is used “off-label” for other cardiovascular disorders such as cardiogenic shock, septic shock and as perioperative cardiac support. In contrast to other positive inotropes, levosimendan has been suggested to exert its effects without increasing oxygen demand (Ukkonen et al. 1997). And furthermore, studies suggest that levosimendan does not increase intracellular calcium concentration and does not activate the sympathetic system (for a review see Antila et al. 2007). Its beneficial effects on heart failure (HF) symptoms include reduced dyspnoea and decreased P-BNP. The use of levosimendan for ADHF is associated with shorter hospital stays compared to dobutamine (Mebazaa et al. 2005).

2.2.5.1 Clinical trials

In clinical trials the effects of levosimendan on heart failure symptoms have been beneficial, whereas the effects on mortality have yielded controversial results. In three clinical trials levosimendan was proven to be superior to dobutamine (LIDO, 203 patients), to placebo (RUSSLAN, 504 patients) and both (CASINO, 299 patients) (Follath et al. 2002; Moiseyev et al. 2002; Lehtonen et al. 2007) In two recently finished large clinical trials levosimendan did not improve mortality compared to placebo (SURVIVE, 1327 patients) and dobutamine (REVIVE II, 600 patients) (Lehtonen et al. 2007; Mebaaza et al. 2007). In one clinical trial 1 mg levosimendan was given orally once or twice a day (PERSIST, 307 patients) and compared with placebo treatments in randomly assigned HF patients with NYHA IIIB-IV symptoms. Levosimendan improved the quality of life score (Minnesota
living with heart failure quality of life score) and reduced the plasma concentration of NT-ProBNP but did not affect the primary composite end-point consisting of repeated symptom assessments, worsening of heart failure and mortality during 60 days (Niemininen et al. 2008).

### 2.2.6 Adverse effects

Levosimendan is well tolerated in general; however some adverse events mainly affecting the cardiovascular system are associated with the use of levosimendan. Hypotension is classed as a very common adverse event (occurring in ≥1/10 subjects), whereas common adverse events affecting the heart include atrial fibrillation, tachycardia, heart failure, cardiac ischemia and extrasystole (occurring in ≥1/100 subjects). In the gastrointestinal system nausea, constipation, diarrhoea, vomiting are commonly occurring adverse events. Other common or very common adverse events include hypokalemia, insomnia, headache, vertigo and decrease in blood haemoglobin.
3 Aims of the study

Diabetes is an independent risk factor for the development of left ventricular hypertrophy, diastolic dysfunction and myocardial infarction. The increasing prevalence demands for better understanding of the molecular mechanisms that contribute to cardiac remodelling. The aim of this study was firstly to characterize the molecular mechanisms of diabetic cardiomyopathy in spontaneously diabetic Goto-Kakizaki (GK) rats. Secondly, we aimed to examine the effects of myocardial infarction (MI) on ventricular remodelling in diabetic GK rats. Thirdly, the aim was to study the effects of the calcium sensitizer levosimendan on post-infarct heart failure and left ventricular remodelling.

For these goals the following specific aims were established:

I. To investigate whether left ventricular remodelling is associated with cardiac dysfunction, apoptosis, left ventricular hypertrophy and fibrosis in diabetic GK rats. And to examine whether longevity-associated and pro-hypertrophy pathways Sirt1 – p53 and - FOXO3a are altered in the myocardium of GK rats.

II. To examine the effect of myocardial infarction on the parameters of left ventricular remodelling stated in aim I, and to further study the role of Akt - FOXO3a, Sirt1 - p53 signalling pathways in the pathogenesis.

III. To examine the effects of oral levosimendan treatment on 1) post-MI left ventricular remodelling and 2) myocardial gene expression profile in the diabetic GK rat.
4 Materials and methods

4.1 Experimental animals

4.1.1 Spontaneously diabetic Goto-Kakizaki rats

The Goto-Kakizaki rat is a model of type 2 diabetes developed by selective inbreeding of glucose intolerant Wistar rats (Goto et al. 1976). The GK rats are non-obese and develop mild hyperglycemia at 4-8 weeks (fP-Gluc up to 8 mMol/L), modest hyperinsulinemia, glucose intolerance, impaired glucose-induced insulin secretion and peripheral insulin resistance spontaneously. The GK rats are further dyslipidemic and show a degree of coagulation disorder. The GK rats develop modest hypertension and cardiac hypertrophy spontaneously, and GK rats have impaired vascular relaxation. The animals are salt-sensitive and develop kidney damage which is reversed by RAS blockade (Cheng et al. 2001). Wistar rats were used as a normoglycemic controls.

4.1.2 Animal welfare

Spontaneously diabetic Goto-Kakizaki rats were purchased from Taconic M&B Laboratory Animals and Services for Research, Ry, Denmark. Nondiabetic Wistar rats were purchased from Harlan, USA. All rats had free access to standard chow (Harlan, Germany) and drinking water.

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

<table>
<thead>
<tr>
<th>Study #</th>
<th>Strain</th>
<th>Group</th>
<th>Treatment</th>
<th>Follow-up (weeks)</th>
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<td>Goto-Kakizaki</td>
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<td></td>
<td>Wistar</td>
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<td>III</td>
<td>Goto-Kakizaki</td>
<td>GK MI</td>
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<td>Goto-Kakizaki</td>
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<td>Goto-Kakizaki</td>
<td>GK SHAM + Levo</td>
<td>Levosimendan 1 mg/kg</td>
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<td>Goto-Kakizaki</td>
<td>GK SHAM + Levo</td>
<td>Levosimendan 1 mg/kg</td>
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In all studies the animals were 8-week old at the start of the protocol.
4.2 Experimental myocardial infarction

Experimental myocardial infarction (MI) was performed by left anterior descending (LAD) coronary artery ligation in male eight-week old GK or Wistar rats as described previously (Palojoki et al 2001). The operation was made under ketamine (65 mg/kg i.p.) and medetomidine (0.5 mg/kg i.p.) anaesthesia. Long-acting insulin (1 IU/animal) was given two hours before anaesthesia to GK rats. Buprenorphine (0.02 mg/kg s.c.) was given twice a day for two consecutive days for post-operative analgesia. The rats that were alive 24 hours after the operation were randomized into study groups.

4.3 Levosimendan dose

Levosimendan (Orion Ltd, Espoo, Finland) was mixed in drinking water (10 mg/l) and given for oral consumption; this produced the daily dose of approximately 1 mg/kg (each animal drank an average of 30 ml/day). The dose was chosen based on our previous experiments where levosimendan produced clinically relevant plasma concentrations and exerted clear therapeutic effects (Louhelainen et al. 2007; Levijoki et al. 2001). Fresh levosimendan solution was prepared daily.

4.4 Echocardiography

Transthoracic echocardiography (Toshiba Ultrasound, Japan) was performed in a blinded fashion by the same technician, with all rats under isoflurane anesthesia (AGA, Riihimäki, Finland). Each animal underwent three separate measurements by detaching the transducer between each measurement, and three pictures were taken. Parameters needed for the calculation of left ventricular (LV) function and LV dimensions were measured from three systole-diastole cycles. A short-axis view of the left ventricle at the level of the papillary muscle was obtained (Gibson method). Two-dimensionally guided M-mode recording through the anterior and posterior walls of the left ventricle allowed us to measure the left ventricle (LV) end-systolic (LVESD) and end-diastolic (LVEDD) dimensions as well as interventricular septum (IVS), anterior wall (AW) and posterior wall (PW)
thickness. LV fractional shortening (FS), ejection fraction (EF), measures of LV systolic function, stroke volume (SV), end-diastolic volume (EDV), end-systolic volume (ESV) and cardiac output (CO), were calculated from the M-mode LV dimensions using the following equations: FS (%): \[
\frac{[(LVEDD-LVESD) / LVEDD]}{100},
\]
EF (%): \[
\frac{SV}{EDV},
\]
SV: \[
EDV – ESV,
\]
EDV: \[
0.52 \times (0.98 \times \left(\frac{LVIDD}{10}\right) + 5.90) \times \left(\frac{LVIDD}{10}\right)^2,
\]
ESV: \[
0.52 \times (1.14 \times \left(\frac{LVIDS}{10}\right) + 4.18) \times \left(\frac{LVIDS}{10}\right)^2,
\]
CO: \[
\text{Heart rate} \times SV.
\]
(LVIDD: Diameter of short axis left ventricle in end-diastole), (LVIDS: Diameter of short axis left ventricle in end-systole).

### 4.5 Blood pressure recordings

Blood pressure was measured using a tail-cuff blood pressure analyzer (Apollo-2AB Blood pressure analyzer, M179-2AB, IITC, Life Sciences, Woodland Hills, CA, USA). Blood pressure recordings were performed by the same technician at the same time of day.

### 4.6 Sample preparations

Urine samples were collected during a 24-hour period when the rats were seated in metabolic cages. Rats were anaesthetized with CO$_2$/O$_2$ (AGA, Riihimäki, Finland) and decapitated. Blood samples were collected in tubes containing ethylenediaminetetra-acetic acid (EDTA). Hearts were excised, washed with ice-cold saline solution and blotted dry. After weighing, the hearts were snap-frozen in liquid nitrogen (molecular biology) or isopentane (immunohistochemistry). All samples were stored in -80°C until assayed.

### 4.7 Biochemical analyses

Blood glucose was determined using a handheld test meter (Glucocard II®, Arkray, Japan and Contour, Bayer, Basel, Switzerland), plasma BNP (BNP-45, Peninsula Laboratories, Belmont, CA, USA), plasma renin activity (Angiotensin I RIA kit, Diasorin, Italy), serum aldosterone (Coat-a-Count Aldosterone RIA kit, DPC Biermann, Bad Nauheim, Germany) and serum insulin (Rat insulin RIA kit, Linco, St. Charles, Missouri, USA) were determined by radioimmunoassay.
according to the instructions of the manufacturers. Urinary noradrenaline was analyzed using the isocratic ion-pair reversed-phase high-performance liquid chromatography method with electrochemical detection. Plasma samples were determined for levosimendan and its active metabolite OR-1896 by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Kivikko et al. 2003).

4.8 Histology

The hearts were fixed in 10% buffered formalin solution for histological analyses. Transversal 5-µm sections were cut from the paraffin-embedded left ventricle and stained with hematoxylin-eosin or picrosirius red. Infarct sizes were determined planimetrically from the picrosirius-red stained histological sections as described elsewhere (Palojoki et al 2001). Collagen volume fraction was determined by computerized software analysis (ImageJ, NIH) from picrosirius red –stained histological sections (x20 magnification). Cardiomyocyte cross sectional area was measured from transverse-cut tissue slides (6 slides from each group) by conventional light microscopy at 40x magnification.

4.9 Immunohistochemistry

Immunohistochemistry was performed on deparaffinised and rehydrated formalin-fixed 5 µm thick myocardial sections as described in detail elsewhere (Finckenberg et al. 2003). For evaluating Sirt1 protein expression and localization primary polyclonal antibody against Sirt1 (Sir2alpha, Rabbit polyclonal, Upstate; 1:200 dilution) and secondary biotinylated anti-rabbit antibody (Vector Laboratories Inc., California, USA) were used. The average percentages of Sirt1-positive cardiomyocytes/total number of cardiomyocytes were calculated from 6 separate fields of transverse left ventricular tissue sections using light microscopy (x40 magnification). Immunoperoxidase staining for p16^{INK4a} was performed on frozen myocardial sections using a primary monoclonal p16^{INK4a} antibody (Santa Cruz BT, Santa Cruz, CA, USA) and peroxidise –conjugated rabbit anti-mouse secondary antibody (DAKO A/S, Glostrup, Denmark).
4.10 Apoptosis microarray

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

4.11 Genome-wide microarray

Total RNA was labelled and hybridized to rat whole-genome arrays (Rat Expression Array 230 2.0, Affymetrix, Santa Clara, CA, USA). Sample labelling, hybridization to chips and image scanning (GeneChip Scanner 3000, Affymetrix) were performed according to the manufacturer’s instructions. Expression values were calculated with the Robust Multichip Average (RMA) method and normalized per chip to the median and analyzed with Genespring 7.2 (Agilent, Santa Clara, CA, USA). Microarray hybridisations were performed using 4 MI samples, 4 sham samples, 3 sham + levosimendan samples and 3 MI + levosimendan samples. Differentially expressed probe sets were selected based on filtering by the parametric statistical analysis not assuming equal variances (Welch-type t test) with P<0.05 as a threshold for significance, followed by filtering for fold change (±1.2 fold) between the compared groups. The list of up- and downregulated probe sets were inspected for the enriched canonical pathways of the Kyoto Encyclopedia of Genes and Genomes (KEGG) among the genes using the ‘David 6.7’ program (NIAD, (NIH), MD, USA). Our analysis identified the canonical pathways that were most significant to the data set. The analysis was performed for up- and down-regulated gene sets separately.
4.12 TUNEL staining

Cardiomyocyte apoptosis was assessed by the terminal deoxynucleotidyl transferase mediated ddUTP nick end labeling (TUNEL) assay as previously described elsewhere (Kytö et al. 2004). In brief, apoptotic nuclear DNA strand breaks were end-labeled with digoxigenin-conjugated deoxy-UTP by terminal transferase and visualized immunohistochemically with digoxigenin antibody conjugated to alkaline phosphatase. The assay was standardized with the use of adjacent tissue sections treated with DNase I to induce DNA fragmentation as a positive control for apoptosis. The proportion of TUNEL-positive cardiomyocytes was calculated from transverse left ventricular tissue sections using light microscopy (×20 magnification) with an ocular grid. Cardiomyocyte origin of the apoptotic cells was identified by the presence of myofilaments surrounding the nucleus. The proportion of apoptotic cardiomyocytes was counted in the border zones of infarct scars and in the remote non-infarct myocardium (Palojoki et al. 2001).

4.13 Western blotting

Myocardial sample lysate from left ventricles of GK and Wistar rats were electrophoretically separated by SDS-PAGE. Proteins were transferred to a PVDF membrane (Immobilon-P®, Millipore, Bedford, MA, USA) and blocked in 5% non-fat milk-TBS - 0.01% Tween-20® buffer. The membranes were probed with the following antibodies:

- anti-caspase-3 (Upstate-Millipore), anti-Bax (Abcam), anti-phospho-FKHRL/FOXO3a (Cell Signaling Technologies (CST)), anti-FKHRL/FOXO3a (CST), anti-Akt (CST), anti-phospho-Akt (CST), anti-p38 (CST), anti-phospho-p38 (CST). Tubulin was used as loading control (Anti-alpha tubulin, Abcam, Cambridge, UK). Horseradish peroxidase-conjugated anti-rabbit secondary antibody (Chemicon) was subjected to enhanced chemiluminescence solution (ECL plus, Amersham Biosciences, Buckinghamshire, UK) and exposed to x-ray film (Hyperfilm-ECL, Amersham). We quantified the relative protein expression in
separate samples from the x-ray film by densitometry (Synoptics, Cambridge, UK).

4.14 Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was used to determine the DNA-binding properties of nuclear FOXO3a transcription factor. Nuclear proteins were extracted by grinding left ventricle samples from the remote area in liquid nitrogen and incubating in a buffer with protease inhibitor (Complete®, Roche), membrane proteins were solubilized with Nonidet P-40 (Roche Diagnostics GmbH, Mannheim, Germany). 5 µg of nuclear extracts were incubated in binding buffer (3 mM Tris pH 7.5, 50 mM NaCl, 5% Glycerol, 1 mmol/l EDTA, 0.01 mg/ml Poly[d(I-C)], 1 mmol/l DTT, 2 mg/ml BSA) and 1.25 fmol of digoxigenin-tagged FOXO3a oligonucleotides: 5´-DIG-ATT GCT AGC AAG CAA AAC AAA CCG CTA GCT TA-3´ and 5´-DIG-TAA GCT AGC GGT TTG TTT TGC TTG CTA GCA AT-3´ (Oligomer, Helsinki, Finland). After 30’ incubation, the samples were electrophoretically separated by 5% SDS-PAGE and blotted to a nylon membrane (Hoefer semiPhor, TE77, Amersham Biosciences). Nuclear proteins were cross linked to the membrane for 10’ in 254 nm UV-light. The membrane was probed with rabbit Anti-digoxigenin-AP-conjugate (Roche) for 30 min. and incubated in detection reagent (CDP Star, Roche) and exposed to an x-ray film (Kodak Inc., Japan). The indication reaction was done by incubating the samples with tagged and untagged oligonucleotides and probing with rabbit anti-FKHRL1 (sc-11351, Santa Cruz, CA, USA).

4.15 Quantitative RT-PCR

Total RNA from the rat hearts were extracted with Trizol® (Gibco, Invitrogen, Carlsbad, CA, USA), treated with DNAses 1 (Deoxyribonuclease 1, Sigma Chemicals Co., St Louis, MO, USA) and reverse transcribed to cDNA by reverse transcription enzyme (Enhanced avian HS RT-PCR kit, Sigma Chemicals Co, Saint Louis, MO, USA). One µl of cDNA was subjected to quantitative real time polymerase chain reaction using the Lightcycler® instrument (Roche diagnostics,
Neuilly sur Seine, France) for detection of atrial natriuretic peptide (ANP), connective tissue growth factor (CTGF), interleukin-6 (IL-6), ProRenin receptor, AT1 receptor, ACE, ACE2, NEP, HO-2, and ribosomal 18S mRNA. The samples were amplified using FastStart DNA Master SYBR Green 1 (Roche diagnostics) according to the protocol of the manufacturer.

4.16 Statistical analyses

Statistics were performed by software program analysis (GraphPad Prism, San Diego, CA, USA). Student’s t-test was used to assess difference in mean values between two groups. One-way analysis of variance (ANOVA) and Neuman-Keuls post test were used to assess the difference in means when three or more groups were compared. A significant difference between groups was considered when the $P$ value was <0.05.
5 Results and discussion

For a summary table of results from studies 1-4, refer to the end of section 5.

5.1 Survival

Ligation of the left anterior descending artery and subsequent myocardial infarction of the anterior wall resulted in a relatively high 24 -hour mortality. 24 – hour survival in GK rats was 35% vs. 56 % in Wistar rats (P<0.05) (study 3). The difference between the groups was significant, and the results indicate that diabetic GK rats have an increased risk of fatal MI. In each study, 1-2 animals died during follow-up. After the follow-up of 4 or 12 weeks the size of the infarct areas were evaluated by planimetry. Levosimendan was introduced 24 hours after experimental MI/sham operation. No differences in infarct sizes between the groups were found, and levosimendan did not influence survival or infarct size.

Discussion

A fairly large meta-analysis recently showed that diabetes is a risk factor for mortality following acute coronary syndromes (UAP, NSTEMI, STEMI) (Donahoe et al. 2007). My results are in line with this notion. A prior study showed that levosimendan decreased mortality 312 days post-MI (Levijoki et al. 2001). My studies did not examine long-term mortality; the longest follow-up was 84 days which may explain the absent effect on survival.

5.2 Plasma concentration of levosimendan

In levosimendan –treated rats the terminal plasma concentration of the stable metabolite OR-1896 was 20.4 ± 4.0 ng/ml (study 3). The same dosing (10 mg/ml p.o. corresponding estimated 1 mg/kg) was used in study 4.
5.3 Blood pressure and cardiac function

In study 1, systolic blood pressure was 8-10 mmHg higher in 10-12 week old GK rats compared to Wistar controls. This finding was confirmed in studies 2 and 3 where systolic blood pressure in 20-week old sham-operated GK rats was increased by 5 mmHg and 8 mmHg respectively. In studies 1-3 systolic blood pressure in GK rats ranged from 138-150 mmHg and in Wistar from 127-144 mmHg. These results confirm the notion that GK rats develop spontaneous hypertension. In studies 2 and 3, MI resulted in a reduction of ca. 18 mmHg in systolic blood pressure in GK rats whereas in study 4, MI induced a 3 mmHg reduction in systolic blood pressure. These differences are explained at least in part by the difference in follow-up time, 12 weeks in studies 2 and 3 vs. 4 weeks in study 4. Levosimendan treatment did not alter systolic blood pressure in post-MI GK rats in study 3 and 4. Levosimendan treatment did slightly but significantly lower systolic blood pressure in sham-GK rats in study 3 but not in study 4. Levosimendan treatment increased heart rate in GK + MI rats in study 3 (12 weeks follow up) but not in study 4 (4 weeks follow-up), and did not influence heart rate in Wistar + MI or sham-operated rats.

Transthoracic echocardiography revealed an increase in diastolic volume in the LV of GK rats compared to Wistar indicating a dilated left ventricle (study 1). Furthermore plasma BNP was increased in GK rats compared to Wistar indicating volume overload. Systolic function was decreased in GK rats compared to Wistar in study 1 (EF% 59 vs. 83). Experimental MI by LAD ligation induced thinning of the anterior wall of the left ventricle in studies 2-4. In GK rats, ejection fraction (EF) decreased to ca. 23% at week 1 and 25% at week 4 post MI (study 3-4) and increased slightly at 12 weeks post-MI to reach 42% (study 2-3) (Figure 7). LAD ligation in Wistar rats produced similar results.
Figure 7. Effects of levosimendan on ejection fraction (EF), a measure of systolic function, 1, 4 and 12 weeks after experimental myocardial infarction (MI) in diabetic Goto-Kakizaki (GK) rats. Values are presented as means ±SEM, * denotes $P<0.05$ vs. GK SHAM, # denotes $P<0.05$ vs. GK MI.

Discussion
Diastolic dysfunction, the hallmark in type 2 diabetes, is a consequence of reduced compliance of the ventricular wall most often caused by LVH and/or fibrosis that results in increased LV end-diastolic pressure (LVEDP) and primarily a reduction in early to late atrial filling (E/A ratio) (For a review see Fang et al. 2004). Earlier studies with GK rats have shown an increased susceptibility to diastolic dysfunction after dietary high sodium intervention and concomitant increase in blood pressure (Grönholm et al. 2005). Systolic dysfunction withholds a degree of diastolic dysfunction and others have shown that systolic dysfunction occurs in STZ-induced diabetes (Joffe et al. 1999; Wold et al 2001; Hoit et al. 1999). In these studies I did not examine LVEDP and E/A by echocardiography; however the results indirectly indicated the presence of diastolic dysfunction in diabetic GK rats, which I observed by increased fibrosis, LVH and systolic dysfunction (study 1). However, systolic dysfunction was not present in sham-GK rats in studies 2-3, providing a notion of the genetic variation in the rat model of the inbred GK rats. This notion is supported by the findings in a study which
proposed that cardiac dysfunction is accelerated in Goto-Kakizaki rats post-MI (Chandler et al. 2007); my studies did not show significant differences in echocardiography parameters after MI in GK vs. Wistar rats. Levosimendan ameliorated systolic dysfunction in post-MI GK rats, with ejection fraction reaching 41% at 4 weeks post-MI (study 4) and 71% 12 weeks post-MI (study 3) (Figure 7). Additionally levosimendan increased cardiac output in post-MI GK rats (study 3-4). In study 3, the increase in cardiac output can be partly explained by the increase in heart rate, however in study 4 levosimendan did not alter heart rate. In Wistar rats Levosimendan increased systolic function less efficiently; ejection fraction was increased from 35% (sham) to 47% (MI) (study 3), cardiac output however was increased from 277 ml/min to 376 ml/min in Wistar + MI without increased heart rate. In sham-operated GK rats levosimendan did not alter echocardiography parameters, whereas in sham-operated Wistar rats EF was increased after levosimendan treatment.

Discussion

The results showed that 4 weeks and 12 weeks of levosimendan treatment efficiently ameliorated cardiac dysfunction in GK rats with post-MI heart failure. Systolic blood pressure was not changed in post-MI rats, however heart rate was increased in the 12 –week follow up but not in the 4 –week study in GK + MI rats. The reason for this was unclear and the increase in heart rate was absent also in Wistar rats 12 –weeks post MI. Levosimendan is a known vasodilator through opening K_{ATP} –channels and inhibiting PDEIII; these factors could explain the increase in heart rate via compensatory sympathetic activation. However, a recent study has shown that in the clinical setting levosimendan does not increase sympathetic activity (Despas et al. 2010). Taken together the inotropic actions of levosimendan were evident and presumably derived from the known effects of sensitization of cardiac troponin C to calcium, without excluding the role of an increase in heart rate.
5.4 Blood glucose and insulin

The non-obese Goto-Kakizaki rat has been shown to exhibit the polygenic features of type 2 diabetes including insulin resistance, abnormal glucose metabolism and spontaneous hyperglycaemia (Goto Y et al. 1976; Galli et al. 1999). In my studies the GK rats developed mild hyperglycemia with fasting plasma glucose values ranging from 7.3 mmol/L to 10.5 mmol/L in 12-20 -week old animals. In control non-diabetic Wistar rats fP-Glucose ranged from 3.3 to 4.9 mmol/L (studies 1-4). Serum insulin levels were slightly increased in GK rats, with values ranging from 2.0 to 4.1 ng/ml compared to 1.6 to 3.4 ng/mL in 12-20 -week old non-diabetic Wistar controls (studies 1-2). In post-MI GK rats, serum insulin was increased compared to Wistar MI (study 2). Experimental myocardial infarction did not alter terminal plasma glucose values. Levosimendan did not influence plasma glucose or serum insulin concentration (study 3 and 4). These results justify the use of the GK rat as a model to study type 2 diabetes and the use of non-diabetic Wistar rats as their controls.

5.5 Left ventricular hypertrophy

Cardiac hypertrophy was evaluated by heart weight to body weight ratio and histologically by examining average cardiomyocyte cross sectional areas in the left ventricle. In the 12 –week old diabetic GK rats cardiac hypertrophy was evident due to increased heart weight to body weight ratio (+33% compared to Wistar) and increased cardiomyocyte cross sectional area (+22%) (study 1). In GK rats the increase in systolic blood pressure (+10 mmHg) may have played a role in the development of LV hypertrophy. In GK rats, experimental myocardial infarction further exacerbated LV hypertrophy 1.4 –fold as revealed by cardiomyocyte cross sectional area of the non-infarct area. In Wistar rats, MI increased LV hypertrophy 1.3 –fold. Average cardiomyocyte cross sectional area in Wistar + MI rats was the same as in GK sham rats. Levosimendan ameliorated LV hypertrophy in GK rats 4 and 12 weeks post-MI (studies 3-4) without changes in blood pressure (Figure 8). Notably levosimendan also ameliorated LV
hypertrophy in sham-operated GK rats; in study 3 this was associated with a decrease in systolic blood pressure whereas in study 4 no change in systolic blood pressure was found. Levosimendan did not decrease cardiomyocyte cross sectional area or heart weight to body weight ratio in Wistar rats. MI resulted in an increase in plasma BNP and ANP mRNA levels in GK rats. Levosimendan markedly reduced BNP in all GK + MI rats and ANP mRNA expression in GK + MI rats in study 3.

Figure 8. Effects of levosimendan on left ventricular hypertrophy 1, 4 and 12 weeks after myocardial infarction (MI) in diabetic Goto-Kakizaki (GK) rats as evaluated by cardiomyocyte cross sectional area. Values are presented as means ±SEM, * denotes \( P<0.05 \) vs. GK SHAM, # denotes \( P<0.05 \) vs. GK MI.

Discussion

The results in these studies showed that diabetic GK rats develop spontaneous LV hypertrophy which is one of the hallmarks of diabetic cardiomyopathy. MI in both rat strains exacerbated LV hypertrophy to a similar extent. These results were in line with a previous study where cardiac hypertrophy increased comparably in GK and WKY rats 8 and 20 weeks post MI (Chandler et al. 2007). The mechanisms that promote LV hypertrophy in diabetes have been proposed to include increased ROS production, increased insulin signalling and RAS activation (for a review see Boudina et al. 2010). My results showed a modest increase in serum insulin concentration in GK rats with MI (study 2) and a modest
increase in systolic blood pressure in GK vs. Wistar rats, both of which may have played a role in the increase in LVH. However, in GK rats with MI, LVH was increased further while systolic blood pressure remained unchanged compared to Wistar, providing an indication of a blood pressure–independent mechanism in GK rats.

Levosimendan reduced LVH in all GK rats. In GK rats with MI, levosimendan did not reduce blood pressure whereas in GK sham–rats levosimendan slightly decreased systolic blood pressure in study 3. Therefore I may propose that the LVH reducing effects of levosimendan in GK rats was mediated both through blood pressure dependent and independent mechanisms. These results are consistent with previous results where levosimendan was shown to reduce LV hypertrophy in salt–sensitive Dahl/Rapp rats (Louhelainen et al. 2007). However the mechanism through which levosimendan was able to reduce LV hypertrophy in sham–operated diabetic GK rats is unclear. The reduction in ANP and BNP further provide indirect evidence that levosimendan acts as a negative regulator of LV hypertrophy. These results bring evidence that levosimendan 1) attenuates post-MI LV remodeling and 2) has beneficial effects on diabetic cardiomyopathy by decreasing LV hypertrophy.

5.6 Calcium handling proteins

The expression of myocardial SERCA2a and NCX proteins were studied in GK and Wistar rats ±MI and ±levosimendan. Myocardial SERCA2 and NCX protein expression were not changed in Wistar vs. GK rat heart at baseline, however SERCA2 protein expression was decreased by 30% and NCX protein expression was increased by 74% after MI in GK rats (SERCA2/NCX ratio 0.47 vs. 1.22, \( P<0.05 \)).

Discussion

A decrease in SERCA2 and/or an increase in NCX protein have been associated with decreased contractility in the failing heart (for a review, see Bers et al. 2009). My results are in line with previous studies showing a reduced SERCA2/NCX ratio in post-MI mice with impaired insulin signalling (Sena et al. 2009). Increased free
ROS and protein modification in the SR of cardiomyocytes has been proposed as a potential mechanism for this effect (Kaplan et al. 2003). Levosimendan prevented the changes in SERCA2 and NCX protein expression in GK + MI rats.

5.7 Sympathetic and renin angiotensin system

Sympathetic nervous system activation was studied by examining 24h urinary noradrenaline excretion (U-NA); a non-significantly lower U-NA content in GK rats at baseline (study 1) was aggravated at 12 weeks after MI, at which time U-NA was significantly decreased in GK + MI rats compared to Wistar + MI. MI increased U-NA in Wistar but not in GK rats. I did not study the effect of levosimendan on U-NA. These results provide an indication of sympathetic nervous system dysregulation in diabetic GK rats.

The circulating RAAS activity was studied by examining plasma renin activity (PRA) and serum aldosterone in GK and Wistar rats. My results showed no changes in PRA or aldosterone in GK vs. Wistar rats at baseline. In GK + MI rats PRA was increased vs. Wistar + MI (study 2). RT-PCR showed an increase in angiotensin converting enzyme mRNA expression in GK rats 4 weeks after MI. S-aldo was further increased after MI in GK and Wistar rats (study 2-4). Levosimendan did not affect PRA or S-aldo in sham or MI operated rats.

Discussion

Others have shown that the GK rat has a modest hyperaldosternism (Grönholm et al. 2005) and my results suggest that the increase in S-aldo is further increased after MI and is associated with a low PRA. The slightly higher aldosterone levels may explain the mild increase in systolic blood pressure in GK rats. Previous studies have suggested that tissue RAAS activation is involved in the pathogenesis of post-MI heart failure (for a review see Sun et al. 2010) and have shown that diabetes is associated with an increase in local renin-angiotensin system activation (Sechi et al. 1994; Khattar et al. 1996; Fiordaliso et al. 2000). I used a microarray based approach to study the whole genome of GK rat myocardial tissue (study 4). In GK + MI rats treated with levosimendan my results showed an increase in angiotensin II receptor 1 (Agtr1), thimet oligopeptidase 1
(Thop1) and chymase 1 (Cma1) mRNA expression. However, the increase in At1r was not confirmed by RT-PCR. My results showed an increase in mRNA expression of the Mas receptor and neutral endopeptidase (Nep) enzyme in levosimendan treated GK + Sham (study 4). The Mas receptor is a target of Ang (1-7) and exerts anti-hypertrophic and vasodilatory effects. Ang(1-7) is produced from AngI by enzymes including Thop1 and Nep and prolyl oligopeptidase (Ferrario et al. 1998). These results suggest that levosimendan may attenuate LV remodelling partly through the induction of the Ang(1-7) - Mas receptor –axis which mediated a decrease in LVH, this was shown in levosimendan treated GK sham rats (studies 3-4). I did not examine the tissue RAS in non-diabetic Wistar rats in this study.

5.8 Apoptosis

The protein expression of caspase-3, one of the main effectors of the apoptotic cascade, was increased in GK vs. Wistar rat hearts together with proapoptotic protein p53 and Bax. Furthermore, apoptosis –specific microarray revealed a marked positive shift in the mRNA expression of apoptotic genes in GK vs. Wistar rat hearts (study 3). However these findings did not translate into an increase in apoptosis since TUNEL staining failed to reveal an increase in apoptotic nuclei in GK rat myocardial samples (study 1). I did however find that the negative regulator of p53, namely Sirt1 deacetylase was overexpressed and that the acetylation level of p53 was decreased in GK rats; this offers a possible explanation why no overt apoptosis was evident in the GK rat heart. MI further increased the expression of Caspase-3 and Bax and produced a positive shift in the profile of apoptotic gene expression in both GK and Wistar rat heart samples. MI increased myocardial apoptosis in the infarct border zone (10 – fold increase) and in the remote area (4 –fold increase) as demonstrated by TUNEL staining (Figure 9). There were a higher number of apoptotic nuclei in both the border zone and in the remote area in GK rats compared to Wistar rats. Levosimendan decreased the amount of TUNEL positive cells in the infarct area and in the remote area in both GK and Wistar rats after MI. Levosimendan
produced a negative shift in the expression of apoptotic genes which was determined by apoptosis–specific microarrays. These results provide evidence that diabetic cardiomyopathy in the GK rat is associated with an increase in apoptotic signalling, this was shown by increased gene expression of the apoptotic cascade and increased caspase-3 and Bax protein expression. The situation was exacerbated by MI when overt sustained cardiomyocyte apoptosis was evident 12 weeks post-MI in the infarct border zone and in the remote area in GK rats compared to non-diabetic Wistar rats.

Discussion

These results are in line with the notion that diabetic cardiomyopathy is associated with an increase in apoptosis. The mechanism behind the increase in apoptotic signalling was not studied here. However, earlier studies have shown that diabetes–induced lipo-and glucotoxicity, mitochondrial dysfunction and RAS activation increases the amount of reactive oxygen species (ROS) which in turn activates the apoptotic signalling process (for reviews, see Santos et al. 2003; Rolo et al. 2006 and Dobrin et al. 2010).

![Figure 9. Effects of levosimendan on myocardial apoptosis in the remote area and infarct border zone 12 weeks after myocardial infarction (MI) in diabetic Goto-Kakizaki (GK) rats as evaluated by TUNEL-staining. Values are presented as means ±SEM, * denotes P<0.05 vs. GK SHAM, # denotes P<0.05 vs. GK MI.](image)
12 weeks of levosimendan –treatment was sufficient to decrease the amount of apoptotic cells in the border zone and in the remote area in post-MI GK and Wistar rats. Maytin and Collucci have shown that levosimendan opposes cardiomyocyte apoptosis, a feature that was absent when glibenclamide was used to inhibit K\textsubscript{ATP}-channels (Maytin et al. 2005). These findings are consistent with \textit{in vitro} studies which show that opening of mitochondrial K\textsubscript{ATP} channels protects from ischemia reperfusion injury through following reduction in reactive oxygen species and subsequent inhibition of apoptosis (Akao et al. 2001). I may speculate that the beneficial role of levosimendan in GK rats may be, at least in part explained by the baseline mitochondrial dysfunction that is ameliorated by beneficial effects mitochondrial on K\textsubscript{ATP} –channels.

5.9 Fibrosis

The results showed that the mRNA expression of connective tissue growth factor (CTGF) was increased in GK rat hearts and that fibrosis was accelerated after MI in GK rat LV tissue compared to Wistar. Increased CTGF mRNA expression in GK rats compared to Wistar rats (study 1) was further increased by MI (studies 2-3). IL-6 mRNA expression was slightly increased in GK + MI rats (study 2). Collagen volume fraction measurement from the remote area in myocardial tissue slides revealed a higher degree of interstitial and perivascular fibrosis in GK vs. Wistar rats at 12 weeks post MI (study 2). In levosimendan –treated rats, the increase in collagen volume fraction was absent after MI (study 4) and levosimendan tended to decrease collagen volume fraction in sham GK rats (Study 3). Levosimendan reduced CTGF mRNA expression in GK + MI rats but not in Wistar + MI rats (studies 3). In addition, IL-6 mRNA expression was decreased in levosimendan –treated GK + MI rats compared to GK + MI rats (study 3).

\textit{Discussion}

Studies in humans have shown increased collagen deposition correlating with diastolic dysfunction in type 2 diabetic patients (Regan et al. 1977; Gonzalez-Vilchez et al. 2005). Animal studies of type 2 diabetes have indicated that
increased cardiac fibrosis may occur spontaneously already at the pre-diabetic stage (Mizushige et al. 2000). My results are in line with these studies. Furthermore, the beneficial effects of levosimendan on reduction of cardiac fibrosis were evident. Levosimendan was able to reduce the increase in post MI - CTGF and IL-6 mRNA expression and reduce interstitial fibrosis as measured by collagen volume fraction. Studies have shown that an increase in free ROS after MI increases apoptotic and necrotic cell death and following fibrosis by activating several cytokines including TNF-alpha and IL-6 (for a review, see Hori et al. 2009). The beneficial effects of levosimendan on apoptosis and fibrosis may be, at least in part, attributed to the opening of mitochondrial $K_{\text{ATP}}$ -channels and the subsequent beneficial effect on energy homestasis and reduction of build-up of reactive oxygen species. Although other mechanisms cannot be excluded.

### 5.10 Senescence

I studied the senescence in the myocardial tissue samples from GK and Wistar rats with MI treated with levosimendan. In GK + MI rats the mRNA expression of $P16^{\text{INK4a}}$ was increased 3 -fold compared with GK sham rats. Levosimendan reduced the MI –induced increase in $P16^{\text{INK4a}}$ mRNA expression in GK rats. In Wistar rats, MI did not influence $P16^{\text{INK4a}}$ mRNA expression.

**Discussion**

The marker of senescence studied here, namely $P16^{\text{INK4a}}$, is a known tumour suppressor and increases apoptosis in response to aging (Sharpless 2004). I showed here that a sustained increase in cardiomyocyte apoptosis was associated with an increase in $P16^{\text{INK4a}}$ in post –MI GK rats. These findings are interesting and provide an indication that the ability of levosimendan to decrease cardiomyocyte apoptosis may be mediated at least in part by suppression of the $P16^{\text{INK4a}}$ pathway in the diabetic myocardium.

### 5.11 Akt and FOXO3a

I studied the activity and post-translational modification of FOXO3a, a transcriptional regulator downstream of the insulin receptor - PI3K/Akt kinase
pathway in GK and Wistar rat hearts. Data from study 2 showed that in the GK rat heart phosphorylation of FOXO3a was reduced compared to Wistar hearts. Further, my results showed that the nuclear localization and DNA binding of FOXO3a was increased in GK rat myocardium (study 1). In study 2 I examined the phosphorylation and localization of FOXO3a and the phosphorylation of Akt kinase and protein expression of the Akt kinase deactivator protein PTEN in GK and Wistar rats 12 weeks after MI. The results showed that Akt –kinase was dephosphorylated in GK + MI rats compared to Wistar + MI and that this was associated with increased FOXO3a nuclear translocation and DNA binding in GK + MI rats compared to sham-operated GK rats and Wistar + MI rats. Furthermore I showed that the phosphatase PTEN protein expression was increased in the GK + MI rat heart (study 2). These results provided evidence that Akt dephosphorylation was associated with FOXO3a dephosphorylation and nuclear translocation in GK rat myocardium after MI. The increase in nuclear, DNA bound FOXO3a could explain, at least in part, the increase in cardiomyocyte apoptosis found in the post-MI GK rat. These results were confirmed in study 3, where microarray analysis of apoptotic gene expression showed an increase in the expression of FOXO3a –associated apoptotic genes such as Bcl2l11, Fadd and Bad in GK + MI vs. GK sham rat hearts (study 3). In levosimendan –treated GK + MI rats, the nuclear localization of FOXO3a was decreased compared to untreated GK + MI rats (study 3). Furthermore, levosimendan suppressed the mRNA expression of FOXO3a related apoptotic genes Bcl2l11 and Tnfrsf12a when compared with GK + MI rats.

Discussion

Earlier studies have shown that the diabetic GK rat heart is insulin resistant with decreased insulin receptor and IRS-1 protein content and decreased PI3K phosphorylation upon insulin stimulation (Desrois et al. 2004). However the same study showed that Akt phosphorylation is not decreased in GK rat hearts. These results were consistent with my results that showed no change in Akt phosphorylation between sham –operated Wistar vs. GK rats (study 2). However,
I showed here that after MI the situation changes, as Akt phosphorylation was decreased in the GK rat heart 12 weeks post operation. This could prove important since Akt plays a key role as an antiapoptotic stress response protein when activated through phosphorylation. The decrease in Akt phosphorylation could be explained, at least in part, by the finding that GK + MI rat hearts showed an increase in PTEN protein expression, a phosphatase protein that dephosphorylates PIP3 and consequently Akt. The mechanism for the increase in PTEN protein expression was not studied further and remains unclear.

Increase in cardiomyocyte apoptosis, Akt-FOXO3a dephosphorylation and FOXO3a translocation in GK rat hearts were consistent with the notion that phosphorylated FOXO3a is translocated to the cytoplasm whereas dephosphorylated FOXO3a is localized to the nucleus where it may take part in regulation of apoptotic gene expression (van der Heide et al. 2004). My results showed that overt apoptosis was increased in the remote area of the myocardium in post-MI GK rats and that the GK rat without MI showed increased apoptotic gene expression and increased Caspase-3 and Bax protein expression (study 2). The increase in nuclear FOXO3a translocation and DNA binding was associated with an increase in FOXO3a-regulated proapoptotic gene expression in GK + MI rat hearts compared to Sham (study 3). Earlier studies have shown that stress stimuli such as oxidative stress, heat shock or growth factors increase nuclear localization of FOXO3a (Brunet et al 2004). Nuclear localization of FOXO3a is associated with induction of the superoxide dismutase 2 (SOD2) gene, the product of which reduces toxic superoxide to hydrogen peroxide. The ability of FOXO3a to detoxify ROS is important in an environment with high oxidative stress such as diabetes, and this may provide a mechanism which enables the increased nuclear FOXO3a shuttling seen here. One potentially adverse effect of nuclear FOXO3a is the increased expression of apoptotic genes. FOXO3a-regulated increase in apoptotic gene expression is enhanced by acetylation of nuclear FOXO3a by CBP and p300 in oxidative stress states (Brunet et al. 2004; Huang et al. 2007). Sirt1, a histone deacetylase and longevity factor has been
shown to inhibit FOXO3a–mediated apoptotic gene expression by deacetylating FOXO3a (Brunet et al. 2004; Motta et al. 2004). It has been proposed that modulation of FOXO3a transcriptional regulation by Sirt1 is one of the mechanisms for Sirt1–mediated increase in cell survival. My results showed here that Sirt1 protein expression is increased in diabetic GK rat hearts. Furthermore I showed that one of the targets of Sirt1, namely the proapoptotic p53 tumor suppressor was deacetylated in GK rat hearts (study 1) and GK + MI rat hearts (study 2).

Data from study 3 showed that levosimendan was able to suppress the nuclear localization of FOXO3a. Previously I have shown that in levosimendan–treated rats cardiomyocyte apoptosis was decreased markedly. These results were confirmed by the notion that mRNA expressions of Bcl2-like 11 (Bcl2l11) and the tumor necrosis factor receptor subfamily 12a (Tnfrsf12a) were reduced. In conclusion, levosimendan, by mechanisms that are not entirely clear, was able to partly inhibit the nuclear localization of FOXO3a and suppress the increase in FOXO3a–induced apoptotic gene expression.

5.12 Sirtuin 1 and P53

My results showed that Sirtuin 1 (Sirt1) protein expression was increased in diabetic GK rat myocardial tissue compared to Wistar (study 1). This finding was consistent with the results in study 2 which showed an increase in Sirt1 protein in myocardial tissue from sham-operated GK rats vs. Wistar sham. The study further showed that p53 protein expression was increased in GK rat myocardium 1.9 fold. The increases in Sirt1 and p53 protein expressions were associated with a decrease in p53–acetylation at the Sirt1–preferred lysine sites K373/K382 in GK (study 1). As indicated earlier, GK rats showed no overt cardiomyocyte apoptosis, and therefore Sirt1–mediated inhibition of p53 and thus attenuation of apoptosis, may have played a role in the myocardium in these rats. However since Sirt1 induces growth its anti-apoptotic effect may have come with the cost of increased cardiomyocyte hypertrophy, although other factors such as hypertension are likely involved.
Furthermore, Sirt1 protein was overexpressed in GK + MI rats compared to Wistar MI (study 2) and localized to the nucleus which I demonstrated using western blots and immunohistochemical analysis. Study 2 showed that Sirt1 protein expression is upregulated both in the remote area and in the infarct area in GK + MI rats. The acetylation of the p53 protein was decreased in GK + MI rats consistent with the increase in Sirt1 protein. In GK + MI rats however, Sirt1 – mediated p53 deacetylation was unable to suppress the increase in cardiomyocyte apoptosis as which I tested by TUNEL staining.

**Discussion**

Sirt1 protein expression has been shown to be increased in response to oxidative stress and in LV hypertrophy in dogs and rats (Alcendor et al. 2004; Alcendor et al. 2007). Increased oxidative stress in myocardium of GK rats has been shown in various studies, and the main contributing factors are hyperglycemia, increased FA, decreased antioxidant capability and defects in cardiomyocyte mitochondria (Bitar et al. 2004; Santos et al. 2003). Sirt1 acetylase activity is increased by a high NAD⁺/NADH ratio present in the oxidative stress environment. My results are consistent with this notion as I showed that Sirt1 protein overexpression was associated with increased Sirt1 deacetylase activity observed by the decrease in acetylated p53. Furthermore, hyperglycemia –induced increase in ROS is known to initiate p53 –mediated cardiomyocyte apoptosis (Frustaci et al. 2000; Fiordaliso et al. 2001). Accordingly my results provided evidence that in the GK rat myocardium p53 protein overexpression was associated with Sirt1 –mediated p53 deacetylation and no overt cardiomyocyte apoptosis. However, 12 weeks after MI, Sirt1 – p53 deacetylation still occurred although cardiomyocyte apoptosis was increased. This indicates that other factors than p53 were responsible for the sustained increase in apoptosis in GK + MI rats in this setting.

**5.13 p38 Mitogen activated protein kinase**

Other researchers have suggested that p38 MAPK phosphorylation in response to oxidative stress in insulin resistant skeletal muscle is increased in diabetes (Henriksen et al. 2010). The results here showed that total p38 protein was
increased in GK and Wistar rats after MI compared to Wistar. Furthermore, data revealed for the first time that p38 MAPK phosphorylation is markedly increased in GK rat myocardium 12 weeks after MI but not in Wistar + MI rats (Study 2).

**Discussion**

Studies have previously shown that activation of the p38 MAPK increases apoptosis, fibrosis and the expression of CTGF and IL-6 mRNA (Tenhunen et al. 2006). In addition p38 MAPK phosphorylation has been associated with decreased contractility (Liao et al 2002). Consistent with these studies my results showed an increase in apoptosis and fibrosis and non-significant modest increases in CTGF and IL-6 mRNA expression in GK + MI rats compared to Wistar + MI. Furthermore, my results are in line with other studies which have shown the involvement of p38 MAPK in hypertrophy (Kompa et al. 2008). Taken together these results provide evidence that the diabetic GK rat heart is susceptible to sustained p38 MAPK phosphorylation in response to myocardial infarction; a potentially adverse effect that could have been a cause for the increased apoptosis, hypertrophy and fibrosis seen here.

**5.14 Levosimendan and myocardial gene expression profile**

In levosimendan –treated GK + MI rats the most significantly enriched pathway was the renin-angiotensin system pathway ($P=0.025$) represented by three upregulated genes, namely the Agt1r, Cma1 and thop1 genes. The second most significantly enriched pathway was the glycerolipid pathway ($P=0.036$) with three downregulated genes, namely diacylglycerol kinase gamma (Dgkg), carboxyl ester lipase (Cel) and diacylglycerol kinase iota (Dgki). In levosimendan treated GK sham rats, the most enriched pathways included the purine metabolism pathway ($P=0.02$) with 1.21-1.37 -fold upregulations of adenosine kinase (Adk), guanine monophosphate synthetase (Gmps), phosphodiesterase subtypes 3A and 4A and 4B (PDE3A/4A/4B), phosphoribosyl pyrophosphate amidotransferase (Ppat), polymerase II polypeptide H (Pol2rh), RAB11 family interacting protein 3 non-metastatic cells 4 (Nme4), similar to Adenylosuccinate synthetase isoenzyme 1 (LOC684425) and 0.81-0.69 –fold downregulation of nucleoside phosphorylase
(Np) and polymerase epsilon (Pole). The second most significantly ($P=0.014$) enriched cell cycle pathway included $0.83-0.55$ -fold downregulation in the following genes: cell division cycle 2, G1 to S and G2 to M (Cdc2 or Cdk1), minichromosome maintenance complex component 3 (Mcm3), minichromosome maintenance deficient 7 (Mcm7), E2F transcription factor 1 (E2f1), protein kinase, DNA activated catalytic polypeptide (Prkdc), anaphase promoting kinase inhibitor 1B (Anapc1) and $1.20-1.24$ fold upregulation of cyclin dependent kinase inhibitor 1B (Cdkn1b) and origin recognition complex, subunit 3-like (Orc31). The third pathway included 8 significantly downregulated genes in the pathways in cancer with $0.71-0.80$ –fold changes in colony-stimulating factor 1 receptor (Csf1r), GLI-Kruppel family member GLI1 (Gli1), Collagen type IV, alpha 1 (Col4a1), Collagen type IV alpha 2 (Col4a2), laminin, alpha 4 (Lama4), E2F transcription factor (E2F1), MutS homolog 3 (E.coli) (Msh3), mechanistic target of rapamycin (mTOR).

Levosimendan targeted common genes in MI and Sham rats; these included upregulation of 10 common genes including Arl6ip2, Atp5s, Cmbl, Cxcl11, LOC360830, LOC684270, Mlx, Mrpl41, RGD1309651, Vps28 and downregulation of 4 common genes namely Etv5, Lphn1, Paxip1 and Tln1.

When I analysed which genes were downregulated in MI / Sham and upregulated by levosimendan in MI I found two genes, namely the carboxymethylenebutenolidase homologue (Cmbl) and the pleckstrin homology domain containing family f (Plekhf1) that fulfilled these criteria. Conversely one gene was shown to be downregulated by levosimendan that was upregulated in MI / Sham rats, namely the hydroxyprostaglandin dehydrogenase 15 (Hpgd) which encodes the NAD\(^+\)-dependent 15-dehydroprostaglandin dehydrogenase enzyme (15-PGDH); a key inactivator of prostaglandins (Niesen et al. 2010).

**Discussion**

Diabetes is associated with an increase in fatty acid oxidation in the heart and MI is known to further impair myocardial energy metabolism (for a review, see Lopaschuk et al. 2002). The effect of levosimendan on genes
Table 4. Summary of results

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</tr>
<tr>
<td>(EF%)</td>
<td></td>
<td></td>
<td>↑/↓</td>
<td>↑/↓</td>
</tr>
<tr>
<td>LVH*</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>P-BNP</td>
<td>↑</td>
<td>N/A</td>
<td>↓</td>
<td>↑/↓</td>
</tr>
<tr>
<td>ANP mRNA</td>
<td>N/A</td>
<td>↑/↓</td>
<td>↓</td>
<td>↑/↓</td>
</tr>
<tr>
<td>Apoptosis (TUNEL)</td>
<td>↑/↓</td>
<td>↑</td>
<td>↓</td>
<td>↑/↓</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Apoptosis#</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
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<td></td>
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<td></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Fibrosis*</td>
<td>↑/↓</td>
<td>↑</td>
<td>↑/↓</td>
<td>↑/↓</td>
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<td>↑</td>
<td>↑/↓</td>
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<tr>
<td>CTGF mRNA</td>
<td>↑</td>
<td>↑/↓</td>
<td>↓</td>
<td>↑/↓</td>
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<tr>
<td></td>
<td>N/A</td>
<td>N/A</td>
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</tr>
<tr>
<td>pAkt/Akt protein</td>
<td>N/A</td>
<td>↓</td>
<td>N/A</td>
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<td></td>
<td>N/A</td>
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<tr>
<td></td>
<td>Study 1</td>
<td>Study 2</td>
<td>Study 3</td>
<td>Study 4</td>
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<tr>
<td></td>
<td>GK vs. Wistar</td>
<td>GK MI vs. Wistar MI</td>
<td>GK MI + Levo vs.</td>
<td>GK MI + Levo vs.</td>
</tr>
<tr>
<td></td>
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<td>GK MI + SHAM + Levo vs. GK MI</td>
<td>GK MI + SHAM + Levo vs. GK MI</td>
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<tr>
<td>pFOXO3a/FOXO3a</td>
<td>↓</td>
<td>↓</td>
<td>N/A</td>
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<tr>
<td>DNA-bound FO XO3a</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
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<tr>
<td>Sirt1 protein</td>
<td>↑</td>
<td>↑</td>
<td>N/A</td>
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<tr>
<td>Acetyl p53/p53</td>
<td>↓</td>
<td>↓</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>p38 MAPK phosphorylation</td>
<td>N/A</td>
<td>↑</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

↑ denotes increase, ↓ denotes decrease, ↑/↓ denotes no change, N/A (not available) parameter was not examined in the particular study, *as evaluated by average cardiomyocyte cross sectional area, ‡as evaluated by protein expression of Caspase-3, Bax or microarray of apoptosis specific gene expression, §as evaluated by collagen volume fraction, ¶as evaluated by electrophoretic mobility shift assay (EMSA), for a list of abbreviations refer to page 7.
implicated in energy metabolism such as the ATPase subunit were interesting and provide a new insight into the role of levosimendan in energy metabolism. Further, the mammalian target of rapamycin (mTOR) is a PI3K/Akt–activated transcription factor implicated in increased hypertrophy in cardiomyocytes (Shioi et al. 2002). Downregulation of mTOR by levosimendan in sham GK rats may, at least in part, explain the decrease in LVH seen here.
6 General discussion

This study provides deeper insight into the molecular mechanisms of diabetic cardiomyopathy in the GK rat. The results showed that diabetic cardiomyopathy in GK rats was associated with some of the hallmarks found in diabetic subjects; namely left ventricular hypertrophy (LVH), apoptosis and fibrosis. In the studies I did not study diastolic dysfunction specifically, however study 1 indicated that systolic dysfunction may develop spontaneously in diabetic GK rats. The two other studies (2-3) showed no changes in systolic function between sham-operated GK and Wistar however. These conflicting results show the difficulties and dispersion of results when using an inbred rat strain with a heterogenous genetic background in comparison to using e.g. single gene knock-out strains. Another issue with using spontaneously diabetic GK rats was that they developed modest hypertension at 8-9 weeks of age. This may have contributed to the development of LVH and the other parameters of diabetic cardiomyopathy. However the difference in blood pressure was modest and was absent in post-MI rats. Interestingly, systolic blood pressure was not affected by levosimendan in post-MI rats but did decrease slightly in sham-GK rats. However, these results provide the notion that the beneficial effects of levosimendan on LV remodelling were, at least in part, blood pressure independent.

There are few studies regarding the involvement of stress response pathways FOXO3a and Sirt1 in diabetic cardiomyopathy. My results provided the first notion that FOXO3a and Sirt1 may be involved in the development of diabetic cardiomyopathy. Knowing that Sirt1 promotes growth, LVH in GK rats may be at least in part attributed to Sirt1 overexpression. Others have shown that insulin resistance in GK rats is associated with decreased phosphorylation of downstream kinases (Bitar et al. 2004; Desrois et al. 2004). Here I showed that this was associated with decreased cardioprotective Akt phosphorylation and concomitant proapoptotic FOXO3a activation in response to heart failure post-MI. These pathways may play important roles in the etiology of diabetes-induced
ventricular remodelling and provide part of the answer why diabetic subjects have increased morbidity and mortality after MI compared to non-diabetic ones. Earlier studies have shown the importance of ROS in the pathogenesis of GK rats (Bitar et al. 2004). One shortcoming of the current study was that I did not measure Sirt1 activation or the NAD+/NADH ratio in the cells that regulates Sirt1. Also I did not study the expression of Sirt1 in other metabolically crucial organs deeply involved in the diabetic pathogenesis such as skeletal muscle and liver. However, the results showed that p53-deacetylation at the Sirt1 –specific site was reduced indicating an increase in Sirt1 deacetylase activity was associated with increased protein expression. I did not study the acetylation of FOXO3a here, which should be done in future investigations.

Levosimendan exerted several beneficial effects on ventricular remodelling such as decreased LVH (sham and MI rats), decreased apoptosis and senescence and decreased CTGF mRNA expression. Interestingly these changes were more prominent in diabetic GK rats. One may speculate that the underlying diabetic cardiomyopathy in GK rats which includes mitochondrial dysfunction, is an appreciative target for levosimendan which opens mitochondrial ATP –sensitive potassium channels. Also the involvement of PDE inhibition cannot be fully excluded. This finding warrants further research. The finding that levosimendan induced the Mas receptor and NEP enzyme and downregulated mTOR in sham-operated GK rats was interesting; however the implications of these results were not totally clear. On the one hand, increased Ang(1-7) - Mas receptor activation and on the other downregulation of mTOR are, at least in theory, means by which levosimendan may have attenuated left ventricular hypertrophy seen here in GK sham rats. However, a shortcoming in the 4th study was that I did not examine the levels of tissue angiotensin II in the heart. A number of studies have shown the adverse effects of ROS accumulation in response to increased lipid oxidation in diabetes and in the post-MI condition. Here I found that levosimendan downregulated the gene expression of the Hpgd gene implicated in prostaglandin inactivation. Interestingly, the PPARgamma agonists, i.e. thiazolidinediones,
including pioglitazone have been shown to inhibit Hpgd (Cho et al. 2002). Together with the notion that levosimendan was able to reduce LV remodelling in the diabetic rat this finding warrants further examination into the potential anti-diabetic effects of levosimendan.

In conclusion I have shown here that spontaneously diabetic GK rats exhibit the traits required to examine diabetic cardiomyopathy in an animal model. The data indicate that cardioprotective mechanisms in the GK rat heart are impaired including changes in Akt – FOXO3a -signaling and p38 MAPK phosphorylation. Furthermore my results provide the notion that in the diabetic GK rat myocardium cardioprotective and prohypertrophic Sirt1 signalling is increased. Oral levosimendan for 4 and 12 weeks reduces ventricular remodelling and restores systolic function, by already known mechanisms and by its actions related to gene expression changes in the myocardium.

6.1 Clinical implications and future perspectives

Clinical implications of this study involve novel targets in the search of better treatment of complications due to diabetic cardiomyopathy, including FOXO3a, Sirt1 and p38. Here I have shown beneficial effects of oral treatment with levosimendan in reducing ventricular remodelling in diabetic cardiomyopathy. Since marketing authorisation, the use of levosimendan has increased steadily and is now approved in 47 countries worldwide. Today levosimendan is widely used in cardiac emergency units and is recognised as an alternative for the management of decompensated heart failure. Its off-label use is also gaining ground, including use in states such as septic shock, perioperative cardiac support and ischemic heart disease and cardiogenic shock.
7 Conclusions

The aims of the studies were to investigate in more detail the molecular mechanisms of diabetic cardiomyopathy in spontaneously diabetic Goto-Kakizaki rats, a model of type 2 diabetes. Specifically I examined the role of Sirt1 – p53 and Akt – FOXO3a pathways in the advent of ventricular remodelling. Ventricular remodelling in diabetic rats was further aggravated by experimental myocardial infarction. In this model of post-MI heart failure and ventricular remodelling I studied the cardiovascular effects of oral calcium sensitizer therapy with levosimendan. The main findings in the studies were:

I. Ventricular remodelling in diabetic GK rats was associated with increased LVH, systolic dysfunction and increased apoptotic signalling and activation of the FOXO3a pathway. The study further suggested a role for Sirt1 in growth and counteracting apoptosis in diabetic cardiomyopathy.

II. Post-myocardial infarction in GK rats was associated with increased LVH, sustained cardiomyocyte apoptosis and interstitial fibrosis. This study established the initial finding that FOXO3a activation was associated with Akt dephosphorylation after MI and that Sirt1 – p53 deacetylation was increased post-MI. Furthermore, the data indicated that p38 MAPK protein phosphorylation was increased after MI in GK rat hearts.

III. A 12–week regimen of oral levosimendan exerted beneficial effects on ventricular remodelling by decreasing cardiomyocyte apoptosis, hypertrophy and markers of cellular senescence. The study provided a role for levosimendan in the treatment of diabetes-induced ventricular remodelling in post-MI heart failure.

IV. The beneficial effects of levosimendan on systolic function and ventricular remodelling were evident at 4 weeks post-MI. The effects were associated with changes in the gene expression profile in the diabetic GK rat myocardium including downregulation of the hydroxyprostaglandin dehydrogenase 15 (Hpgd) gene and the mammalian target of rapamycin (mTOR) gene.
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The studies were carried out during the 6-year period between 2004 and 2010 at the department of Pharmacology, Institute of Biomedicine, University of Helsinki. I would like to express my gratitude to my supervisor and mentor Eero Mervaala from whom I received the initial spark for science and the resilience to continue. I would like to thank professor emeritus Heikki Vapaatalo for valuable advice throughout the years and for reviewing my thesis. I want to express my sincere thanks to Esa Korpi, professor and head of the department, for urging me to finish my thesis. Docent Risto Kerkelä and docent Pasi Tavi are acknowledged for reviewing my thesis.

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Erik Vahtola in Espoo, February 2011.
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10 Original publications