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MOLECULAR MECHANISMS OF HYPERTENSION-INDUCED HEART FAILURE

Experimental studies with special emphasis on local renin-angiotensin system, cardiac metabolism and levosimendan

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications (studies I-IV) and some unpublished data:

I.   Mervaala E, **Biala A***, Merasto S*, Lempiäinen J*, Mattila I, Martonen E, Eriksson O, Louhelainen M, Finckenberg P, Kaheinen P, Muller DN, Luft FC, Lapatto R, Oresic M. Metabolomics in angiotensin II-induced cardiac hypertrophy. *Hypertension 2010; 2: 508-515.*

II.   **Biala A**, Tauriainen E, Siltanen A, Shi J, Merasto S, Louhelainen M, Martonen E, Finckenberg P, Muller DN, Mervaala E. Resveratrol induces mitochondrial biogenesis and ameliorates Ang II-induced cardiac remodeling in transgenic rats harboring human renin and angiotensinogen genes. *Blood Press. 2010; 3: 196-205.*

III. **Biala A**, Martonen E, Kaheinen P, Levijoki J, Finckenberg P, Merasto S, Louhelainen M, Muller DN, Luft FC, Mervaala E. Levosimendan improves cardiac function and survival in rats with angiotensin II-induced hypertensive heart failure. *Hypertens Res. 2010; 10: 1004-11.*

IV.   **Biala A**, Finckenberg P, Korpi A, Löytäinen M, Martonen E, Levijoki J, Mervaala E. Cardiovascular effects of calcium sensitization and AT1 receptor blockade combination in hypertensive Dahl/Rapp rats. *Submitted to Journal of Physiology and Pharmacology.*

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MAIN ABBREVIATIONS

ACE	angiotensin-converting enzyme
ACEI	angiotensin-converting enzyme inhibitor
Ang	angiotensin
ANP	atrial natriuretic peptide
ARB	angiotensin II receptor blocker
AT	angiotensin II receptor
ATP	adenosine-5'-triphosphate
BP	blood pressure
COX	cytochrome c oxidase
dTGR	double transgenic rat
GFR	glomerular filtration rate
HF	heart failure
HS	high salt
K$^+_{ATP}$	adenosine triphosphate-sensitive potassium channel
LVH	left-ventricular hypertrophy
LS	low salt
MCP-1	monocyte chamoattractant protein 1
MHC	myosin heavy chain
MI	myocardial infarction
NAD$^+$	nicotinamide adenine dinucleotide
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NCX	Na$^+$/Ca^{2+} exchanger
NO	nitric oxide
NRF	nuclear respiratory factor
PPAR	peroxisome proliferator-activated receptor family
PGC-1α	transcriptional co-activator of peroxisome-proliferator-activated-receptor (PPAR) gamma
RAAS	renin-angiotensin-aldosterone system
ROS	reactive oxygen species
SBP	systolic blood pressure
SD	Sprague-Dawley
SERCA2a	serco/endoplasmatic reticulum Ca^{2+}-ATPase 2a
SIRT1	silent mating type information regulation 2 homolog 1
SNS	sympathetic nervous system
TFAM	transcription factor A, mitochondria
Tn	troponin
TNF	Tumor Necrosis Factor

ABSTRACT

Hypertension is a major risk factor for stroke, ischaemic heart disease, and the development of heart failure. Hypertension-induced heart failure is usually preceded by the development of left ventricular hypertrophy (LVH), which represents an adaptive and compensatory response to the increased cardiac workload. Biomechanical stress and neurohumoral activation are the most important triggers of pathologic hypertrophy and the transition of cardiac hypertrophy to heart failure. Non-clinical and clinical studies have also revealed derangements of energy metabolism in hypertensive heart failure. The goal of this study was to investigate in experimental models the molecular mechanisms and signalling pathways involved in hypertension-induced heart failure with special emphasis on local renin-angiotensin-aldosterone system (RAAS), cardiac metabolism, and calcium sensitizers, a novel class of inotropic agents used currently in the treatment of acute decompensated heart failure.

Two different animal models of hypertensive heart failure were used in the present study, i.e. hypertensive and salt-sensitive Dahl/Rapp rats on a high salt diet (a salt-sensitive model of hypertensive heart failure) and double transgenic rats (dTGR) harboring human renin and human angiotensinogen genes (a transgenic model of hypertensive heart failure with increased local RAAS activity). The influence of angiotensin II (Ang II) on cardiac substrate utilization and cardiac metabolomic profile was investigated by using gas chromatography coupled to time-of-flight mass spectrometry to detect 247 intermediary metabolites.

It was found that Ang II could alter cardiac metabolomics both in normotensive and hypertensive rats in an Ang II receptor type 1 (AT$_1$)-dependent manner. A distinct substrate use from fatty acid oxidation towards glycolysis was found in dTGR. Altered cardiac substrate utilization in dTGR was associated with mitochondrial dysfunction. Cardiac expression of the redox-sensitive metabolic sensor sirtuin1 (SIRT1) was increased in dTGR. Resveratrol supplementation prevented cardiovascular mortality and ameliorated Ang II-induced cardiac remodeling in dTGR via blood pressure-dependent pathways and mechanisms linked to increased mitochondrial biogenesis. Resveratrol dose-dependently increased SIRT1 activity *in vitro*. Oral levosimendan treatment was also found to improve survival and systolic function in dTGR via blood pressure-independent mechanisms, and ameliorate Ang II-induced coronary and cardiomyocyte damage. Finally, using Dahl/Rapp rats it was demonstrated that oral levosimendan as well as the AT$_1$ receptor antagonist valsartan improved survival and prevented cardiac remodeling. The beneficial effects of levosimendan were associated with improved diastolic function without significantly improved systolic changes. These positive effects were potentiated when the drug combination was administered.

In conclusion, the present study points to an important role for local RAAS in the pathophysiology of hypertension-induced heart failure as well as its involvement as a regulator of cardiac substrate utilization and mitochondrial function. Our findings suggest a therapeutic role for natural polyphenol resveratrol and calcium sensitizer, levosimendan, and the novel drug combination of valsartan and levosimendan, in prevention of hypertension-induced heart failure. The present study also provides a better understanding of the pathophysiology of hypertension-induced heart failure, and may help identify potential targets for novel therapeutic interventions.

1. INTRODUCTION

Heart failure (HF) is a pathophysiological condition in which the heart is unable to pump blood at the rate commensurate with the requirements of the metabolizing tissues or can do so only from an elevated filling pressure. (Dickstein *et al.*, 2008) Hypertensive HF emerges as a consequence of hypertension and subsequent changes, and is typically characterized by compromised diastolic function, but preserved systolic function. An important step is the transition from cardiac hypertrophy to HF, characterized by the failure of this maladaptive mechanism to maintain sufficient cardiac output. (Tocci, 2008)(Meredith and Ostergren, 2006) Examination of hospitalized patients has revealed HF to be associated with many pre-existing major risk factors such as ischemic heart disease, myocardial infarction (MI), hypertension, cardiac valvular disease, peripheral vascular disease, chronic renal failure, diabetes. (Lee, 2004)(Schocken *et al.*, 2008)) In addition, minor risk factors contributing to failure of the heart are smoking, high dietary salt intake, sedentary lifestyle, obesity. (for review see (Dupree, 2009)) Moreover there are genetic and behavioral as well as psychosocial factors which infuence the development of HF. (for review see (Spruill, 2010))

There are an estimated 23 million people with HF worldwide and there has been an increase in the number of hospitalization incidents with a resulting increase in health care expenditures. (for review see (Dupree, 2009)) The number of adults suffering from HF is 2 % whereas 6-10 % rate is observed in elderly people living in developed countries. (Dickstein *et al.*,2008)

In 75 % of cases, acute decompensation occurs in a patient with known chronic HF; 25 % have new-onset HF (Fonarow, 2003). It is important to note that more than one-third of patients experiencing acute decompensated HF will have preserved systolic function, though the prognosis of patients with preserved systolic function (ejection fraction (EF) >45-50 %) seems to be similar to those with decreased systolic function. (Tsuyuki *et al.*, 2001)(Bhatia *et al.*, 2006)(Dickstein *et al.*, 2008) Pure diastolic HF occurs in elderly patients, predominantly in women, and is mostly associated with preexisting LVH systemic hypertension and preserved EF. (Francis, 2001)(for review see (Franklin and Aurigemma, 2005))

Surveys have established that hypertensive HF patients requiring care are older, with a mean age between 70 and 75 years, commonly female and have a greater number of concomitant diseases, making them more susceptible to decompensation, more difficult to treat and more prone to recurrent events. (for review see (Lee, 2004)) Appropriate strategies to effectively fight the development of HF during its asymptomatic stages, or at least when early structural and functional cardiac abnormalities have been identified, need to be investigated in individuals with hypertension

or who display a 'high' cardiovascular risk profile as recommended by current guidelines on hypertension and HF. (Tocci, 2008) Even though over the past 20 years, there have been major advances and declining mortality due to novel ways of treating HF with angiotensin-converting-enzyme inhibitors (ACEi), angiotensin II receptor blockers (ARBs), aldosterone antagonists, β-blockers, diuretics and cardiac resynchronization therapy, this disease is still associated with an annual mortality rate of 10 %. (Dickstein *et al.*,2008)(for review see (Neubauer, 2007))

The increasing understanding of cellular and molecular pathways has opened novel types of approaches and revealed new targets for the treatment of diseases. Cardioprotection is one novel therapeutic approach for the treatment of cardiovascular diseases, especially HF, i.e. targeting the molecular pathways associated with the progression of the disease. Cardioprotective salvage pathways include sustained mitochondrial function, corrected cardiac energy metabolism, improved myocyte contractility, suppressed local renin-angiotensin-aldosterone system (RAAS), reduced apoptosis and decelerated cellular senescence. The exact molecular mechanisms of hypertensive HF still remain unclear; therefore studying the signaling pathways known or suspected to be involved in this detrimental process is a prerequisite before one can develop the novel treatments to stop, delay or even reverse progression of established disease. (Slama *et al. ,* 2002) (for review see (Gradman and Alfayoumi, 2006)(Varagic and Frohlich, 2002)(Murray *et al.*, 2007)(Gradman and Wilson, 2009))

This study aimed at characterizing the molecular pathways associated with angiotensin II (Ang II) overactivity. Rat models of hypertension-induced HF were used to examine cardioprotective effects and mechanisms of compounds such as the Ang II receptor antagonist valsartan, the calcium sensitizer levosimendan and the silent mating type information regulation 2 homolog 1 (SIRT1) activator resveratrol, all of which may exert beneficial actions.

2. REVIEW OF THE LITERATURE

2.1 Hypertension as a risk factor for cardiovascular disease

Elevated blood pressure (BP) is known to correlate with increased cardiovascular risk. A higher BP means a higher risk of cardiovascular diseases (CVD) such as stroke, MI, renal disease which eventually can lead to hypertension-induced HF. Due to its life-preserving role, BP must be tightly regulated to maintain a constant perfusion of all organs. (for review see (Lifton *et al.*, 2001)) An increment of as little as 20/10 mmHg starting from BP level of 115 mmHg results in an increased risk of CVD. (for review see (Landmesser and Drexler, 2007) The increase in systolic blood pressure (SBP) develops gradually and often concomitantly with other diseases such as dyslipidemia, insulin resistance, and abdominal obesity. (for review see (Carretero, 2000)) SBP elevation results in a hypertrophic reponse in the arterial smooth muscle layer, decreasing the diameter of the vessel lumen. A consequent increase in afterload contributes to cardiac muscle overgrowth as an adaptation, since the heart needs to pump against a higher resistance. These compensatory mechanisms become maladaptive, increasing the risk of other target organ damage such as in the vascular endothelium, kidney and brain, and thus cardiovascular events. (McEniery *et al.*, 2006)(for review see (Gradman and Alfayoumi, 2006)(Landmesser and Drexler, 2007)(Gradman and Wilson, 2009))

The exact pathogenetic pathways of essential hypertension remain largely unknown and the detailed mechanisms have proved elusive. To date, a significant involvement in this process has been assigned mostly to genetic, environmental and demographic factors. Essential hypertension is shown to be a polygenetic disorder with contribution from variety of risk factors (25-65 % of the total risk). Genes accounting for the onset of hypertension are those predisposing to its progression especially, if the dietary regimen, such as high salt, low potassium or low calcium intake, is imbalanced. (McEniery *et al.*, 2006) (for review see (Gradman and Alfayoumi, 2006)(Landmesser and Drexler, 2007)) In addition to genetic factors, excessive alcohol intake, salt intake (in salt-sensitive patients), aging, sedentary lifestyle, stress, low potassium and calcium intake predispose towards an increase in BP and in that way to CVD. (Carretero, 2000)

2.1.1 Definition and classification of hypertension

BP is defined as force exerted upon vessel wall by circulating blood. When high BP appears to be due to reasons other than secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, essential or primary hypertension is used. (Mancia *et al.*, 2007)

The evaluation of hypertension is based on repeated BP measurements. (Mancia *et al.*, 2007) Depending on the advancement of the condition, different categories can be assigned to the patient. Prehypertension describes the patient at a high risk of developing hypertension. These individuals are encouraged to change the lifestyle and diet to reduce the risk of hypertension in the future. Further stages in the classification of hypertension, defining systolic and diastolic increase in BP (SBP and DBP respectively) for adults are shown in Table 1. The treatment goal for individuals with hypertension is <140/85 mm Hg. (Finnish Current Care Guidelines 2009)

BP classification	SBP (mmHg)	DBP (mmHg)	
Optimal	< 120	and	< 80
Normal	< 130	and	< 85
Pre-hypertension	130-139	and	85-89
Slightly elevated	140-149	or	90-99
Moderately elevated	160-179	or	100-109
Significantly elevated	≥ 180	or	≥ 110
Hypertensive crisis	≥ 200	or	≥ 130

Table 1. A classification system for hypertension for adults. (Finnish Current Care Guidelines 2009)

2.1.2. Importance of dietary salt as key risk factor for hypertension

The link between salt-sensitivity and BP interactions depends on genetic and environmental factors. Genetic factors determine the extent of the BP increase or decrease required to restore and maintain the salt and water balance, indicating that the BP fluctuations of some individuals may be sensitive or resistant to a high salt intake. (for review see (Karppanen, 2006)(Ruilope *et al.*, 2001)) Dietary salt intake is a major contributor to the development of high BP, and even a modest decrease in dietary Na^+ can lower BP. Humans are genetically adapted to consume 0.25 g of salt daily, however nowadays the average daily consumption equals 3-4 g daily. (for review see (Karppanen *et al.*, 2005)(Karppanen, 2006)) The salt excreting mechanisms of the kidney, which are evolutionarily adapted to handle lower amounts of Na^+, become quickly saturated and the kidney is not able to excrete the excess NaCl. This leads to elevated BP, which is an adaptive mechanism allowing the kidney to secrete more salt by using pressure-natriuresis. The magnitude of the BP increase, which is

needed to eliminate a given amount of salt and water, depends strongly on the sodium-handling mechanisms of the kidneys. (for review see (Karppanen, 2006)(Ruilope *et al.*, 2001)) The development of sodium deficiency and decreased extracellular fluid volume during a prolonged period of very small sodium intake or losses due to gastrointestinal causes, sweating, or blood loss, can be effectively prevented by decreasing the BP. By lowering BP, the body is able to prevent renal sodium and fluid excretion completely. On the other hand, in the case of high salt intake the body, is able to effectively prevent salt and fluid accumulation by raising the BP to such an extent that pressure-induced increase in salt and water excretions matches the intakes. (for review see (Karppanen, 2006)) Interestingly, in the presence of normal genetic variations in salt-handling pumps and channels, the excretion of excess sodium is markedly improved by increased intakes of potassium, calcium, and magnesium. Hence, a given amount of excess salt and water can be excreted in at a lower BP than needed with a low intake of the above mineral nutrients. (for review see (Karppanen, 2006))

2.1.3. Subclinical target organ damage in hypertension

2.1.3.1 Endothelial dysfunction and alterations in the vascular wall

Hypertension is associated with abnormal endothelial function in the peripheral, coronary and renal circulation. Endothelial dysfunction is both a consequence of hypertension, and a factor predisposing towards hypertension. Endothelial dysfunction has been implicated in the pathophysiology of macro- and microvascular beds. Thus, it is directly linked to arterial stiffness, coronary atherosclerosis and renal dysfunction in hypertension. (Simko and Pechanova, 2010)(for review see (Landmesser and Drexler, 2007))

Endothelium-derived nitric oxide (NO) relaxes blood vessels and thus regulates vascular tone and structure. It prevents platelet aggregation and adhesion, limits oxidation of low-density lipoprotein (LDL) cholesterol, inhibits the proliferation of vascular smooth muscle cells, and decreases the expression of pro-inflammatory genes that promote atherogenesis. (for review see (Fostermann *et al.*, 2010)(Pechanova and Simko, 2009)(Landmesser and Drexler, 2007)) NO is synthetised by endothelial nitric oxide synthase (eNOS) in the vasculature in the presence of L-arginine. Impairment in NO production leads to changes in BP, decreased shear stress and pulsatile stretch, thrombosis, and atherosclerosis. These changes evoke also disorganization in the structure of vasculature such as vascular smooth muscle cell hypertrophy. (for review see (Landmesser and Drexler, 2007)) Increased reactive oxygen species (ROS) concentrations are known to reduce the amount of bioactive NO by chemical inactivation to form toxic peroxynitrite, which in turn can "uncouple" eNOS to become a

dysfunctional superoxide-generating enzyme that contributes to the vascular oxidative stress. (for review see (Fostermann *et al.*, 2010)(Pechanova and Simko, 2009))

The endothelium is known to produce not only vasodilating NO, but also vasoconstricting factors. These include prostanoids, prostacyclins, acetylcholine and bradykinin, which play a crucial role in the pathogenesis of vessel damage, i.e. they are involved in the processes leading to cardiovascular diseases including HF when in abundance. Of these factors, endothelin-1 seems to be one of the most powerful force. Endothelin is secreted in a paracrine manner in the abluminal direction. It facilitates vasoconstriction hence elevating BP. This increase in BP takes place in the vasculature of smooth-muscle cells with no inevitable alteration in the systemic circulation. (Spanikova *et al.*, 2008)(Simko and Pechanova, 2010)(for review see (Oparil *et al.*, 2003) Tumor necrosis factor-α (TNF-α) in turn, as well as interleukins mediates pro-inflammatory responses in the endothelium. (Zhang *et al.*, 2010) (for review see (Boos and Lip, 2006))

Peripheral vascular resistance is elevated in hypertension, contributes to remodeling of vessels and to high BP and is associated with target organ damage. (Luo *et al.*, 2010)(for review see (Oparil *et al.*, 2003)) Vascular remodeling comprises of adaptive and/or maladaptive changes in the mechanical properties of vasculature. (Kwon et al., 2006)(for review see (Sonoyama *et al.*, 2007)) These kinds of changes may be classified as vessel enlargement (outward remodeling), diminution (inward remodeling), alternatively as adaptive, or maladaptive. (for review see (Herity *et al.*, 1999)) In eutrophic inward remodeling, the diameter of the small arteries lumen decreases and wall thickness increases with no changes in cross-sectional areas. (for review see (Sonoyama *et al.*, 2007)) A disturbed myogenic reflex or further elevated pressure may disrupt the autoregulatory adaptive ability of the vessel. When the vessel wall pressure stress is further increased, eutrophic remodeling is replaced by hypertrophic outward remodeling with reduced ability to contract, namely the myogenic reflex. If this reflex is non-functional or if accelerated pressure overwhelms the blood vessel's ability to autoregulate, then it will be unable to withstand the increased wall stress and it replaces eutrophic inward remodeling with hypertrophy. (for review see (Sonoyama *et al.*, 2007))

Vascular remodeling may also have direct consequences on the heart. Hypertension induced aortic stiffness accelerates aortic pulse wave velocity, which in turn results in a premature wave return in systole. This increases LV afterload and central pulse pressure contributing to cardiac hypertrophy. A decrease in diastolic pressure occurs in parallel, as a result of the concomitant decrease in diastolic BP, reducing coronary perfusion. (for review see (Nagashima and Kasanuki, 2003)(Gradman and Alfayoumi, 2006)(Gradman and Wilson, 2009)(Raman, 2010))

2.1.3.2 Hypertrophy and cardiac remodeling

The main structural adjustment of the heart to pressure overload is LVH. Cardiac hypertrophy is defined as an abnormal increase in heart mass, resulting in increased wall thickness with compromised chamber volume. (for review see (Nadar et al., 2006)(Yip et al., 2009)) Elevated SBP, volume overload and prolonged ejection time, at the expense of reduction of diastole duration and myocardial relaxation, lead to LVH. (for review see (Yip *et al.*, 2009)) LVH predisposes towards coronary heart disease, stroke, cardiac arrhythmias, myocardial ischemia, sudden death, carotid atherosclerotic plaques, and ultimately HF. (for review see (Yip *et al.*, 2009)(Frohlich, 1999)(Phillips *et al.*, 1998))

Cardiac remodeling is associated with the growth and alterations of cardiac myocytes and interstitial matrix, including accumulation of fibroblasts and macromolecules such as collagen, elastin and fibronectin to the extracellular space. (for review see (Finckenberg and Mervaala, 2010)) Loss of myofilaments and myocyte apoptosis are also involved. These changes are accompanied by abnormalities in intracellular calcium handling, initiation of inflammatory processes, and triggring of complex changes in cardiac gene expression encoding ion channels, growth factors and metabolic enzymes. (for review see (Mancia *et al.*, 2007)(Meredith and Ostergren, 2006)(Yip *et al.*, 2009) These detrimental alterations lead to a disproportional growth in the cardiac muscle. (for review see (Yip *et al.*, 2009)) They facilitate diastolic dysfunction, which in turn promotes the progression of systolic dysfunction. (Mancia *et al.*, 2007)(for review see (Moncrieff, 2004)(Meredith and Ostergren, 2006))

As the cardiac remodeling progresses, various types of LVH develop over time. Three major types of LVH are known. Concentric remodeling, in pressure overload conditions, is characterized by sarcomeres which become organized in parallel, adding to the cardiomyocyte thickness and non-dilated thick walled left ventricle, which increases more than the LV cavity volume. This remodeling is associated with a normal ejection fraction and unchanged endocardial stress-shortening relationship. (for review see (Yip *et al.*, 2009) In eccentric remodeling, which is related to volume overload, the sarcomeres become organized in series to promote myocyte lengthening, which results in a proportional increase in LV mass and volume, resulting in ventricular dilation with relatively normal wall thickness but reduced LV systolic function. (for review see (Mancia *et al.*, 2007)(Yip *et al.*, 2009)) MI which combines both of these remodeling types can be considered as the third type of LVH. (for review see (Yip *et al.*, 2009))

Non-hemodynamic factors, such as adrenergic, RAAS and endothelin signaling, are known to activate important pathways, causing downregulation of myocardial β-receptors, inhibition of sarcolemmal

calcium release channels and exaggerated activation of extracellular matrix metalloproteinases (MMPs). (for review see (Mancia *et al.*, 2007)(Gradman and Alfayoumi, 2006)(Subramaniam and Lip, 2009)) Dysregulation of MMPs and their inhibitors (the tissue inhibitors of metalloproteinases, TIMPS) is typical for LVH facilitating the degradation of normal type collagens, which are then replaced by fibrous intestinal deposits of incorrectly crosslinked collagens. This promotes dilatation of the ventricle. In addition, the digestion of extrecellular matrix components by MMPs causes a reactive increase in the production of other factors, including transforming growth factor β (TGF-β), insulin-like growth factor and fibroblast growth factor. (for review see (Diamond and Phillips, 2005))

The increased wall tension evolves into cardiac hypertrophy under the influence of sequencing pathophysiological pathways. These pathways involve intracellular calcium release, as an early response to myocyte stretch and humoral stimuli such as Ang II, phenylephrine and endothelin. The increase in intracellular calcium results in activation of the phosphatase calcineurin, which dephosphorylates a transcription factor, nuclear factor of activated T cell (NFAT3). This results in NFAT3 translocation to the nucleus to initiate transcription of genes that lead to myocyte hypertrophy, such as β-myosin heavy chain and β-skeletal actin. (for review see (Diamond and Phillips, 2005)) Physiological hypertrophy of the heart is generally associated with the induction of α-myosin heavy chains (MHC) expression; however, during pathologic pressure and volume overload hypertrophy, β-MHC is increased at the expense of α-MHC, demonstrating adenosine triphosphate (ATP) management deprivation. (Gupta, 2007) This shift in the myosin- isoform arrangement as well as cross-bridging cycling alterations in the sarcomere plays a significant role in controlling the shortening velocity of cardiac muscle fibers. (Gupta, 2007) Other pathways upregulated genes (such as phospholamdan) and pathways interacting with the calcineurin–NFAT pathway to regulate cardiac myocyte growth, like the mitogen-activated protein kinase (MAPK) pathway. (Rysä *et al.*, 2005)(Rysä *et al.*, 2006)(for review see (Diamond and Phillips, 2005)) Other pathways are known to interact with calcineurin-NFAT pathway and thus regulate cardiac myocytes growth. The MAPK pathway regulates calcineurin via the c-jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs). Targeting these pathways occurs to promote LV mass regression. (for review see (Diamond and Phillips, 2005))

2.1.3.3 Kidney dysfunction

The renal system is responsible for maintaining fluid and electrolyte balance, and therefore playing a crucial role in the development of cardiovascular diseases (CVDs). On average, 20 % of the cardiac output goes to the kidneys. (for review see (Rea, 2008)) The presence of mild renal insufficiency in

hypertensive subjects is accompanied by higher initial levels of both SBP and DBP. (for review see (Ruilope *et al.*, 2001)) Elevated systemic and increased glomerular BP both contribute to nephropathies. (for review see (Cohuet and Struijker-Boudier, 2006)) Hydrostatic and oncotic pressure gradients and permeability of the glomerular basement membrane impact on the glomerular filtration rate (GFR). Neurohumoral mechanisms such as RAAS, anti-diuretic vasopressin and endothelin have a vasoconstrictive role in the kidney. Sympathetic nervous system (SNS) activation decreased NO levels and the resulting increase in oxidative stress causes a constriction of afferent and efferent arterioles, decreasing renal blood flow and GFR. The vascular damage is followed by vascular stiffening, intense neuronal activation, venous congestion, reduced blood flow facilitating the endothelium and media damage; thrombosis, inflammation, hemorrhage and edema. It is crucial that all the regulatory systems in the kidney remain balanced, including the continuous monitoring of the sodium concentration in the distal tubule. These regulatory systems maintain renal hemodynamics and correct GFR and they are controlled by tubuloglomerular feedback loop within the juxtaglomerular apparatus. (for review see (Rea, 2008))

Renal damage mostly can be traced to renal arterioral thickening, fibrinoid deposition in the glomeruli and proteinuria (for review see (Cohuet and Struijker-Boudier, 2006)) Microalbuminuria, or the development of overt proteinuria progresses in parallel with the development of hypertension. There is a concomitant increase in serum creatinine concentration or a decrease in creatinine clearance. Both elevated serum creatinine levels and a decrease in GFR (creatinine clearance, 30-90 ml/min) and elevated urinary excretion of albumin below crucial limits i.e. (microalbuminuria, 30 to 300 mg/day) or above (macroalbuminuria, 300 mg/day), serve as good markers of the development of kidney dysfunction. (for review see (Ruilope et al., 2001)(Johnson, 2008)) Moreover, additional electrolyte imbalances occur; hyponatremia, hypomagnesemia, and hypokalemia in parallel with increased blood urea nitrogen (BUN). (for review see (Francis, 2001))

2.1.4 Hypertension-induced target organ damage

2.1.4.1 Stroke

Cerebrovascular disease is one of the most common consequences of hypertension. This may result in haemorrhagic and ischemic strokes, as well as vascular dementia. (for review see (Aronow and Frishman, 2004)(Veglio, 2009)) Stroke is associated with 9 % mortality worldwide and with 2-4 % of total health care expences. There are several stroke-related risk factors e.g. hypertension, diabetes, and smoking. (for review see (Donnan *et al.*, 2008)) A quarter of all stroke patients have a history of some kind of symptomatic coronary event. One of the major risk factor is atrial fibrillation,

contributing to nearly 10 % of all ischemic strokes. (for review see (Bernstein, 2010)(Armario and de la Sierra A, 2009))

Strokes can be divided into haemorrhagic (intracerebral haemorrhage) and ischemic. The management of stroke is type-dependent. (for review see (Donnan *et al.*, 2008)) Ischaemic strokes account for 80 % of all strokes. The mechanism involves vessel occlusion, impaired cellular perfusion, functional cerebral impairments creating an area called the penumbra. The penumbra is associated with imbalances in energy and ion homeostasis, the presence of free radicals, necrosis, apoptosis and onset of inflammatory processes. These cascades in the penumbra can be arrested and thus cells can be saved. However the infarct core itself consists of tissue which has been fatally damaged. (for review see (Donnan *et al.*, 2008)) Heamorrhigic strokes are commonly associated with hypertension and the mechanism is directly related to hypertensive small vessel disease. Systemic hypertension predisposes to aneurysm, blood-filled dilation. When the size of the aneurysm increases, the risk of its rupture is also increased, leading severe complications or even death. (for review see (Donnan *et al.*, 2008))

The prognosis for patients with stroke is poor; approximately a quarter of stroke patients will die, a third by six months, and a half by a year. (for review see (Donnan *et al.*, 2008)) Antihypertensive therapy can reduce stroke events by 40 % and the recurrence of stroke by 28 %. (for review see (Aronow and Frishman, 2004)(Veglio, 2009)) In particular, rapid and aggressive management for acure stroke such as fibrinolytic agents may be needed to reduce mortality and improve patient recovery. Recombinant tissue plasminogen activator must be injected urgently within 3-4.5 hours after onset of acute stroke. (Otwell *et al.*, 2010) (for review see (Goldemund and Mikulik, 2010))

2.1.4.2 Coronary heart disease

Atherosclerosis is the process predominantly involved in vessel diseases. The predisposition to coronary heart disease is influenced by both- genetic and environmental factors. The heritability of atherosclerosis exceeds 50 % and is subjected to the presence of complex factors such as lipoprotein levels, body fat and others. (for review see (Lusis, 2000)) In addition to hypertension, dyslipidemia and cigarette smoking contribute to its development. (for review see (Veglio, 2009)) The atherosclerotic process comprises vessel remodeling including coronary arteries comprising also inflammatory responses. (for review see (Ross, 1999)) First, there is a compensatory enlargement of the cross-sectional areas of the vessels and changes in the structure and functions. The atherosclerotic plaque develops and occupies 30 % to 40 % of the vessel space. (for review see Palluzzoli *et al.*, 2010)(Francis, 2001)) This vascular stiffness causes major hemodynamic alterations,

these being associated with arterial pulse widening and increases in cyclic arterial flow. The ventricular-arterial stiffening results in changes in cardiovascular reserve, BP variations, diastolic dysfunction, altered coronary and peripheral flow regulation, and mechanical signaling as well as endothelial dysfunction. These changes are common in subjects having cardiac hypertrophy with increased end-systolic chamber stiffness progressed into HF with preserved ejection fraction. (Kawaguchi *et al.*, 2003)(for review see (Chen, 1998))

The progression of atherosclerosis includes disruption of the endothelium, which in turn predisposes lipids and fibrous elements to combine, to form complicated plaques and accumulate in the large arteries. (for review see (Veglio, 2009)(Lusis, 2000)) Plaques are mostly found in large vessel bifurcations and bends where the blood flow is impaired. (for review see (Veglio, 2009) Subendothelial aggregation of LDL cholesterol, which on the contrary to high-density lipoprotein (HDL) is strongly promoting atherosclerosis, accumulates inside congested macrophages (foam cells). Subsequently, the fatty streaks formed are precursors of more advanced fibrous lesions, such as smooth muscle cells and lipid-rich necrotic cells. More extensive alterations may occur e.g. calcification, luminal surface ulceration and hemorrhage from small vessels, which may grow into the lesion from the media of the blood vessel wall. This change may even result in an interruption the block of blood flow, leading to thrombus of blood clot and in the end to infarction, HF or stroke. (Lehtonen-Smeds *et al.*, 2005)(for review see (Lusis, 2000)(Kovanen and Pentikainen, 2003))

Concomitant inflammation triggered by oxidized LDL, is associated with the recruitment monocytes and lymphocytes to the artery wall. Oxidized LDL reduces NO production, thus causing vasoconstriction. Haemodynamic forces, sex, hormones and infections can also regulate the inflammatory process, which further promotes vascular stiffness. (for review see (Lusis, 2000))

2.1.4.3 Myocardial infarction and ischemic heart disease

MI is defined as a necrotic locus in the heart. This area has low tissue perfusion due to symptomatic cardiac cell death. (Zornoff, 2009)) Commonly the rupture of atherosclerotic plaque in the wall of the coronary artery leads to the interruption of the blood supply to the heart. The triggering event for MI is the disruption of a sufficiently large atherosclerotic coronary plaque with the consequent production of a thrombus (blood clot). This onset may start due to internal circadian, external physical or emotional factors. (for review see (Hammoudeh and Alhaddad, 2009)) Cardiac arrhythmias such as tachycardia can enhance the formation of an atherosclerotic plaque and later its disruption. (for review see (Orso *et al.*, 2009) Coronary arteries undergo compensatory outward remodeling in relation to the plaque area over the years. Therefore the development of MI may be

clinically silent for a very long time. Ultimately MI may lead to ischemia and its consequences i.e. lack of oxygen in the myocardium and metabolic dysregulation. (Otwell *et al.*, 2010) (for review see (Misra *et al.*, 2009)

Infarcted myocardial muscle displays an increased stiffness independently of the volume due to the development of myocardial contracture, interstitial edema, fibrocellular infiltration and scar. In case of large infarcts, LV remodeling and dilation progress further. If these detrimental changes continue to develop, the failure of relaxation intensifies and will be accompanied by concentric hypertrophy resulting in post-infarct remodeling. Tachycardia is capable of reducing the duration of diastole, therefore raising ventricular volume; bradycardia in turn has the opposite effect but both conditions worsen the ischemic state. Successful treatment of ischemia improves diastolic relaxation and lowers both diastolic and pulmonary venous pressures, thereby reducing dyspnea. (Braunwald *et al.*, 2008) (for review see (Gajarsa and Kloner, 2010)

2.1.4.4 Hypertension-induced heart failure

HF represents the final common pathway of many different cardiac diseases of different etiologies as described earlier. (Tocci, 2008)(for review see (Dickstein, 2005)) When the ventricle becomes hypertrophied and stiffened, it fills abnormally and fails to relax completely. These chronic changes predispose the individual to diastolic dysfunction but these may remain asymptomatic, until they ultimately progress into symptomatic HF. At first a shift towards increased diastolic pressure-volume overload enhances increased end-diastolic pressure, left atrial and pulmo-capillary pressure and greater stroke volume (for review see (Mandinov *et al.*, 2000)(Meredith and Ostergren, 2006)) but with time, even this mechanism starts to fail. Then the LV filling worsens even more, resulting in inadequate filling even though elevation in diastolic pressure. This decreases end-diastolic volume, which in turn depresses stroke volume (for review see (Mandinov *et al.*, 2000) leading to pulmonary congestion. Furthermore, due to the continuous accumulation of extracellular matrix, cardiac stiffness and vascular resistance, diastolic dysfunction becomes worse and evolves into overt systolic reduction, where cardiac output is decreased. (for review see (Mandinov *et al.*, 2000)(Meredith and Ostergren, 2006) Primarily systolic function of heart such as left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) are sustained in diastolic HF, nonetheless it develops further into systolic HF with reduction in systolic function of the heart and concomitant increase in ventricular volume. (for review see (Kitzman *et al.*, 2002)(Kostis, 2003)(Zile and Brutsaert, 2002)) The development of hypertension-induced HF in depicted in Figure 1.
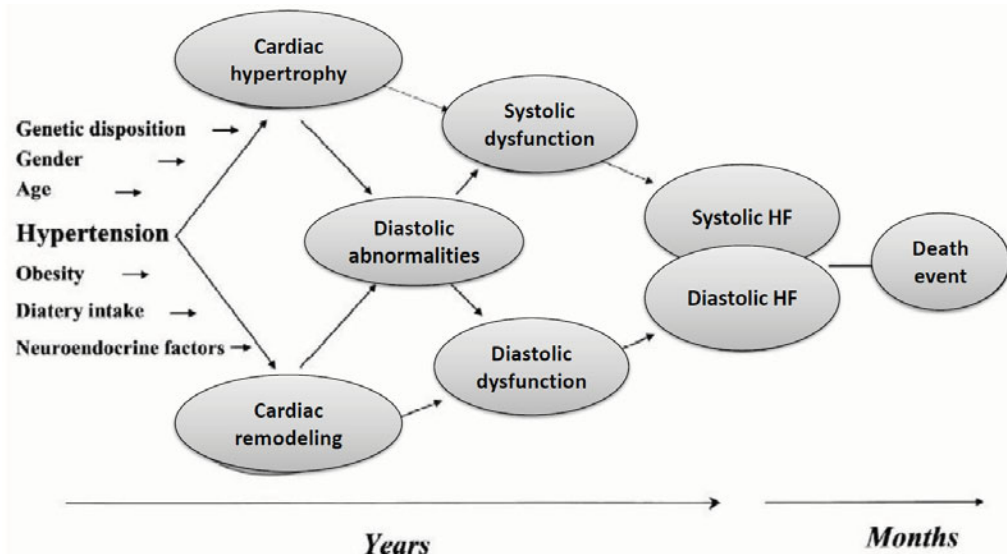
Figure 1. The development of diastolic HF concomitant with systolic HF. As the disease progresses and congestive symptoms become more apparent, thus result can be sudden cardiac death or end-stage failure. Modified from (Mandinov *et al.*, 2000)

The compensatory mechanisms in heart and kidney are associated with overactivity of SNS and overstimulation of RAAS. These systems provide inotropic support to the failing heart, increasing stroke volume and peripheral vasoconstriction in attempts to maintain mean arterial perfusion pressure. However, these alerations are deleterious to cardiocirculatory homeostasis during the late stages of the disease. (for review see (Palazzuoli, 2010))

Acute decompensated HF may be challenging to diagnose. (Lee, 2004) The clinical signs vary depending whether there is pulmonary congestion, organ perfusion, or the presence of coronary disease, fluid retention and systemic pressure. (for review see (Palazzuoli, 2010)) The evaluation of the level of congestion and tissue perfusion thus the disease appear to be the most appropriate ways to achieve the correct diagnosis and treatment. (Lee *et al.*, 2004)

The symptoms and signs of HF include predominantly:

• HF with SBP greater than 140 mmHg (50 %);

• HF with SBP 90 mmHg to 140 mmHg (48 %)

• HF with SBP less than 90 mmHg (2 %)

• Cardiogenic shock (< 1 %)

• Dyspnea (90 %)

• Pulmonary congestion (74 %)

• Pulmonary edema (< 3 %). (Lee *et al.*, 2004)

HF is mostly clinically characterized by fluid overload due to filling pressures and paroxysmal nocturnal dyspnea and orthopnea (shortness of breath) with apparent fatigue and cough. The symptoms best indicating HF are a distended internal jugular vein, tachycardia, and displaced apical impulse. In addition swelling in ankles, legs and abdomen area (ascites) occur being a sign of HF. (Dickstein *et al.*, 2008)(Lee et al., 2004)(for review see (Francis, 2001))

Elevated levels of natriuretic peptides, namely brain natriuretic peptide (BNP) and N-terminal pro-BNP, can serve as diagnostic tools to differentiate HF from non-heart failure- related symptoms. (for review see (Onwuanyi and Taylor, 2007)(Francis, 2001)(Meredith and Ostergren, 2006)) The BNP is produced by the heart ventricles in response to mechanical overload and increased wall stretch, and thus elevated levels of BNP can serve as a good indicator of HF. (for review see (Vuolteenaho *et al.*, 2005)) There are results that BNP reflects the remodelling process in hypertension. (Uusimaa *et al.*, 2004)

Correct and early diagnosis and choice of treatment strategy are still challenges for scientists and clinicians, both partly because there is no comprehensive understanding of the mechanisms contributing to HF development as well as the unsatisfactory availability of drug regimens.

2.1.4.5 Kidney failure

Kidney function is also greatly disturbed and its dysfunctions are driven by the RAAS and the SNS. (for review see (Schrier and Fassett, 1998)) The efferent arteriole of the kidney constricts at the low blood flow levels and the kidney attempts to re-absorb more salt and water, but it excretes more potassium in attempt to maintain proper tissue perfusion. This triggers peripheral edema and imbalanced electrolyte homeostasis. Afferent arteriolar vasodilatory hormones such as prostaglandins and natriuretic peptides, which initially are able to balance glomerular efferent vasoconstricting mechanisms, later fail to maintain renal blood flow. The GFR is usually adequate in the early stages of HF. However due to the decreases in cardiac output and renal blood flow, it fails to remain stable. (for review see (Francis, 2001))

Once there is the presence of renal injury or inflammation a vicious circle ensues to enhance detrimental processes. Not only vasoconstriction (renal artery, efferent and afferent arterioles) and reduced total renal and glomerular blood flow, but there is also a reduction in the number of nephrons processes. The remaining nephrons exhibit glomerular capillary hypertension accompanied by hyperfiltration proteins and other molecules due to increased permeability of the glomerular capillary wall, a decrease in the sodium filtration rate, which ultimately will lead to hyaline deposition, fibroplastic intimal thickening of small arteries, glomerulosclerosis, tubular damage, inflammation and tissue scarring. (for review see (Frohlich, 2001)(Cohuet and Struijker-Boudier, 2006)(Ruilope *et al.*, 2001))

2.2. Pathophysiology of hypertension-induced heart failure

Transition from cardiac hypertrophy to cardiac decompensation is an overt sign of HF. Extrinsic and intrinsic stimuli providing an adaptive response in cardiac hypertrophy start to fail in the maintenance of cardiac output. Over time this decompensation in the heart leads to cardiac failure. (for review see (Lorell, 2000)) At this point the heart has an elevated left ventricular mass with extensive fibrosis and significantly impaired contractility. (for review see (Kitzman *et al.*, 2002)(Kostis, 2003)(Zile and Brutsaert, 2002)) These maladaptive changes resulting in diastolic and followed by systolic HF are driven primarily by neurohumoral stimulation. The low blood supply triggers responses that had developed in order to regulate BP, blood flow and organ perfusion, including RAAS and sympathetic activation. Both of these systems are involved in induction of detrimental molecular pathways. The important molecular pathways associated with HF are impairments in cardiac calcium handling, mitochondrial dysfunction and deprivation in energy utilization, generation of ROS, induction of cell death pathways, and cellular senescence. (for review see (Finckenberg and Mervaala, 2010))

2.2.1. Neurohumoral activation

2.2.1.1 Renin-Angiotensin-Aldosterone System

RAAS regulates sodium balance, extracellular fluid volume, vascular resistance, and, ultimately, arterial BP. (for review see (Harrison-Bernard *et al.*, 2009)(Danilczyk, 2006)) It has been proposed that the RAAS has evolved in order to respond to changes in the level of blood volume and salt intake, which may occur under natural conditions. (for review see (Karppanen, 2006)) Its main effector, Ang II is produced by enzymatic activity of renin. Renin release is stimulated by three mechanisms: 1) intrarenal baroreceptors within the afferent arteriole, 2) alterations in the delivery of

sodium chloride to the macula densa cells, and 3) the influence of sympathetic nerves in the arterioles of the juxtaglomerular apparatus. (for review see (Harrison-Bernard *et al.*, 2009)) The RAAS cascade is presented in the Figure 2.
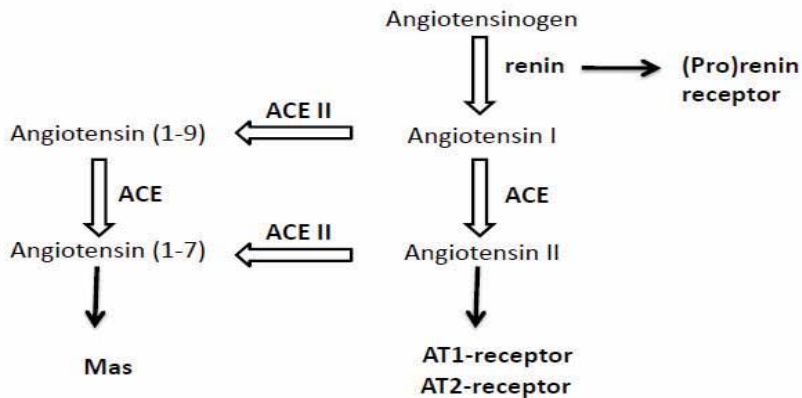
Figure 2. Novel insights into RAAS system and many novel components. Modified from (Bader and Ganten, 2008)

Renin cleaves Ang I from angiotensinogen (Ao). Ang I is then converted to Ang II by ACE. ACE is a circulating enzyme found in the endothelial cells of lungs, vascular endothelium, and cell membranes of the kidney, heart, and brain. ACE also degrades bradykinin into inactive fragments, reducing the serum levels of this endogenous vasodilator. (for review see (Cassis *et al.*, 2010)) Ang II is not only generated in the circulation but also locally in several organs and tissues including kidney, vessels, heart, adrenal gland, eye, testis, and brain. The local RAAS amplifies the actions of circulating Ang II with important implications for physiology and pathophysiology of cardiovascular diseases (for review see (Bader and Ganten, 2008)) It is well known that all components required for Ang II production are available in the heart, and cardiac Ang II formation seems to be regulated independently of circulating RAAS. (for review see (Ferreira *et al.*, 2008)) Ang II causes an increase in systemic and local BP via its vasoconstrictive effect. It influences renal tubuli to retain sodium and water through the hemodynamic regulation of renal blood flow and glomerular filtration rate, tubular epithelial cell sodium chloride and water transport mechanisms. It also stimulates aldosterone release from adrenal gland. (for review see (Harrison-Bernard *et al.*, 2009)) Since renin

secretion followed by Ang II production helps to maintain the correct level of BP, there is a feedback control mechanism, which in response to elevated BP suppresses plasma renin activity almost completely. Hence in hypertensive patients low plasma renin levels may be a predictive factor for cardiovascular (stroke, heart attack, HF) and their renal sequelae, and often correlate with a high sodium intake. In contrast, a low sodium intake/excretion correlates with reduced incident of heart disease and strokes. (Schmieder, 1996)(for review see (Cohen, 2007)) Ang II, known to play a prominent role in the transition of cardiac hypertrophy to HF, is related to a progressive impairment of ventricular function, as well as myocardial fibrosis in humans; however its mechanisms still remain to be resolved. It impairs mechanical and humoral regulation in HF, increasing cardiac wall tension and sympathetic hyperactivity. Sympathetic activity, by activating β-adrenergic receptors (β-AR), stimulates renin and Ao synthesis in fibroblasts and Ao synthesis in cardiac myocytes. (for review see (Ferreira *et al.*, 2008))

Ang II exerts its effects via G-protein-coupled receptors type1 (AT_1) and type2 (AT_2) in many cardiovascular and other tissues. The majority of the cardiac and renal actions of Ang II are mediated by the AT_1 receptor, including vascular smooth muscle contraction, aldosterone secretion, dipsogenic responses, adrenergic stimulation, renal sodium reabsorption, and pressor and chronotropic responses. (for review see (Danilczyk, 2006)(Bader and Ganten, 2008)) AT_1 serves also as a mediator of normal aging processes by stimulating both cytosolic and mitochondrial ROS increasing oxidative stress damage to mitochondria. (for review see (Cassis *et al.*, 2010)) It has been also demonstrated that disruption of the gene encoding AT_{1A} receptor subtype can prolong lifespan in mice. (Benigni *et al.*, 2009) The AT_2 receptor in turn antagonizes many effects of AT_1. (for review see (Cassis *et al.*, 2010)) Binding of Ang II to the AT_2 receptor evokes vasorelaxation that is largely mediated by bradykinin and NO. (for review see (Danilczyk, 2006)) These agents reduce resistance artery remodeling, promote cardiovascular protection against ischemia-reperfusion injury and acute MI, inhibit cardiac fibrosis, and protect the kidney from ischemic injury. (for review see (Cassis *et al.*, 2010))

Aldosterone triggers sodium and fluid retention resulting in increases in intravascular volume and suppression of endothelial function. In addition, aldosterone, similarly to Ang II, exerts non-hemodynamic effects. They comprise direct pro-inflammatory and pro-fibrotic effects of vascular bed, kidneys and heart, thus contributing to target-organ damage, followed by the development of cardiovascular events. In subjects with hypertension or HF, the plasma aldosterone level correlates negatively with systemic arterial compliance as calculated from intra-arterial BP and cardiac output. (for review see (Gaddam *et al,* 2009)(Cassis *et al.*, 2010)(Harrison-Bernard *et al.*, 2009))

In addition to the classical RAAS components, several new participants have been discovered lately; a homolog of ACE- ACE2, which degrades Ang II to Ang-(1-7). The Mas proto-oncogene is a receptor for this peptide and the ACE2-Ang-(1-7)-Mas axis can counter-regulate the above mentioned cardiovascular actions of the classical RAAS. Furthermore, a protein which binds and activates renin and prorenin in tissues, the (pro)renin receptor or (P)RR has been discovered. The physiological role of these new RAAS components is not totally clear. (for review see (Bader and Ganten, 2008))

2.2.1.2 Sympathetic nervous system

Sympathetic hyperactivity is a hallmark of the early stages of hypertension and HF. It has been associated with higher cardiovascular risk and target-organ damage in heart, blood vessels and kidneys. (Ferreira *et al.*, 2008) (for review see (Malpas, 2010)(Charkoudian and Rabbitts, 2009)) SNS causes a plethora of cardiovascular actions: increased heart rate (HR) (positive chronotropism), elevated cardiac contractility (positive inotropism), increases in the rate of relaxation (positive lusitropism), and impulse conduction through the atrioventricular node (positive dromotropism). (for review see (Triposkiadis *et al.*, 2009)(Charkoudian and Rabbitts, 2009) SNS is a critical component of neurohumoral response observed in HF. In the early stages of the syndrome, an intrinsic decrease in myocardial function leads to an increase in sympathetic activity. Altered cardiac structure, increased cardiac output, stroke volume and peripheral vasoconstriction are at first effective compensatory mechanisms to maintain arterial perfusion pressure overload. However over time they fail, eventually leading to failure of the myocardial muscle. (Mancia *et al.*, 2007) Chronic exposure of the heart to elevated levels of catecholamines released from sympathetic nerve terminals and the adrenal gland may evoke further pathological changes in the heart. These can result in continued elevation of sympathetic tone and a progressive deterioration in cardiac structure and function, leading to hypoxia, increased sarcolemmal permeability, calcium overload, oxidative damage, fibrosis and apoptosis**.** (For review see (Triposkiadis *et al.*, 2009)) Moreover, cardiovascular reflexes are disturbed: inhibited aortic arch, carotid, cardiopulmonary baroreflexes, as well as sympathoexcitatory reflexes, such as augmented cardiac sympathetic afferent reflexes and arterial chemoreflexes. (Chan, 2010)(Ferreira *et al.*, 2008) The increased sympathetic signaling in conjunction of RAAS worsens the pathological effects on the cardiovascular system. It has been claimed that Ang II activates central sympathetic outflow and norepinephrine secretion from adrenergic nerve terminals and reduces adrenergic receptor responsiveness. (for review see (Grassi, 1998))

The effects of SNS are mediated in many sites e.g. sinoatrial node, myocardium and peripheral vasculature via the β_1 and β_2 beta-adrenergic receptors (ARs). In the failing heart, the elevated level

of noradrenaline downregulates β-adrenergic receptors, mostly β_1-AR, and causing uncoupling of the β_2-subtype. The imbalanced β_1/β_2-AR ratio results in impairment of the myocardial contractile response as well as structural changes involved in the transition from compensated cardiac hypertrophy to decompensated HF. (for review see (Triposkiadis *et al.*, 2009)(Charkoudian and Rabbitts, 2009)) In the human heart, activation of β_1- and β_2-ARs is the most powerful physiologic mechanism to increase cardiac in the short term performance. The human heart also expresses α_{1A}- and α_{1B}-ARs, but at lower levels. (for review see (Triposkiadis *et al.*, 2009)) It is unknown whether cardiac α_1-ARs play any significant role under physiological conditions. Moreover, the α_1-ARs heavily populate the major arteries (including the aorta, pulmonary arteries, mesenteric vessels, and coronary arteries) and activation of these receptors by noradrenaline (NA) and adrenaline (ADR) and the resulting vasoconstriction is a major contributor to the regulation of blood flow. α_1-ARs may function in a compensatory fashion to maintain cardiac inotropy in the heart and are involved in both developmental cardiomyocyte growth as well as pathological hypertrophy. In the presence of pressure overload or in cases of MI, activation of α_1-ARs also appears to produce important pro-survival effects at the level of the cardiomyocyte and helps to protect against maladaptive cardiac remodeling and decompensation to HF. There is recent evidence to suggest that α_2-AR deficiency can be associated with elevated catecholamine levels, cardiac hypertrophy, fibrosis, and eventually HF. (for review see (Triposkiadis *et al.*, 2009))

The exposure to high levels of circulating catecholamines has been reported to be toxic to cardiac myocytes, leading to myofibrillar degradation and increased cardiac collagen volume fraction mediated by β-AR stimulation. The deleterious cardiac effects of sympathetic overactivity seem to be related mainly to the activation of β_1-AR pathway. (for review see (Brum *et al.*, 2006)

The role of β_3-AR is still unclear, however they have been postulated to be inactive under physiological conditions or to exert a negative inotropic effect due to increased NO production and inhibition of calcium transients. (for review see (Triposkiadis *et al.*, 2009)) β-adrenergic effects are mediated via protein kinase A (PKA) related pathways. (for review see (Triposkiadis *et al.*, 2009)(Charkoudian and Rabbitts, 2009))

In kidneys, activation of the SNS causes vasoconstriction, decreasing renal blood flow and reducing the glomerular filtration rate, increasing the risk of renal ischemia. Sympathetic nervous activity prevents the kidney from excreting sodium. (for review see (Rea, 2008))

2.2.2. Derangement in cardiac excitation-contraction coupling

Calcium plays a vital role in coupling the depolarization process in the cell membrane to muscle cell contraction. This process is called the excitation-contraction coupling (EC-coupling) and it is responsible for the contractile force of each cardiomyocyte and in the end for all cardiac muscle. (for review see (Birkeland *et al.*, 2005)) Calcium ions enter cardiac myocyte cells via L-type calcium channels and these channels are known to contribute to electrical and contractile heart function. They regulate the duration of the action potential, enabling calcium to enter the cardiomyocytes, to trigger contraction, and regulate growth-related signaling in the heart. (for review see (Satin and Schroder, 2009)) The presence of intracellular Ca^{2+} causes myosin, a motor protein present in the sarcomere, to bind to actin filament consuming ATP, resulting in sarcomer shortening. During relaxation, tropomyosin covers the binding sites for myosin on actin. In addition to the tropomyosin filament, the troponin complex, which consists of troponin-I, -T and -C, is found in the sarcomere. In the presence of Ca^{2+}, which binds to troponin-C, the troponin complex will move tropomyosin so that the myosin heads can bind to the actin filament, and the sarcomere can contract. When the Ca^{2+} concentration in the cytosol declines, tropomyosin again covers the binding sites for myosin on actin, and the contraction terminates. Inside the cell Ca^{2+} is stored in the sarcoplasmic reticulum (SR). The contraction is initiated by the opening of ryanodine receptors (RyRs) on the SR. Ca^{2+} removal from cytosol to SR by plasma membrane Ca^{2+} ATPases (PMCAs), SERCA, and out of the cell by Na^+/Ca^{2+}-exchanger (NCX), causes relaxation. (for review see (Birkeland *et al.*, 2005)) The calcium interplay is depicted in Figure 3.
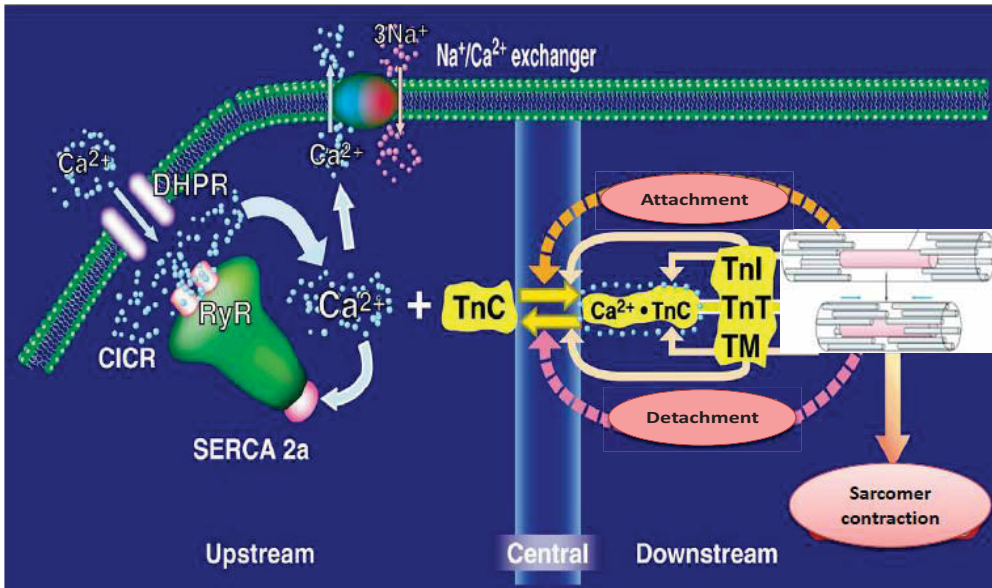
Figure 3. Regulation of calcium interactions in cardiac action. DHPR, denotes dihydropyridine receptor (L-type Ca^{2+} channel); CICR- Ca^{2+}-induced Ca^{2+} release mechanism; RyR, denotes ryanodine receptor; SERCA2a- sarco-endoplasmic Ca^{2+}-ATPase; TnC- troponin C; TnI- troponin I; TnT- troponin T; TM- tropomyosin. Modified from (Endoh, 2008)

In HF, the heart is not capable of pumping enough blood to satisfy the needs of the metabolic tissues, without increasing left ventricular filling pressure. Systolic dysfunction may be due to too few cardiomyocytes, or to reduced contractile function of the cardiomyocytes. In the latter situation the myocardial function is impaired and this condition is called myocardial failure. The pathophysiological mechanisms behind myocardial failure are not known, but it is believed that there may be a defect in L-type Ca^{2+} current and EC-coupling. Many of the changes at the molecular level represent a transition to the fetal phenotype. At the functional level, altered EC-coupling has been demonstrated in cardiomyocytes. (for review see (Birkeland *et al.*, 2005)(Endoh, 2008))

The cardiac isoform of the SERCA2a is a Ca^{2+} pump powered by ATP hydrolysis. SERCA2a transfers Ca^{2+} from the cytosol of the cardiomyocyte to the lumen of the sarcoplasmic reticulum during muscle relaxation, and thus plays a key role in cardiomyocyte Ca^{2+} regulation. In both experimental models and human HF, SERCA2a expression is significantly decreased, which leads to abnormal Ca^{2+} handling and an impaired contractile state. A large number of studies in isolated cardiac myocytes and in small

and large animal models of HF have revealed restoration of SERCA2a expression by gene transfer can correct the contractile abnormalities and improve energetics and electrical remodeling. (for review see (Lipskala *et al.*, 2010)) Beyond its role in contractile abnormalities in HF, SERCA2a overexpression has beneficial effects in a host of other cardiovascular diseases. (for review see (Kawase, 2008)) Similarly the RyR Ca^{2+}-channel, which controls the SR Ca^{2+}-release, has been shown to have an important role in HF and cardiac hypertrophy. The levels or activity of these key Ca-handling proteins are known to be altered in cardiomyopathies, and these changes have been linked to the deteriorations in cardiac function and deranged. Furthermore, genetic variants in these SR Ca-cycling proteins have been identified, which may predispose to HF or fatal arrhythmias. (for review see (Kranias, 2007))

2.2.3 Reactive oxygen species

Reactive oxygen species (ROS) is a collective term that describes O^{2-} derived free radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), peroxyl ($RO_2^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals, as well as O^{2-} derived non-radical species such as hydrogen peroxide (H_2O_2). (Dai and Rabinovitch, 2009)(for review see (Circu and Aw, 2010)) A small amount of ROS is vital for the organisms since these agents are involved in many physiological processes such as cell-cell communication, cell respiration and energy production; phagocyte derived engulfment and digestion alien particles (anti-inflammatory properties), mediation ROS-signaling pathways, programmed cell death-apoptosis, necrosis as well as aging. (for review see (Pourova *et al.*, 2010)(Puzanowska-Tarasiewicz *et al.*, 2009)) They are therefore involved in the development of cardiovascular diseases, hypertension, atherosclerosis and HF. (for review see (Pourova *et al.*, 2010))

Oxidative stress is involved in cardiac remodeling in patients with chronic HF, and increasing oxidative damage may be a marker of worsening functional capacity within this disease. These changes have been shown to be in line with enhanced neurohumoral activity in HF. (Rodanovic *et al.*, 2008) ROS display high reactivity with lipids, proteins and nucleic acids. Different cell types including vascular endothelial, vascular smooth muscle, fibroblast and tubular epithelial cells are being damaged. This happens not only due to generation of both NO and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived superoxide in the cytosol, but also to stimulation by Ang II, which triggers eNOS uncoupling, switching from NO to superoxide production. (for review see (Cassis *et al.*, 2010)) These events contribute vasoconstriction and increased cardiac loading. (for review see (Seddon *et al.*, 2007)) Oxygen deprivation resulting from stress conditions and which can be accompanied by ROS formation is characterized as an oxidative stress response. (Wang *et al.*, 2008)(for review see (Marin-Garcia, 2008))

Two main physiological sources regulate oxidative response in the cell; NADPH oxidases (Noxes) and mitochondria as a major intracellular source of ROS. Moreover ROS is produced by enzymes such as xanthine oxidase, cyclooxygenases, lipoxygenases, myeloperoxidases, haem oxygenases, as well as cytochrome P450-type enzymes. (for review see (Pourova *et al.*, 2010)(Circu and Aw, 2010)(Dai and Rabinovitch, 2009)) Excessive production of NO is associated with peroxynitrite (ONOO⁻) production. Thus particularly cytotoxic factor is created from the diffusion-controlled reaction between NO and a superoxide anion. It serves as a key element in resolving the contrasting roles of NO in physiology and pathology. (for review see (Pacher *et al.*, 2007))

The cell ROS defence system includes glutathione (GSH), vitamins C and E, enzymes such as superoxide dismutase (SOD), manganese superoxide dismutase (MnSOD), catalase and peroxidases. (for review see (Pourova *et al.*, 2010)(Dai and Rabinovitch, 2009)) The potent mechanisms of ROS signaling are presented in Figure 4.
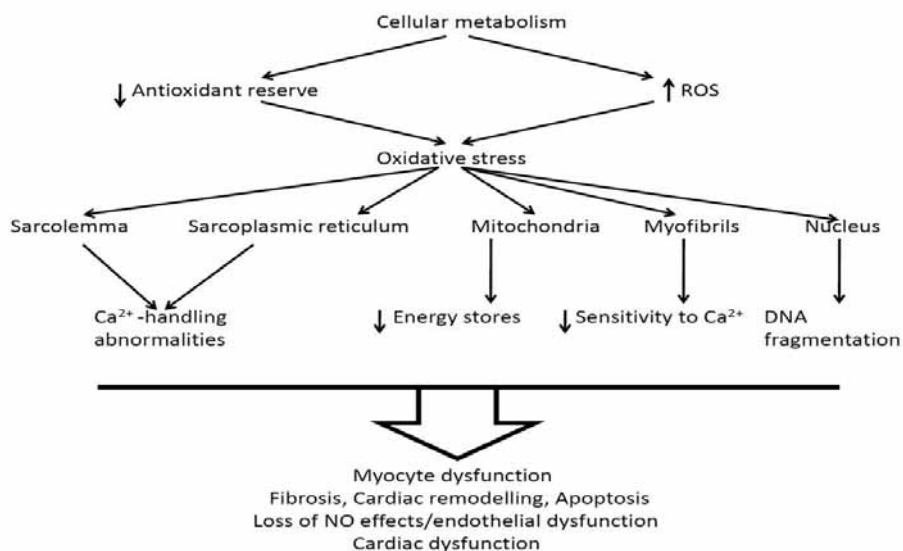

Figure 4. A graph demonstrating involvement of reactive oxygen and nitrogen species in defects insubcellular organelle, involved in cardiovascular dysfunctions. Modified from (Dhalla, 2000) and (Seddon *et al.*, 2007)

2.2.4. Altered myocardial energetics and mitochondrial dysfunction

2.2.4.1 Myocardial energetics

Deprivation of cardiac energy utilization makes a significant contribution to HF. The heart consumes more energy than any other organ, and it cycles about 6 kg of ATP every day, 20 to 30 times of its own weight. (for review see (Neubauer, 2007)) The normal, healthy adult heart utilizes diverse substrates as fuels; lactate, amino acids, ketones, and notably free fatty acids (FFA) (60-90 %) and glucose (Turer, 2009) to generate ATP for cardiac performance. The source of the fuel depends on physiological/patophysiological conditions. This work of the actin-myosin interaction of myofibrils comprises contraction (pump function), Ca^{2+} uptake into the sarcoplasmic reticulum, and maintenance of the sarcolemmal ion gradients. (for review see (Huss, 2004)) As the heart has a limited storage capacity of substrates, the balance between energy demand and energy supply must be maintained, (for review see (Huss and Kelly, 2005)) even with large increases in cardiac output as during intense exercise. (for review see (Stanley *et al.*, 2005)) In non-ischemic conditions, ATP production originates from mitochondrial oxidative phosphorylation (95 %). The FFA β-oxidation pathway, the citric acid cycle, and with a minor contribution from the pyruvate dehydrogenase reaction and glycolysis which result in generation of the reduced form of nicotinamide adenine dinucleotide (NADH) and the reduced form of flavin adenine dinucleotide ($FADH_2$) to create a proton gradient in the electron transportation chain, the reaction coupled with ATP production. (for review see (Huss, 2004)) Mitochondrial creatine kinase catalyzes the transfer of the high-energy phosphate bond in ATP to creatine to form phosphocreatine and ADP. Phosphocreatine rapidly diffuses from the mitochondria to the myofibrils, where myofibrillar creatine kinase catalyzes the reformation of ATP from phosphocreatine. The free creatine, formed by the removal of phosphate from phosphocreatine, diffuses back to the mitochondria. An important function of the creatine kinase system is to act as an energy buffer. (for review see (Neubauer, 2007))

Much of the metabolic regulation of these processes is directly linked to the expression of genes controlling the utilization of cellular FFAs; switch from glucose to FFAs in newborns occurs under O_2 availability and dietary fat consumption. In contrast, in pressure- or volume overload–induced hypertrophy, the mitochondrial oxidative capacity is reduced, enhancing cardiac glucose metabolism, resembling the fetal metabolic pattern. (for review see (Huss, 2004)) During the development of cardiac hypertrophy and in the failing heart, the chief myocardial energy source switches from fatty acid beta-oxidation to glycolysis: a reversion to the fetal energy substrate preference pattern. For review see (Barger *et al.*, 1999) Impairments in HF-related energy metabolism occur in all three core points: substrate utilization (cellular uptake and breakdown), oxidative phosphorylation, and high-

energy phosphate metabolism. (for review see (Neubauer, 2007)) Schematic pathways related to energy utilization are depicted in Figure 5.
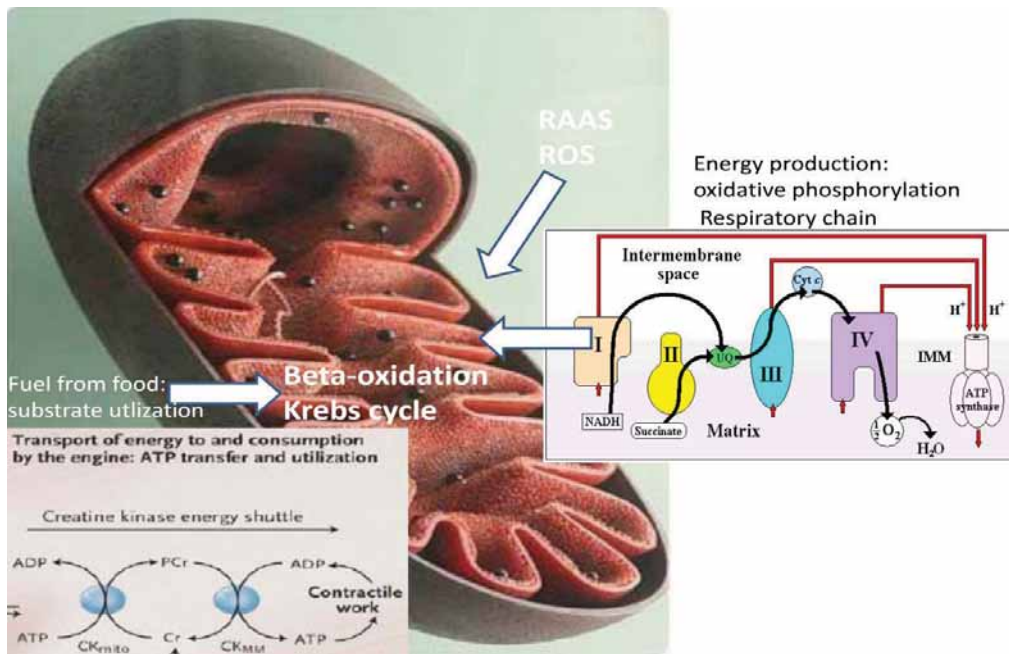
Figure 5. Cardiac energy utilization. Substrate utilization- beta-oxidation and glycolysis resulting in formation of CoA, which is fed later into the Krebs cycle, which in turn produces nicotinamide adenine dinucleotide phosphate (NADP) and CO_2; Oxidative phosphorylation and production energy via respiratory chain and electrons transfer from NADH to oxygen. The emerging gradient provides ATP synthase by phosphorylation of adenosine diphosphate (ADP), and in case of uncoupling proteins-heat rather than ATP; Energy transfer and utilization, the transport of energy and usage by myofibrillar ATPase and other reactions (sarcolemmal/ sarcoplasmic reticulum ion pumps). ATP transfer is obtained by the creatine kinase energy shuttle. Creatine, which is not produced in the heart, is taken up by the creatine transporter. Modified from (Neubauer, 2007)

When the energy demand exceeds the energy supply, the phosphocreatine level falls, keeping ATP at a normal level. The reduced phosphocreatine/ATP ratio limits cardiac performance during metabolic stress conditions and can be one way to predict early stage of HF. (for review see (Neubauer,

1997)(Neubauer, 2007)) The free ADP level is elevated, which in turn inhibit the function of many intracellular enzymes, causing failure of the heart's contractile mechanism. Thus, a metabolic derangement in the cardiac myocyte can occur when phosphocreatine levels fall and free ADP levels rise, even though ATP levels remain unchanged. (for review see (Neubauer, 2007))

In HF both changes in glycolytic and mitochondrial enzyme expression occur, as well as alterations in nuclear and mitochondrial transcription rates which are known to be crucial in the metabolic profiling. (for review see (Stanley *et al.*, 2005)) The peroxisome proliferator-activated receptors (PPAR) family of nuclear receptor transcription factors has been shown to regulate cardiac fuel metabolism at the gene expression level. The three PPAR family members (α, β/δ and γ) serve as transducers of developmental, physiological, and dietary cues that influence cardiac fatty acid and glucose metabolism. (for review see (Madrazo and Kelly, 2008)) In cardiac hypertrophy the expression of PPAR(α) is decreased in proportion to the depression of fatty acid utilization. For this reason, the down-regulation of PPAR(α) is thought to be the main mechanism underlying the switch in substrate utilization from fatty acids to glucose. This switch is a typical characteristic of the hypertrophied heart. A nuclear-receptor coactivator, PPAR(γ) coactivator-1 (also known as PCG-1(α)), is a master regulator of metabolic function in mitochondria since it can activate several genes that are responsible for fatty acid uptake and oxidation and for oxidative phosphorylation. These genes include PPAR(α) and PPAR(β) and nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2). There are experimental studies revealing that the inhibition of PCG-1(α), probably as a direct consequence of high plasma catecholamine levels, leads to down-regulation of mitochondrial gene expression. In this way, it contributes to the impairment of oxidative phosphorylation in the failing heart. The development of HF is accelerated by PCG-1(α) deficiency, suggesting that this coactivator may have a cardioprotective function. (for review see (Neubauer, 2007))

Genetically manipulated mice with selective knockout of genetic components have been created to study altered energetic profiles in HF. The deletion of a variety of genes that encode specific metabolic components related to substrate utilization, oxidative phosphorylation, and high-energy phosphates has evoked a loss of contractile reserve, overt HF, cardiac hypertrophy, tachyarrhythmias, or even bradyarrhythmias. These genetic studies show that a fully integrated metabolic machine is important for normal cardiac function and that selective deficiency of components of energy metabolism can cause early or advanced HF. (for review see (Neubauer, 2007))

2.2.4.2 Mitochondrial biogenesis

Mitochondrial biogenesis is a process of growth and division of pre-existing mitochondria. Mitochondria are organelles derived from α-proteobacteria, which during the process of endosymbiosis became established in the host cells. They have their own genome and autoreplication capacity. Mitochondrial proteins are encoded by both the nuclear and mitochondrial genomes. (for review see (Rimbaud, 2009)(Ventura-Clapier, 2008)) Owing to the dynamic properties of fusion and fission, controlled by GTPases and mitofusins, and translocations, mitochondria are able to divide and organize during the biogenesis process, including accompanied size, number, mass variations, as well as ensuring proliferation of mitochondrial membrane. Mitochondrial biogenesis is enhanced in the presence of environmental stress factors- oxidative stress, exercise, cold exposure, caloric restriction, cell division/renewal and differentiation. (for review see (Ventura-Clapier, 2008)) The continual cardiac contractile activity requires sustained energy production. Mitochondrial respiration accounts for 95 % of heart energy supply and depends on both dynamics and function of mitochondria. (for review see (Rimbaud, 2009)) and alterations in mitochondrial biogenesis can result in cardiac hypertrophy or failing heart. (for review see (Ventura-Clapier, 2008))

Interactions between transcriptional in the mitochondrial and nuclear genome allows co-ordination of biogenesis. The transcriptional co-activator PGC-1α, a master regulator of mitochondrial biogenesis, is abundantly present in cardiac tissue and is activated in the energy demand state. PGC-1α regulates mitochondrial biogenesis and controls mitochondrial content in the normal heart. During compensated hypertrophy, PGC-1α expression levels are maintained and mitochondrial biogenesis is increased in proportion to the increase in cell size. In HF, PGC-1α becomes down-regulated and its oxidative capacity is reduced, leading to energy starvation and cardiac dysfunction. PGC-1α lacks DNA binding activity, but it undergoes an interplay with NRF-1 and NRF-2, increasing the transcriptional activities of these genes, and hence the respiration rate. (for review see (Ventura-Clapier, 2008)) Nuclear encoded mitochondrial transcription factor A (Tfam) promoter possesses recognition sites for NRFs, thus ensuring that mitochondrial biogenesis is coordinated at the mitochondrial and nuclear levels. (for review see (Ventura-Clapier, 2008)) In addition by binding and activating other promoters encoding nuclear genes, NRFs activate oxidative phosphorylation system (OXPHOS). (Garnier, 2003) PGC-1α also interacts with and co-activates PPARγ, encoding the major regulator of the fatty acid oxidation pathway, and enabling fatty acid oxidation (FAO) increase in parallel with mitochondrial biogenesis. The transcription factors PPARγ family is expressed in the heart, and plays a pre-dominant role in the regulation of mitochondrial FAO enzymes and transport proteins. (for review see (Ventura-Clapier, 2008))

Hypertrophied and failing heart is characterized by down-regulated FAO enzymes and other mitochondrial proteins, which result in cardiac inability to maintain normal heart function due to the down-regulation of the mitochondrial biogenesis transcriptional pathway. (for review see (Rimbaud, 2009)(Ventura-Clapier, 2008)) There is also a decrease in oxidative capacity.

Experiments conducted with PGC-1α knock-out mice demonstrated normal mitochondrial volume density in the heart, but blunted expression of mitochondrial genes. When chronic haemodynamic overload had been produced, these mice developed accelerated cardiac dysfunction progressing to heart failure, suggesting that low PGC-1α level can be considered as a predisposing factor to HF. (for review see (Ventura-Clapier, 2008)) An *in vivo* rat model of chronic HF has exhibited down-regulation of PGC-1α, NRFs, Tfam, as well as impaired mitochondrial respiratory chain function as subunits cytochrome c oxidase 1 and 4 (cox1 and cox4), regulating mitochondrial capacity. (Garnier, 2003) Respiratory chain dysfunction is known to play a crucial role in a deprived mitochondrial ATP production rate. (Hansson *et al.*, 2004)

2.2.5 Apoptosis and ER stress

Activation of cellular pathways of programmed cell death may lead to LVH, and cardiac dysfunction. (for review see (Ginzalez *et al.*, 2006)) In cardiac failure, the heart is suffering from progressive myocyte loss. (for review see (Kang, 2000)(Kitsis, 2008)) In the adult, healthy heart, apoptosis has no physiological role, nonetheless it plays a crucial role in forming the cardiac valves and outflow track during cardiac development. (for review see (Dorn, 2009)) There are several death programs that can operate in a cell, necrosis, perhaps autophagy and perhaps the best understood- apoptosis, a kind of cell suicide process. (for review see (Kitsis, 2008)) Apoptosis is characterized by shrinkage of the cell membrane, condensation of nuclear chromatin, cellular fragmentation, and finally the engulfment of the apoptotic bodies by neighboring cells. (for review see (Kockx, 2000)) This process is mediated by two inter-connected pathways. (for review see (Kitsis, 2008)(Movassagh, 2008))

In the extrinsic pathway, extracellular ligands bind to the tumor necrosis factor (TNF) superfamily, death receptors; Fas ligand (FasL), TNF-related apoptosis inducing ligand (TRAIL), TNF ligand superfamily member 10 (TNFSF10) or TNF-α. Binding to death receptors of extrinsic factors triggers apoptosis by receptor oligomerization and Fas Associated Death Domain (FADD)-containing protein and the initiating caspase 8, resulting in the formation of the death-inducible signaling complex (DISC). These reactions are followed by proteolytic degradation (caspase 8 and 10) of intracellular proteins and cleavage of nuclear DNA. The extrinsic pathway may directly result in apoptosis due to

activation of caspase 3 or may be associated with the intrinsic pathway. (for review see (Gerald *et al.*, 2009)(Movassagh, 2008))

The intrinsic pathway is activated by nutrient shortage, survival factors, oxygen supply, oxidative stress, physical and chemical toxins, DNA damage and misfolded proteins. (for review see (Kitsis, 2008)(Movassagh, 2008)) Outer mitochondrial membrane permeabilization (MOMP) occurs due to the formation of pores, which enhance the release of cytochrome c and intermembrane space proteins e.g. second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein (smac/Diablo). This in turn allows caspase activation which then causes structural and regulatory cytoplasmic and nuclear proteins to disintergrate. The process of cell apoptosis in this pathway is coordinated by pro- and antiapoptotic B-cell lymphoma 2 (Bcl-2) protein family members like Bak and Bax. (for review see (Parsons, 2010)) Bak and Bax, as well as p53 are pro-apoptotic factors that stimulate MOMP. P53 is known to trigger apoptosis directly by stimulating Bax or Bak or through binding to Bcl-2 and Bcl-xL. Bcl-2, Bcl-xL and induced myeloid leukemia cell differentiation protein (Mcl-1) prevent MOMP. (for review see (Movassagh, 2008)) Pro-apoptotic Bcl-2 induced in hypertrophied heart results in activation of the caspase cascade, impaired respiration and reduced ATP production. (for review see (Dorn, 2009)(van Empel, 2005))

The endoplasmic reticulum (ER) apoptotic pathway signaling starts by impairments Ca^{2+} signaling which disturb intracellular Ca^{2+} homeostasis and then there is impaired protein folding and glucose deprivation, which provoke ER stress thus trigerring apoptosis. (for review see (Okada, 2004)(van Empel, 2005)(Movassagh, 2008)) The ER stress response is an adaptive mechanism of cardiac myocytes to the prolonged need for excess protein synthesis of both structural and natriuretic peptides. (for review see (Toth *et al.*, 2007)) The unfolded protein response (UPR) results in increased levels of G-protein coupled receptor 78 (GPR78)/binding immunoglobulin protein (BiP). (for review see (Brostrom and Brostrom, 2003)) This ER chaperone binds to unfolded proteins and regulates the activity of upstream proteins such as serine/threonine-protein kinase/endoribonuclease IRE1 (IRE1), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). (for review see (Tabas, 2010)) It can also bind to other chaperones of higher specificity such as GRP94, calnexin and calreticulin, in the folding of selected proteins. (Brostrom *et al.*, 2001)(for review see (Brostrom and Brostrom, 2003)

Virtually every myocardial disease is characterized by programmed cardiomyocyte death, resulting in pathological processes. (Jugdutt and Sawicki, 2004) HF for example is characterized by a significantly

elevated apoptosis level of myocytes in 0.08-0.25 % of patients with dilated cardiomyopathy compared to only 0.001-0.002 % of healthy patients. (for review see (Movassagh, 2008))

2.2.6 Autophagy

Autophagy is a conserving process of degradation and recycling of cytoplasmic components, such as proteins and organelles, including selective elimination of damaged mitochondria as a cytoprotective mechanism, and maintenance of ER. As damaged mitochondria release pro-apoptotic factors such as cytochrome c, autophagy can prevent the activation of apoptosis. Constitutive cardiomyocyte autophagy is required for protein quality control and normal cellular structure and function under the basal state. The accumulation of abnormal proteins and organelles, especially mitochondria, may directly cause cardiac dysfunction. In the heart, autophagy is important for the turnover of organelles at low basal levels under normal conditions and it is upregulated in response to stresses such as ischemia/reperfusion and in cardiovascular diseases such as HF. The level of autophagy as well as triggering upstream signaling pathway determines whether either it has a protective or a detrimental function. (for review see (Nishida *et al.*, 2009)) Autophagy has been observed in hypertrophied myocardium, failing myocardium caused by dilated cardiomyopathy by valvular disease and by ischemic heart disease, in patients with terminal HF. Nonetheless, the role of autophagy in cardiac hypertrophy remains to be elucidated. (for review see (Nishida *et al.*, 2009))

Autophagy has a dual purpose. It is involved in cellular survival i.e. during ischemia or in nutrient-deprived cells, or is a part of cell death at stages when progressive myocytes alterations are beyond repair. (for review see (Dhesi *et al.*, 2010)(Nishida *et al.*, 2009)) Multiple cross-linking signaling pathways are involved in mediating autophagy, apoptosis and necrosis, thus making them challenging to define and distinguish. (for review see (Dorn, 2010))

Three forms of autophagy have been described to date; macroautophagy (most prevalent form), microautophagy and chaperon-mediated autophagy. Autophagy results from double-membrane autophagosome engulfing proteins and organelles. Subsequently, the autophagosome fuses with lysosomes forming the autolysosome. (for review see (Nishoda *et al.*, 2009)

One of the most important upstream regulatory pathways comprises class III phosphoinositide 3-kinase (PI3K), which phosphorylates serine/threonine protein survival serine/threonine protein kinase Akt/PKB (provides mitochondrial protection) and thus activates the mammalian target of rapamycin (mTOR) protein kinases (a key regulator of cell growth and proliferation). Recently AMP-activated protein kinase (AMPK) pathway has been suggested to be involved in autophagy. (for review see (Wang *et al.*, 2010)

Autophagy is controlled downstream by autophagy- related genes (Atgs), many of which are involved in autophagosome formation. Beclin1 (Atg6) and PI3K are needed for the vesicle (called isolation membrane) nucleation step of autophagy. (for review see (Nishida *et al.*, 2009)) Formation of autophagosome is controlled by 2 conjugated systems; Atg12-Atg5 and the conjugation of phosphatidylethanolamine to Atg8 (microtubule-associated protein 1 light chain 3; LC3)-phosphatidylethanolamine systems. (for review see (Nishida *et al.*, 2008))) The conjugation leads to the conversion of the soluble form of LC3B (LC3-I) to the autophagic vesicle-associated form (LC3-II), which is used as a marker of autophagy. (for review see (Nishida *et al.*, 2009)(Dhesi *et al.*, 2010))

In Atg5-deficient mice autophagy has been observed to be a protective mechanism in terms of pressure overload. In the failing heart, however this mechanism is believeded to be unable to undertake the repair during cardiomyocyte remodeling. (for review see (Nishida *et al.*, 2009))

2.2.7 Cellular senescence

Cellular senescence is the irreversible growth arrest process of mitotic cells. Accumulation of damaged DNA, telomere attrition and ultimately chromosomal damage can manifest itself as an increase in senescent cells in tissue and organs, followed by the dysfunctions. Accumulated premature senescent cells in the heart can be associated with the progressive heart degeneration, followed by the cardiac failure. (for review see (Bergmann *et al.*, 2008)(Wong *et al.*, 2010)(Dai and Rabinovitch, 2009))

Various stressors are known to induce cellular senescence; RAAS, imbalanced ROS production, increased mitochondrial membrane permeability, followed by altered mitochondrial respiratory chain- derived oxidative stress, and impaired ATP production. (for review see (Dai and Rabinovitch, 2009)(Juhaszova *et al.*, 2005)(Sawyer, 2000)) Imbalanced calcium handling proteins, connected to altered β-adrenergic signaling, is linked to decline in SERCA2a, and the compensatory increase in NCX proteins impair Ca^{2+} intracellular transients. (Janczewski *et al.*, 2002)(for review see (Josephson *et al.*, 2002)(Juhaszova *et al.*, 2005)) These mechanisms orchestrate the decrease in the force of myofilaments, prolonged relaxation and contraction of heart muscle, predisposing it to cellular senscence. (for review see (Dai and Rabinovitch, 2010))

Cellular senescence can be triggered by various mechanisms such as telomere-shortening (replicative senescence), p16INK4a expression (telomere-independent senescence), oxidative stress/DNA damaging agents (stress-induced premature senescence) and activated oncogenes such as Ras (oncogene-induced senescence). The role of each of these mechanisms in inducing cellular

senescence in tissues remains to be elusive. (Krishnamurthy *et al.*, 2004) (for review see (Bernhard and Laufer, 2008))

Furthermore senescent cells are characterized by elevated levels of cell cycle inhibitors like p21 and p16/INK4a and p53-pathway are upregulated. (for review see (Bernhard and Laufer, 2008)) p16Ink4a enhances and p19Arf attenuates the onset of cellular senescence and ageing in these tissues, however surprisingly in cultured cells p19Arf is known to be effector of senescence. (Baker *et al.*, 2008)) These 2 tumor suppressor molecules are encoded by the Ink4a/Arf locus. (Krishnamurthy *et al.*, 2004) Expression of these principal aging mediators increases with age in both rodents and human tissues. (Baker *et al.*, 2008)

Mice lacking p16INK4a have an increased capacity for regeneration, whereas constitutive p16INK4a expression blocks endogenous regeneration. In addition, there is claimed to be an association between single nucleotide polymorphisms in the INK4a/ARF locus and early onset diabetes and vascular heart disease. (for review see (Sharpless and DePinho, 2007)) The p16INK4a expression tightly correlates with hypertensive tissue damage both in experimental models and human samples. Treatment prevents both organ damage and p16INK4a expression. (for review see (Bergmann *et al.*, 2008))

2.2.8 Sirtuin- related pathways

The sirtuins are a highly conserved family of nicotinamide adenine dinucleotide (NAD^+) dependent histone deacetylases (HDACs) that removes acetyl groups from acetyl-lysine residues in histones and non-histone proteins such enzymes and transcription factors. Seven members of sirtuin family (1-7) have been described so far. (for review see (Finkel, 2009)(Schemies *et al.*, 2009))

The silent mating type information regulation 2 homolog 1 (SIRT1), a nuclear protein, mediates lifespan extension (for review see (Alcendor, 2007)) and regulates numerous physiological and pathological processes (for review see (Lu *et al.*, 2009)) like cardiovascular and metabolic diseases, diabetic nephropathy, metabolic diseases, aging, cancer and many other in experimental animal models. The cardioprotective effects of SIRT1 have been observed in HF, hypertension, inflammation and endothelial NO imbalance. (for review see (Zeng *et al.*, 2009)(Li *et al.*, 2009)(Schemies *et al.*, 2009))

In particular, the activity of SIRT1 has been associated with apoptosis, energy production, respiration, antioxidant defenses, as well as adaptation to environmental stresses by adapting cellular responses to the energetic state of the cell. These mitochondrial-related responses are crucial in cardiovascular

biology, highlighting the important functions of SIRT1 in regulating vascular growth, shape, and function; however SIRT1 mechanisms remain to be resolved. (for review see (Lu *et al.*, 2009)(Gurani *et al.*, 2010)) Recent studies have also elucidated new substrates for SIRT1 in metabolic regulation such as AMPK. (for review see (Tang and Chua, 2010))

Various factors serve as SIRT1 substrates e.g. pro-apoptotic and carcinogenic p53, forkhead box class O which is involved in cell growth, proliferation, differentiation, and longevity, PGC-1α, transcriptional coactivator and metabolic regulator. (Vahtola *et al.*, 2010)(for review see (Puigserver, 2003)) SIRT1 is also known to induce NO signaling; eNOS, an enzyme that generates NO, is atheroprotective and promotes blood vessel relaxation. (for review see (Haigis, 2010)) SIRT1 overexpression in transgenic mice has been found to be associated with its anti-aging properties and protection against oxidative stress, which may be a novel cardioprotective strategy against aging and certain types of cardiac stress, such as oxidative stress. (for review see (Chiao *et al.*, 2008))

In contrast, inhibition of SIRT1 reverses these cardioprotective actions. Its deactivation results in depletion of NO bioavailability, inhibits endothelium dependent vasorelaxation, causes induction of ROS and inflammation- mediated premature senescence of endothelial and smooth muscle cells and it can cause an imbalance in normal vascular function in the presence of ischemia. (for review see (Haigis, 2010))

Even mild to moderate (up to 7.5-fold increase) expression of SIRT1 retards aging, delays or can prevent cardiac hypertrophy, apoptosis, fibrosis, cardiac dysfunction, expression of senescence markers, and induces resistance to oxidative stress. On the contrary to the situation after a 7.5-fold increase, a high level of SIRT1 (12.5-fold increase) induces cardiac remodeling and impairs cardiac functions. However, although high levels of SIRT1 increase oxidative stress, moderate expression of SIRT1 induces resistance to oxidative stress and apoptosis in transgenic Sirt1 mice. (Alcendor, 2007) SIRT1 is activated under caloric restriction, exercise, and by treatment with compounds such as resveratrol, and ican be nhibited by nicotinamide and sirtinol. (for review see (Haigis, 2010))

2.3 Management of hypertension-induced heart failure

Lowering BP to the recommended guideline levels of 140/85 mmHg with utilizing antihypertensive medication could considerably reduce the morbidity and mortality of cardiovascular disease, including the incidence of myocardial ischemia and infarction, congestive HF, stroke, renal failure, and retinal damage. (for review see (Francis, 2001)) To date there are no specified guidelines how to treat HHF in particular, however these defined by European Society of Cardiology (ESC) consist of

treatment regimens also for HHF. Currently available therapies aim to prevent the development of HF, as well as to reverse already coexisting symptoms. Nonetheless due to incompletely understood mechanisms, the current available treatments still require further development and improvement in order to prevent and treat HF patient with more efficient outcomes. Most antihypertensive agents described by ESC belong to the following five major drug classes: diuretics, β-blockers, digoxin, hydralasine comined with isosorbide dinitrite and two drug classes that inhibit components of the RAAS. These drugs may efficiently control BP, but they do not reduce the risk of cardiovascular disease to the levels in normotensive individuals. (for review see (Rajko *et al.*, 2007)) In conjunction with pharmacological treatment, and adherence to a diet regimen, exercise plays a crucial role in preventing cardiovascular events.

2.3.1 Non-pharmacological treatment of hypertension-induced heart failure

In addition to pharmacological treatment, self-education and understanding the etiology of the HF is crucial. Self-monitoring of BP is a practical approach of controlling the progress of the disease. Prevention of cardiac incidents consists of adoption of healthy lifestyles, such as a healthy low-salt diet, rich in fruits and vegetables and the avoidance of saturated fats and excessive alcohol consumption. Other efficient methods to prevent CVD are regular physical activity, rest and adequatel sleep. Only 20-60 % of patients with HF follow the pharmacological and non-pharmacological treatments and recommendations. Therefore it will benefit these individuals if they are aware of their condition and the benefits of adherence to pharmacological treatments, as well as self-care management-related issues. (Dickstein *et al.*, 2008)(Mancia *et al.*, 2007)(Graham *et al.*, 2007)(for review see (He and MacGregor, 2007))

2.3.2 Current drug therapy for hypertension-induced heart failure

2.3.2.1 Angiotensin-converting enzyme inhibitors (ACEIs)

The mechanism of action in case of ACEIs is linked to inhibition of the Ang II-related effects. ACEIs are used primarily in the treatment of hypertension, though they are also sometimes used in patients with cardiac failure, renal disease or systemic sclerosis ACEIs can also be used to treat diabetic nephropathy and left ventricular hypertrophy. ACEIs are a group of drugs used in symptomatic HF with decreased systolic function, unless they are not tolerated or contraindicated. Their mechanism of action depends on ACE inhibition, preventing Ang II production; therefore ACEIs exert cardio- and renoprotective functions, also in diabetics**.** (Dickstein *et al.*, 2008) These drugs reduce pre- and afterload in the heart, prevent ventricular remodeling, and even reverse atherogenic changes in the

vessel walls of patients with congestive HF, acute MI, or coronary artery disease. (for review see (Katragadda and Arora, 2010)) Potential adverse effects of ACEIs include renal function impairment, hyperkalaemia, symptomatic hypotension and cough. In those cases where ACEIs are not tolerated, AT_1 receptor blockers (ARBs) should be used. (Dickstein *et al.*, 2008)

2.3.2.2 AT_1 receptor antagonists

ARBs block specifically Ang II binding to the AT_1 receptor. Inhibitors antagonizing RAAS components seem to be efficient in regulating BP rates and reducing functional and structural abnormalities common in cardiovascular diseases. (for review see (Rajco *et al.*, 2007)) ARBs are used in hypertensive patients, displaying LV hypertrophy, as well as in those with nephropathy with type 2 diabetes. The group has seven representatives: candesartan, eprosartan, irbesartan, losartan, olmesartan, telmisartan, and valsartan. These drugs tend to be effective and well-tolerated. In patients with essential hypertension, ARBs are known to be as effective as ACEIs, however they do not produce bradykinin-mediated cough, a common side effect in patients treated with ACEIs. Due to their equivalence with ACEIs in the reduction of cardiac outcome, ARBs can be used in cases when ACEIs are contraindicated. (Mancia *et al.*, 2007)(Graham *et al.*, 2007)(for review see (Igic and Behnia, 2007)) In addition, due to the development of tolerance ARBs can confer more beneficial effects than ACEIs in patients with a greater degree RAAS stimulation. (for review see (Ravandi and Teo, 2009)) The cardio- and vasoprotective effects of ARBs are not mediated only via their blood-pressure lowering- mediating properties. By blocking the effects of AT_1 receptors, ARBs highly selectively reduce effects mediated by AT_1 which are involved in cardiovascular disease. This mode of action prevents feedback inhibition of renin release leading to RAAS overstimulation, which would result in elevated Ang II levels. The ability of these drugs to block AT_2 receptor activation, may possibly be another source of cardioprotective effects.

Numerous clinical trials have proved the potency of ARBs in reducing blood pressure, promoting HF survival as well as improving left ventricular dysfunction, and inhibiting microalbuminuria. In diabetic patients these drugs can inhibit the appearance of diabetic nephropathy, and they offer protection against MI, atrial fibrillation, atherosclerosis, stroke and kidney damage. (for review see (Igic and Behnia, 2007)(Fitchett, 2007)(Siragy, 2010))

Since ACEIs, ARBs, and aldosterone antagonists can all increase potassium levels, this may represent a dangerous combination if used together. (for review see (Chavey, 2008)) As the ONTARGET study has demonstrated no greater benefits were obtained after combining ACE's with ARB's, however

worsened renal outcomes have been recorded. (for review see (Ravandi and Teo, 2009)(Perret-Guillaume *et al.*, 2009))

2.3.2.3 Aldosterone antagonists

Aldosterone antagonists are known to produce a reduction in remodeling of the vascular smooth muscle cells and myocytes and thus an improvement of endothelial cell dysfunction in heart failure. These changes slow down the progression of LV dysfunction and end-organ damage**.** In addition aldosterone receptor blockade improves heart rate variability in heart failure in humans due to restoration of the baroreceptor reflex. In mammals, aldosterone is the principal regulator of sodium and potassium homeostasis and hence is central to the control of extracellular fluid volume and BP. These effects improve vascular compliance and slow down the progression of left ventricular dysfunction and end-organ damage. Aldosterone receptor blockade also restores the baroreceptor reflex, improving heart rate variability in heart failure in humans. (for review see (Ovaert *et al.*, 2010))

Aldosterone antagonists block the effects of aldosterone. They are often combined with ACEIs in patients with failing hearts. (for review see (Chavey, 2008)) As revealed in the RALE study, in addition to standard therapy, spironolactone by blocking aldosterone receptors significantly reduces the risk of progressive HF and sudden death from cardiac causes, contributing to decrease mortality rates. (Pitt *et al.*, 1999)(Kulbertus, 1999)

Aldosterone antagonists reverse aldosterone-mediated actions in kidney and heart, as well as in the vasculature such as sodium and water retention and potassium excretion**.** Aldosterone blockade produces potassium-sparing, causing hyperkalaemia, especially when combined with ACEIs, ARBs, and exogenous potassium (for review see (Chavey, 2008)) These actions are associated with adverse effects, such as impaired synthesis of the vasodilator NO; promotion of vasoconstriction, endothelial dysfunction, inflammation, and fibrosis in the vasculature; and ventricular hypertrophy, collagen deposition, fibrosis, and remodeling in the heart. (for review see (Struthers, 2004)) Spironolactone and eplerenone may induce hyperkalaemia, worsening renal function, and possible breast tenderness or enlargement. (Dickstein *et al.*, 2008)(Mancia *et al.*, 2007)(Graham *et al.*, 2007) (Dickstein *et al.*, 2008)

2.3.2.4 β-Blockers

Antagonism of β-receptors is known to cause anti-hypertensive effects due to exertion of their negative chronotropic and inotropic effects, resulting in reduced cardiac output. In addition β-

blockers reduce renin release in kidney and reduce sympathetic activity SNS. (for review see (Goldsmith, 2004)) β-blockade is recommended in patients with HF caused by systolic dysfunction, except in those who are dyspneic at rest with signs of congestion or hemodynamic instability, or in those who cannot tolerate β-blockers. It has been recommended that β-blockers, such as carvedilol, metoprolol, bisoprolol should be added when patients are stable to diminish the progression of the disease. The β-blockade therapy is not recommended as a rescue therapy in decompensating patients. (Mancia *et al.*, 2007)(Graham *et al.*, 2007)(Dickstein *et al.*, 2008) Similarly to ACEIs, β-blockers improve ventricular function in patients with symptomatic HF with LVEF≤40 %. β-blockers are prescribed to the patients with mild to severe symptoms with asymptomatic left-ventricular systolic dysfunction after MI. With the special caution, they may also be administered to recently decompensated patients. . (Mancia *et al.*, 2007)(Graham *et al.*, 2007)(Dickstein *et al.*, 2008)

Indications for β-blockers are patients who have had a previous MI, acute coronary syndromes, congestive HF, ventricular arrhythmias, supraventricular tachyarrhythmias, diabetes mellitus, after coronary artery bypass graft surgery etc. Possible adverse effects consist of hypotension, worsening of HF and bradycardia. Adjustment of the dose or increasing the dose of the diuretics or discontinuation may be required. (Dickstein *et al.*, 2008)

Although the data related to β-blockers therapy in primary hypertension is controversial, the European Society of Hypertension guidelines state that diuretics, β-blockers, ACEIs, ARBs and calcium channel blockers do not significantly differ in their ability to lower BP and to exert cardiovascular protection in both elderly and younger patients. Nowadays novel β-blockers, carvedilol and nebivolol are believed to be effective at reducing cardiovascular events but nonetheless further clinical investigations are still required. (for review see (Aronow, 2010))

2.3.2.5 Diuretics

Diuretics are classified as a group of drugs used in moderate to severe HF with clinical signs of congestion. (Dickstein *et al.*, 2008) They are indicated for volume overload due to sodium and water retention. (for review see (Jhund *et al.*, 2000)) Diuretics reduce blood volume, which in turn depletes the effective circulating volume and reduces cardiac output. (for review see (Francis, 2001))

Diuretics however produce an ion imbalance such as potassium and magnesium wasting, therefore monitoring of electrolytes is very important. This group also elevates RAAS activity, hence must be combined either with ARBs or ACEIs. Most patients receive loop diuretics such as furosemide to obtain benecitial effects such as dilation of peripheral vessels, followed by a decrease in LV filling

pressure, reduced cardiac output and relieve symptoms of pulmonary congestion. (for review see (Jhund *et al.*, 2000) (Somberg and Molnar, 2009))

Loop diuretics inhibit sodium reabsorption in the ascending tubule of the nephron, followed by water retention. Loop diuretics inhibit the $Na^+/2Cl^-)/K^+$ cotransporter of the thick ascending loop of Henle, resulting in decreased sodium and chloride reabsorption from the urine and subsequent diuresis. Loop diuretics also induce the synthesis of prostaglandins, resulting in renal and peripheral vascular smooth muscle relaxation and venodilatation. (for review see (Jentzet *et al.*, 2010)) Loop diuretics serve as a baseline therapy for HF and often being combined with ACE inhibitors, β-blockers, spironolactone, and digoxin, however they are associated with acute and chronic distal tubular compensation i.e. renal hypertrophy. Their mechanism of action is due to dilation of peripheral vessels, which produces a decrease in LV filling pressure. (for review see (Jhund *et al.*, 2000)) Combining a loop diuretic with a thiazide diuretic increases potency by minimizing distal tubular compensation. (Mancia *et al.*, 2007)(Graham *et al.*, 2007)(Dickstein *et al.*, 2008) (for review see (Chavey, 2008)) Thiazide diuretics inhibit sodium-chloride symporters at the distal portion of the ascending limb and in the early part of the distal tubule, thereby increasing the excretion of Na^+, Cl^-, and water across the renal tubular epithelium. Although they are less potent than loop diuretics, they have synergistic effects with loop diuretics via sequential segmental nephron blockade. Potassium-sparing diuretics in turn produces increases the secretion of water and Na^+, and decreases in the excretion of K^+, by competing for the aldosterone- sensitive Na^+/K^+ channel in the distal tubule of the nephron. Since sodium exchange in the distal tubule is low, potassium-sparing diuretics are generally relatively weak diuretics. (for review see (Felker, 2010))

2.3.2.6 Digoxin

Digoxin, a cardiac glycoside, is known to act through inhibition of the sarcolemmal Na^+-K^+ ATPase pump, which in turn leads to increased intracellular Na^+ that is then exchanged with Ca^{2+}. This intracellular Ca^{2+} produces the inotropic effect of the drug. The beneficial hemodynamic effects of digoxin are attained and providing absence of any hypotension or tachycardia and with associated favorable effects on neurohormones including a decrease in the sympathetic drive and reninangiotensin- aldosterone activation and an increase in vagal stimulation. Few studies have assessed the effects of digoxin in patients with acute HF. Digoxin use is commonly in patients with chronic HF (approximately 50–65% of patients overall, range 55–91%). (for review see (Teerlink, 2009)) Digoxin is also used in patients with atrial fibrillation and to correct sinus rhythm, however it does not reduce cardiovascular or all-cause mortality. (Dickstein *et al.*, 2008)(for review see (Thadani,

2008)) Digoxin alone is not always sufficient though it does seem to alleviate the symptoms and decrease hospitalization rates in symptomatic patients and is a supplemental drug in addition to diuretics, ACE inhibitors, or β-blockers. There are reports of increased mortality among women with HF under digoxin. Therefore monitoring the correct dose and effects of digoxin is essential. Discontinuation of digoxin should be done with caution due to the possible clinical deterioration. (Dickstein *et al.*, 2008)(for review see (Thadani, 2008)) It is important to stress that digoxin has no benefits in the conversion to sinus rhythm compared to placebo, although it has a rate-controlling effect (for review see (Khoo and Lip, 2009)) However, as long as providing toxicity is avoided, digoxin is remarkably free of side effects and improves quality of life in patients with atrial fibrillation. (for review see (Cleland and Cullington, 2009))

2.3.2.7 Hydralazine and isosorbide dinitrate (H-ISDN)

H-ISDN comprises of hydralasine, which is vascular smooth muscle relaxant and a vasodilator isosorbide dinitrite. This combination lowers BP, reduces cardiac filling pressures, increases cardiac output, reduces pulmonary vascular pressures, and improves in symptoms and exercise tolerance in patients with heart failure. Vasoconstriction and NO deficiency in the vasculature play a role in aggravating the symptoms of HF, especially in patients of African-American origin. (for review see (Thadani, 2008)) These patients are known to display lower bioavailability of NO and less RAAS activity than whites. (for review see (Vivian, 2006)) Therefore direct acting vasodilators such as H-ISDN are used more frequently in blacks, when intolerance for ARB or ACEIs occurs in the treatment of HF. Their usage can decrease the number of hospitalized patients due to worsening of HF, they improve ventricular function and exercise capacity, and they may serve as additional therapy for patients receiving ACEI, ARB, or β-blocker, reducing the risk of death. (Dickstein *et al.*, 2008) Possible adverse effects consist of symptomatic hypotension, which may disappear with time and therefore it may not require intervention, but there may be also muscle and joint pain, rash or fever. (Dickstein *et al.*, 2008) Treatment with high doses of isosorbide dinitrate (ISDN) has been shown to increase symptom-free walking time, but tolerance to the hemodynamic effects of ISDN develops rapidly. Experimental data suggests that hydralazine, given concomitantly, attenuates the development of hemodynamic tolerance to ISDN and may increase the bioavailability of NO in the vasculature. (for review see (Thadani, 2008))(Dickstein *et al.*, 2008)

2.3.3. Levosimendan

Levosimendan (((-)-0-cyano-N-{4-[(4R)-4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl]phenyl}methanecarbohydrazonoyl cyanide)) a novel inotropic compound used in hospitalized patients as intravenous infusions for the treatment of acute decompensated HF; however its long-term effects are still largely unknown. Even though calcium sensitizers are not included in either European Society of Cardiology or American Heart Association guidelines as therapy for HF, these drugs may constitute an important part of the treatment of patients with failing hearts due to their peculiar ability to increase cardiac output without causing severe hypotension when compared to phosphodiesterase inhibitors (PDE III) such as milrinone. (for review see (Triposkiadis *et al.*, 2009)(Pollesello and Papp, 2007)) Inotropic agents, such as dobutamine and milrinone, that work through the β-receptor-cAMP-protein kinase A pathway, increase cardiac contractility at the expense of increased intracellular concentrations of calcium and cAMP. They increase myocardial oxygen demand and also induce myocardial ischemia or malignant ventricular tachyarrhythmias. Therefore they may cause to increased HR, hypotension, arrhythmias, and mortality. These adverse effects are inextricably linked to their inotropic mechanism of action. (for review see (Teerlink, 2009)(Fotbolcu and Duman, 2010))

Calcium sensitizers are a novel pharmacological group possessing inotropic properties. (for review see Fotbolcu and Duman, 2010) Levosimendan has been reported to increase myocardial contractility, improves hemodynamics and it also dilates peripheral and coronary vessels. (for review see (Antila *et al.*, 2007)) Its mechanism of action is primarily due to binding in a calcium concentration dependent manner to the N-terminal domain of cardiac troponin C (TnC). This in turn facilitates inhibition of the effects of troponin I (TnI) effects and thus it can prolong the actin-myosin cross-bridging action. This positive inotropic effect is obtained without increasing the intracellular calcium concentration or significantly increased myocardial oxygen demand, seen with other inotropic agents. (for review see Fotbolcu and Duman, 2010)(for review see (*et al.*, 2009)) In addition, levosimendan opens ATP-sensitive potassium channels (K^+_{ATP}) in vascular smooth muscle cells contributing thereby to vasodilatation, decreasing preload and afterload, improving coronary and systemic blood flow. Levosimendan opens also mitochondrial K^+_{ATP} channels, which are believed to mediate cardioprotective mechanism linked to the preconditioning in response to oxidative stress. (for review see (Fotbolcu and Duman, 2010)) Recently, levosimendan administered as a coronary bolus has been reported to counteract programmed cell death, specificly autophagy in anesthetized pigs with regional myocardial ischemia. (Grossini et al., 2010) The role of inhibition of PDE III still needs to be clarified. (for review see (Antila *et al.*, 2007))

Although levosimendan has a short half-life (about 1.5 hours) if given orally, its active metabolite OR-1896 has a longer half-life, approximately 80 hours. Due to the long half-life of the active metabolite, the effects last for up to 7 to 9 days after discontinuation of a 24-hour infusion of levosimendan. (for review see (Fotbolcu and Duman, 2010))

There are several clinical observations indicating that levosimendan can improve hemodynamics even in patients with cardiogenic shock and sepsis-induced myocardial and pulmonary dysfunction. Future large-scale multicenter clinical trials are needed to clarify whether levosimendan can improve the overall outcome of patients with sepsis and septic shock. (for review see (Fotbolcu and Duman, 2010))

Levosimendan evokes fewer adverse effects (mostly nausea, dizziness, headache and hypotension) and lower risk of arrhythmias compared with traditional inotropics or inodilators. (for review see (Fotbolcu and Duman, 2010)(Parissis, 2009))

Another calcium sensitizer, however with no vasodilation property, is pimobendan, approved only in Japan. It was found that pimobendan could reduce the Ca^{2+} level required for actin sliding in an *in vitro* motility assay using reconstituted thin filaments. Clinical data showed benefits on exercise capacity but also a trend toward increased mortality. (for review see (Anttila *et al.*, 2007))

2.4 Other potential therapies

2.4.1 Resveratrol and SIRT1-mimetics

Resveratrol (3,5,4'-trihydroxystilbene), a polyphenol obtained from grapes, and thus an ingredient of red wine, is synthesized by plants in response to attacks by fungi, bacteria, or other injurious substances. (for review see (Sadruddin and Arora, 2009)) Resveratrol mimicks the effects of calorie restriction by prolonging the lifespan and has been claimed to confer protection against cardiovascular disease. (for review see (Das, 2010)(Lippi *et al.*, 2010)) Light to moderate red wine consumption is said to protect from atherosclerotic processes, atherogenesis, vessel occlusion and oxidative stress as well as to inhibit lipoproteins oxidation, macrophage cholesterol accumulation, foam-cell formation and to increase levels of HDL cholesterol thus enhancing cholesterol efflux from vessel walls. As a free radical scavenger and anti-oxidant, resveratrol, is responsible mainly for these effects and is known to mediate also the increase in NO availability hence improving structure and the function of the endothelium, decreasing blood viscosity. Resveratrol is also known to possess anti-thrombotic, fibrinolytic activity and to improve insulin sensitivity. (Sadruddin and Arora, 2009)(for review see (Lippi *et al.*, 2010)) It provides cardioprotection in association with catalase

activity upregulation and by balancing redox homeostasis in mammalian systems by regulating several antioxidant enzymes including glutathione peroxidase, glutathione-S-transferase and glutathione reductase. (Ungvari *et al.*, 2009)(for review see (Das, 2010)) Resveratrol has been demonstrated to regulate process of apoptosis via Bax, p53 and TNF receptor associated factor. In addition, in earlier studies, resveratrol has been proposed to downregulate the expression of tumorigenic and pro-inflammatory nuclear factor NF-κB and it has displayed anti-inflammatory properties due to inhibition of cyclooxygenase-1 and -2 (COX1 and COX2) expression. (for review see (Das, 2010)) Moreover, resveratrol contributes to lifespan extension due to activation of the longevity gene SIRT1, (for review see (Sadruddin and Arora, 2009)) and by acting not only as an anti-aging compound, (for review see (Das, 2010)) but also by suppressing AT_1R expression via SIRT1 activation *in vivo* and *in vitro*. (Miyazaki, 2008) Lately however it has been claimed that regulation of enzymes and molecular pathways involved in lifespan extension and disease protection are not necessarily connected to SIRT1 activation. (for review see (Das, 2010)) The AMPK- resveratrol regulated pathway may be also involved in PGC-1α activity, increased mitochondrial number, and improved motor function in obese mice. (for review see (Baur, 2010)) Nonetheless the same PGC-1α activation can be induced via a SIRT1-related pathway, evoking mitochondrial biogenesis. (Lagouge *et al.*, 2006)(for review see Guarente, 2007)) In addition, several genes such as Sirt3, Sirt4, FoxO1, Foxo3a associated with cardioprotection are also induced by resveratrol. (for review see (Das, 2010)) Other positive SIRT1 regulators, SRT1720, SRT501, SRT2104 are currently under investigation to determine whether or not they possess or not resveratrol- like effects. (for review see (Baur, 2010)) Isonicotinamide can relieve inhibition of Sir2 activity by competing with nicotinamide for binding in the same pocket within the enzyme. Since isonicotinamide cannot participate in the reverse reaction, the intermediate is stabilized and more likely to complete the deacetylation step. Isonicotinamide is therefore predicted to be an activator of sirtuins under normal physiological conditions. (for review see (Baur, 2010))

Nicotinamide, produced by sirtuin enzymes, is a potent inhibitor of their activity. (for review see (Baur, 2010)) Nicotinamide has been claimed to be as a direct negative regulator of cellular Sir2 function, an analog of SIRT1 in humans. (Jackson *et al.*, 2003) Sir2 deacetylation reaction generates two products: O-acetyl-ADP-ribose and nicotinamide, a precursor of nicotinic acid and a form of niacin/vitamin B3. Physiological concentrations of nicotinamide noncompetitively inhibit both Sir2 and SIRT1 by abolishing silencing heterochromatin and therefore shortening lifespan. The degree of inhibition by nicotinamide is equal to or better than the most effective known synthetic inhibitors of

this class of proteins. It has been reported that nicotinamide inhibits deacetylation by binding to a conserved pocket adjacent to NAD$^+$, thereby blocking NAD$^+$ hydrolysis. (Bitterman, 2002)

3. AIMS OF THE STUDY

Hypertension-induced left ventricular hypertrophy represents an adaptive and compensatory response to increased workload and represents an independent risk factor of cardiovascular events. Biomechanical stress and neurohumoral activation are the most important triggers of pathologic hypertrophy and the transition of cardiac hypertrophy to HF. The aim of this study was to investigate the molecular mechanisms and signalling pathways of hypertension-induced HF and, thus, to identify putative novel targets for drug development. In our experimental studies conducted in two different models of hypertension-induced heart failure special emphasis was placed on local renin-angiotensin system, cardiac metabolomics and calcium sensitizers.

The specific objectives of this thesis were the following:

I.   To investigate by using tissue-based metabolomics approach and a hypertensive rat model with increased local RAAS activity (dTGR) whether Ang II alters cardiac substrate utilization and cardiac metabolomic profile. The effects of AT_1 receptor antagonist treatment on cardiac metabolomic profile both in hypertensive and normotensive animals were also examined.

II.   To investigate whether resveratrol, a natural polyphenol derived from grapes, could prevent Ang II-induced cardiovascular damage. The possible mechanisms of action mediating the beneficial effects of oral resveratrol treatment were also examined.

III.   To examine whether oral levosimendan treatment could prevent Ang II-induced cardiovascular mortality and cardiac remodeling in dTGR. The influence of oral levosimendan treatment on cardiomyocyte hypertrophy, cardiomyocyte apoptotic signaling, inflammatory response, as well as myocardial expression of calcium-handling proteins was also examined.

IV.   To investigate whether oral treatment with levosimendan or valsartan can prevent cardiovascular mortality and hypertension-induced cardiac remodeling in Dahl/Rapp rats, a low-renin model of salt-sensitive hypertension and heart failure. The cardiovascular effects of the drug combination were also investigated.

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Experimental animals

The 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.

The double transgenic rats (dTGR) harboring human renin and human angiotensinogen genes were purchased from Biotechnology and Animal Breeding Division, Füllingsdorf, Switzerland, Sprague-Dawley (SD) rats- from Charles River, Dahl/Rapp SS rats- from Harlan. The animals had free access to chow; dTGR and SD to standard chow (SDS Special Diet Services), and Dahl/Rapp SS free access to high salt chow, which was produced by adding NaCl (8 %) (Honeywell Riedel-de-Haen) to commercial low salt diet (Na 0.3 %, K 0.8 %, Mg 0.2 %; Harlan). Wistar rats (E17) for *in vitro* study were purchased from Harlan.

4.1.2 Study design

The study protocols were designed as showed in tables 2-7.

Table 2. Design for *in vivo* study I.

Study I (*in vivo*)				
Experimental group	Rat strain	Treatment	Dose	Follow-up period (weeks)
dTGR	dTGR			4
dTGR+VAL	dTGR	Valsartan (in food)	30 mg/kg	4
SD	SD			4
SD+VAL	SD	Valsartan (in food)	30 mg/kg	4

Table 3. Design for *in vivo* study II.

Study II (*in vivo*)				
Experimental group	**Rat strain**	**Treatment**	**Dose**	**Follow-up period (weeks)**
dTGR	dTGR			4
dTGR+Resv	dTGR	Resveratrol (by gavage)	800 mg/kg	4
dTGR+NAM	dTGR	Nicotinamide (i.p.)	400 mg/kg	4
SD	SD			4

Table 4. Design for *in vitro* study II.

Study II (*in vitro*)				
Experimental group	**Cell line**	**Treatment**	**Concentration**	**Incubation time (hours)**
Control	Wistar rats (E17)			48
Ang II	Wistar rats (E17)	Angiotensin II	100 nM	48
Ang II + Los	Wistar rats (E17)	Angiotensin II Losartan	100 nM 10 µM	48
Ang II+PD 123319	Wistar rats (E17)	Angiotensin II PD123319	100 nM 10 µM	48

Table 5. Design for *in vivo* study III.

Study III- 4 week follow-up (*in vivo*)				
Experimental group	**Rat strain**	**Treatment**	**Dose**	**Follow-up period (weeks)**
dTGR	dTGR			4
dTGR+Levo	dTGR	Levosimendan (in drinking water)	1 mg/kg	4
SD	SD			4
SD+Levo	SD	Levosimendan (in drinking water)	1 mg/kg	4

Table 6. Design for *in vivo* survival study III.

Study III (*in vivo*)				
Experimental group	**Rat strain**	**Treatment**	**Dose**	**Follow-up period (weeks)**
dTGR	dTGR			∞
dTGR+Levo	dTGR	Levosimendan (in drinking water)	1 mg/kg	∞

Table 7. Design for *in vivo* study IV.

Study IV (*in vivo*)					
Experimental group	Rat strain	Content of salt in a diet	Treatment	Dose	Follow-up period (weeks)
HS	Dahl/Rapp SS	NaCl=8 %			8
HS+Levo	Dahl/Rapp SS	NaCl=8 %	Levosimendan (in drinking water)	1 mg/kg	8
HS+Vals	Dahl/Rapp SS	NaCl=8 %	Valsartan (in food)	30 mg/kg	8
HS+Levo+Vals	Dahl/Rapp SS	NaCl=8 %	Levosimendan (in drinking water) Valsartan (in food)	1 mg/kg 30 mg/kg	8
LS	Dahl/Rapp SS	NaCl=0.3 %			8

4.2 Medicines and compounds used in experiments

4.2.1 *In vivo* studies

4.2.1.1 Valsartan

Valsartan ((S)-3-methyl-2-[N-({4-[2-(2H-1,2,3,4-tetrazol-5-yl)phenyl]phenyl}methyl)pentanamido]butanoic acid) (a kind gift from Orion Pharma) was administered at the dose of 30 mg/kg either by mixing with food (study I and IV), or by gavage after diluting in hydroxymethylcellulose (study III). Fresh solutions were prepared daily.

4.2.1.2 Resveratrol

Resveratrol (trans-3,5,4 ' -trihydroxystilbene, Orchid Chemicals & Pharmaceuticals Ltd.) was given as dissolved in hydroxymethylcellulose, at the dose of 800 mg/kg by gavage (Study II). Fresh solutions were prepared daily.

4.2.1.3 Nicotinamide

Nicotinamide (NAM, 3-pyridinecarboxamide, Sigma-Aldrich, Germany; 400 mg/kg i.p.) was diluted in physiological saline (0,9 % NaCl) administered intraperitoneally (i.p.) at a dose of 400 mg/kg. Fresh solutions were prepared daily.

4.2.1.4 Levosimendan

Levosimendan ((-)-0-cyano-N-{4-[(4R)-4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl]phenyl}methanecarbohydrazonoyl cyanide) (a kind gift from Orion Pharma) was given orally via drinking fluid to produce an approximate daily dosage of 1 mg/kg as in our previous rat experiments. (Louhelainen *et al.*, 2007)(Louhelainen *et al.*, 2009) Fresh levosimendan solutions were prepared daily (Study III and IV). Due to the dipsogenic effect of Ang II, (Fregly *et al.*, 1992)(Fregly Rowland, 1995) the water intake in dTGR is approximately 3-fold higher compared to SD rats. We therefore adjusted the levosimendan concentration in drinking fluid at three-fold higher concentration for SD rats (levosimendan concentration in drinking fluid 3 mg/L for dTGR and 10 mg/L for SD rats).

4.2.2 *In vitro* studies

4.2.2.1 Angiotensin II

Angiotensin II (Sigma-Aldrich) was used as a 100 nM water solution. (Neuss, 1994)

4.2.2.2 Losartan

Losartan ((2-butyl-4-chloro-1-{[2'-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl}-1H-imidazol-5-yl)methanol) (Sigma-Aldrich), was used for cell incubation after dissolving in water at the concentration of 10 µM. (Gao *et al.*, 2009)

4.2.2.3 PD123319

PD123319 (S-(+)-1-[4-(Dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid di(trifluoroacetate) salt hydrate) (Naito *et al.*, 2010)(Rabey *et al.*, 2010) was used as 10 nM water solution of PD123319, as described previously (Okada, 2010) to treat neonatal rat cardiomyocytes.

4.3 Methods

4.3.1 Blood pressure and heart rate measurement

SBP and HR were measured using a tail-cuff blood pressure analyzer (Apollo-2AB Blood Pressure Analyzer, model 179-2AB, IITC Life Science). The measurements were taken in triplicates.

4.3.2 Sample preparation

Urine samples were collected over 24-hour period in metabolic cages for albumin measurement. Urine volumes and water intakes were measured gravimetrically. Rats were anesthetized with CO_2/O_2 (95 %/5 % AGA) and decapitated. Blood samples were collected for using ethylenediaminetetra-acetic acid (EDTA) as an anticoagulant. The hearts and kidneys were excised, washed with ice-cold saline, blotted dry, weighed, and snap-frozen in liquid nitrogen or isopentane (-35 °C). All of the samples were stored at -80 °C until assayed.

4.3.3 Histology and cardiomyocyte cross-sectional area

The heart and kidneys were fixed in 10 % buffered formalin solution for histological analysis. Transversal 5-μm-thick sections of the paraffin-embedded left ventricle were cut and stained with Masson's trichrome or picrosirius red. The cross-sectional area was evaluated in a blinded fashion and was analyzed using the ISIimaging software (Image Solutions, Inc) as described earlier. (Vahtola *et al.*, 2008)

4.3.4 Myocardial and kidney morphology

Tissue morphology was evaluated from hematoxylin eosin (H&E) stained cardiac and renal sections in a blinded fashion. The severity of observed lesions was graded with numerical values denoting to the degree of damage at the whole tissue level. The following system of severity grading according proliferative, degenerative, necrotic and inflammatory changes was used: (0, no abnormalities detected) 1, minimal; 2, mild; 3, moderate; 4, marked; or 5, severe. (Herbert *et al.*, 2002)

4.3.5 Echocardiography

Transthoracic echocardiography (Toshiba Ultrasound) was performed on all rats under isoflurane anaesthesia (AGA) in a blinded fashion by the same technician during the last study week as described previously. (Vahtola *et al.*, 2008) Parameters needed for the calculation of cardiac function and cardiac dimensions were measured from three systole-diastole cycles. A short-axis view of the left ventricle at the level of the papillary muscles was obtained by a two-dimensional imaging

method (Gibson method), using a 15-MHz linear transducer. Two-dimensionally guided M-mode recording through the anterior and posterior walls of the left ventricle was used to measure the left ventricle (LV) end-systolic (LVESD), and end-diastolic (LVEDD) dimensions. In addition, interventricular septum (IVS) and posterior wall (PW) thickness were measured. LV fractional shortening (FS) and ejection fraction (EF) were calculated from the M-mode LV dimensions using the following equations:

$$FS\ (\%) = \{(LVEDD\text{-}LVESD) / LVEDD\} \times 100$$

$$EF = SV/EDV$$

$$SV = EDV - ESV$$

$$EDV = 0.52 \times (0.98 \times (LVEDD/10) + 5.90) \times (LVEDD/10)2$$

$$ESV = 0.52 \times (1.14 \times (LVEDS/10) + 4.18) \times (LVEDS/10)2$$

LVEDD = Diameter of the short-axis left ventricle in end diastole. LVEDS = Diameter of the short-axis left ventricle in end systole. Diastolic dysfunction was assessed by measuring the isovolumic relaxation time (IVRT) using color Doppler imaging. IVRT was measured as the interval between the aortic closure click and the start of mitral flow.

Diastolic dysfunction was assessed by measuring the isovolumic relaxation time (IVRT) using color Doppler imaging. IVRT was measured as the interval between the aortic closure click and the start of mitral flow.

4.3.6 Western blotting

Cardiac samples from the left ventricle were electrophoretically separated by 8 % SDS-PAGE (20 µg total protein of the whole cell lysate per lane). Each lane corresponded to one rat and all 4 groups were run on one gel. Proteins were transferred to a PVDF membrane (Immobilon-P®, Millipore) and blocked in 5 % non-fat milk-TBS-0.01 % Tween-20® buffer. The membranes were probed with the following primary antibodies; anti-Sir2alpha, 1/1000 (Upstate); anti-p53, 1/1000 (Chemicon); anti-acetyl-p53 firma, 1/1000 (K373/K382) (Upstate), anti-Serca2a, 1/1000 (Abcam); anti-NCX, 1/5000 (Immunodiagnostics); anti-LC3B, 1/500 (Cell Signalling Technology); anti-Bip, 1/1000 (Cell Signalling Technology). Tubulin was used as the loading control (Antialpha tubulin, 1/2000, 1/3000; Abcam). Horseradish peroxidase-conjugated anti-rabbit secondary antibody (Chemicon) was subjected to enhanced chemiluminescence solution (ECLplus, Amersham Biosciences). We quantified the relative

protein expression in separate samples from the membranes with Fluorescent Image Analyzer (FUJIFILM Corp). The measurements were repeated three times, and the data were presented as means ± standard error of the mean (SEM) of these experiments.

4.3.7 Quantitative real-time RT-PCR

Total cardiac mRNA was collected with Trizol® (Gibco, Invitrogen). 1 µg of isolated RNA was treated with DNAse1 (Deoxyribonuclease 1, Sigma Chemicals Co.) and reverse transcribed to cDNA by RT enzyme (reverse transcriptase-polymerase chain reaction)(Enhanced avian Hot Start RT-PCR kit (Sigma Chemicals Co or Im-Prom-II RT system, Promega). One µg of cDNA was subjected to quantitative real time PCR using the Lightcycler ® instrument (Roche Diagnostics) for detection of ANP, SIRT1, monocyte chamoattractant protein 1 (MCP-1), PGC-1α, NRF-1, Tfam, cox4, Bax, α-MHC, β-MHC, p16^{INK4a}, p19^{ARF}, and ribosomal 18s. The samples were amplified using FastStart DNA Master SYBR Green 1 (Roche Diagnostics) according to the manufacturer's protocol. The quantities of the PCR products were quantified with an external standard curve amplified from purified PCR products.

Table 8. RT-PCR primer sequences.

	Forward Primer	Reverse Primer	Reference
18S	CATCCAAGGAAGGCAGCAG	TTTTCGTCACTACCTCCCCG	(Wellner *et al.,* 2005)
ANP	CCGATAGATTCTGCCCTCTTGAA	CCCGAAGCAGCTTGATCTTC	(Heyen *et al.,* 2002)
SIRT1	GCAGACGTGGTAATGT	ACACTCTCCCCAGTAG	
PGC-1α	GGTCCCCAGGCAGTAG	CTCCATCATCCCGCAG	Louhelainen *et al.,* 2010
NRF-1	GCTTGCGTCGTCTGGAT	GCACCGTGTCGCTCAT	Louhelainen *et al.,* 2010
Tfam	AGACCTCGGTCAGCATATAACA	GCGACGGATGAGATCACTT	Louhelainen *et al.,* 2010
Cox4	TGGGAGTGTTGTGAAGAGTGA	GCAGTGAAGCCGATGAAGAAC	
Bax	CGGCGAATTGGAGAGATG	GGTCCCGAAGTAGGAG	
α-MHC	CTGAAAACGGCAAGACGGT	ACTTATAGGGGTTGACGGTG	
β-MHC	GCCCGGCATGATTGCG	TGGCGTCCGTCTCATACT	
p16[INK4a]	AACACTTTCGGTCGTACCC	GTCCTCGCAGTTCGAATC	Bastide *et al.,* 2008
p19[ARF]	CAGGAGCATGGGTCGCAGGT	GGTCGGGAGAGGGTGGTGGG	Bastide *et al.,* 2008
MCP-1	GCAGGTCTCTGTCACGCTTCT	GGCTGAGACAGCACGTGGAT	

4.3.8 Gas chromatography coupled with time-of-flight mass spectrometry

GC×GC-TOF/MS was used to detect intermediary metabolites from cardiac tissue. The instrument used was a GC×GC-TOF mass spectrometer (Pegasus 4D, Leco) with an autosampler (6890N GC and Combi PAL, Agilent Technologies). The metabolites were identified using an in-house reference compound library, as well as by searching the reference mass spectral library. Mass spectra from the GC×GC-TOF/MS analysis were searched against the Palisade Complete Mass Spectral Library, 600K Edition (Palisade Mass Spectrometry). (Oresic, 2009)

4.3.9 Respiratory chain functions

The respiratory chain activities of complex I (reduced nicotinamide-adenine dinucleotide dehydrogenase), complex II (succinate dehydrogenase), and complex IV (cytochrome C oxidase) were quantitated by measuring the oxygen consumption polarographically with a Clark-type electrode (Instech).

4.3.10 Cardiomyocyte cells studies

Neonatal rat cardiomyocytes were isolated from hearts of the embryonic Wistar rats (E17). Rats were decapitated and hearts were quickly excised. Hearts were minced and enzymatically digested in a buffer containing 120 mM NaCl, 1 mM NaH_2PO_4 , 20 mM HEPES, 5.5 mM glucose, 5.4 mM KCl, 0.8 mM MgSO4, 1 mg/ml collagenase IV (Worthington Biochemical Corp) and 2.5 % trypsin (Sigma-Aldrich). Tissue was digested by incubating in a 37 °C water bath for 20 min and supernatant with cells was collected, centrifuged and re-suspended in fetal bovine serum (FBS). This cycle was repeated five times until all tissue was digested. After digestion, supernatant from digestion cycles was combined, centrifuged and resuspended in growth medium containing Dulbecco's modified Eagle's medium (DMEM), 5 % FBS and 10 % horse serum (all from Gibco). In order to remove non-myocyte cells, the culture was plated on a cell culture dish for 90 min in growth medium and non-adherent cells (mostly myocytes) were collected and plated on 0.2 % gelatin-coated 12-well plates for experiments. After 24 h, cells were washed and growth medium was replaced with a serum-free medium and incubated for another 24 h before treatments. The cell isolation method is the same as that described by Chlopčíková *et al*. (Chlopčíková *et al.*, 2001) with the exception of the addition of 1 mg/ml collagenase IV (Worthington) to the digestion buffer. Cells were treated with Ang II (100 nM, Sigma-Aldrich) alone and Ang II with angiotensin II type 1 receptor (AT_1R) antagonist losartan (10 µM, Sigma-Aldrich) or with AT_2R antagonist PD123319 (10µM, Sigma-Aldrich) for 48 h. After incubation, cells were collected with Laemmli sample buffer (Biorad) for analyzing SIRT1 protein level in cells.

4.3.11 Electron microscopy

Electron microscopy from samples taken from left ventricle. Fixation of mitochondria was performed by adding 1 % glutaraldehyde directly to the suspension. Samples were embedded, sectioned, and post-fixed with osmium tetroxide, dehydrated, and embedded in epoxy resin. Thin sections were stained with lead citrate and uranyl acetate. The sections were viewed in a Jeol 1200 transmission electron microscope at a magnification of ×10,000.

4.3.12 Biochemical determinations

Serum creatinine and electrolytes as well as liver enzymes were measured by routine laboratory techniques. Serum aldosterone (Coat-a-Count Aldosterone RIA kit, DPC Biermann) was determined by radioimmunoassay according to the manufacturers' instructions. Creatinine and electrolytes from plasma and urine as well as plasma lipids and liver enzymes were measured by routine techniques. Urinary albumin was measured by ELISA using rat albumin as the standard (Calltrend). Plasma samples were analyzed for levosimendan and OR-1896 by liquid chromatography-tandem mass spectrometry (LC-MS/MS). (Kivikko, 2003)(Lewis *et al.*, 2008)

4.3.13 Measurement of SIRT1 activity

SIRT1 Fluorimetric Drug Discovery Kit (AK-555, Enzo Life Sciences Int.) was used to measure SIRT1 activity. Human recombinant SIRT1 was incubated together with 25 µM Fluor de Lys-Sirt1, deacetylase substrate and 25 µM NAD^+ (37 °C), in the absence (Control) or presence of resveratrol (2, 10, 20, 40 and 100 µM) to produce deacetylated product. In the second step, treatment with Developer II/2 mM nicotinamide produced a fluorophore, which was detected on a fluorometric plate reader (Victor 2 Multilabel Counter, PerkinElmer, Ex. 355 nm, Em. 460 nm).

4.3.14 Statistical analysis

Data are presented as the mean ± SEM. Statistically significant differences in mean values were tested by analysis of variance (ANOVA) and the Tukey post hoc test or the Newman-Keul's post-hoc test for comparison of multiple groups. Two-tailed Student's t -test was used when comparing only two groups. The differences were considered significant when $p < 0.05$. For cardiac metabolomic profiles, R statistical software (http://www.r-project.org/) was used for data analyses and visualization. The concentrations were compared using the Wilcoxon rank-sum test, with P values <0.05 considered significant. In order to take into consideration the problem of multiple comparisons, false discovery rate was estimated as the maximum q value in the set of significant differences for the metabolomic data set. False discovery rates were computed using the R package q value. The significance of the group differences was evaluated by the P value for the fixed-effect parameter estimate of group differences. The Kaplan-Meier test was used for survival analysis. The Log Rank test was used to compare survival distributions. The Pearson correlation co-efficients were calculated to measure the correlation between two variables. Linear regression curves were calculated by the partial least squares method.

5. RESULTS

5.1 Survival (All studies)

The dTGR rats develop fulminant hypertension and severe target-organ damage, with an increased mortality rate at the age of 7-9 weeks. During the 4 week follow-up period at about 8 weeks of age, the dTGR rats exhibited total mortality of 55-75 %, significantly more than the mortality rate of normotensive SD rats (Figure 6a). During this time period, valsartan and resveratrol completely prevented the excess mortaliy in dTGR (Study I and II), whereas levosimendan partially prevented Ang II-induced cardiovascular mortality in dTGR (survival rate 73 %) (Figure 6a, 6b and 6c respectively). In a separate survival study, a lifelong levosimendan treatment, when initiated at the age of 4 weeks, increased mean survival time in dTGR by 58 % (Study III). Nicotinamide did not affect the mortality rate in hypertensive dTGR (Study II).

Cardiovascular mortality of untreated Dahl/Rapp SS rats was 30 % at postnatal weeks 13. All rats from valsartan, levosimendan, and the combination group as well as those from low-salt group survived the entire follow-up period (8 weeks) (Figure 6d). There was 1 death (7 %) in the levosimendan treated Dahl/Rapp rats consuming the high-salt diet.
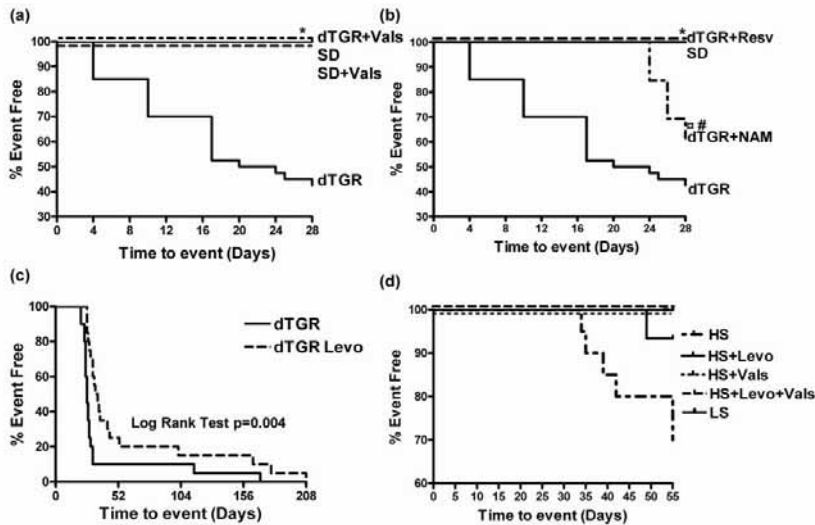
Figure 6. Survival curves of dTGR and the effects of (a) valsartan (30 mg/kg), (b) resveratrol (800 mg/kg) and nicotinamide (400 mg/kg), and (c) levosimendan (1 mg/kg) vs SD rats; and of (d) Dahl/Rapp SS rats on high salt diet (Dahl/Rapp HS) treated with levosimendan, valsartan and the combination of valsartan and levosimendan vs Dahl/Rapp rats (Dahl/Rapp LS). * denotes p<0.05 compared to dTGR or Dahl/Rapp rats on high salt diet, ¤ denotes p<0.05 compared to dTGR+resveratrol, # denotes p<0.05 compared to SD.

5.2 Blood pressure and heart rate (All studies)

Overexpressed RAAS contributes to the development of fulminant hypertension in dTGR rats when compared to normotensive SD rats; 200 mmHg vs 97 mmHg (p<0.05, Study I), 200 mmHg vs 115 mmHg (p<0.05, Study II), 205 mmHg vs 132 mmHg (p<0.05, Study III) (Figure 7a, 7b and 7c respectively). Both valsartan and resveratrol significantly decreased SBP (Study I and II). SBP in dTGR treated with nicotinamide remained at the level of dTGR i.e. at 215 mmHg (p<0.05, study II). Levosimendan did not produce any changes in SBP (Figure 7c), nor did it affect heart rate in dTGR (450 beats per minute (bpm)) (data not shown, Study III)).

High-salt diet evoked fulminant hypertension in Dahl/Rapp SS rats; 228 mmHg vs 158 mmHg (p<0.05, Study IV) (Figure 7d). Levosimendan alone did not decrease systolic blood pressure, whereas valsartan reduced it moderately but significantly. The drug combination significantly reduced the development of hypertension to the level found in LS Dahl rats. At week 13, the heart rate was not

affected by the treatments during the 7-week follow-up period and i.e. it was at 448 bpm in high salt Dahl/Rapp SS (data not shown).
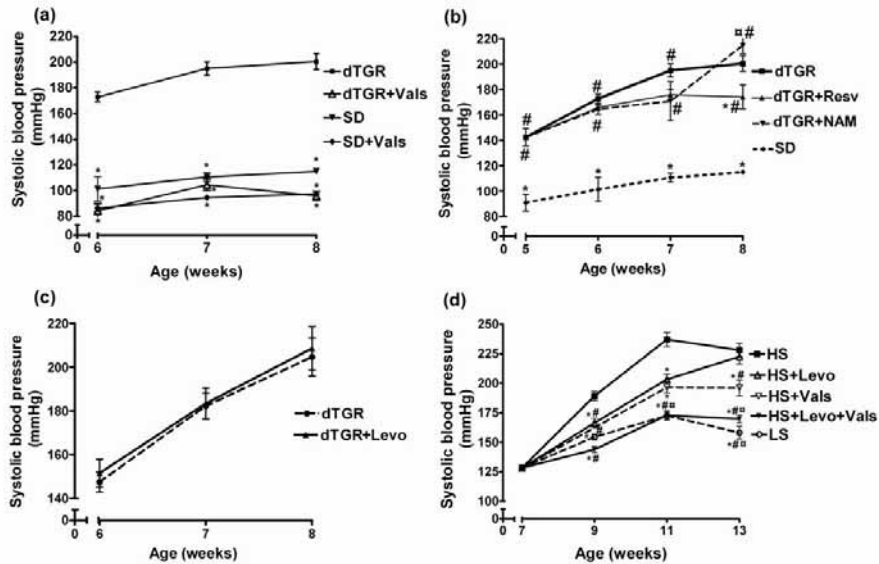

Figure 7. Systolic blood pressure (SBP) development in dTGR treated with (a) valsartan, (b) resveratrol and nicotinamide, (c) levosimendan vs SD rats; and in (d) Dahl/Rapp rats on a high salt diet treated with levosimendan, valsartan and the combination of levosimendan and valsartan vs LS Dahl/Rapp. * denotes $p<0.05$ compared to dTGR or Dahl/Rapp HS, # denotes $p<0.05$ compared to SD or high salt Dahl/Rapp rats + levosimendan, ¤ denotes $p<0.05$ compared to dTGR+resveratrol or HS+valsartan, §$P<0.05$ compared to HS+levosimendan+valsartan (See Figure 6 for doses).

5.3 Cardiac functions (Study III and IV)

RAAS induced deterioration of cardiac functions and LVH; however there was no difference in systolic function measured as ejection fraction or as fractional shortening between dTGR and SD rat strains at the age 8 weeks (Figure 8a and 8b). Levosimendan increased ejection fraction and fractional shortening, both in dTGR and SD rats (Study III). Color Doppler imaging revealed increased IVRT in dTGR as compared to SD rats, indicating impaired diastolic relaxation in dTGR. Levosimendan did not correct the Ang II-induced diastolic dysfunction in dTGR (IVRT 27.1±1.2 ms, $p>0.05$).

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Seven weeks of high salt intake induced a slight increase in systolic function as measured via EF and FS at post-natal week 13 in Dahl/Rapp rats (Figure 8c and 8d). While valsartan decreased EF and FS variables, treatment with levosimendan or the combination did not significantly increase cardiac function at systole (Study IV). Diastolic function assessed as isovolumic relaxation time (IVRT) was prolonged in the high-salt group indicating diastolic dysfunction (Figure 7 e). Both levosimendan and the combination of the drugs significantly shortened IVRT comparably to low-salt rats. Valsartan did not correct diastolic dysfunction i.e. early diastolic filling to late diastolic filling (E/A ratio) remained unchanged (Figure 8f).
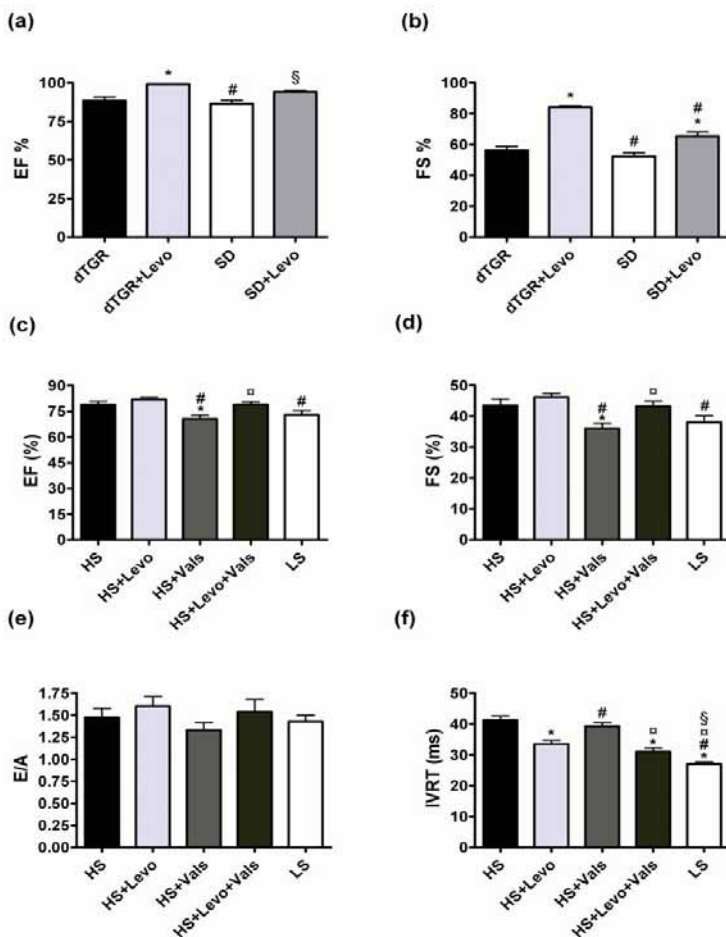
Figure 8. Systolic function assessed as (a) and (c) ejection fraction and (b) and (d) fractional shortening respectively in dTGR treated with levosimendan, vs SD rats, and in Dahl/Rapp rats treated with levosimendan, valsartan and duotherapy, vs LS Dahl/Rapp; diastolic function in Dahl/Rapp rats on high salt diet treated with levosimendan, valsartan and the combination of levosimendan and valsartan, assessed as (e) isovolumic relaxation time and (f) E/A waves compared to Dahl/Rapp LS. * denotes $p < 0.05$ compared to dTGR or Dahl/Rapp HS, # denotes $p < 0.05$ compared to dTGR+levosimendan or Dahl/Rapp HS + levosimendan, §$P < 0.05$ compared to SD or Dahl/Rapp HS + levosimendan+valsartan, ¤ denotes $p < 0.05$ compared to Dahl/Rapp HS +valsartan (See Figure 6 for doses).

5.4 Cardiac hypertrophy and myocyte cross-sectional area (All studies)

The dTGR rats demonstrated clearly elevated heart weight-to-body weight ratio compared with SD controls; 4.9 mg/g vs 3 mg/g ($p < 0.05$, Study I and Study II), 5.2 mg/g vs 3.4 mg/g ($p < 0.05$, Study III) (Figure 9a, 9b, 9c respectively). Treatment with valsartan and with resveratrol was able to significantly improve LVH (3.2 mg/g and 4.3 mg/g respectively), whereas neither levosimendan nor nicotinamide treatment influenced HW/BW ratio (5.0 mg/g and 4.7 mg/g respectively).

Unmedicated Dahl/Rapp rats consuming the high-salt diet displayed pronounced cardiac hypertrophy expressed via the heart weight-to-body weight ratio, when compared with low-salt Dahl/Rapp (4.4 mg/g vs 3 mg/g, $p < 0.05$, Study IV) (Figure 9d). The combination of levosimendan and valsartan prevented cardiac hypertrophy more effectively than the monotherapies (3.2 mg/g, vs. 3.8 mg/g or 3.7 mg/g, levosimendan and valsartan respectively).
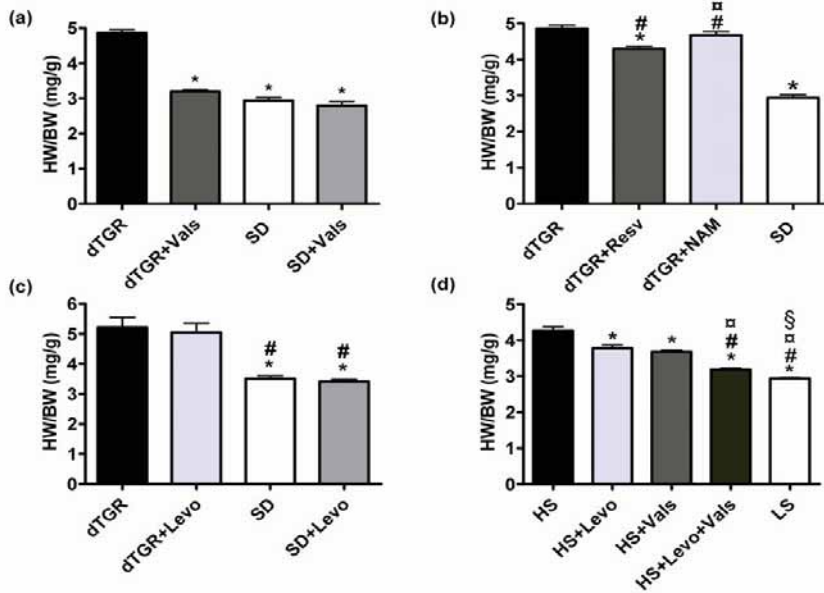
Figure 9. Cardiac hypertrophy estimated as heart weight-to-body weight ratio in dTGR treated with (a) valsartan, (b) resveratrol and nicotinamide, (c) levosimendan vs SD rats, and in (d) Dahl/Rapp HS treated with levosimendan, valsartan and combination of levosimendan and valsartan vs LS Dahl/Rapp. * denotes $p<0.05$ compared to dTGR or Dahl/Rapp HS, # denotes $p<0.05$ compared to SD, dTGR+levosimendan or Dahl/Rapp HS + levosimendan, §P<0.05 compared to HS+levosimendan+valsartan, ¤ denotes $p<0.05$ compared to dTGR+resveratrol or HS+valsartan (See Figure 6 for doses).

Cardiomyocyte cross-sectional areas were significantly enlarged in dTGR rats in comparison with SD rats; 308 μm^2 vs 193 μm^2 (Figure 10a, 10b, 10c) ($p<0.05$, Study I), 308 μm^2 vs 186 μm^2, ($p<0.05$, Study II), 251 μm^2 vs 217 μm^2 ($p<0.05$, Study III). Valsartan and resveratrol, but not levosimendan, prevented cardiac hypertrophy (225 μm^2, 270 μm^2, 261 μm^2 respectively), whereas nicotinamide evoked a significant enlargement (335 μm^2).

High-salt Dahl/Rapp SS rats showed markedly elevated cardiomyocyte cross-sectional areas, 237 μm^2 vs 181 μm^2 ($p<0.05$, Study IV) (Figure 10d), and in this animal model, levosimendan together with valsartan, significantly decreased the high-salt-induced increase in cardiomyocyte cross-sectional areas to the level of 181 μm^2, more efficiently than levosimendan (196 μm^2) or valsartan (197 μm^2).
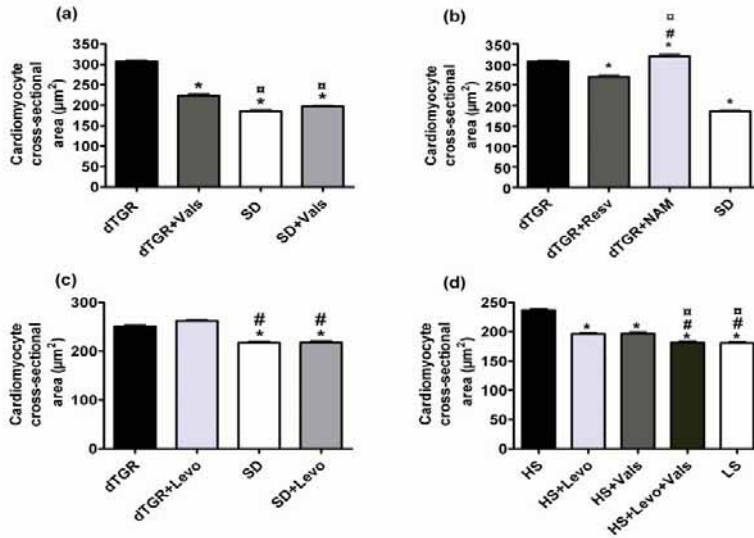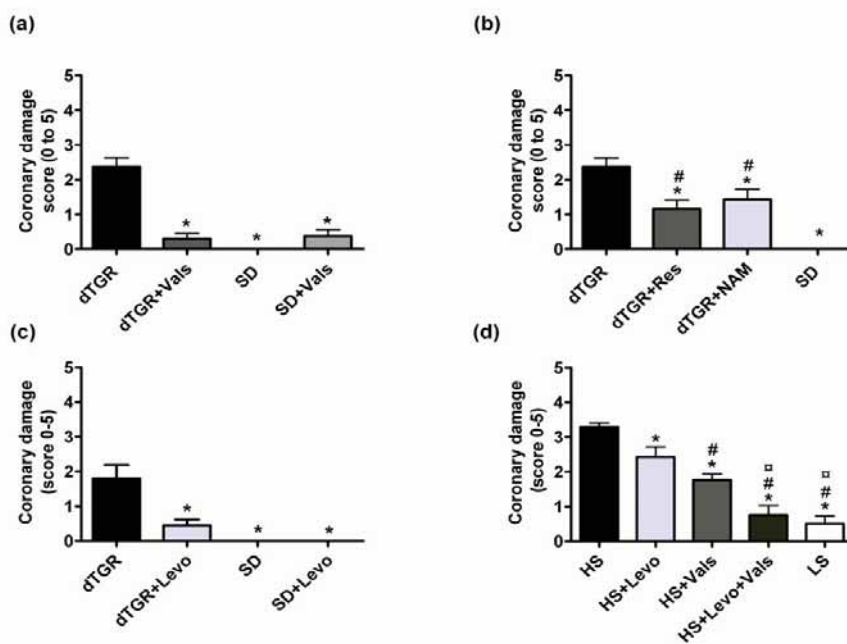
Figure 10. Cardiac hypertrophy estimated as cardiomyocyte cross-sectional area in dTGR treated with (a) valsartan, (b) resveratrol and nicotinamide, (c) levosimendan vs SD rats, and in (d) Dahl/Rapp HS treated with levosimendan, valsartan and the combination of levosimendan and valsartan vs LS Dahl/Rapp. * denotes p<0.05 compared to dTGR or Dahl/Rapp HS, # denotes p<0.05 compared to SD or dTGR+levosimendan or Dahl/Rapp HS + levosimendan, ¤ denotes p<0.05 compared to dTGR+valsartan or dTGR+resveratrol or HS+valsartan (See Figure 6 for doses).

5.5 Cardiac morphology (All studies)

The severity of cardiac damage was graded as either coronary artery damage or myocardial damage. In the dTGR heart samples, the lesions in the cardiac arteries varied from minimally thickened media and adventitia to moderate hyperplasia of intimal/medial layers and fibrosis of the adventitia with inflammatory cell infiltration. Lesions in the myocardial muscle were seen as accumulations of excess connective tissue and foci of inflammatory cells with elevated coronary damage and myocardial damage scores. Coronary and myocardial damage scores averaged 1.8 to 2.3 and 1.2 to 1.9 in the dTGR (Figure 11a, 11b, 11c). Valsartan markedly, whereas resveratrol and levosimendan partially prevented Ang II-induced coronary (0.3, 1.2, 0.4 respectively) and myocardial damage in dTGR (0.0, 0.2, 0.3 respectively)(Figure 12a, 12b, 12c)(Study I, II, III).

Dahl/Rapp on HS exhibited lesions in the coronary arteries ranged from minimally thickened media and a slight increase of connective tissue around the arteries up to severe hyperplasia of

intimal/medial layer and necrosis of the arterial wall with perivascular inflammatory cell infiltration. Lesions in the myocardium ranged from a focal increase of slender connective tissue bundles up to necrotic foci in the myocardium with inflammation. Coronary and myocardial damage scores averaged 3.2 and 2.0 in high salt Dahl/Rapp rats (Figure 11d and Figure 12d)(Study IV). Although levosimendan and valsartan provided some tissue protection at the histological level (coronary damage scores 2.4, 1.8 and myocardial damage scores 1.3, 0.5 respectively), the duotherapy seemed to be superior in its ability to completely prevent the cardiac damage (coronary and myocardial damage scores 0.8, 0.2). Furthermore, therapy did not reach the level of the tissue protection achieved with valsartan alone.

Figure 11. Coronary artery damage in dTGR treated with (a) valsartan, (b) resveratrol and nicotinamide, (c) levosimendan vs SD rats, and in (d) Dahl/Rapp HS treated with levosimendan, valsartan and combination of levosimendan and valsartan vs LS Dahl/Rapp rats. * denotes $p<0.05$ compared to dTGR or Dahl/Rapp HS, # denotes $p<0.05$ compared to SD or Dahl/Rapp HS + levosimendan, ¤ denotes $p<0.05$ compared to HS+valsartan (See Figure 6 for doses).
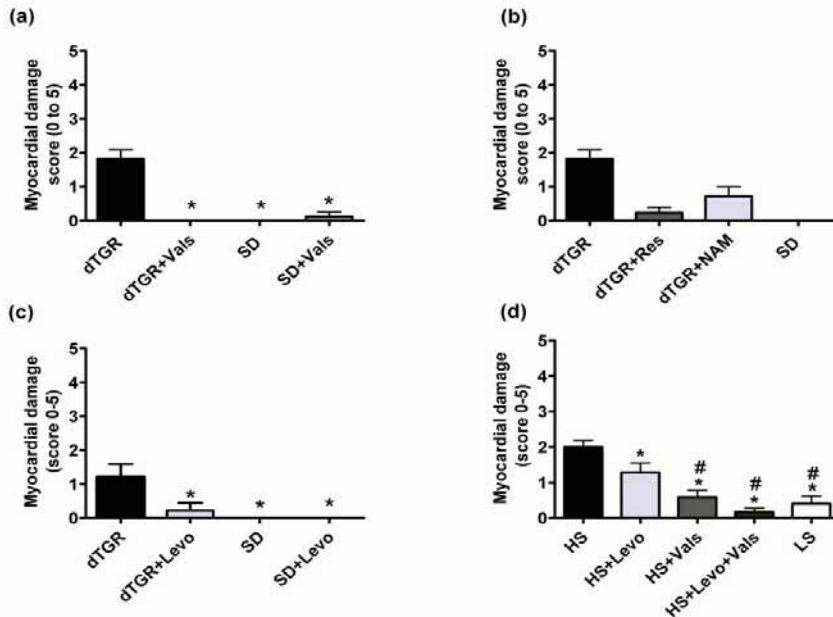
Figure 12. Myocardial damage in dTGR treated with (a) valsartan, (b) resveratrol and nicotinamide, (c) levosimendan vs SD rats, and in (d) Dahl/Rapp HS treated with levosimendan, valsartan and combination of levosimendan and valsartan vs LS Dahl/Rapp rats. * denotes p<0.05 compared to dTGR or Dahl/Rapp HS, # denotes p<0.05 compared Dahl/Rapp HS + levosimendan (See Figure 6 for doses).

5.6 Kidney morphology (Study III and IV)

The renal lesions of dTGR ranged from a slight thickening of the arteries and tubular dilatation with proteinaceous casts to diffuse arterial and glomerular necrosis with tubular atrophy/regeneration (2.5, 1.5, 1.9 kidney artery, glomerular and tubular damage scores respectively)(Study III). Levosimendan did not significantly influence kidney morphology (1.7, 1.4, 1.6 kidney artery, glomerular and tubular damage scores respectively, p<0.05).

In high salt Dahl/Rapp rats, the lesions were manifested as variable degrees of arterial and glomerular necrosis (4.1 and 4.0 damage scores)(Study IV), tubular atrophy and dilatation containing proteinaceous casts with diffuse inflammatory infiltrates affecting the parenchyma (3.7 damage score). The renoprotective effect of valsartan was greater than that of levosimendan (2.2 vs 2.8, 2.1 vs 3.0, 2.1 vs 3.0 kidney artery, glomerular and tubular damage scores respectively, p<0.05). An

additive renoprotective effect was found by the drug combination (1.2, 1.3, 1.4 kidney artery, glomerular and tubular damage scores respectively, p<0.05). Both cardiac and renal damage correlated very closely with systolic blood pressure.

5.7 Expression of hypertrophy- assossiated genes (All studies)

Myocardial ANP mRNA expression, a pressure overload marker in untreated dTGR was markedly increased as compared with SD controls (Figure 13a, 13b, 13c). Valsartan decreased ANP mRNA expression by 92 % (Study I), resveratrol by 66 % (Study II); however, the difference did not quite reach statistical significance. Levosimendan produced a 56 % decrease in dTGR when compared to hypertensive dTGR (p<0.05, Study III).

Cardiac ANP mRNA expression, a pressure overload marker, was increased 25-fold in untreated Dahl/Rapp rats on high-salt diet (Figure 13d). Levosimendan combined with valsartan produced a significant decrease in ANP mRNA level of approximately 81 % (p<0.05, Study IV) and this treatment was more effective than levosimendan or valsartan treatments separately (significant 50 % and 60 % decrease respectively).
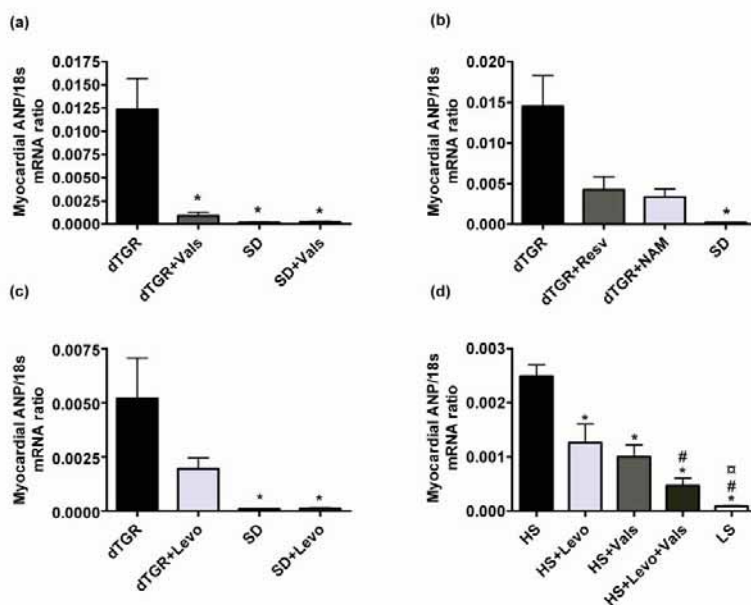

Figure 13. Expression of ANP mRNA in dTGR with (a) valsartan, (b) resveratrol and nicotinamide, (c) levosimendan vs SD rats, and in (d) Dahl/Rapp HS treated with levosiemendan, valsartan and combination of levosimendan and valsartan vs LS Dahl/Rapp rats. * denotes p<0.05 compared to

dTGR or Dahl/Rapp HS, # denotes p<0.05 compared to Dahl/Rapp HS + levosimendan, ¤ denotes p<0.05 compared to HS+valsartan (See Figure 6 for doses).

There was no difference between the treatment groups in cardiac α-MHC or β-MHC mRNA expression. Levosimendan produced a non-significant 31 % decrease in β-MHC in dTGR, and a 51 % decrease in SD (data not shown).

5.8 Cardiac metabolic profile in AngII-induced hypertrophy (Study I)

The GC×GC-TOF/MS data set comprised of 247 intermediary metabolites. All the metabolites were detected by multivariate analysis (partial least squares discriminant analysis) out of which 112 were identified from myocardial samples that differed statistically significantly between the groups after correction for multiple comparisons (false discovery rate q <0.05); 64 cardiac metabolites differed significantly between dTGRs and SD rats (P<0.05), 32 metabolites differed between dTGR and dTGR+valsartan treatment (P<0.05), and 34 metabolites differed between SD and SD valsartan treatment (P<0.05). In general, the metabolomic profile of valsartan-treated dTGRs resembled SD rats more than untreated dTGRs. Nonetheless, the metabolomic profile of valsartan-treated SD rats also visibly differed from untreated SD controls as revealed by partial least-squares discriminant analysis. Several decreases in cardiac fatty acid concentrations in dTGRs were recorded such as lower myocardial octanoic acid, oleic acid, and linoleic acid levels (all parameters P<0.001). There were also markedly increased ketogenic amino acid tyrosine levels and decreased tryptophan levels in dTGRs; otherwise, the drug effect dominated the amino acid changes. The dTGRs demonstrated several changes in organic acid levels. Moreover, decreases in hippuric acid, malic acid, and γ-hydroxybutyric acid concentrations were recorded, whereas the level of β-hydroxybutyric acid in dTGRs was elevated. The higher cardiac hypoxanthine level in dTGRs was found to be assossiated with an increase in purine degradation. Interestingly, valsartan treatment decreased the cardiac hypoxanthine level by 70 %. Supplementary data is attached in the section ''ORIGINAL ARTICLES''. The metabolic profiling is depicted in Figure 14.
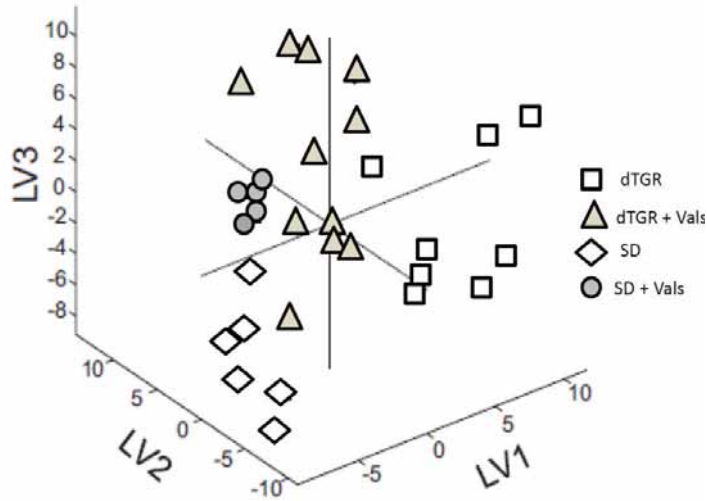
Figure 14. Graph presenting partial least-squares discriminant analysis including all 247 intermediate metabolites detected by GC×GC-TOF/MS to detect 247 intermediary metabolites. Each spot represents a single animal and the whole information of the 247 metabolites measured. Model properties 3 LVs, Q2=65%.

5.9 Expression of markers of mitochondrial biogenesis (Study I-III)

There was no difference between dTGR and SD rats in the mRNA expressions of mitochondrial biogenesis markers nuclear respiratory factor 1, transcription factor A mitochondrial, or PGC-1α. Valsartan increased mitochondrial biogenesis markers in SD rats, whereas the expression of mitochondrial biogenesis markers in valsartan-treated dTGRs was slightly but significantly lower compared with control dTGRs. Interestingly, resveratrol produced a significant increase in cardiac PGC-1α, Tfam and cox4 mRNA levels, NAM did not influence these factors, and levosimendan had no effect on dTGR or SD rats biogenesis marker PGC-1α (Figure 15a, 15b, 15c, 15d, 15e, 15f).
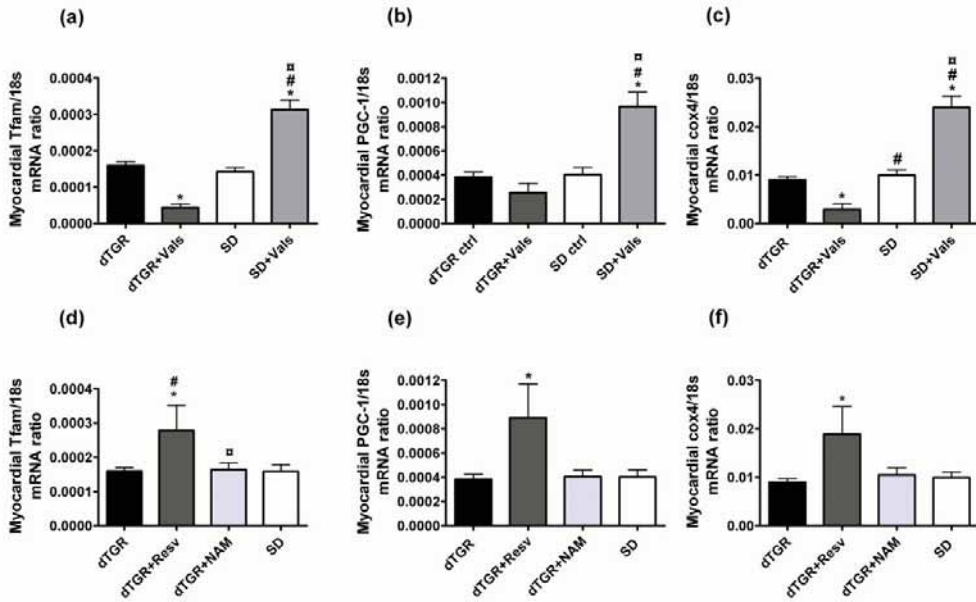
Figure 15. Expression of mitochondrial biogenesis markers Tfam (a,d), PGC-1α (b,e), cox4 (c,f) in dTGR treated with valsartan (a, b, c) and resveratrol (d, e, f) vs SD rats. * denotes p<0.05 compared to dTGR or Dahl/Rapp HS, # denotes p<0.05 compared to SD or dTGR+valsartan, ¤ denotes p<0.05 compared to dTGR+valsartan or dTGR+resveratrol (See Figure 6 for doses).

5.10 Expression of markers of mitochondrial respiratory chain (Study I)

The activity of cytochrome c oxidas was decreased in dTGRs than in SD rats. The AT$_1$-R antagonist, valsartan, partly reversed this change. The activities of other respiratory chain enzymes did not vary between groups as shown in Figure 16.
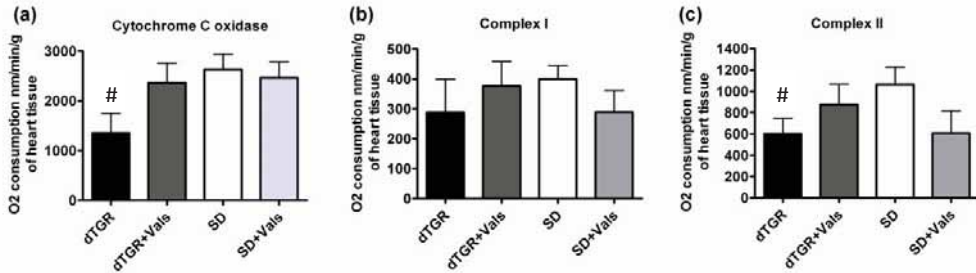
Figure 16. Bar graphs showing mitochondrial respiratory chain activities in dTGR treated with valsartan compared to SD rats. # denotes $p < 0.05$ vs SD rats (See Figure 6 for doses).

5.11 Ultrastructure of cardiac mitochondria

Changes in the cellular metabolic status influence the balance between fission and fusion leading to alterations in mitochondrial morphology. It was therefore decided to investigate the ultrastructure of cardiac mitochondria in dTGRs. Electron microscopy revealed that cardiac mitochondria from dTGRs, but not from control animals, frequently formed interconnected tubes (Figure 17a) but this was not observed in healthy rats (Figure 17b). This finding suggests that alterations in the cardiac metabolism of dTGRs promote mitochondrial fusion.
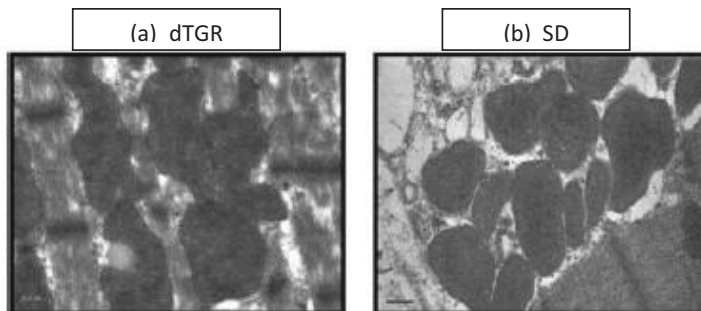

Figure 17. Representative photomicrograph from transmission electron microscopy from dTGR and SD rat heart.

5.13 Expression of inflammatory markers (Study III)

Cardiac MCP-1 expression was increased by 5-fold in dTGR when compared to SD rats (0.000065 vs 0.000012, $p<0.05$). Levosimendan did not affect MCP-1 mRNA overexpression.

5.14 SIRT1-related salvage pathways (Study I and II)

SIRT1 levels were associated with increased apoptosis, hypertrophy, and decreased cardiac function. Cardiac SIRT1 expression was moderately increased in untreated dTGRs compared with SD controls, with no differences in the cardiac SIRT1 mRNA expressions.

Cardiac SIRT1 protein expression was higher in untreated dTGR compared with SD controls, whereas there was no difference in cardiac SIRT1 mRNA expression indicating changes in post-transcriptional regulation. Resveratrol decreased SIRT1 protein expression by 42 % but increased cardiac SIRT1 mRNA expression. NAM decreased cardiac SIRT1 protein expression to the same extent as resveratrol, but did not influence cardiac SIRT1 mRNA expression (Figure 18c).

The effect of Ang II on SIRT1 protein level was studied using rat neonatal cardiomyocytes. There was no significant difference in SIRT1 protein level between Ang II and vehicle treated cells. Blockade of AT_1R by losartan or AT_2R by PD123319 did not influence SIRT1 protein level in rat neonatal cardiomyocytes (Figure 18d).

In our *in vitro* studies, resveratrol increased SIRT1 activity by 17-fold in a dose-dependent manner. NAM decreased SIRT1 activity by more than 70 % (data not shown).

A supplementary experiment with Dahl/Rapp SS rats on high and low-salt diet did not evoke any SIRT1 protein expression between the groups (Figure 18b).
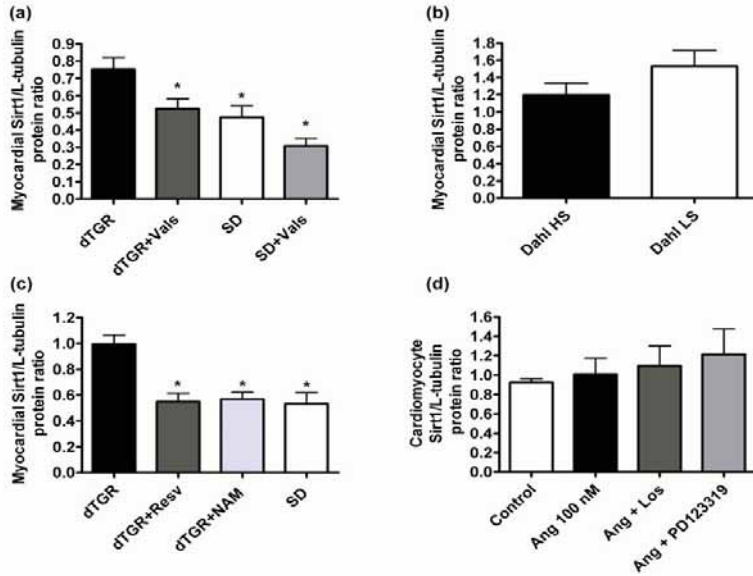
Figure 18. SIRT1 protein expression in dTGR treated with valsartan (a), Dahl/Rapp HS vs Dahl/Rapp LS (b), resveratrol and NAM (c) and in (d) neonatal cardiomyocytes. * denotes p<0.05 compared to dTGR rats (doses as in Figure 6).

5.15 Apoptotic pathways (study II and III)

Since p53 is a target of SIRT1, it was decided to determine whether or not p53 could be de-acetylated. There were no differences between dTGR and SD rats in protein expression of cardiac P53, acetylated-P53 or acetylated-P53-to-P53 ratio and treatment with resveratrol or NAM did not have any influence on these parameters (Figure 19a, 19b, 19c).
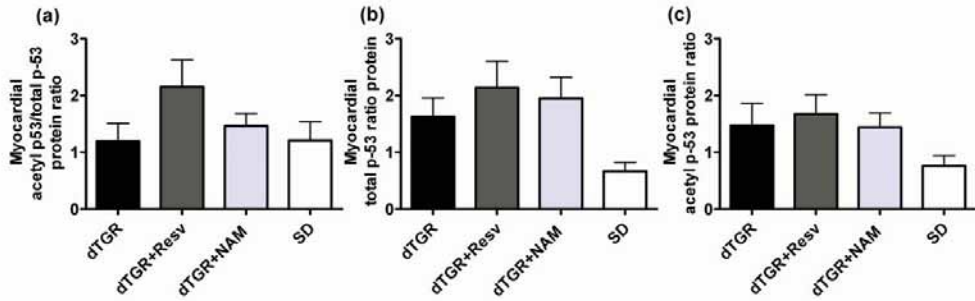
Figure 19. Cardiac protein p53 expression in dTGR treated with resveratrol (800 mg/kg/day for 4 weeks) compared to SD rats, (a) acetyl-p53, (b) total p53, (c) acetyl p53/total p53.

The mRNA expression of the pro-apoptotic factor Bax was slightly increased in myocardium of dTGR as compared to SD rats ($p < 0.05$) and levosimendan did not influence cardiac Bax mRNA expression in dTGR or SD rats (Figure 20).
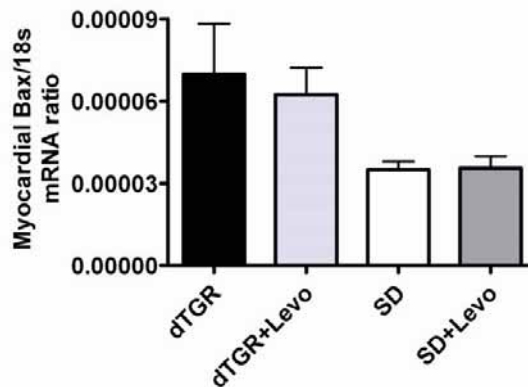

Figure 20. Expression of pro-apoptotic Bax mRNA in dTGR treated with levosimendan (1 mg/kg for 4 weeks) vs SD rats.

5.16 Premature cardiomyocyte senescence (Study II)

We observed no statistically significant differences in the markers of cellular senescence INK4A and ARF between unmedicated dTGR and SD controls, although ARF mRNA expression was 48 % greater in untreated dTGR than in SD rats.

5.17 Calcium handling proteins (Study III)

Impaired calcium handling is known to be associated with the development of cardiac dysfunction and it was possible to detect lower cardiac SERCA2 and NCX protein expressions in dTGR as compared to SD rats. Levosimendan did not influence SERCA2 or NCX expression in dTGR, but increased cardiac SERCA2A in SD rats (Figure 21a, 21b).
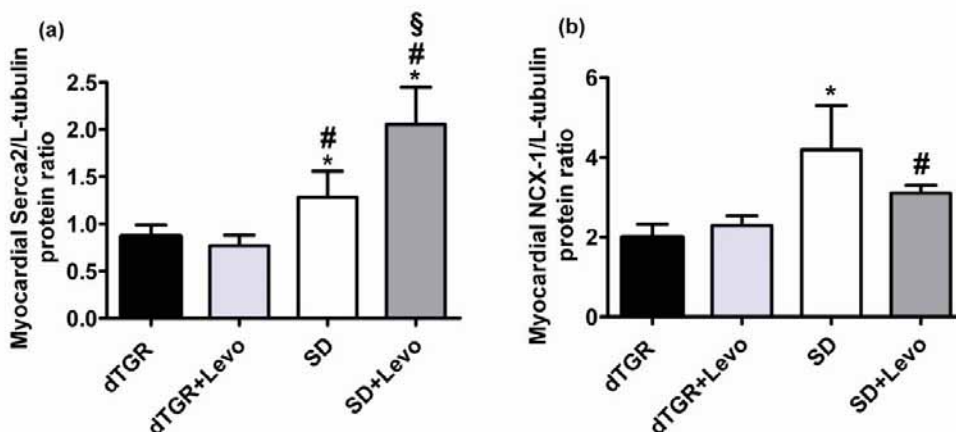

Figure 21. Bar graphs showing the effects of 4-week levosimendan treatment (1 mg/kg/day) on myocardial protein expression of (a) SERCA2a and (b) NCX-1. *P<0.05 compared to dTGR; #P<0.05 compared to dTGR+Levo; § P<0.05 compared to SD.

5.18 Cardiomyocyte autophagy and endoplasmic reticulum stress (Study IV)

A high salt diet did not induce cardiomyocyte autophagy measured as LC3B, 14 kDa/16 kDa ratio. Neither levosimendan nor valsartan treatment affected cardiomyocyte autophagy, whereas the drug combination tended to increase cardiac autophagy; however this increase was not statistically significant. Diet and drug regimens did not influence cardiac BiP protein expression.
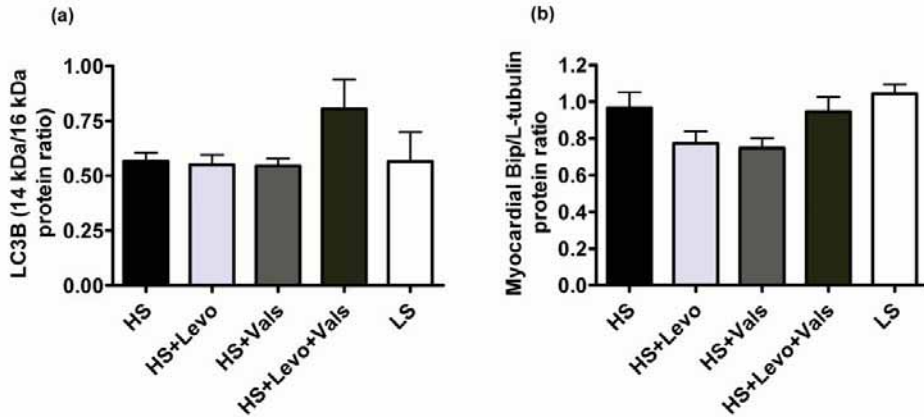
Figure 21. Bar graphs showing the effects of 8-week treatments with levosimendan, valsartan on myocardial protein expression (a) LC3B (14/16 kDa unit) protein expression; (b) BiP/L-tubulin in Dahl/Rapp rats on high salt diet.

5.19 Biochemical and hormonal analysis (Study II and III)

The albumin excretion was 40-fold increased in dTGR when compared to SD rats (8274 µg/24h vs 229 µg/24h respectively, p<0.05) and there was a seven-fold increase in serum aldosteron level in dTGR (1507 pg/ml vs 207 pg/ml, p<0.05). Resveratrol non-significantly decreased albuminuria by 25 % (6204 µg/24h) and was able to decrease the serum aldosterone level by 50 % (798 pg/ml, p<0.05)) compared with untreated dTGR. Nicotinamide did not influence either albumin excretion or aldosterone levels in dTGR (p>0.05). Levosimendan did not have any effect on albumin excretion in dTGR (p>0.05).

5.20 Serum electrolytes, creatinine and liver enzymes (Study III and IV)

The 24-h urinary excretion rates of potassium and phosphorus in dTGR were 2,313 mmol/24h, 0,1294 mmol/24h respectively) and levosimendan did not influence these parameters. Furthermore, there was no difference between dTGR nor SD rats (p>0.05, Study III). There was however a statistically significant decrease in sodium, chloride, calcium and creatinine clearance levels in dTGR when compared to SD rats (1,772 vs 0,9543 mmol/24h; 2,442 vs 1,560 mmol/24h; 0,1719 vs 0,01359 mmol/24h; 0,4524 vs 1,018 ml/min, respectively, p<0.05), however levosimendan did not evoke any changes in both animal strains (p>0.05, Study III).

HS did not produce any changes in serum levels of potassium, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), or alkaline phosphatase (AFOS) when compared to LS Dahl/Rapp rats(6,893 mmol/l; 52,97 μmol/l; 67,21 U/l; 283,2 U/l; 102,1 U/l respectively, p>0.05, Study IV). Serum sodium and chloride concentrations were significantly increased by consumption of a high salt diet (i.e. 149,8 mmol/l and 113,5 mmol/l respectively) when compared to LS dahl/Rapp rats (144,0 mmol/l and 102,3 mmol/l respectively). Levosimendan significantly decreased the concentration of sodium in serum to the level of LS dahl/Rapp rats. With respect to chloride levels valsartan, levosimendan and the combination therapy achieved comparable values to the level of LS Dahl/Rapp rats and were significantly lower when compared to HS Dahl/Rapp rats (p<0.05).

5.21 Plasma drug concentration (Study III and IV)

The daily dosage of levosimendan was calculated weekly and averaged 1.04 ± 0.03 mg/kg in dTGR (range 0.9-1.15) and 1.1 ± 0.05 mg/kg in SD rats (range 0.73-1.3). The average daily dose of levosimendan In HS Dahl/Rapp rats was approximately 0.69 ± 0.03mg/kg (range 0.31-0.72 mg/kg) whereas in the combination with valsartan 0.52 ± 0.03 mg/kg (range 0.3-0.57 mg/kg).

Terminal plasma levosimendan concentrations in dTGR were 5.5 ± 1.2 ng/ml. The level of OR-1896, the stable metabolite of levosimendan was 31.7 ± 5.6 ng/ml. In SD rats treated with levosimendan, plasma levosimendan and OR-1896 concentrations were approximately 7.6 ± 3.1 ng/ml and 43.4 ± 5.2 ng/ml respectively. The terminal plasma concentration of levosimendan in HS Dahl Rapp rats were found to be 16.65 ± 3.25 ng/ml, and the levosimendan and valsartan combination, 6.91 ± 1.42 ng/ml. The concentration of the stable metabolite of levosimendan- OR-1896 was 17.36 ± 1.73 ng/ml in HS Dahl Rapp rats, and 4.15 ± 0.38 ng/ml in HS Dahl Rapp rats when the drug combination was given.
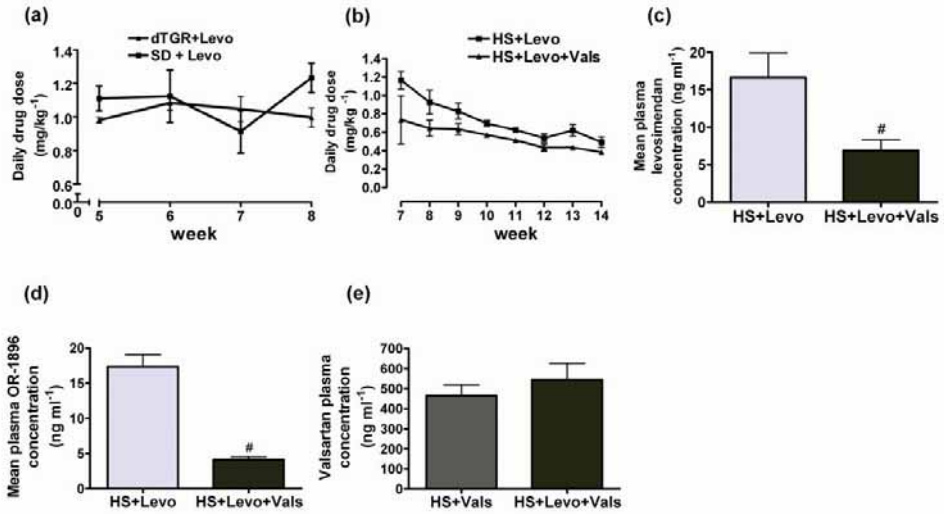
Figure 22. The average weekly levosimendan doses calculated from water consumption during the 4-week experimental period in (a) dTGR, (b) Dahl/Rapp HS, (c) mean plasma concentration of levosimendan and (d) OR-1896 in Dahl/Rapp HS, and valsartan plasma concentration in HS Dahl/Rapp rats. # denotes p<0.05 compared to HS+levosimendan.

6. DISCUSSION

The mechanisms responsible for the onset and progression of HF still remain to be characterized. The current treatments are not fully satisfactory due to their limited efficacy, adverse effects or loss of their therapeutic efficacy with time. Furthermore the numbers of patients suffering HF and other related cardiovascular disorders increases every year and also the number of deaths related to HF continuous to rise, as does the prevalence of contributing risk factors. (Wright *et al.*, 2008)

This study revealed the impairment of cardiac substrate utilization and the cardiac changes in the metabolic profile due to an overactive RAAS. The treatments with valsartan, levosimendan, their combination as well as resveratrol examined here proved to confer cardioprotective effects and prevented cardiovascular mortality. The beneficial effects of valsartan were associated with BP dependent and independent mechanisms. Resveratrol improved mitochondrial biogenesis and prevented Ang II- induced excess mortality. Levosimendan ameliorated systolic functions in Ang II-induced HF model, dTGR. In salt-dependent hypertensive HF, levosimendan combined with valsartan evoked superior effects than the monotherapies with valsartan or levosimendan due to the greater decrease in SBP and improved cardiac functions and reduced hypertension induced cardiac remodeling.

6.1 Methodological aspects

6.1.1 Experimental animal models

Hypertension-induced HF is a common disease that develops over years. LVH is an inappropriate adaptation response to high BP, which eventually fails as a compensatory mechanism causing congestive HF. (Meredith and Ostergren, 2006) Animal models have been created in order to study hypertensive HF with preserved systolic function, a common occurence in humans. (Ganten *et al.*, 1992)(Rapp and Dene, 1985)

6.1.1.1 Double transgenic rat harboring human renin-angiotensinogen genes (dTGR)

These studies were conducted with dTGR rats. These represent a strain of animals which overexpress RAAS, offering a unique possibility to study the function and regulation of these human genes *in vivo*. It is important that the translational products human renin and human angiotensinogen do not cross-react enzymatically with rat RAAS and vice versa. Furthermore this model can be used to study human-specific drugs such as the renin inhibitor aliskiren to reveal their advantages and limitations with RAAS. (Ganten *et al.*, 1992)(for review see (Pool, 2007)) As Wellner *et al.* revealed with tissue

Doppler measurements the peak early (Ea) to late (Aa) diastolic expansion is reduced in dTGR, consistent with terminal HF from diastolic dysfunction. (Wellner *et al.*, 2005) The animals also showed pronounced cardiomyocyte hypertrophy, preserved systolic function, increased matrix protein expression, downregulation of several genes involved in the mitochondrial respiratory chain and lipid metabolism, and distinct patterns in the expression profile of genes encoding transcription factors, coagulation, cardiac remodeling, immune system, and metabolic pathways. (Wellner *et al.*, 2005)

Due to overactive local RAAS our dTGR rats developed hypertension, cardiac hypertrophy, impaired diastolic filling (increase in IVRT), vascular inflammation, proteinuria, and renal and cardiac failure with a mortality rate of 50-60 % at the age of 8 weeks, i.e. they are very different from SD rats as described earlier. (Ganten *et al.*, 1992)(Mervaala *et al.*, 1999)(Luft *et al.*, 1999)(Muller *et al.*, 2000)(Muller et al., 2002)(Helkaama et al., 2003)

6.1.1.2 Dahl/Rapp salt-sensitive (SS) rat

The inbred Dahl/Rapp salt-sensitive (SS) rat, a model of salt-induced hypertension, develops diastolic HF with relatively preserved systolic function, resulting in an increased mortality rate. (Chang, 2009)(Solomon *et al.*, 2010)(Louhelainen *et al.*, 2009) In Dahl/Rapp rats, on the contrary to Dahl salt-sensitive rats, inbreeding consists of brother-sister breeding for 20 or more generations, providing homozygosity. In Dahl salt-sensitive rats, inbreeding has been conducted for only few generations, followed by the introduction of foreign stock to reduce inbreeding depression. (Rapp and Dene, 1985) Fulminant hypertension with concomitant LVH and increased chamber stiffness appears when there is addition of 8 % NaCl to the diet of 6 weeks old animals (Kim *et al.*, 2001)(Klotz *et al.*, 2006) and thus is followed in the proceeding 11-12 weeks by passive diastolic dysfunction with reductions in end-diastolic volume, and enhanced end-systolic function. (Klotz *et al.*, 2006) Salt-induced hypertension and vascular remodeling are initially driven by salt and water retention though there is some degree of hypertrophy, and the later phase is partially mediated through the RAAS. (Bayorh *et al.*, 2005) Dahl/Rapp rats on a high salt diet have traditionally been considered as a low renin volume overload model, where salt intake suppresses RAAS. (for review (Pinto, 1998)) Indeeed, experiments with Dahl/Rapp rats (Bayorh *et al.*, 2005)(Liang and Leenen, 2007) have revealed that even though the activity of the circulatory RAAS is attenuated, significant increases in components of the local tissue RAAS in heart, kidney and brain can be demonstrated, resulting in hypertension, cardiovascular hypertrophy, interstitial and perivascular fibrosis in ventricles and the aorta, as well as

other target-organ damage. (Liang and Leenen, 2007)(Jun *et al.*, 2003)(Zhao *et al.*, 2001)(Kreutz, 1995)

Since the Dahl/Rapp rat is a model of preserved cardiac systolic function, developing gradually hypertension- induced heart failure, it exhibit prolonged isovolumic relaxation time, compared to low salt (0.3 % NaCl) Dahl/Rapp rats and high mortality rates, i.e. similarly as noted in these studies studies, where there was a mortality rate of 30 % at week 14. (Louhelainen *et al.*, 2007)(Tian, 2007) These detrimental changes have been found to be linked to impaired calcium handling. (Louhelainen *et al.*, 2007) Moreover as earlier studies have shown, renal damage in Dahl rats, which involves salt-induced oxidative stress, inflammation and glomerular podocyte injury as well as local RAAS production, might have been involved in the development of HF. (Nagase *et al.*, 2006)(Datla and Griendling, 2010)

6.2 Cardiovascular effects of calcium sensitizers, AT$_1$ receptor blockers and resveratrol

6.2.1 Mortality

To date no satisfactory HF treatment can be considered as effectively preventing the development of hypertensive heart disease and reducing the subsequent mortality due to HF, stroke, arrhythmias, and sudden death. (for review see (Erhardt, 2003)(Shu, 2009))

In the present study, hypertensive dTGR exhibited an Ang II- induced excess mortality rate when compared to normotensive SD rats. The causes of death in dTGR rats consisted of heart and kidney failure, sudden death and stroke. (Luft *et al.*, 1999) In addition to BP-dependent effects, Ang II mediates also BP-independent effects such as end-organ damage, inflammation, and cellular growth. (Mervaala *et al.*, 2000)(Cassis *et al.*, 2010) Dahl/Rapp rats when fed with the HS diet in turn are known to develop moderate heart failure with preserved ejection as wel as end-organ damage, which was observed in our studies. (Louhelainen *et al.*, 2007)

Interestingly both valsartan and resveratrol completely, and levosimendan prevented death events in dTGR. In addition to reducing BP and aldosterone secretion due to Ang II type 1 receptor blockade, AT$_1$ receptor antagonists exert BP independent effects. (Mervaala *et al.*, 2000) In these present studies valsartan prevented mortality which was associated with improved cardiac remodeling, mitochondrial function and biogenesis. As hypothesized previously, the anti-inflammatory, anti-oxidative, and sympatho-inhibitory effects of sartans might have played a role in these dTGR rats. (Mervaala *et al.*, 2000)(Balt, 2002)

It was also discovered that the nutraceutical compound resveratrol was equally effective in the prevention of Ang II- induced mortality as valsartan. This reveals that a nutraceutical agent may have cardioprotective effects comparable to a drug treatment. Its beneficial effects on lifespan were BP dependent and associated with cardioprotection and improved mitochondrial biogenesis. Even though resveratrol is known to evoke SIRT1 longevity gene activation (for review (Das, 2010)), in our studies these effects were minor, pointing other, BP- independent actions of resveratrol, such as its anti- oxidative, and/or anti-inflammatory properties being involved.

On the contrary to valsartan and resveratrol, levosimendan decreased mortality in dTGR in a BP independent manner, emphasizing the importance of other salvage pathways. It is postulated that levosimendan exerted its cardioprotective effects due to its inotropic and vasodilatory properties, possibly combined with anti-inflammatory effects due to opening of the mitochondrial K^+_{ATP} channels in cardiomyocytes. (Louhelainen *et al.*, 2007) It has been shown that levosimendan may decrease the mortality rate not only due to improved diastolic function and hemodynamics, as seen in the studies with dTGR rats, but also by conferring protection from stroke events and arrhythmias. (Meyer, 2008)(Erhardt, 2003) This could be the case also in the studies conducted here with Dahl/Rapp rats, in which the cardioprotective effects of duotherapy of a Ca^{2+} sensitizer and an AT_1 receptor antagonist were studied. The combination completely prevented salt- induced cardiovascular mortality, similarly to the monotherapies. These findings were associated with a reduction in SBP, improved cardiac and renal structure as well as improved diatolic function. As compared to the monotherapies, the drug combination provided the best protection against hypertension-induced target organ damage. However a longer follow-up period would have been required in order to reveal survival differences between the treatment groups.

6.2.2 Cardiac remodeling and cardiac functions

Hypertension followed by cardiomyocyte hypertrophy and cardiac fibrosis, leads to diastolic stiffness and abnormal relaxation- related filling of the left ventricle which is a characteristic of HF with preserved systolic function. (Wellner *et al.*, 2005)

In this present study, the dTGR exhibited Ang II-induced cardiomyocyte hypertrophy and increased heart weight-to-body weight ratio with co-existing fibrosis, underlining the detrimental effect of local RAAS in this animal model. It was decided to investigate putative pathways involved in the transition from hypertrophied heart into HF. Myocardial pressure/volume overload and a hemodynamic marker, ANP mRNA expression were significantly higher in untreated dTGRs than SD rats. In addition, an imparment of excitation-contraction coupling in dTGR has been described by Wellner et al.

(Wellner *et al.*, 2005) Here the dTGR levels of SERCA2a protein were lower compared to healthy SD rats, indicative maladaptive calcium handling. Moreover, Wellner *et al.* reported detrimental shift from α-MHC to fetal β-MHC phenotype in cardiomyocytes, implying cardiac hypertrophy and derangements in energy metabolism in hypertrophied heart. (Krenz and Robbins, 2004) This kind of change was not observed here between the dTGR and SD rats.

In these experiments, valsartan in dTGR reduced hypertension, cardiac remodeling and pressure/volume overload, thus suggesting prevention of hypertensive HF development, which is in line with the findings of Siragy. (for review (Siragy, 2010)) Valsartan is an AT_1 receptor antagonist and widely used to treat hypertensive patients. By blocking the binding of angiotensin II to AT_1, valsartan dilates blood vessels, which results in lowered pressure/volume overload imposed to the heart, reduced the risk of congestive HF, MI and stroke. (Verdecchia *et al.*, 2010) Other actions of valsartan possibly involved in cardioprotection due to AT_1 receptor blockade independent properties such as anti-inflammatory, anti-aggregatory and anti-growth effects. (for review (Schmidt *et al.*, 2004))

Ang II is known to evoke oxidative stress mostly via a protein kinase C-dependent pathway followed by NADPH oxidase activation, resulting in peroxynitrite formation. Ang II can induce a decrease in endothelial NO production thus promoting the development endothelial dysfunction (for review see (Doughan, 2008)) vasoconstriction, cardiac muscle overgrowth and inflammation and it predisposes to aging. (for review see (Cassis *et al.*, 2010)(Wright *et al.*, 2008)) These maladaptive Ang II-dependent responses can be reversed by AT_1 receptor blockers in a BP independent manner (for review see (Cassis *et al.*, 2010)(Doughan, 2008)), as could have been the case in these present studies with valsartan. It is known that the differences in lipid solubility and the variation in the molecular structure of the ARBs determine differences in the binding affinity to the receptor and pharmacokinetic profiles. (for review see (Israili, 2000))

Studies with resveratrol have indicated that it activates sirtuin deacetylases and thus modulation of the downstream SIRT1 pathways related to improved mitochondrial function, reduced apoptosis, inflammation and aging. (Lagouge *et al.*, 2006)(Baur *et al.,* 2006)(for review see (Baur and Sinclair, 2006)) In addition resveratrol possesses free-radical and anti-oxidative properties. These pharmacological pathways affected by resveratrol can be translated into the attenuation of platelet aggregation, promotion of vasorelaxation, suppression of atherosclerosis, reduced lipid peroxidation as well as corrected serum cholesterol and triglyceride concentrations. There is growing evidence to indicate that resveratrol may have a significant effect in prevention or delaying the onset of heart disease. (for review see (Baur and Sinclair, 2006))

In the dTGR experiments, resveratrol improved cardiac structure and prevented the development of hypertrophy. In particular, resveratrol could have exerted these cardioprotective effects in dTGR due to prevention from Ang II-induced activation of NADPH oxidase and xanthine oxidase. (Mervaala *et al.*, 2001) Since resveratrol exerted similar effects to valsartan, this might be considered as evidence of that the AT-1 blockers possess significant anti-oxidative effects as described earlier by Cassis et al. (for review see (Cassis *et al.*, 2010)

Levosimendan, a novel inodilator, acts both via calcium sensitization of myocardial contraction and opening of K^+_{ATP} channels in cardiomyocytes. These properties of levosimendan result in increased cardiac force of contraction and decreases in preload and afterload and thus contributing to cardioprotection. Levosimendan is used for acute decompensated HF and given usually intravenously; however this drug is also well absorbed when administered orally. Long- term effects of levosimendan still remain to be elucidated. Even though the effects of levosimendan in the dTGR were BP-independent, moderate protection was observed against Ang II-induced cardiomyocyte and the vascular damage and this was associated with a prominent decrease in pressure/volume overload. This could have been due to vasodilatation and improved microcirculation and overall coronary flow in the heart. (Grossini *et al.*, 2009, Pataricza *et al.*, 2003, Erdei *et al.*, 2006)(Honish *et al.*, 2009) Moreover, no alterations were observed in two calcium- handling proteins, SERCA2a and NCX, in levosimendan treated dTGR, though the amounts of SERCA2a were increased in levosimendan treated SD. In the latter case, PDE III inhibition as a mediator of the beneficial effects of levosimendan could have been involved, however this hypothesis remains to be confirmed. There was also a tendency in levosimendan treated dTGR towards an increase in the level of α-MHC and a corresponding decrease in that of fetal β-MHC, when compared to dTGR. Nonetheless, the treatment period might have been too short to reveal entirely the significance of this trend.

In the study with Dahl/Rapp rats consuming a high salt diet, levosimendan and valsartan duotherapy significantly corrected pressure overload, cardiac hypertrophy, cardiac damage and as well as improved cardiac diastolic function compared to the monotherapies. It is noteworthy, that these beneficial effects of the duotherapy were largely due to its pronounced anti-hypertensive effects. Even though the dose of levosimendan in the combined therapy was lower due to the Ang II-dependent dipsogenic effect blockade encountered after valsartan treatment, the duotherapy produced superior effects to monotherapies. Valsartan is known to attenuate an overactive tissue RAAS and salt-overload related tissue damage (Liang and Leenen, 2008) evidence of a significant role of Ang II mediated pathology in this low-renin model. Levosimendan improved cardiac function, discussed below, and due to opening of sarcolemmal K^+_{ATP} channels of vascular smooth muscle cells

resulted in vasodilation and corrected the pre- and after-load, which could be postulated to improve coronary flow, providing better perfusion to the heart (Louhelainen *et al.*, 2007)(for review see (Parissis, 2009) even though BP was not significantly affected by levosimendan alone. The levosimendan-valsartan combination produced a major decrease in ANP, reflecting a decrease in myocardial tension and the degree of cardiac overload, which might result in improved microcirculation in the heart. This improvement in hemodynamics may have resulted from improved renal damage and function, most effectively with the drug combination. Since the valsartan mechanism could have been related to local RAAS inhibition and its anti-inflammatory properties, levosimendan could have evoked increased blood flow to the renal medulla and decreased renal medullary/cortical vascular resistance due to the K^+ATP channel opening, anti-inflammatory properties, and a reversal of Ang II-mediated mesangial cell contraction. (Yilmaz *et al.*, 2007)

Cardiac diastolic dysfunction and preserved systolic function is characteristic for hypertension induced HF. (Zile, 2004)(for review see (Zile and Brutsaer, 2002)) The rat strains used here represent models of HF with preserved systolic functions. (Wellner *et al.*, 2005)(for review see (Solomon *et al.*, 2010)) However, the ratio of diastolic velocities, E/A-ratio in dTGR, when assessed by tissue Doppler imaging, was reduced, indicative of diastolic dysfunction, as demonstrated earlier. (Wellner *et al.*, 2005)(Rudko *et al.*, 2008)(for review see (Gary and Davis, 2008)) Deterioration of active relaxation, namely prolonged isovolumetric relaxation time (IVRT) in Dahl/Rapp rats, was also observed with echocardiographic measurements as described by Rudko *et al*. (Rudko *et al.*, 2008)

When tested in the dTGR levosimendan improved cardiac function at systole even though it did not restore preserved systolic functions when compared to SD rats. This finding underlines the involvement of calcium sensitization mechanism, resulting in reduction in diastolic stiffness and an improvement of abnormal relaxation filling of left ventricle. However levosimendan did not correct Ang II-deteriorated diastolic cardiac functions in dTGR, on the contrary to the findings with Dahl/Rapp rats consuming a high salt diet. With or without addition of valsartan, levosimendan was equally effective at improving cardiac function at diastole in Dahl/Rapp rats receiving the high salt diet, as assessed as by the shortened IVRT. This occurred largely via blood pressure-dependent mechanisms, which implies that it can lower risk for diastolic HF. This finding is in line with a previous clinical study showing that levosimendan could improve diastolic function and shorten IVRT in patients with acutely decompensated heart failure. (Parissis *et al.*, 2005)(Yontar *et al.*, 2010) The putative mechanism behind diastolic improvement (Louhelainen *et al.*, 2007) and a possible increment in coronary blood flow. (Grossini *et al.*, 2010) This may be related to the increase in SERCA2 expression in cardiac sarco/endoplasmic reticulum in Dahl/Rapp rats evoked by

levosimendan, as shown earlier by Louhelainen et al. (Louhelainen *et al.*, 2007) It needs to be emphasized that the combination therapy provided the most efficient cardioprotection

Systolic parameters, namely fractional shortening and ejection fraction in untreated high salt Dahl/Rapp rats were increased possibly as a result of the development of hypertrophy; levosimendan treatment insignificantly tended to augment systolic function. Nonetheless, neither in dTGR nor in Dahl/Rapp rats consuming a high salt diet was levosimendan able to prevent changes in HR, which can be speculated to exclude a potential role of PDE III inhibition as the mechanism involved in positive inotropic effects. (Gruhn *et al.*, 1998)

6.2.3 Metabolic profiling of Ang II-induced cardiac hypertrophy

The failing heart is characterized by imbalanced energy sources such as fatty acid and glucose. (Wellner *et al.*, 2005) Metabolomics is a novel method which aims to study dynamics, composition, interaction and responses to various interventions, both in healthy and diseased organs. (Oresic, 2009) The metabolomic approach seems to be a promising tool as a marker of tissue-damage, including cardiac disorders. The quantification of small molecules leaking from injured myocardial cells is the basis for metabolomic studies. (for review see (Gregory *et al.*, 2008)) Thus detection of the shift from fatty acids to carbohydrates may be a sign of failing myocardium and a therapy aimed at modulating energy metabolism could be a new alternative approach to the improvement of a failing myocardium. Drugs that are able to shift energy metabolism away from fatty acids to carbohydrates have been developed and are currently in clinical trials. (for review see (Fragasso *et al.*, 2008)(Horowitz *et al.*, 2010)(Kaddurah-Daouk *et al.*, 2008))

In these studies, the AT_1 receptor blocker, valsartan was used as a tool to investigate the effects of Ang II, and its blockade, on cardiac energy metabolism. With a novel method GC×GC-TOF/MS 247 different intermediary metabolites were recognized in the heart. Interestingly, valsartan was able to change the metabolomic profile not only in dTGR but also in normotensive SD rats. In valsartan-treated SD rats, the blockade of physiological AT_1 signalling was associated with a disturbed metabolic profile, without affecting hemodynamic parameters when compared with normotensive SD. This is evidence that Ang II-mediated actions are strongly related to metabolic profiling also in healthy rats.

Moreover, as shown by Wellner *et al.*, the dTGR exhibit a dysregulation in the genes involved in energy metabolism. The changes in these genes correlated with contractile alterations in the heart, such as dysfunctional contractility and Ang II-induced LVH, as well as with cardiac energy demand. In

the advanced stages of HF, many key enzymes involved in myocardial energy substrate metabolism display various degrees of downregulation. The altered metabolic phenotype consists of reduced cardiac fatty oxidation, increased glycolysis and glucose oxidation and rigidity of the metabolic response to changes in workload. (Wellner *et al.*, 2005) This is in line with the present findings. It was found that dTGR rats exhibited a decrease in medium-chain fatty acids, namely octanoic, oleic and linoleic acids, indicative of a downregulation of fatty acid synthesis in the presence of overproduced Ang II. Furthermore markedly decreased octanoic acid, involved in the synthesis of lipoic acid, an endogenous sulfur-containing coenzyme is required for the mitochondrial dehydrogenase reactions leading to ATP formation, this being reflected as an imbalance in aerobic metabolism. In addition, there was evidence of changes in the cardiac hypoxanthine level indicating purine degradation. This finding is in parallel with our earlier studies with dTGR demonstrating xanthine oxidoreductase overexpression. (Procopciuc *et al.*, 2010) In addition, heart converts the chemical energy stored in fatty acids and glucose into the mechanical energy of actin-myosin interaction of myofibrils. The period of LVH that precedes decompensated HF is characterized by alterations in myocardial bioenergetics have been considered to play an important role in this transition. The ratio of phosphocreatine to ATP (PCr/ATP) reflects the energetic cardiac state and is significantly decreased in hearts with LVH reflecting impaired mitochondrial function, and is accompanied by a decrease in creatine kinase flux and alterations in substrate utilization in LVH hearts. These changes result in impaired pressure-overload, volume overload, cardiac output, eventually leading to LVH and HF. (for review see (Jameel and Zhang, 2009))

Remodelled cardiac samples from septal MI in humans have exhibited alterations in pyrimidine metabolism levels, the tricarboxylic acid cycle, and in the pentose phosphate pathways (Lewis *et al.*, 2008) Furthermore as an adaptation mechanism in atrial fibrillation, increased levels of β-hydroxybutyrate, a ketogenic amino acid, were recorded. (Mayr *et al.*, 2008) Altered patterns were also found in the expression profiles of genes encoding transcription factors and factors involved in coagulation, cardiac remodeling, immune system, and metabolic pathways. (Wellner *et al.*, 2005) It was confirmed that these findings in failing hearts of the dTGR. They exhibited altered glucose utilization resulting in excessive production of ketogenic acids.

6.2.4 Mitochondrial biogenesis and mitochondrial functions

The transition from LVH to HF is strictly connected to impaired mitochondrial biogenesis, mitochondrial respiratory chain function and oxidative phosphorylation. (for review see (Stanley et al., 2005) Mitochondria generate energy, regulate apoptosis, and produce ROS and contribute to the

development of HF by promoting apoptosis, senescence and inflammation. (Hossain *et al.*, 2009)(Lemieux *et al.*, 2010)(for review see (Ventura-Clapier *et al.*, 2010)

The mitochondria of dTGR were fused, suggesting a possible dysfunctional energy metabolism. Wellner et al reported the downregulation in mitochondrial respiratory chain in dTGR. (Wellner *et al.*, 2005) Furthermore it was found that cytochrome c oxidase (complex IV), an enzyme responsible for adenosine triphosphate production, was significantly decreased. This could explain the defects in contractile mechanisms in HF. (Quigley, 2000) In this study valsartan, an AT_1 receptor blocker was able to prevent these changes, further highlighting the important role of RAAS in the regulation of mitochondrial functions. Ang II-mediated mitochondrial dysfunction is linked to protein kinase C-related pathway, reduced form of NADPH oxidase and further- ROS formation. (Doughan, 2008) Disruption of AT_1 receptor in mice has been shown to prolong lifespan due to a decrease in oxidative stress and induction of mitochondrial biogenesis. (Benigni *et al.*, 2009) However its exact influence on mitochondrial biogenesis remains to be determined due to the complex results obtained in dTGR and SD rats, such as the failure to detect any difference between both rat strains.

On the contrary to valsartan, resveratrol dTGR treatment resulted in increased mitochondrial biogenesis through PGC-1-dependent pathway as shown earlier. (for review see (Lagouge *et al.*, 2006)) There was a significant increase in mRNA expression of transcription factors regulating mitochondrial function and biogenesis such as PGC-1 α (peroxisome proliferator-activated receptor [PPAR]- γ coactivator 1 α), cox4 and Tfam (mitochondrial transcription factor) when compared to hypertensive rats. These findings are in line with the previous experiments conducted with aged animal models (for review see (Vina *et al.*, 2009)) and suggest that the cardioprotective effects of resveratrol in dTGR were linked to mitochondrial biogenesis, which in turn balanced energy metabolism and possibly improved cardiac performance.

In contrast to the situation with resveratrol changes were observed in dTGR treated with levosimendan, even though one mode of action of levosimendan is to open mitochondrial K^+_{ATP} channels. In order to reveal the involvement of mitochondrial K^+_{ATP} channels, there may be a need to use specific mitochondrial K^+_{ATP} channels blockers in the future. This could be one way to clarify the effect of levosimendan on mitochondrial function.

6.2.5 SIRT1 activation and SIRT1-related salvage pathways

Sirtuin 1 (SIRT1) constitutes a longevity gene. Since it is an energy sensor, cardioprotective, anti-apoptotic and anti-inflammatory factor (Nisoli, 2007)(for review see (Finkel, 2009)) SIRT1 is highly

expressed in HF (Alcendor et al., 2004) comprising a compensatory mechanism. Due to the induction of mitochondrial activity in the NO-dependent pathway, it reduces ROS production, protecting from cardiovascular events. (for review see (Guarente, 2008)) Deacetylation of target proteins such as p53, FOXO transcription factor and PGC-1α results in cardioprotection by balancing energy metabolism, cellular senescence, inflammation *in vivo* and *in vitro* and by promoting DNA repair. (Alcendor, 2007)(Wang *et al.*, 2008)(for review see (Borradaile and Pickering, 2009))

The differences of expression in these rat strains were screened and it was noted that cardiac SIRT1 mRNA levels remained unchanged between SD and dTGR strains, indicating post-translational modifications of the protein. These findings stimulated a further investigation of the role of SIRT1 in dTGR. Surprisingly our dTGR showed more SIRT1 protein expression level than normotensive SD rats. In order to examine in detail, whether SIRT1 protein expression could be mediated by pressure overload or by Ang II or by both factors, Dahl/Rapp rats consuming high and low salt diet, a model of low systemic RAAS. Although exhibiting high BP in Dahl rats on high salt diet, no differences in SIRT1 were found between screened hypertensive and normotensive groups. Our study with neonatal cardiomyocyte cells incubated with Ang II also did not reveal any alterations in SIRT1 protein levels. This finding may be associated with marginal amounts of AT_1 receptors in cardiac neonatal cells as demonstrated by Porello et al. (Porrello *et al.*, 2009) These results also imply that both hypertension and Ang II together are required for altered SIRT1 expression in postnatal period. Therefore, it was hypothesized that SIRT1 can be considered as a marker of Ang II-induced cardiac remodeling.

It has been reported that overexpression of cardiac SIRT1 (>12.5 fold) is detrimental and triggering apoptosis, hypertrophy, fibrosis and cardiac dysfunction through induction of mitochondrial dysfunction, ultimately leading to the development of HF. (Alcendor, 2007))(for review see (Guarente, 2008) Thus, a moderate increase in SIRT1 protein expression points to compensatory salvage mechanism in dTGR. Valsartan treated dTGR exhibited a relatively low SIRT1 protein level, comparable with SD and SD rats treated with valsartan. This may have contributed to the cardioprotection, which could have resulted from a decrease in apoptosis, senescence, hypertrophy and impaired cardiac functions as reported earlier by Alcendor et al. (Alcendor, 2007)

The SIRT1 protein was examined in detail by administering resveratrol, proposed to be a SIRT1 activator. In dTGR, resveratrol normalized the expression of SIRT1 protein to the level of normotensive SD rats. However, when investigated further no activity *in vivo* was observed in resveratrol treated dTGR and no differences between dTGR and SD rats downstream SIRT1-regulated pathways such as anti-apoptotic and anti-senescent p53. This finding points to other properties of

resveratrol involved in cardioprotection in dTGR. In addition to inducing mitochondrial biogenesis, resveratrol could have mediated its effects due to oxidative stress reduction or free radical-scavenging features but those properties were not estimated in these experiments.

6.2.6 Inflammation and apoptotic pathways

Ang II is known to exert inflammatory effects in a BP-independent manner. This process is linked to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1), redox-sensitive transcription factors, and oxidative stress. (Muller *et al.*, 2000)(Muller *et al.*, 2002)(Mervaala *et al.*, 2001)

Levosimendan, a calcium sensitizer and inodilator, was found to exert anti-inflammatory, anti-apoptotic and anti-senescent effects in Dahl/Rapp rats consuming a high salt diet as well as in spontaneously diabetic Goto-Kakizaki rats with post-infarction HF. (Louhelainen *et al.*, 2007)(Louhelainen *et al.*, 2009) *In vitro* levosimendan also has been shown to reduce NF-κB-associated, iNOS promoter transcriptor followed by synthesis of the enzyme which then evokes NO formation. (Sereila *et al.*, 2008)

Due to the overactivated RAAS, dTGRs develop inflammation. (Wellner *et al.*, 2005) Nonetheless levosimendan was not able to prevent the progression of inflammation in dTGR, nor could it protect from apoptosis, as assessed via the levels of MCP-1 mRNA and Bax mRNA. This indicates that other salvage pathways are involved in levosimendan-mediated cardioprotection.

It was earlier reported that cardiac remodeling has been associated with ER stress and a disturbance in autophagy in patients with heart failure (Nishida *et al.*, 2009) Even though AT_1 receptor blockade was previously noted to reduce ER stress and thus prevent cardiac hypertrophy, (Tang and Chua, 2008) valsartan was unable to produce any major changes in high-salt loaded Dahl/Rapp rats. Neither the drug combination nor levosimendan alone affected ER stress even though they have previously been found to induce LC3B expression and autophagy in pig myocardium. (Grossini *et al.*, 2010) There was however a tendency towards activation of cardiac autophagy indicative of enhanced cellular clearance mechanism.

6.2.7 Clinical relevance

These studies evaluated the importance of metabolomic profile of the failing heart in a hypertensive animal model with overactive RAAS. Since there is no satisfactory treatment for this condition and associated disorders, the pathways involved, especially those in energy utilization may represent a

very important target area in the development of novel, more effective treatments. Earlier studies highlighted the significance of adequate metabolism in sustaining correct cardiac function and structure (Sabatine *et al.*, 2005)(for review see (Knuuti *et* Tuunanen, 2010)(Stanley *et al.*, 2005)) Perhaps in the future it will be possible to devise an animal model making a direct link between tissue damage and concentrations in serum and/or urine samples. This kind of bioindicator could be helpful if it could be extrapolated and relevant humans as indicating particular disorder or severity of disease in a patient.

The commonly used AT_1 receptor blocker valsartan, was found to be linked to improved energy balance in dTGR. The induction of biogenesis in mitochondria may be a useful target for correcting the existing derangements in the failing heart.

Resveratrol, which is a natural polyphenol obtained from grapes, can be considered a novel approach in prevention or treatment of hypertension, cardiac remodelling and resulting cardiovascular disorders. Resveratrol, provided as a dietary supplement, may be able to confer significant cardioprotection and achieve a reduction in cardiovascular events, if administered regularly. It may possibly represent an alternative therapy for hypertensive subjects. (for review see (Lekli, 2010)(Das and Das, 2007)) Moreover, currently there are molecules in addition to resveratrol, known to activate SIRT1. These compounds such as SRT2104, SRT2379 and SRT501 are produced by Sirtris Pharmaceuticals Inc. and are already undergoing clinical trials. (for review see (Camins *et al.*, 2010))

To date there is not enough satisfactory data related to chronic oral treatment with levosimendan, a drug that is more commonly used as a short term (24 h) intravenous infusion in patients with acute HF. (Bergh *et al.*, 2010) These studies demonstrated that long-lasting levosimendan treatment did alleviate mortality, mainly due to inotropic and local vasodilatory actions. Therefore it is hypothesized oral levosimendan regimen could be both safe and beneficial, i.e. it may possess clinical potential.

Ultimately the addition of levosimendan to valsartan therapy seems to confer hemodynamic improvements and cardioprotective benefits indicative of possible symptomatic and survival benefits for the HR patient. This treatment was shown to be significantly superior to monotherapies and provided blood pressure- dependent protection against target organ damage in the heart and kidneys. Moreover, levosimendan, alone and in combination with valsartan, could correct hypertension- induced diastolic dysfunction. Perhaps, in the future medicating patients intermittently with levosimendan may become a common treatment in addition to the daily drug

regimen. However, further studies are warranted to elucidate the central actions of the drug combination as well as their effects on renal blood flow and kidney function.

This present study did not reveal any possible interactions between levosimendan and valsartan in the combined therapeutic group. In particular, the dose of ingested levosimendan when it was administered via the drinking water in the drug combination was significantly lower than that achieved by levosimendan therapy alone. Further studies need to be conducted in order to clarify the mechanisms behind the cardioprotective effects of the levosimendan and valsartan combination.

7. CONCLUSIONS

The aim of the present study was to investigate the molecular mechanisms and signalling pathways of hypertension-induced heart failure with a special emphasis on the local renin-angiotensin-aldosterone system, cardiac metabolomics and the putative therapeutic role of oral administration of the calcium sensitizer levosimendan. Exprimental studies were conducted in two different models of hypertension and hypertension-induced heart failure with preserved systolic function, i.e. in double transgenic rats harboring human renin and human angiotensinogen genes (dTGR), and in salt-sensitive Dahl/Rapp rats. The main findings and conclusions of the present non-clinical cardiovascular studies were as follows:

I. Ang II altered cardiac substrate use and the metabolomic profile in both normotensive and hypertensive animals. In Ang II-induced cardiac hypertrophy a distinct substrate use from fatty acid oxidation towards glycolysis could be detected by using a novel tissue-based metabolomics method. The altered cardiac metabolomic profile in dTGR was associated with mitochondrial dysfunction.

II. Resveratrol prevented cardiovascular mortality and ameliorated Ang II-induced cardiac remodelling in dTGR. The beneficial effects of resveratrol were mediated by blood pressure-dependent pathways and appear to be linked to increased mitochondrial biogenesis. Resveratrol dose-dependently increased SIRT1 activity *in vitro*.

III. Oral levosimendan treatment improved cardiac function and survival in rats with Ang II-induced hypertensive heart failure. The beneficial effects of levosimendan in dTGR are mediated via blood pressure-independent mechanisms and involved improved systolic function and amelioration of Ang II-induced coronary and cardiomyocyte damage.

IV. Oral levosimendan treatment improved survival, produced a modest decrease in systolic blood pressure, and prevented hypertension-induced cardiac remodelling in hypertensive Dahl/Rapp rats on high salt diet. Levosimendan slightly improved systolic function and shortened IVRT, evidence of improved diastolic function. In Dahl/Rapp rats, valsartan decreased blood pressure more effectively than levosimendan, prevents cardiovascular mortality and cardiac remodelling, but it did not improve systolic or diastolic function. An additive antihypertensive effect was found when levosimendan and valsartan were combined.

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