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Susceptibility to Acute Decompensated Heart Failure in Two Common Mouse Strains

MEDIHA BECIROVIC-AGIC

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Abstract

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Heart failure is a clinical syndrome characterized by an inability of the heart to meet oxygen demands of the body. During the initial stage of heart failure development compensatory mechanisms are activated to help the heart sustain proper function. Over time these compensatory mechanisms become inadequate resulting in decompensation. Acute decompensated heart failure is characterized by rapidly escalating heart failure symptoms, such as dyspnea and congestion, which require urgent treatment. The pathophysiology of decompensation and role of genetic background on this process is not completely understood. The aim of this thesis was to investigate the role of genetic background on susceptibility to develop acute decompensated heart failure.

Balb/CJ and C57BL/6J mice are two common mouse strains that we found have different susceptibility to angiotensin II and high-salt diet (AngII+Salt) induced decompensation. Balb/CJ treated with AngII+Salt develop massive edema associated with anuria and high mortality within 4-6 days of treatment, while C57BL/6J mice do not. Due to the clinical symptoms of heart failure we hypothesized that Balb/CJ develop acute decompensated heart failure, and that the genetic background of this strain is responsible for the increased susceptibility to heart failure. AngII+Salt increased pulmonary and systemic vascular resistance, reduced left ventricle filling, and increased sodium and water retention in Balb/CJ mice. Increased pulmonary vascular resistance correlated with a higher angiotensin II response in isolated pulmonary arteries from Balb/CJ compared to C57BL/6J. Cardiac output was lower in Balb/CJ than C57BL/6J during AngII+Salt treatment even though they retained more sodium and water. This indicated that AngII+Salt impairs cardiac function in Balb/CJ mice. Oxidative stress was shown to play a role in AngII+Salt induced acute decompensation since treatment with an antioxidant reduced oxidative stress but impaired cardiac function and increased mortality in both strains. A linkage study was performed to reveal genes that are with high probability related to AngII+Salt induced decompensation in Balb/CJ mice. Quantative trait loci (QTLs) on chromosome 3 and 12 were linked to cardiac dysfunction and QTLs on chromosome 2 and 3 were linked to sodium and fluid balance. Foxo1 was found to be one of candidate genes for further study.

Taken together, the data in this thesis shows that genetic background does play a large role in the development of acute decompensated heart failure. It reveals several candidate genes that could be studied in the setting of acute decompensated heart failure. Finally, it describes a new mouse model that could potentially be used for studying the pathophysiology of decompensation and identifying new drug targets.

Mediha Becriovic-Agic, Department of Medical Cell Biology, Box 571, Uppsala University, SE-75123 Uppsala, Sweden.

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Becirovic-Agic M.**, Jönsson S., Tveitarås MK., Skogstrand T., Karlsen TV., Lidén M., Leh S., Ericsson M., Nilsson SK., Reed RK., Hultström M. (2019) Time-course of decompensation after angiotensin II and high-salt diet in Balb/CJ mice suggests pulmonary hypertension-induced cardiorenal syndrome. *Am J Physiol Regul Integr Comp Physiol.*

II **Becirovic-Agic M.***, Jönsson S.*, Isackson H.,Tveitarås MK., Skogstrand T., Karlsen TV., Lidén M., Leh S., Ericsson M., Nilsson SK., Reed RK., Hultström M. Angiotensin II and salt-induced decompensation in Balb/CJ mice is associated with genetic differences in glutathione transferase activity. *Manuscript*

III **Becirovic-Agic M.**, Jönsson S., Hultström M. Quantitative Trait Loci (QTL) associated with angiotensin II and high-salt diet induced acute decompensation in Balb/CJ mice. *Submitted*

IV **Becirovic-Agic M.**, Hultström M. Release of a contractile factor and reduced nitric oxide from isolated pulmonary resistance vessels from BalB/CJ mice cause higher reactivity to angiotensin II compared to C57BL/6J mice. *Manuscript*

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* These authors contributed equally to the study.

Contents

Abbreviations

AngII	Angiotensin II
ANP	Atrial natriuretic peptide
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
BNP	B-type natriuretic peptide
CO	Cardiac output
EC50	Half maximal effective concentration
EDV	End-diastolic volume
EF	Ejection fraction
ESV	End-systolic volume
GFR	Glomerular filtration rate
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
IVRT	Isovolumic relaxation time
LOS	Log of odds
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NOS	Nitric oxide synthase
QTL	Quantitative trait locus
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphisms
SNS	Sympathetic nervous system
SV	Stroke volume

Introduction

The present thesis studies the relationship between the heart, kidneys, vasculature and genetics in development of heart failure. The text bellow gives a brief introduction to the different organs and systems, and their role in heart failure.

The heart

The main role of the heart is to provide all cells of the body with sufficient amount of oxygen and nutrients, necessary for cellular metabolism and constant internal environment (147). The heart constitutes of cells called myocytes. These cells are characterized by the ability to relax and contract resulting in the pumping function of the heart (136). The heart can be thought of as two pumps operating in series, where the right atrium and ventricle are pumping oxygen depleted blood from the systemic veins into the pulmonary circulation and the left atrium and ventricle are pumping oxygenated blood delivered via pulmonary veins into the systemic circulation (Figure 1). The atrioventricular valves separate the left atrium and ventricle (mitral valve), and the right atrium and ventricle (tricuspid valve). The semilunar valves separate the left ventricle from the aorta (aortic valve) and the right ventricle from the pulmonary artery (pulmonary valve). Oxygen depleted blood is delivered to the right atrium via vena cava superior and vena cava inferior. From right atrium the blood flows into the right ventricle and pulmonary artery. In the lungs the blood is oxygenated and delivered to the left atrium via pulmonary veins. The oxygenated blood flows from left atrium into left ventricle followed by ejection into the aorta and systemic circulation (70). Since mean arterial pressure of systemic circulation (~100 mmHg) is higher than mean arterial pressure of pulmonary circulation (8-19 mmHg), left ventricle has 3-4 times the mass of right ventricle (8, 40, 51, 78). This allows the left ventricle to produce the high pressure that is necessary for aortic valve to open and for the blood to be ejected into systemic circulation. The heart is enclosed in a noncompliant fibrous sac called pericardium (18).

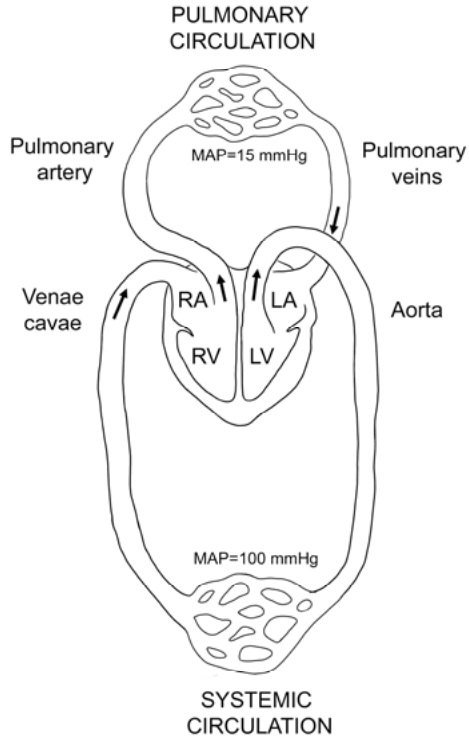

Figure 1. Pulmonary and systemic blood circulation. RA: Right atrium, RV: Right ventricle, LA: Left atrium, LV: Left ventricle.

The cardiac cycle can be divided into diastole and systole. Diastole is the period during which ventricles fill with blood and systole is the period during which ventricles eject blood into their respective circulations (Figure 2). Diastole starts with isovolumic relaxation which occurs after the semilunar valves close. During this phase the atrioventricular valves are also closed, meaning that the ventricles are relaxing without change in volume. Filling of the ventricles starts when atrial pressure exceeds ventricular pressure. At this point the valves open and the early phase of filling starts during which the blood flows from atria to the ventricles because of a difference in pressure. During the late phase of filling atria contracts and delivers an additional volume of blood to the ventricles. Thereafter the myocardium starts contracting and systole begins. Early during systole, the intraventricular pressure exceeds the pressure of atria and atrioventricular valves close to prevent backflow of blood from the ventricle to the atrium. Systole starts with isovolumic contraction during which the myocardium contracts without ejecting blood. When

intraventricular pressure reaches the pressure in systemic and pulmonary circulation semilunar valves open and blood is ejected into respective circulation (30, 45, 70). The efficiency of ventricular filling is determined by venous return, end-systolic volume (ESV) and the intrinsic ability of the heart to relax and fill. The ejection on the other hand is determined by outflow resistance, end-diastolic volume (EDV) and the intrinsic ability of the heart to contract (69, 83, 116).

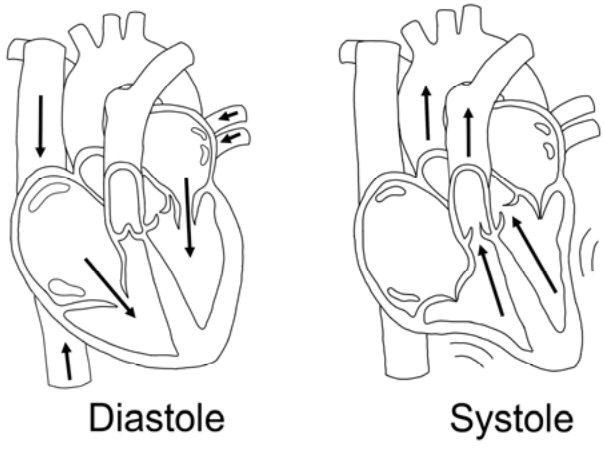

Figure 2. Heart in diastole and systole. During diastole blood flows into the ventricles and during systole blood is ejected into the pulmonary artery and aorta.

The volume of blood that the heart ejects during a defined period of time is called cardiac output (CO). It is a product of stroke volume and heart rate (SV*HR). Stroke volume is the volume of blood ejected by the ventricle during one heartbeat, and is affected by preload, afterload and contractility of the heart. Preload is defined as the amount of cardiomyocyte stretch at the end of diastole, which in turn determines contractility and work output of the heart (30). Thus, a large EDV causes larger cardiomyocyte stretch and therefore higher contraction force of the heart. This is known as the Frank-Starling mechanism, which states that the amount of blood that is ejected by the ventricle depends on the amount of blood that reaches the ventricle. Thus, larger amount of blood causes more forceful myocardial contraction and therefore higher CO (73). Afterload is defined as the resistance that the ventricle must overcome to open the pulmonary and the aortic valve, and eject blood. Afterload is mainly determined by pulmonary and systemic vascular resistance (73, 147). Finally, contractility is the intrinsic ability of the heart to contract independently of preload and afterload (73), which depends on the intrinsic state of the myocardium and the neurohormonal state; e.g., increased sympathetic activity results in a more forceful heart contraction independent of cardiomyocyte stretch (73, 147).

Heart failure

Heart failure is a clinical syndrome characterized by an inability of the heart to pump sufficient amount of blood to meet metabolic needs of the body. As a consequence, patients are unable to perform daily activities without symptoms of dyspnea or fatigue (36, 73). Typical signs of heart failure are dyspnea, reduced exercise tolerance, fatigue and edema (100). Heart failure is commonly divided into heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF). The primary cause of HFrEF is systolic dysfunction where myocardial contractility is impaired either due to loss of cardiomyocytes or decreased function of the viable cardiomyocytes. As a result the heart is unable to eject sufficient amount of blood and CO is reduced (124). HFpEF, also called diastolic heart failure occurs when the ventricles are unable to fill with and adequate volume of blood at normal filling pressures. This may be a result of impaired ventricular relaxation or ventricular stiffness. The symptoms of HFpEF are more often pronounced during exercise since these patients are unable to increase their stroke volume by increasing EDV (15, 75, 140).

In addition to systolic and diastolic dysfunction, either right or left or both ventricles may fail. The incidence of left and right ventricular failure is similar, each affecting 1 in 20 of the population (133). However, the difference lies in left ventricular failure more often being a chronic progressive disease, while right ventricular failure may be an acute or chronic condition depending on the underlying cause (43). Since pulmonary circulation is a low resistance system, the right ventricle pumps same stroke volume as the left ventricle but with less effort (<25%) (17, 90). As a consequence, the right ventricle adapts poorly to sudden increases in afterload (90), and may therefore more often result in acute heart failure.

Ventricular interdependence

The left and right ventricle are separated by a shared wall called the interventricular septum. In normal cases, the interventricular septum moves towards the left ventricle during systole and aids left ventricular ejection. Due to the shared interventricular septum and the heart being contained in a noncompliant pericardium, there is a high degree of ventricular interdependence (115). Ventricular interdependence is mostly prominent during diastole, because dilation of one ventricle may impair filling of the other ventricle (115, 149, 150) Increase in left ventricular EDV moves the interventricular septum towards the right ventricle and in that way increases the end-diastolic pressure and impairs filling of the right ventricle. Similarly, increase in right ventricular EDV

moves the interventricular septum towards the left ventricle, resulting in decreased filling and volume of the left ventricle (134).

The kidney

The main role of the kidney is to produce urine and excrete waste products. Since this organs is able to regulate what leaves the body, it is also responsible for regulating electrolyte and fluid balance. All cells in the body are dependent on a stable concentration of electrolytes for proper function. The functional units of the kidneys are called nephrons. A nephron consists of a glomeruli, Bowman's capsule, proximal tubule, loop of Henle, distal tubule and the collecting duct. The glomeruli consists of a vascular bed where all contents of the blood except red blood cells and proteins are filtered into Bowman's capsule and proximal tubule. During a single day, the kidneys filter 180 liters of blood but excrete only 2 liters of urine. The high filtration is necessary for the kidneys to be able to precisely regulate fluid and electrolyte content of the body. Most of the electrolytes, valuable molecules, such as glucose and amino acids, and water will be reabsorbed in the proximal tubule. The loop of Henle, distal tubule and the collecting duct are responsible for fine adjustment of the urine that is to be excreted (126).

For the kidneys to properly carry out their function glomerular filtration rate (GFR) must be kept constant. GFR is defined as volume of plasma completely cleared from a substance during a defined period of time. It is kept constant through several auto regulatory mechanisms, including tubuloglomerular feedback. A decrease in renal blood flow due to e.g. a reduced cardiac output reduces GFR, which in turn reduces sodium load delivered to the distal tubule. The lower sodium load is sensed by specialized cells in the distal tubule called macula densa, which in turn stimulate renin release from juxtaglomerular cells (105). Renin cleaves the angiotensinogen to angiotensin I. Angiotensin I is further cleaved by angiotensin converting enzyme to angiotensin II (AngII), a hormone that stimulates vasoconstriction and increases GFR (73). In order to restore GFR, AngII also increases blood pressure and blood volume to ensure an adequate renal perfusion and restoring of GFR (25, 53, 68). The role of AngII and the entire renin-angiotensin-aldosterone system (RAAS) is covered in more detail below.

The renin-angiotensin-aldosterone system

To ensure proper perfusion of all organs mean arterial blood pressure is highly regulated. One system that is involved in regulating blood pressure is the RAAS, which when activated increases blood pressure through vasoconstriction and increased blood volume. As mentioned above renin is necessary

for AngII production. AngII acts as a vasoconstrictor and can quickly increase blood pressure by increasing vascular resistance. AngII also stimulates sodium and water retention in the kidneys. Moreover, it stimulates release of aldosterone from adrenal glands which further increase sodium and water retention. AngII stimulates release of vasopressin, a hormone that causes aquaporin 2 mediated water retention and at high doses vasoconstriction (16, 73). These hormones together result in increased blood volume and therefore higher arterial pressure (6, 119).

Compensatory mechanisms activated during heart failure

As previously noted, heart failure is defined as a state during which the heart is not able to meet oxygen demands of the body (73). Usually, during heart failure CO is reduced resulting in reduced arterial pressure, tissue perfusion and lower oxygen delivery (64, 97, 108). The unmet oxygen demand activates compensatory mechanisms intended to restore CO and oxygen delivery. CO can be restored by increasing preload, heart rate or contractility. During heart failure, increased sympathetic nervous system (SNS) activity increases heart rate and contractility, which in turn increases CO. Reduced renal blood flow due to decreased CO, and increased SNS signaling activates the RAAS (73). Increased activity of the RAAS increases ventricular preload by increasing blood volume and CO through the Frank-Starling mechanism (6, 119). In addition the increased vasoconstriction allows proper tissue perfusion and blood supply. In response to increased workload ventricular hypertrophy is induced. Hypertrophy is characterized by increased size of the myocytes and therefore increased thickness of the cardiac wall. An increase in muscle size results in increased contraction force and therefore increased cardiac output (48, 85, 114).

Acute decompensated heart failure

Initially the compensatory mechanism serve to restore tissue perfusion, and oxygen and nutrient delivery in order to meet metabolic demands of the body (73). In the long run ventricular hypertrophy may progress to ventricular dilation and impaired contractility, while activation of neurohormonal systems may cause congestion and edema (73). In addition, prolonged activation of the SNS and the RAAS causes myocardial fibrosis, impaired relaxation and cell death, negatively affecting cardiac performance (1, 29, 32, 33). Further, reduced capillary density and blood flow of the hypertrophic cardiac muscle (13, 59), as well as fibrosis which produces a physical diffusion barrier for delivery

of nutrients and oxygen, is believed to reduce oxygen supply and result in myocardial dysfunction (13, 29). Thus prolonged activation of the compensatory mechanisms is responsible for worsening cardiac function and may result in decompensation (73, 119). Decompensation occurs when the compensatory mechanisms become inadequate for the heart to balance venous return and cardiac output, and patients present with rapidly progressing heart failure (64, 81). Acute decompensation of heart failure is defined as a syndrome characterized by sudden or gradual onset of heart failure symptoms, such as dyspnea and congestion, which rapidly escalates and requires urgent treatment (93). Acute decompensated heart failure is a complex syndrome associated with high mortality and a pathophysiology not completely understood (2, 46, 88, 106, 110).

Cross-talk between the kidney and the heart

Given the kidneys role in regulating blood volume and arterial pressure, it is understandable that there is a constant crosstalk between the heart and the kidneys. Under normal conditions, the kidneys receive approximately 25% of the CO. During heart failure renal blood flow is reduced approximately by 70% and glomerular filtration rate approximately by 35% (4). Activation of the compensatory mechanisms during heart failure has a deleterious effect on renal function (117). Increased activity of both the SNS and RAAS reduce renal blood flow and increase tubular sodium reabsorption, which in turn increases oxygen consumption and may through hypoxia cause acute kidney injury (49, 64). Indeed, a certain degree of renal dysfunction has been observed in majority of heart failure patients (91), and is believed to be a cause of neurohormonal activation as well as increased oxidative stress and inflammation (2, 47, 72, 106, 142). Coexistence of renal and heart failure, referred to as cardiorenal syndrome, is a major contributor to increased mortality (34, 58, 117). The natriuretic peptides, atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP), are produced in response to myocardial stretch and provide the most important counterregulatory system in situations of fluid overload. ANP and BNP stimulate sodium and water excretion in the kidneys, in an attempt to reduce blood volume and fluid overload. However, during heart failure the response to the natriuretic peptides in the kidney is blunted, resulting in stronger response to the SNS and RAAS and therefore sodium and water reabsorption becomes the dominant process (64, 76).

Cross-talk between the heart and the vessels

Arterial resistance in pulmonary and systemic circulation contributes to afterload and therefore has large effect on cardiac function. A vessel constitutes of

three layers; the tunica intima, a monolayer of endothelial cells located closest to the lumen of the vessel; the tunica media, the middle muscular layer constituting of smooth muscle cells; and the tunica adventitia, a connective tissue layer surrounding the vessel. The arteries in the pulmonary circulation have thinner muscular layer because they are exposed to a lower pressure compared to the systemic circulation arteries (22, 50). Noradrenaline, a major player of SNS acts directly on the muscular layer to stimulate contraction and induce vasoconstriction (50). AngII on the other hand may induce vasoconstriction by direct interaction with smooth muscle cells or by release of endothelin-1 which in turn causes vasoconstriction (23, 38, 60, 138). Acetylcholine, is a neurotransmitter which stimulates vasodilatation by acting on the endothelial cells to release nitric oxide which in turn causes smooth muscle cells to relax (50). It is the balance between vasoconstriction and vasodilatation that determines vascular tone and consequently circulatory pressure. In heart failure, endothelial dysfunction plays a central role in disease development and progression (89). Endothelial dysfunction is characterized by reduced endothelial production of vasodilators resulting in an imbalance between relaxing and constricting factors and consequentially vasoconstriction. Endothelial dysfunction of coronary vessels has been connected to reduced blood flow and oxygen delivery of myocardium which in turn accelerates cell death and the progress to decompensation (89, 95, 111). Further, endothelial dysfunction of systemic and pulmonary arteries results in higher resistance and workload for the heart (89, 98, 139).

The effect of angiotensin II in heart failure-beyond sodium and water retention

AngII exerts most of its known functions by binding to the AngII type 1 receptor (AT1R) or AngII type 2 receptor (AT2R) (28, 145). AT1R are responsible for the effect of AngII resulting in vasoconstriction and sodium and water retention (26, 55). Signaling through AT2R is believed to mainly counteract the effects of AT1R signaling (77, 118, 130). Besides the well-known effects of AngII it is also involved in regulation of cell growth, cell death and inflammation (151), all three processes highly involved in heart failure. AngII increases cell growth which causes both hypertrophy and fibrosis (61, 62, 118), and induces apoptosis of cardiomyocytes (19, 67, 84, 123, 151) and may therefore be involved in the decompensation of heart failure. Further, AngII triggers inflammatory processes in the heart failure by activating tumor necrosis factor α and nuclear factor κB, as well as increasing production of endothelial chemokines (92, 129, 144, 151) .

Dietary sodium intake in heart failure

Patients with heart failure are recommended to restrict their sodium intake, because excessive sodium in the diet is associated with fluid retention (20, 54, 112). As previously noted, fluid overload is associated with worse outcomes in heart failure. However, in several studies restriction of sodium intake has not been shown to improve outcomes in patients with heart failure (54). Indeed in some studies sodium restriction was associated with higher mortality. One reason may be that dietary sodium restriction reduces cardiac output which further increase SNS and RAAS activity (24, 27, 94).

Oxidative stress in heart failure

Reactive oxygen species (ROS) are unstable substances formed by reduction of molecular oxygen. They react quickly with surrounding molecules and may directly damage proteins, lipids and DNA. Increased production of ROS have been linked to progression of several diseases, including heart failure. The most common ROS are the superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicas ($OH^{\bullet}$). These molecules are produced as a byproduct of ATP production in the mitochondrion, but can also be produced by NAD(P)H oxidases and NOS uncoupling (151). Recent evidence have shown that reactive oxygen species play an important role in normal physiological cell signaling, such as gene expression, cell growth, apoptosis and oxygen sensing (10). Activity of many cellular proteins is regulated by reduction and oxidation of their thiol groups, making ROS a regulator of cellular pathways (151). Further, ROS are also involved in inflammation and host defense against microbes. There are several antioxidant defense mechanisms, including superoxide dismutase, catalase and glutathione peroxidase, which serve to neutralize the reactive oxygen species and prevent oxidative stress (152).

ROS are increased both in humans and animals during heart failure (137), and have been implicated in several processes that contribute to heart failure development, such as vasoconstriction, cardiac hypertrophy, apoptosis, fibrosis and inflammation (71, 128). However, reduction of ROS with antioxidant treatment has not demonstrated a clear beneficial effect on heart failure (135, 151). Contrary, studies have shown that overexpression of molecules producing ROS can have protective effects in mouse hearts subjected to pressure overload (153). Further, we have shown that treatment with the antioxidant N-acetylcysteine increases mortality in AngII and high-salt diet treated mice (65). The explanation to why antioxidant treatment has not been proven beneficial in heart failure, may be because the antioxidant treatment inhibits both the beneficial and detrimental ROS signaling (151).

Genetics of heart failure

The human genome consists of approximately 20 000-25 000 genes which encode different proteins. The proteins in turn are responsible for dictating all processes related to cellular function. Genes consist of protein coding regions called exons and non-coding regions called introns. The genome contains millions of genetic variants called (SNPs), believed to be responsible for large parts of individual differences, such as curly hair, obesity, drug response and susceptibility to diseases. An SNP is thus only one nucleotide change in one single position which may be non-synonymous (change the encoded amino acid) or synonymous (silent). Non-synonymous SNPs may in turn affect the function of the encoded protein, resulting in gain of function or loss of function. Further, SNPs localized to the non-coding regions, may instead affect binding of transcription factors and therefore gene expression or splicing of the mRNA which may result in production of non-functional protein (87, 121). Heart failure is a complex disease interacting both with the genetic background of the individual as well as the environment. Most forms of heart failure are multifactorial, meaning that interaction of multiple genes are necessary to develop a disease. Thus, the size effect of an individual variant is small, but an interaction of several variants results in disease development (5, 14, 57, 125). Even though multiple genes are responsible for disease development, Heart failure is largely heritable where the offspring of individuals with heart failure has 70% higher risk of developing heart failure themselves (80, 80, 82, 125, 127).

Genetics of different mouse strains

For several decades inbred mouse strains have been of significant importance in biomedical research. The main reason for this is the genetic homogeneity and stability of a mouse strain which allows for well controlled and reproducible research experiments, as well as the possibility of genetic engineering (9, 21). The inbred mouse strain is homozygous for at least 99% of their genes and all mice within a strain are genetically identical. However, what makes inbred mouse strains so attractive also causes a genetic difference between the strains which may affect their susceptibility to certain diseases (9). The naturally occurring genetic variations in a mouse strain are of special interest because they are believed to be more similar to the subtle variations responsible for human disease (99). This strain difference in susceptibility to a disease has previously been used to identify quantitative trait loci (QTL) and genes associated with disease pathogenesis (9, 12, 31, 79, 131, 132). In this thesis, C57BL/6J and Balb/CJ mice are used to study genetic susceptibility to acute decompensated heart failure. These two strains are widely used inbred mouse strains shown to be genetically very diverse (74). The genetic difference is

evident from the studies in this thesis as well as other studies where these two strains have shown different susceptibility both to infectious diseases and development of diabetes mellitus (42, 44, 63).

Aims

We have observed that Balb/CJ and C57BL/6J mice react completely differently to combined treatment with AngII and high-salt diet (AngII+Salt). In Balb/CJ mice AngII+Salt induces massive edema, anuria and high mortality. In C57BL/6J mice AngII+Salt induces hypertension, with absence of edema formation and most importantly the high mortality. The overall aim of papers presented in this thesis was to identify the physiological and genetic causes contributing to the high mortality in Balb/CJ. The specific aims of each paper are presented below.

Paper I

- To study the effect of AngII+Salt on cardiac function, blood pressure and sodium and fluid balance in Balb/CJ and C57BL/6J mice.

Paper II

- To study the effect of AngII+Salt on cardiac gene expression in Balb/CJ and C57BL/6J, and to verify the findings.

- To investigate the individual effects of AngII and Salt on cardiac gene expression and function.

Paper III

- To identify QTLs linked to AngII+Salt induced response in Balb/CJ.

Paper IV

- To study the effect of AngII on isolated pulmonary vessels from Balb/CJ and C57BL/6J mice.

Materials and methods

Animals

Male Balb/CJ and C57BL/6J mice were used in Paper I, Paper II and Paper IV. Both strains are healthy inbred mouse strains widely used in preclinical research. In Paper II, male first filial generation (F1) and male second filial generation (F2) mice were used. The F1 mice were derived from a cross between Balb/CJ and C57BL/6J. Since F1 mice inherit one gene from each parent, they are heterozygous for all their alleles and all individuals exhibit same phenotype. F2 mice were derived from a backcross between Balb/CJ and F1 mice. The F2 mice have larger genetic variation and as a consequence exhibit a spectrum of different phenotypes. Further, the large genetic and phenotypic variation enables us to link a chromosome region to a specific phenotype. Two different types of F2 cross can be used (Figure 3), a backcross or a balanced intercross. A balanced intercross is derived from a cross between two F1 animals and generates larger genetic variation than a backcross. An intercross displays three genotypes for an allele while a backcross displays only two. In linkage analysis where a chromosome region is connected to a phenotype, the balanced intercross is beneficial since it results in a smaller region containing lower number of candidate genes compared to a backcross. However, a balanced intercross requires a larger number of animals to obtain enough animals that display the phenotype of interest. A backcross would be more often used if the phenotype of interest does not show strong penetrance in the F1 generation, while an intercross would be used with a phenotype that does show strong penetrance.

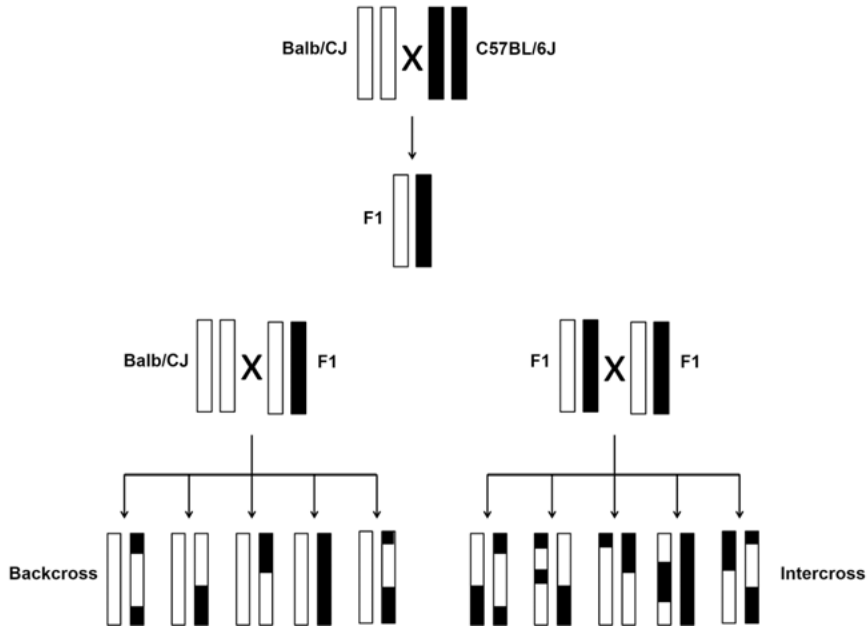

Figure 3. Genotype variation in first filial generation and second filial generation derived from a backcross or an intercross.

Treatments

The animals were treated with angiotensin II (AngII), high-salt diet (Salt), a combination of angiotensin II and high-salt diet (AngII+Salt) or a combination of angiotensin II, high-salt diet and the antioxidant N-acetylcysteine (AngII+Salt+NAc). Angiotensin II was infused using osmotic minipumps. These pumps are miniature infusion pumps that are implanted subcutaneously. They release a substance in a continuous manner ensuring constant plasma levels of the substance. Angiotensin II is often used to induce hypertension in experimental animals. The high-salt diet contained 3% sodium in all experiments except the preliminary experiment in Paper I where sodium content was 4%. High-salt diet can also be used to induce hypertension in salt sensitive experimental animals. Combination of AngII and high-salt diet accelerates the development of hypertension and aggravates renal and vascular lesions more than single treatment (39). Control animals received standard pelleted food and regular tap water. N-acetylcysteine (NAc) was administered in the drinking water. N-acetylcysteine works as an antioxidant by stimulating synthesis of the endogenous antioxidant glutathione and by direct neutralization of ROS (7, 11, 152).

Paper I:	Control and AngII+Salt
Paper II:	Control, AngII, Salt, AngII+Salt, AngII+Salt+NAc

Paper II: Control, AngII+Salt
Paper IV: Control

Experimental design

Paper I

In the preliminary study Balb/CJ and C57BL/6J were treated as controls or with AngII+Salt for five weeks. Tail-cuff blood pressure was measured at baseline and five weeks after starting the treatment or just before sacrifice in edematous mice.

In the follow-up study Balb/CJ and C57BL/6J were treated with AngII+Salt for seven days. Sodium and fluid balance was measured with metabolic cages, blood pressure with tail-cuff plethysmography and cardiac function with echocardiography (Figure 4). Blood pressure and cardiac function were measured at baseline and every day during the seven day treatment period. Sodium and fluid balance was measured at baseline, and during treatment when one of following criteria were fulfilled: (1) >20% reduction in cardiac output estimated with echocardiography, (2) clinical symptoms of decompensation are present, mainly development of edema. To balance the groups, every time a Balb/CJ mouse was put in a metabolic cage a randomly picked C57BL/6J mouse was also put in a metabolic cage. For same reason, every time a Balb/CJ mouse died or had to be sacrificed, a randomly picked C57BL/6J mouse was sacrificed.

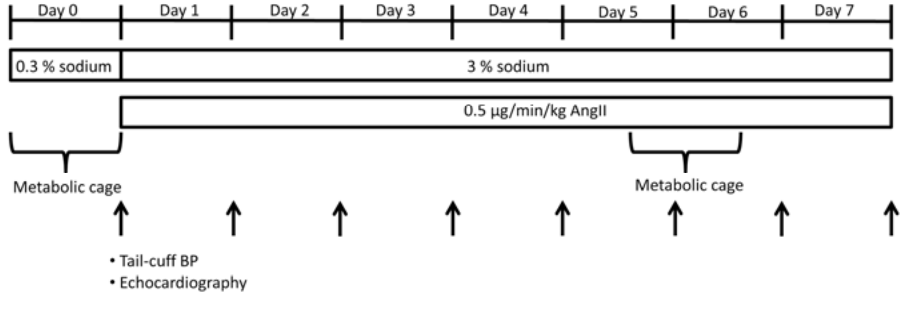

Figure 4. Experimental design of the follow-up study in Paper I. BP = Blood pressure.

Paper II

Echocardiography was performed at baseline and on the fourth day of treatment with AngII, Salt, AngII+Salt or AngII+Salt+NAc. Cardiac tissue for microarray was collected on fourth day of treatment in controls, AngII, Salt, and AngII+Salt treated animals. Controls in the microarray received osmotic minipums filled with saline instead of Ang II to control for the effect of surgery on gene expression.

Paper III

Sodium and fluid balance was measured with metabolic cages, blood pressure with tail-cuff plethysmography, renal function by conscious glomerular filtration rate measurement, and cardiac function with echocardiography. These measurements were performed at baseline and on the fourth day of treatment with AngII+Salt (Figure 5).

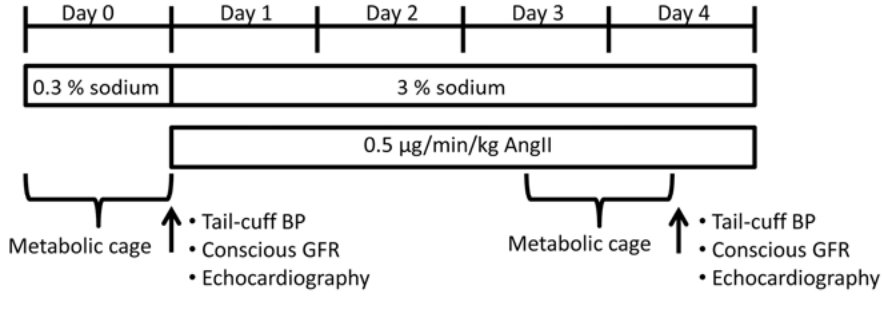

Figure 5. Experimental design in Paper III. BP = Blood pressure. GFR = Glomerular filtration rate

Paper IV

The animals were anaesthetized with isoflurane and sacrificed by cervical dislocation. Thereafter the lungs were collected for isolation of pulmonary resistance vessels and *in vitro* experiments.

Echocardiography (Paper I, II and III)

In this thesis cardiac function was measured using echocardiography, a method that uses ultrasound to image the heart. It is a well-established method for evaluation of cardiac function both in clinical and preclinical setting. Echocardiography relies on reflected pulses of sound to localize different parts of the heart. Since the mouse heart is small and beats 400-600 per minute, utilization of high frequency ultrasound is necessary to obtain high resolution images. High frequency ultrasound results in high-resolution images but has limited depth of penetration (102, 122). However, in a mouse, this is not a problem since penetration of more than 1.5-2 cm is not necessary.

CO, SV and ejection fraction (EF) were quantified in parasternal long-axis view. By measuring the left ventricle area in end-diastole and end-systole, SV, CO and EF can be calculated. SV is calculated by subtracting ESV from EDV (Figure 6). CO is calculated by multiplying heart rate (HR) with SV. EF equals (EDV-ESV)/EDV. CO is used as a measurement of global cardiac function and EF is used as a measurement of cardiac contractility. Reduced EF indicates systolic dysfunction.

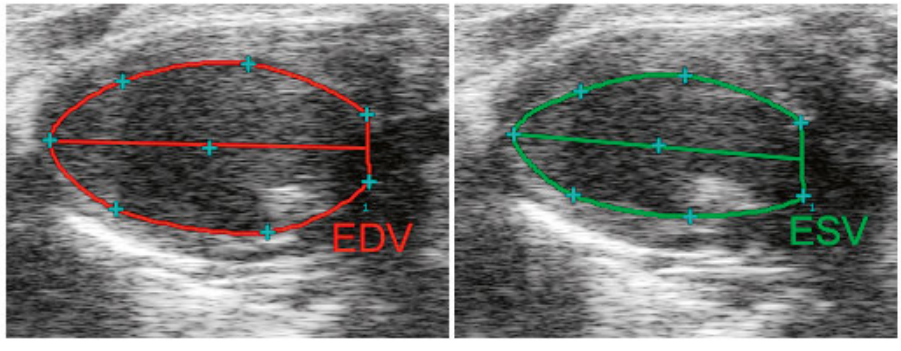

Figure 6. End-diastolic volume (EDV) and end-systolic volume (ESV) in parasternal long-axis view.

Pulsed wave Doppler was used to assess mitral valve flow and pulmonary artery flow. Pulsed wave Doppler is able to detect moving objects since moving objects change the characteristic of sound waves. The mitral valve flow measures the flow between left atria and ventricle, and is used to measure diastolic function and thereby filling of left ventricle. Mitral valve flow is characterized by two peaks, early peak (Peak E) which measures passive filling of left ventricle, and late or atrial peak (Peak A) which measure filling aided by atrial contraction (Figure 7 left). Diastolic dysfunction is characterized by reduction in Peak E and increase in Peak A, resulting in decreased E/A ratio.

Isovloumic relaxation time (IVRT) can also be estimated from the mitral valve flow (Figure 7 left). In this thesis reduced peak E or E/A ratio together with increased IVRT was used to define diastolic dysfunction.

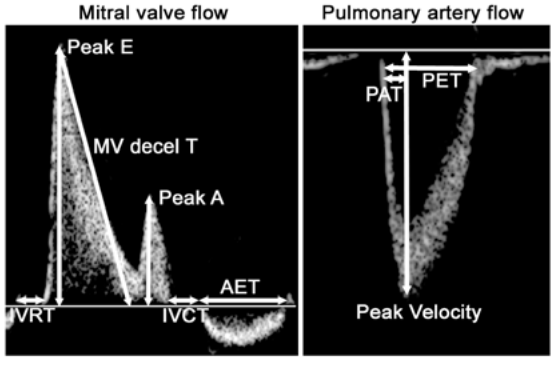

Figure 7. Pulsed wave Doppler of mitral valve flow and pulmonary artery flow.

Pulmonary artery flow was measured in parasternal long-axis view at the right ventricle outflow tract. Pulmonary artery flow is characterized by one peak divided in acceleration time and ejection time (Figure 7 right). Acceleration time is the time from start of ejection to peak velocity, and ejection time is the whole time during which ejection occurs. When pulmonary artery flow is restricted as in cases of high pulmonary pressure, acceleration time is reduced and ejection time may be increased, resulting in reduced acceleration time to ejection time ratio (AT/ET).

Cardiac wall thickness is used as a measurement of remodeling and hypertrophy. Left ventricle posterior wall thickness can be estimated from an M-mode (motion mode) (Figure 8). M-mode is used to detect moving structures in the body. A single beam of ultrasound produces a one-dimensional picture of the moving structure showed in a wave-like manner (96). An M-mode can be thought of as a cross section of the heart at the beam position.

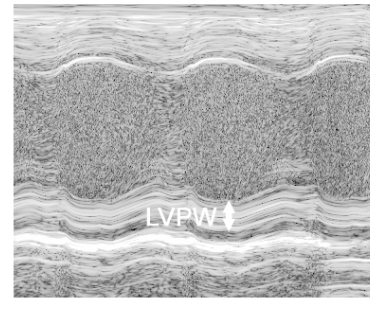

Figure 8. M-mode of the left ventricle. The white arrow represents left ventricle posterior wall (LVPW) thickness.

Tail-cuff blood pressure (Paper I and III)

Blood pressure was measured using tail-cuff plethysmography. The method works similar to bed side blood pressure where a cuff is inflated around the arm of the patient to stop the blood flow and then released until the first pulse is detected bellow the cuff (Figure 9). In mice, instead of pulse detection volume pressure detection is used. The method works by first pushing back the blood from the tail using an occlusion cuff and volume pressure sensor cuff, and then slowly releasing the blood back into the tail. The volume of the blood returning to the tail is detected with the volume pressure sensor cuff. The pressure at which the tail blood volume starts to increase is systolic blood pressure. The pressure at which the blood flow in and out of the tail is equal represents diastolic blood pressure (37). It is important to acclimatize the animals to a temperature of 32-35 ºC to increase blood flow to the tail, and not deviate from the temperature to have reliable measurements. Further, animals are restrained during the procedure which causes stress and increased blood pressure. Therefore the animals should be trained two to three days before starting collection of data. In papers included in this thesis, animals were not trained; however, they were allowed to acclimatize to the restraining tube covered with a dark blanket for 10-15 minutes before starting blood pressure measurements. Further, first three measurements were considered acclimatization measurements and were always discarded. Five following measurements were averaged to obtain systolic and diastolic blood pressure, and heart rate.

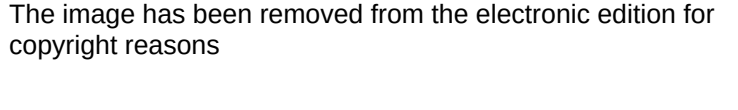

Figure 9. Tail-cuff blood pressure setup.

Conscious GFR (Paper III)

GFR is under tight autoregulation to allow the kidneys to maintain control of water and salt balance. It is considered to be the optimal measurement of kidney function. In this thesis GFR was measured by inulin clearance. It is considered the gold standard method of GFR measurement. Inulin is a polysaccharide that is freely filtered in the kidneys, and is not reabsorbed or secreted in the tubules. Labeled inulin is injected into the tail-vein of the mouse and

repeated blood samples are taken over period of time. As kidneys clear the plasma from inulin, plasma concentration of the polysaccharide decreases. Plasma concentration of inulin is plotted over time and area under curve is calculated. GFR equals clearance of inulin which can be calculated using the formula $Dose_{inulin}/AUC$.

Metabolic cages (Paper I and III)

Sodium and fluid balance was measured using metabolic cages. A metabolic cage is divided into two parts, an upper part where the animal is kept, and a lower part where faeces and urine is collected. To get a good measurement of sodium and urine excretion, urine is collected for 24 hour. Sodium content in urine is measured and multiplied by urine volume to obtain 24 hour sodium excretion. During the time in metabolic cages, food and water intake is measured and related to sodium and urine excretion.

Lung water content and heart weight (Paper I)

Lung edema is a common finding in heart failure. Lung water content was measured by drying the lungs and calculating water content in the original tissue. Size of atria is increased in cases of increased filling pressures and wall thickness of ventricles in cases of ventricular hypertrophy. In this thesis weight of left and right atria, and left and right ventricle was used to see if the size of atria and wall thickness was increased. Heart weight was corrected for tibia length because size of the heart is dependent on size of the animal. In heart failure, weight is not corrected for because of edema development and muscle wasting.

Oxidative stress (Paper III)

Since ROS are highly reactive they are difficult to measure in a direct manner. Therefore most often indirect measurement methods of ROS are used, such as 8-isoprostane and thiobarbutiric acid reactive substances (TBARS). In this thesis TBARS was used as a biomarker of oxidative stress. TBARS are formed as byproducts of lipid peroxidation, a process in which ROS react with fatty acids. In most TBARS assays malondialdehyde is measured as was the case in this thesis. Malondialdehyde gives an indication of the amount of oxidative stress, however it is not the only byproduct of lipid peroxidation, nor is it produced specifically by lipid peroxidation. Therefore, 8-isoprostane is a better measurement, but it requires stabilization of 8-isoprostane during storage which should be planned for before starting data collection.

Microarray analysis (Paper II)

Gene expression can be used to understand changes in organ or tissue function. Gene expression starts with transcription of the gene to mRNA which is then translated to the protein. The proteins in turn are responsible for dictating all cellular processes. The idea is that the higher level of a certain protein the more effect it will have on cellular function. However not all mRNA is transcribed and the protein function can be regulated by posttranslational modification such as phosphorylation or reduction and oxidation as previously mentioned in the context of oxidative stress. Microarrays used in Paper II measure expression of thousands of genes at the same time and allows us to get a global picture of what is happening in that tissue. Instead of looking at only one or few genes, gene expression data from microarrays may be used to look at molecular pathways and see if any pathways is more upregulated in a condition of interest. The method is based on using beads conjugated to specific oligonucleotides, and thousands of copies of the oligonucleotide are bound to the beads. RNA is isolated from the tissue and converted to biotin labeled cRNA. The cRNA is hybridized to the beads and the biotin is fluorescently stained. If the gene is highly expressed, more cRNA will be present in the samples resulting in more cRNA binding to the beads. Consequently, more biotin will be labeled resulting in higher intensity of the signal. Thus, genes that are highly expressed will result in a stronger signal than genes that are lowly expressed.

Quantitative trait locus analysis (Paper III)

In linkage studies we are trying to connect a specific part of the chromosome to a phenotype of interest, to find candidate genes that are important for expressing a phenotype. To be able to perform linkage analysis you need to have two organisms expressing different phenotypes, for example one mouse strain that develops heart failure and another mouse strain that does not develop heart failure. These two mouse strains are crossed to increase the genetic variation and consequently the he phenotypic variation of your animals. In paper III we used a second generation backcross to increase the genetic variation. The animals are phenotyped and the genome sequenced using genetic markers. In paper III, known common SNPs in the mouse were used to genotype the animals. QTL analysis is a statistical method that links the phenotype to a genotype. For each marker a probability score is calculated, which tells us how large the probability is that molecular marker is correlated to a phenotype. These probability scores, often expressed as log of odds (LOD) score are plotted on the y-axis and chromosome region on the x-axis to obtain a QTL Manhattan plot. High peaks indicate a larger probability that the marker is linked to the phenotype of interest. A LOD score of three would mean that there is 1000 higher risk to develop a disease if you have a certain marker. Usually, a

95% confidence interval is calculated for the top marker to estimate a chromosome region, also called a QTL that is linked to a phenotype. These QTLs usually contain hundreds of genes and any of these genes could be connected to the phenotype. Gene expression can be combined with the QTL-analysis to narrow down number of candidate genes.

Immunohistochemistry (Paper III)

Immunohistochemistry allows us to visualize a protein in the tissue using antibodies which selectively bind to the protein of interest. In Paper III, a DAB (3,3'-diaminobenzidine) based method was used. The method is based on two steps, first localization of the protein using a primary antibody, and second, localization of the primary antibody using horseradish peroxidase (HRP) conjugated secondary antibody. HRP is an enzyme that catalyzes the oxidation of DAB by hydrogen peroxide. Oxidized DAB forms a brown precipitate at the location of secondary antibody resulting in brown coloring of the tissue.

Wire myography (Paper IV)

Paper IV is solely based on wire myography, an *in vitro* method invaluable for studying vascular response to contracting and relaxing agents in isolated vessels. A vessel is mounted on two wires which are attached to the jaws of the myograph (Figure 10). One of the jaws is attached to a force transducer while the other is attached to an adjustable arm controlled by a micrometer. Prior to starting the experiments vessels are normalized to an internal circumference at which the vessels are fully relaxed and have the same transmural pressure as the circulation from which they are isolated. For instance, vessels from systemic circulation would be normalized to a transmural pressure of 100 mmHg and vessels from the pulmonary circulation would be normalized to a transmural pressure of 15 mmHg. During the experiment cumulative increasing doses of vasocontractile or vasorelaxing factors are added to the chamber, and concentration curves are performed. A contracting vessel pulls on the wires resulting in a force that is recorded. Since the vessels are fully relaxed, they must be preconstricted before vasorelaxants are added. A relaxing vessel will then reduce the pull on the wires resulting in a lower force that is recorded.

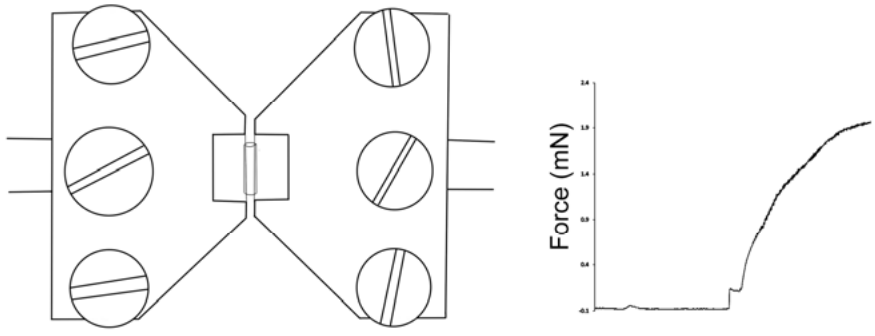

Figure 10. Schematic figure showing a vessel mounted on to wires and attached to myograph jaws (left). When the vessel contracts it pulls on the wire which is registered as force (right).

Statistical analysis

All data fulfilling the assumption of independency were analysed using two-way ANOVA and Tukey's post-hoc test. Data not fulfilling the assumption of independency (repeated sodium and fluid balance measurements, echocardiography and blood pressure measurements) were analysed with linear mixed effects model using a restricted maximum likelihood fit. Individual contrasts of least-squares means were adjusted using Tukey's method. $p<0.05$ was accepted as significant for all papers. Kaplan-Meier survival analysis was performed using death or premature sacrifice as end-point censoring animals sacrificed at experiment completion. Microarrays were analysed using empirical Bayes (eBayes) and false discovery rate (FDR). Concentration curves obtained in wire myography were analysed by calculating half maximal effective concentration (EC50) and maximal contraction and relaxation. EC50 was analysed using two-way ANOVA or Student's t-test when only two groups were compared.

Results

Paper I

In a preliminary study we observed that AngII+Salt increases mortality in Balb/CJ mice (Figure 11, left). They developed significant subcutaneous edema on the breast, forepaws and neck and had to be sacrificed. Some animals also developed ascites and many were anuric. In contrast, 90% of C57BL/6J mice survived without any symptoms (Figure 11, right).

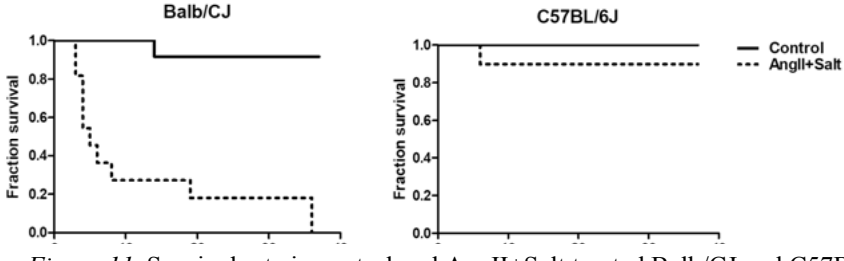

Figure 11. Survival rate in control and AngII+Salt treated Balb/CJ and C57BL/6J mice.

Considering the clinical symptoms of heart failure we hypothesized that AngII+Salt impairs cardiac function and induces cardiac remodelling in Balb/CJ but not in C57BL/6J mice. AngII+Salt increased pulmonary (Figure 12A) and systemic (Figure 12B) resistance in Balb/CJ thus increasing afterload both in right and left ventricle. Lower CO was associated with impaired filling (Figure 13C-D) but preserved EF (Figure 13B). Interestingly, Balb/CJ had lower CO even though they retained more sodium and water (Figure 14). Both strains showed same degree of hypertrophy but only Balb/CJ developed symptoms associated with decompensation. Neither strain developed pulmonary edema.

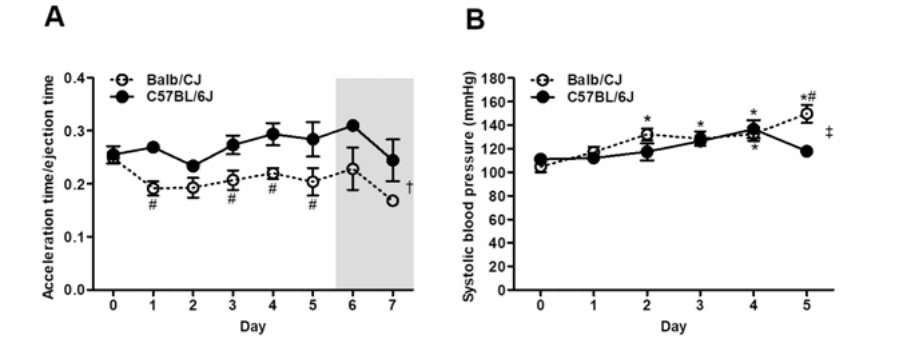

Figure 12. Acceleration time to ejection time ratio (A) and systolic blood pressure (B) in Balb/CJ and C57BL/6J at baseline (Day 0) and over a period of 5-7 days during AngII+Salt treatment (Day 1-7). Because of low power animals after day 5 were not included in statistical analysis. In the graphs this period is colored in grey. Day 6 and 7 for systolic pressure are not shown because of missing data. * indicates $p < 0.05$ treatment vs. control within strain. # indicates $p < 0.05$ between strains with same treatment. † indicates significant strain difference and ‡ indicates significant interaction of time and strain.

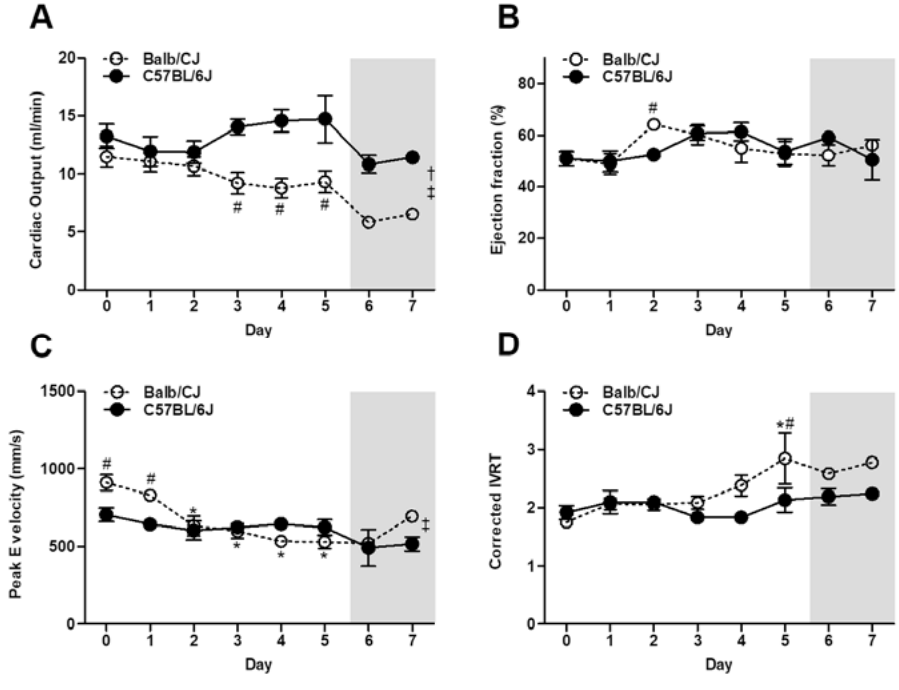

Figure 13. Cardiac output (A), ejection fraction (B), peak E velocity (C) and corrected isovolumic relaxation time (IVRT, D) in Balb/CJ and C57BL/6J at baseline (Day 0) and during AngII+Salt treatment (Day 1-7). Because of low power animals after day 5 were not included in statistical analysis. In the graphs this period is colored in grey. * indicates $p < 0.05$ treatment vs. control within strains. # indicates $p < 0.05$ between strains with same treatment. † indicates significant strain difference and ‡ indicates significant interaction of time and strain.

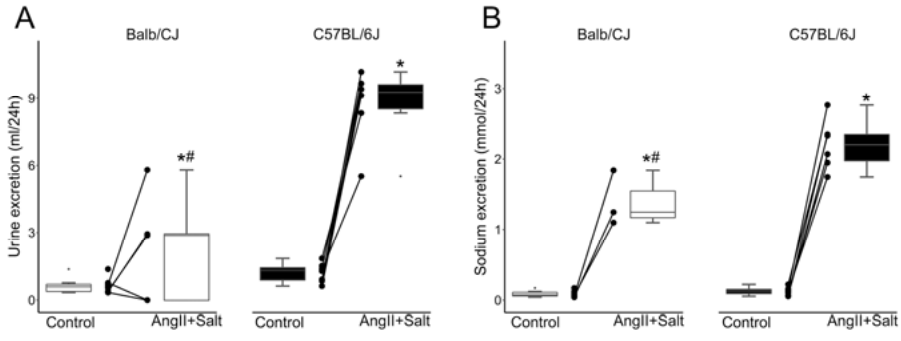

Figure 14. Urine (A) and sodium excretion (B) in control and AngII+Salt treated Balb/CJ and C57BL/6J. * indicates $p < 0.05$ treatment vs. control within strains. # indicates $p < 0.05$ between strains with same treatment.

Paper II

Gene expression in the heart was measured using microarrays. AngII+Salt treatment had stronger effect on gene expression in Balb/CJ than C57BL/6J. In hierarchical clustering of significant differentially expressed genes in Sham vs. AngII+Salt treated animals, AngII and AngII+Salt treated Balb/CJ clustered together indicating that the cardiac gene expression is regulated mainly by AngII (Figure 15). Salt alone had very little effect on cardiac function and cardiac gene expression. Gene ontology analysis indicated that the glutathione transferase system is differentially regulated in Balb/CJ and C57BL/6J mice and that oxidative stress may be involved in the pathogenesis. In a previous study we showed that oxidative stress is lower in AngII+Salt treated Balb/CJ than C57BL/6J mice, and that reducing oxidative stress increases sodium and fluid reabsorption and increases mortality in C57BL/6J (65). In paper II we showed that treatment with the antioxidant NAc in addition to AngII+Salt worsened cardiac function both in Balb/CJ and C57BL/6J mice (Figure 16), which may partially explain the increased mortality seen by Jönsson et al. (65).

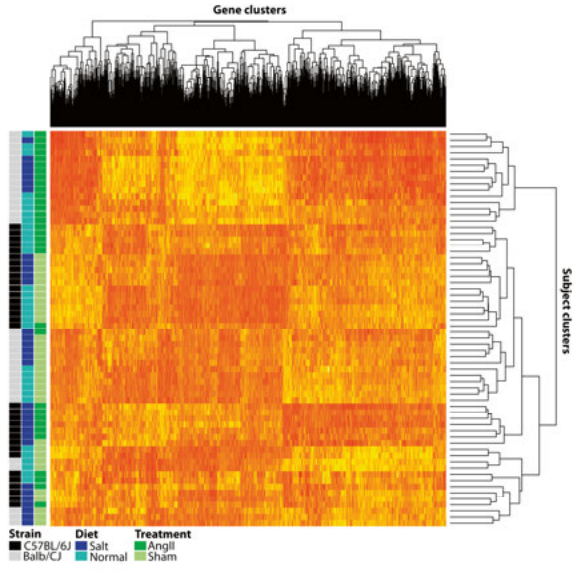

Figure 15. Hierarchical clustering of significant differentially expressed genes in Sham vs. AngII+Salt treated animals.

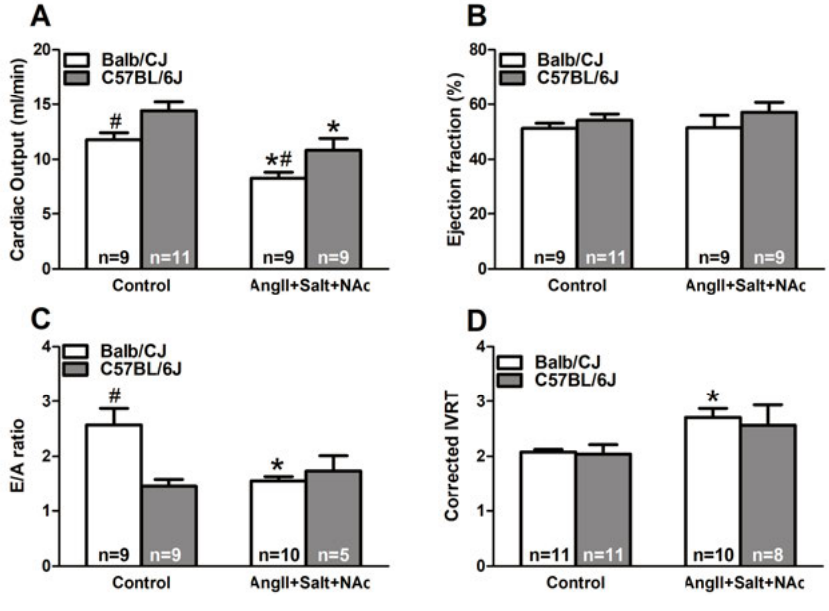

Figure 16. Cardiac output (A), ejection fraction (B), E/A ratio (C) and isovolumic relaxation time corrected for heart rate (E). * indicates $p < 0.05$ treatment vs. control within strains. # indicates $p < 0.05$ between strains with same treatment.

Paper III

Linkage study was performed to identify QTLs associated with AngII+Salt induced decompensation in Balb/CJ mice. CO was linked to a QTL on chromosome 3 (Figure 17) and IVRT was linked to a QTL on chromosome 12 (Figure 18), indicating that AngII+Salt induced cardiac impairment in Balb/CJ is genetically predisposed. QTL on chromosome 3 contained 294 protein-coding genes of which Foxo1 (forkhead box protein O1) was the only gene that matched a previously known coding non-synonymous SNP that differed between Balb/CJ and C57BL/6J in the mouse genome database. To validate the difference in Foxo1 activation between Balb/CJ and C57BL/6J immunohistochemical staining of heart sections was performed. Higher percentage of nuclei were stained in C57BL/6J after AngII+Salt than in Balb/CJ indicating a translocation of Foxo1 from the cytoplasm to the nuclei after AngII+Salt treatment in C57BL/6J but not in Balb/CJ (Figure 19). QTL on chromosome 12 contained 298 protein-coding genes of which Ahr (aryl-hydrocarbon receptor) was the only gene that matched a previously known coding non-synonymous SNP that differed between Balb/CJ and C57BL/6J in the mouse genome database.

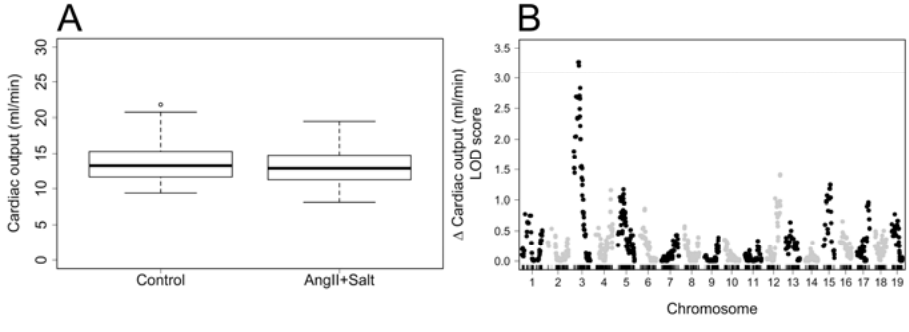

Figure 17. Cardiac output (A) and LOD score for delta cardiac output (B) in F2 mice.

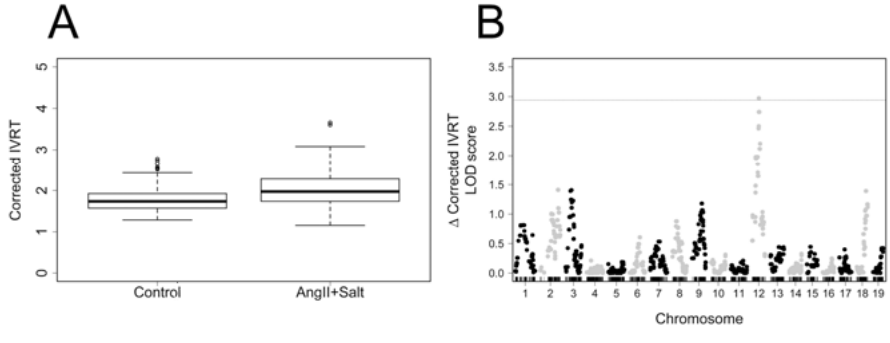

Figure 18. Isovolumic relaxation time (IVRT) corrected for heart rate (A) and LOD score for delta corrected IVRT (B) in F2 mice.

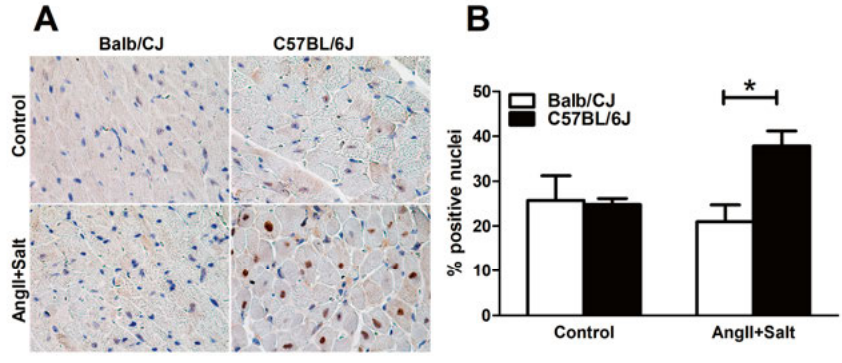

Figure 19. Foxo1 staining in cardiac sections from controls and AngII+Salt treated Balb/CJ and C57BL/6J.

Further, a QTL on chromosome 3 was linked to urine excretion (Figure 20), and QTLs on chromosome 2 and 3 were linked to sodium excretion (Figure 21). The QTL on chromosome 3, linked to urine excretion, contained 130 protein-coding genes with no genes matching previously known coding non-synonymous SNPs that differ between Balb/CJ and C57BL/6J in the mouse genome database. The QTL on chromosome 2, linked to sodium excretion, contained 1513 protein-coding genes of which Serping1 (serine peptidase inhibitor, clade G, member 1), Thbs (thrombospondin 1), Fbn1 (fibrillin 1) and Adam33 (a disintegrin and metallopeptidase domain 33) matched previously known coding non-synonymous SNP that differed between Balb/CJ and C57BL/6J in the mouse genome database. Several QTLs linked to AngII+Salt induced decompensation indicates that this is a multifaceted phenotype.

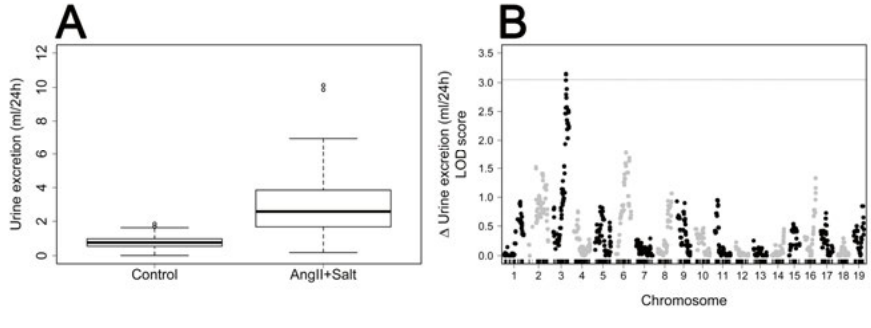

Figure 20. Urine excretion (A) and LOD score for delta urine excretion (B) in F2 mice.

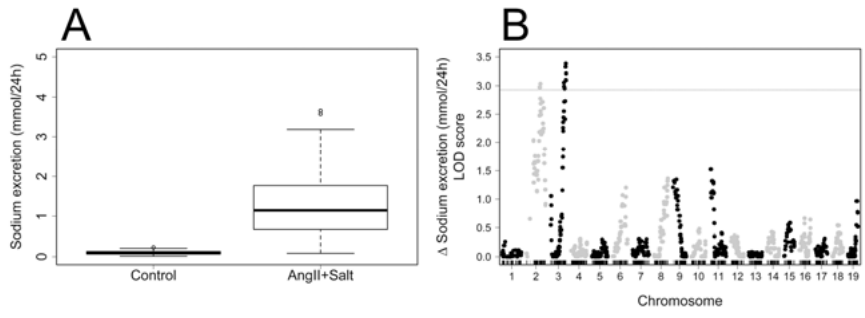

Figure 21. Sodium excretion (A) and LOD score for delta sodium excretion (B) in F2 mice.

Paper IV

AngII induced stronger contraction of pulmonary resistance vessels from Balb/CJ than C57BL/6J (Figure 22A). AngII stimulated contraction in Balb/CJ was dependent on release of a secondary substance. This was evident as transfer of bath solution from AngII stimulated Balb/CJ vessels to previously AngII non-responding untreated C57BL/6J vessels resulted in a vascular contraction that was equal to that observed in Balb/CJ (Figure 22B). Contrary, AngII directly added to C57BL7/6J vessels did not result in a contraction equal to that of Balb/CJ vessels. AngII also potentiated the effect of norepinephrine on Balb/CJ vessels by increasing the maximal contraction (Figure 22C). AngII did not affect the response of C57BL/6J vessels to norepinephrine. Acetylcholine (Ach) induced lower vasorelaxation in Balb/CJ than in C57BL/6J mice indicating lower endothelial nitric oxide production in Balb/CJ (Figure 22D).

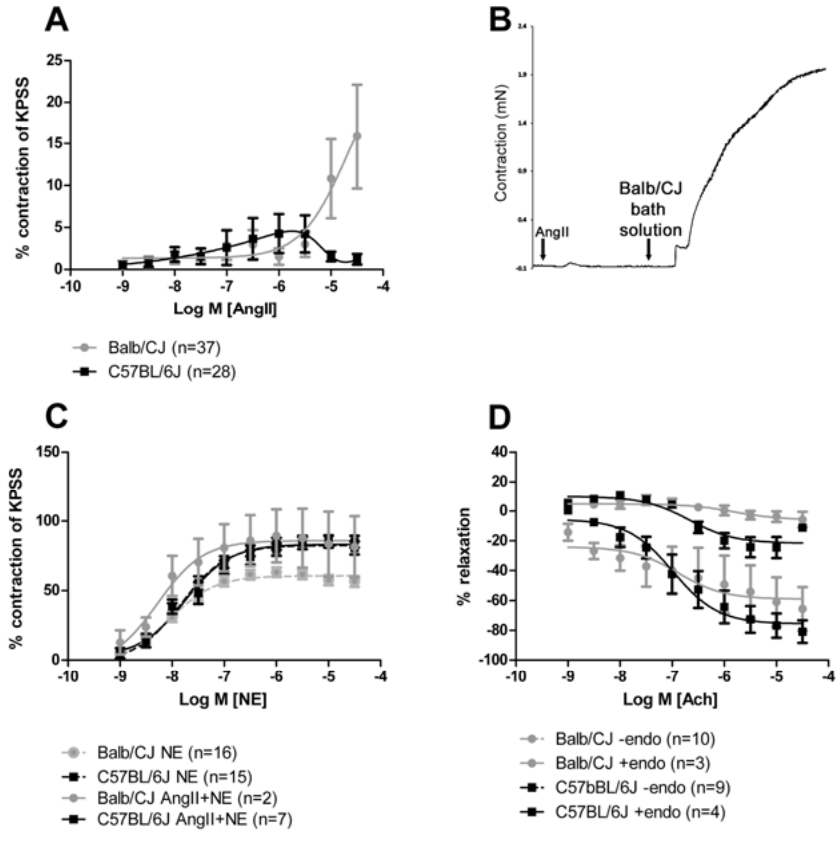

Figure 22. Response of pulmonary resistance vessels to cumulative doses of angiotensin II (A), norepinephrine (C) and acetylcholine (D). B shows response of a C57BL/6J pulmonary resistance vessel to angiotensin II and the same vessel responding to transfer of bath solution from AngII stimulated Balb/CJ vessel.

Discussion

The overall aim of this thesis was to identify the physiological and genetic causes contributing to AngII+Salt induced mortality in Balb/CJ. We hypothesized that AngII+Salt impairs cardiac function and induces remodeling in Balb/CJ but not C57BL/6J mice. Interestingly, AngII+Salt induced cardiac remodeling to the same extent in both strains, but only Balb/CJ showed impaired cardiac function. Impaired cardiac function was linked to higher pulmonary and vascular resistance, impaired left ventricular filling, lower CO and increased sodium and water retention in Balb/CJ compared to C57BL/6J. Further, oxidative stress was shown to play a role in AngII+Salt induced decompensation and several QTLs were linked to the pathology indicating a multifaceted phenotype.

The higher systemic pressure and pulmonary artery resistance indicates increased overall responsiveness to AngII in Balb/CJ mice. Indeed, pulmonary resistance vessels were shown to be more reactive to AngII compared to vessels from C57BL/6J mice. The increase in overall responsiveness may be a result of increased expression of AT1R or decreased expression of AT2R, the latter suggested to have vasodilatory effects (120, 146). The different shape of AngII dose response curve in Balb/CJ and C57BL/6J suggests that lower expression of AT2R may be a part of the explanation. The bell-shaped dose response curve, as observed in C57BL/6J mice, has previously been observed in aorta and femoral resistance arteries (7, 13). It is believed that AT2R are responsible for the bell-shaped curve, since AngII at higher concentrations binds to the AT2R and causes relaxation (14, 17). AngII dose response curve in Balb/CJ mice was exponential indicating a difference in AT2R mediated response between the strains. However, the AngII mediated vessel contraction in Balb/CJ was dependent on release of secondary factor, since transfer of bath solution from an AngII responding Balb/CJ vessel to a C57BL/6J AngII non-responding vessel resulted in contraction as strong as the one observed in Balb/CJ. Since the released contractile factor was very potent it may have opposed the anti-contractile effect of AT2R in Balb/CJ. Further, release of the contractile factor from pulmonary vessels makes it possible that same contractile factor is released from systemic arteries and coronary arteries which may affect both vascular and cardiac function. Future studies should concentrate on identifying the contractile factor and its effect on cardiac function.

Balb/CJ mice showed impaired left ventricular filling with preserved ejection fraction during AngII+Salt treatment. Considering the increased pulmonary vascular resistance, one may hypothesize that the right ventricle is failing and that right ventricular dilation impairs left ventricular filling because of ventricular compression (preload limitation) (3, 52). However, ultrasound images from short-axis view did not reveal right ventricular dilation. Instead it revealed a decrease in both left ventricle and right ventricle dimensions, suggesting that both left and right ventricle filling are decreased. This may be a result of AngII directly impairing left ventricular relaxation since both IVRT and peak E are decreased. A second reason may be that AngII increases vascular permeability (107, 148) resulting in decreased venous return. However, absences of pulmonary edema indicates that vascular permeability is not affected, and that reduced filling is most likely a direct effect of AngII on myocardial cells and the intrinsic capacity of cells to relax.

The increased reactivity of pulmonary resistance vessels to AngII in Balb/CJ raises the question if kidneys are more reactive to AngII as well. Moreover, AngII may stimulate aldosterone release to a bigger extent in Balb/CJ, resulting in higher sodium and water reabsorption (101), and thus making Balb/CJ a model of volume overload-induced heart failure. This is probably not the case since AngII alone stimulates sodium and water reabsorption to same extent in both strains (65), further indicating that fluid reabsorption is probably a compensatory mechanism to decreased cardiac function.

It is of interest that Balb/CJ had lower CO than C57BL/6J during AngII+Salt treatment. This was the case even though Balb/CJ had higher sodium and water retention, which should increase blood volume, venous return and CO (6, 119). It is worth noting though that CO in Balb/CJ was not decreased to an extent that could explain the high mortality. Some animals did have 50-60% decrease in CO but there is a strong survival bias, meaning that those animals that did survive and on which we measured cardiac function were also the animals in best physical condition. The sickest animals were lost to investigation. The condition of Balb/CJ mice treated with AngII+Salt deteriorate really fast, meaning that measurement of cardiac function once per day may not be enough to detect the final progression of decompensation. Therefore a continuous telemetric approach may be warranted in future studies. Further C57BL/6J are able to compensate the effect of AngII+Salt by increasing CO, suggesting that AngII+Salt increases oxygen consumption, and that CO needs to increase to meet metabolic needs of the body. This increase in CO is absent in Balb/CJ indicating that the strain is not able to compensate.

Microarray analysis showed that gene expression in Balb/CJ hearts seem to be driven by AngII in both AngII and AngII+Salt as these cluster together. Salt treatment did not affect cardiac gene expression or cardiac function in any

of the strains. However, worse cardiac function in the combination treatment compared to single treatment with AngII indicates that Salt aggravates the effect of AngII. Increased sodium in the diet stimulates thirst and water intake which increases total blood volume (20) and could therefore be and additional cardiovascular stressor.

Oxidative stress seem to play a role in AngII+Salt induced decompensation in Balb/CJ mice. We have previously shown that oxidative stress is lower in AngII+Salt treated Balb/CJ than C57BL/6J mice (65). Reducing oxidative stress with NAc increased sodium and water retention, as well as mortality in C57BL/6J mice (65). In paper III we showed that treatment with the antioxidant NAc worsened cardiac function in both strains by impairing filling and reducing CO. Increased levels of the antioxidant glutathione has previously been connected to cardiomyopathy and congestive heart failure in transgenic mice expressing mutant alpha B-crystallin (109). Further, antioxidant treatment has been shown to interfere with skeletal muscle adaptation to exercise (104). The finding in this study that NAc impairs diastolic function and reduces cardiac output in C57BL/6J may partially explain the increased mortality during AngII+Salt+NAc treatment. However, we have only measured urinary TBARS which is to a large extent affected by renal ROS production. Therefore, TBARS should be measured in cardiac tissue to see if NAc reduces oxidative stress or if the effect of NAc might be related to other processes such as posttranslational modification (56, 103).

A linkage study was performed to investigate the effect of genetic background on AngII+Salt induced decompensation. A QTL on chromosome 3 including 294 protein-coding genes was linked to change in cardiac output before and after AngII+Salt. Out of these 294 genes, Foxo1 was the only gene that matched a previously known coding non-synonymous SNP that differed between Balb/CJ and C57BL/6J in the mouse genome database. Interestingly, immunohistochemical localization of Foxo1 showed a higher nuclear translocation in C57BL/6J mice than in Balb/CJ mice after AngII+Salt treatment. Foxo1 has been shown to be essential for normal cardiac development and maintenance of cardiac function in the adult heart (113). It participates in the activation of inflammatory cells as well as the regulation of oxidative stress. Both mechanisms are known to be highly involved in the pathology of heart failure (35, 113, 143), and are consistent with our previous findings in both renal (16) and cardiac gene expression.

Linkage analysis of corrected IVRT identified a QTL on chromosome 12. Ahr was the only gene that matched a previously known coding non-synonymous SNP that differed between Balb/CJ and C57BL/6J in the mouse genome database. Interestingly, Ahr has been shown to play a role in cardiac function but has not previously been connected to relaxation time of the left ventricle (141).

In addition, Ahr null mice develop cardiac hypertrophy, which is related to increased endothelin-1, AngII and mean arterial blood pressure (86).

In conclusion, the results indicate that Balb/CJ develop pulmonary and systemic hypertension which increased afterload on both the right and left ventricle. Further, impaired relaxation of left and probably right ventricle reduces filling of the heart, which stimulates fluid retention and edema (Figure 23). The pattern of peripheral edema and unchanged lung fluid indicates that Balb/CJ develop right ventricular failure and that right ventricular failure may be the cause of high mortality in this strain. The effect of AngII on pulmonary resistance vessels is mediated by a released contractile factor which may also be involved in systemic vascular response as well impaired relaxation of the heart. Levels of AngII are increased both in hypertension and in heart failure and individuals that are as reactive to AngII as Balb/CJ, may be at higher risk of developing decompensated heart failure.

The main drawbacks of the studies presented in this thesis is the lack of right ventricular measurements. Unfortunately, because of the way the right ventricle in mice is wrapped around the left ventricle and its position behind the sternum, reliable imaging are very challenging. However, since we know that right ventricle and left ventricle eject exactly the same quantities of blood (41), left ventricular cardiac output measurements can be used to estimate right ventricle cardiac output. Moreover, the condition of Balb/CJ deteriorate really fast, and measurement of cardiac function once per day is probably not enough to detect the final progression of decompensation.

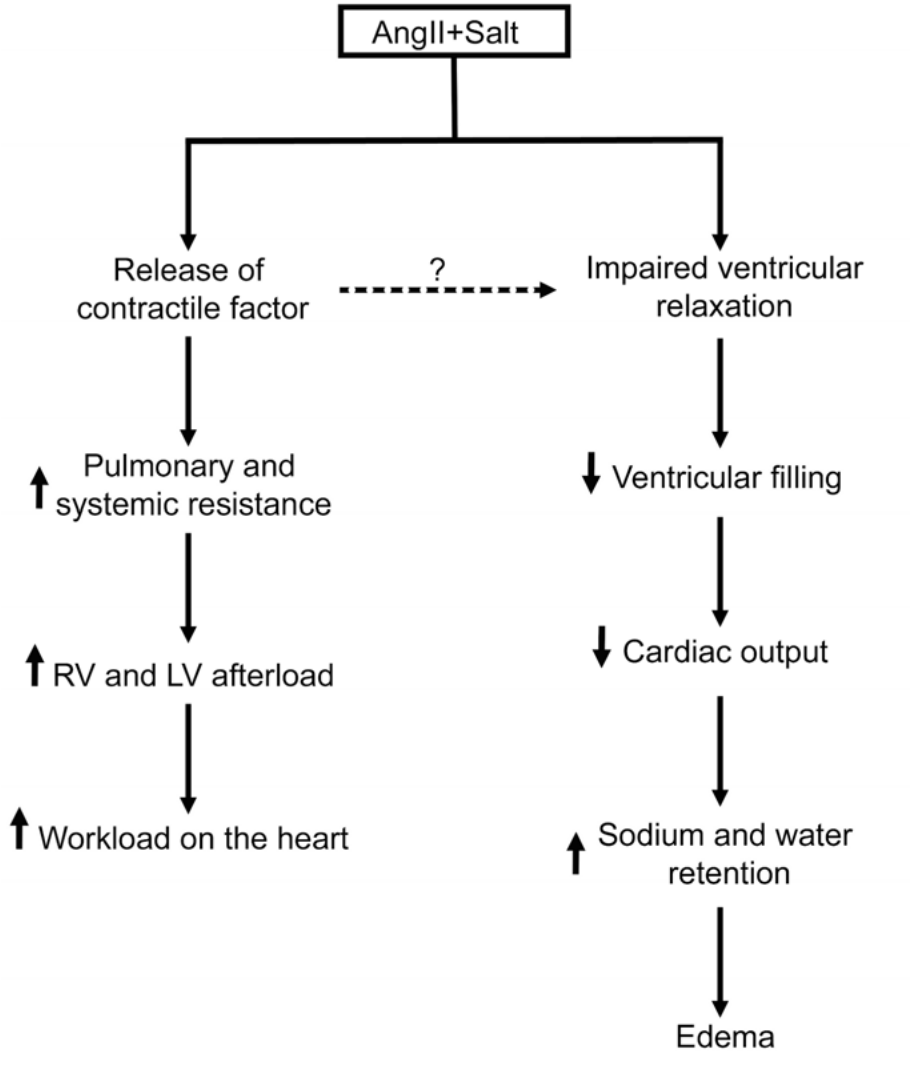

Figure 23. Schematic figure showing the effect of angiotensin II and high-salt diet (AngII+Salt) in Balb/CJ mice.

Conclusions

Paper I

- AngII+Salt induces cardiac remodeling to the same extent in Balb/CJ and C57BL/6J mice. However only Balb/CJ mice display impaired cardiac function.

- Balb/CJ retain more sodium and water compared to C57BL/6J during AngII+Salt treatment.

Paper II

- The different response to AngII+Salt in Balb/CJ and C57BL/6J is connected to differences in glutathione transferase activity and lower oxidative stress in Balb/CJ mice.

- Reducing oxidative stress induces diastolic dysfunction both in Balb/CJ and C57BL/6J mice.

Paper III

- AngII+Salt induced decompensation in Balb/CJ is linked to QTLs on chromosome 2, 3 and 12.

Paper IV

- Pulmonary resistance vessels from Balb/CJ are more reactive to AngII than pulmonary resistance vessels from C57BL/6J.

- AngII induced contraction in pulmonary resistance vessels from Balb/CJ is mediated by a released contractile factor.

Populärvetenskaplig sammanfattning

Denna avhandling behandlar ämnet akut dekompenserad hjärtsvikt, ett komplicerat och dödligt tillstånd. För att öka förståelsen för de vetenskapliga resultaten som denna avhandling presenterar behövs en kortare introduktion till hjärtat och dess uppgifter i kroppen. Nedan följer och kort introduktion samt en förklaring av vad hjärtsvikt och akut dekompenserad hjärtsvikt är.

Hjärtats uppgift är att pumpa blod så att alla delar av kroppen får tillräckligt mycket syre och näring. Syre är en nödvändighet för att alla kroppens celler ska kunna fungera normalt. Hjärtsvikt är en sjukdom där hjärtats förmåga att pumpa blod har blivit nedsatt. Detta kan ske av olika orsaker så som myokardiell infarkt, där en del av hjärtmuskeln dör och den förlorade funktionella muskelmassan gör att hjärtat inte kan pumpa lika mycket blod som innan. Även vid andra typer av sjukdomar, så som högt blodtryck (hypertoni) och sockersjuka (diabetes), kan hjärtats förmåga att pumpa blod bli nedsatt och hjärtsvikt utvecklas. Eftersom det är så viktigt att kroppens celler får tillräckligt mycket syre har kroppen flera kompensatoriska mekanismer vilka hjälper hjärtat att upprätthålla sin pumpförmåga. Dock fungerar dessa mekanismer endast under en viss tid. Slutligen blir de kompensatoriska mekanismerna otillräckliga och hjärtats pumpförmåga blir snabbt sämre. Patienterna ansamlar vätska i ben och armar samt buken (ödem), får svårt att andas (lungödem) och måste snabbt omhändertas av sjukvården för att överleva. Detta kallas för akut dekompenserad hjärtsvikt.

När hjärtat inte klarar av att pumpa tillräckligt mycket blod och syre, påverkas alla kroppens organ. Denna avhandling behandlar kopplingen mellan hjärta, njurar och kärlsystemet i kroppen vid hjärtsvikt. Dessutom undersöker den vikten av den genetiska bakgrunden på känsligheten av att utveckla akut dekompenserad hjärtsvikt. De gener man ärver från sina föräldrar kan göra att vissa individer blir mer känsliga och andra mer resistenta mot att utveckla hjärtsvikt. Vår forskningsgrupp har de senaste åren studerat två olika musstammar, där den ena visat sig vara genetiskt mer känslig för att utveckla akut dekompenserad hjärtsvikt medan den andra musstammen visat sig vara mer resistent. Teorin är att den känsliga musstammen via sin arvsmassa uttrycker proteiner eller aktiverar mekanismer som gör att den inte klarar av att kompensera sin nedsatta hjärtfunktion, medan den resistenta musstammen uttrycker proteiner eller aktiverar mekanismer som gör att den blir mer skyddad

och kan då kompensera för sin nedsatta hjärtfunktion. Om vi kunde förklara vad som händer i den känsliga musstammen och varför den resistenta stammen är skyddad skulle vi kunna hitta sätt att bromsa utvecklingen av dekompenserad hjärtsvikt.

Balb/CJ, som är den känsliga musstammen utvecklar massiva ödem och dör när de utsätts för en kombinationsbehandling med hormonet angiotensin II och en diet som innehåller mycket salt. Angiotensin II ökar blodtrycket och vätskereabsorptionen i njurarna. Det är ett hormon som vanligtvis frisätts vid hjärtsvikt och som kroppen använder sig av för att kompensera och upprätthålla hjärtfunktion. I längden kan dock för mycket angiotensin II leda till att hjärtat blir stelare och dess pumpförmåga minskar. Hög-salt diet ökar också blodtrycket och vätskereabsorptionen i njurarna och patienter som har hjärtsvikt brukar rådas att äta mindre salt. C57BL/6J som är den resistenta stammen får högt blodtryck men de utvecklar inte ödem och de dör inte av behandlingen. I den första studien som presenteras i denna avhandling visar vi att Balb/CJ får högt blodtryck både i systemiska kretsloppet och i lungkretsloppet. Dessutom blir hjärtat stelare och klarar inte av att fyllas på som det ska och eftersom hjärtat inte fylls på kan den inte pumpa samma mängd blod och hjärtsvikt utvecklas därför. Detta leder till att njurarna sparar mer vätska för att hjälpa hjärtat att öka fyllnaden men eftersom hjärtat är stelt kan det inte fyllas på med mer blod och den vätska som njurarna har sparat går ut i vävnaden och mössen utvecklar därmed ödem. C57BL/6J får också högt blodtryck i systemiska kretsloppet, men de får inte högt blodtryck i lungkretsloppet och hjärtat fylls på som det ska.

I studie II visar vi att oxidativ stress kan förklara en del av skillnaden mellan den känsliga och den resistenta musstammen. I kroppen bildas så kallade reaktiva syreradikaler vilka är involverade i normal cellsignalering och är viktiga för kroppens funktion vid normala nivåer. Vid höga nivåer kan reaktiva syreradikaler skada DNA, proteiner och fetter, och därmed ha en negativ inverkan på cellfunktionen. Detta kallas för oxidativ stress och antioxidanter som vi får i oss via maten men som även bildas i kroppen, används för att neutralisera de reaktiva syreradikalerna. I studie II visar vi att oxidativ stress är lägre i Balb/CJ än i C57BL/6J och att behandling med en antioxidant gör att hjärtat blir stelare både i den känsliga och den resistenta musstammen. Därmed kan det inte fyllas på och pumpförmågan minskar, Dessutom ökar dödligheten i den resistenta musstammen. Detta indikerar att oxidativ stress i detta fall kan vara nödvändigt för cellsignalering och för att hjärtat ska kunna kompensera och upprätthålla sin pumpförmåga.

I studie III visar vi att den genetiska bakgrunden som Balb/CJ har är kopplad till den ökade känsligheten för att utveckla akut dekompenserad hjärtsvikt.

Där påvisar vi flera olika kandidatgener som ska studeras mer i detalj i framtiden och som skulle kunna göra att man blir mer känslig eller mer resistent mot att utveckla akut dekompenserad hjärtsvikt.

I studie IV visar vi att lungkärl från Balb/CJ är känsligare för angiotensin II och att detta är kopplat till frisättning av en för närvarande okänd substans. Denna substans skulle möjligtvis kunna påverka blodtrycket i både det systemiska- och lungkretsloppet men också påverka hur stelt hjärtat är och hur bra det fylls på. Framtida projekt kommer att fokusera på att identifiera denna substans. Om vissa individer är lika känsliga som Balb/CJ för angiotensin II skulle det innebära att dessa individer har högre risk att utveckla akut dekompenserad hjärtsvikt.

Sammanfattningsvis presenterar denna avhandling en musmodell för akut dekompenserad hjärtsvikt i vilken vi kan studera förloppet av akut dekompenserad hjärtsvikt samt effekten av nya läkemedel. Dessutom presenteras nya kandidatgener vilka skulle kunna vara involverade i utveckling av akut dekompenserad hjärtsvikt och skulle kunna förklara varför vissa individer har högre risk att utveckla sjukdomen än andra.

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