The Role of Angiotensin II in Experimental Acute Kidney Injury and Cardiorenal Failure

SOFIA JÖNSSON
Blood pressure and fluid regulation are kept constant through the interaction between heart and kidneys. When the systemic blood pressure decreases, the levels of the hormone Angiotensin II increase, leading to vasoconstriction and therefore increased blood pressure. Angiotensin II may act directly on the vessels or cause fluid retention in the kidneys, consequently increasing blood pressure. Dysfunctional heart and kidneys lead to different diseases, such as heart failure and acute kidney injury. A failing heart can cause damage to kidneys and vice versa, leading to cardiorenal syndrome. Due to the severity of these diseases and their increasing prevalence, it is important to investigate them further. Therefore, the aim of this thesis was to evaluate blockage or treatment of Angiotensin II on the effects on renal and cardiac function.

Study I investigates the effect of Losartan, an Angiotensin receptor blocker, on kidney oxygenation as well as the effect on blood pressure, after resuscitated haemorrhage and Norepinephrine administration. It showed that Losartan does not worsen the effects of kidney oxygenation. The blood pressure managing affects of Norepinephrine were also not worsened in rats treated with Losartan.

Study II-IV investigate treatment of Angiotensin II and high salt diet on renal and cardiac function in Balb/CJ and C57BL/6J mice. This treatment showed increased mortality in Balb/CJ mice compared to C57BL/6J. Balb/CJ also retained more fluid and sodium than C57BL/6J and had worsened cardiac function after Angiotensin II and salt treatment. These are signs of heart failure and decompensation. Balb/CJ had lower amount of oxidative stress, compared to C57BL/6J. Treating the latter with the antioxidant N-acetylcysteine, reduced the levels of oxidative stress, but increased the mortality.

In conclusion, Angiotensin II treatment or blockage has different effects on both renal function as well as cardiac function, depending on strain and treatment settings.

Keywords: Heart failure, Acute kidney injury, Angiotensin II, Fluid balance, Cardiac function, Renal function

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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.


IV Jönsson, S., Becirovic-Agic, M., and Hultström, M. Left and right ventricular function in Balb/CJ and C57BL/6J mice after Angiotensin II and Salt treatment using cardiac catheterization. *Manuscript*

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* Authors contributed equally to the study
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**Abbreviations**

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<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ADH</td>
<td>Anti-diuretic hormone</td>
</tr>
<tr>
<td>ADQI</td>
<td>Acute dialysis quality initiative</td>
</tr>
<tr>
<td>AngI</td>
<td>Angiotensin I</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>AKD</td>
<td>Acute kidney disease</td>
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<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>AKIN</td>
<td>Acute kidney injury network</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin receptor blocker</td>
</tr>
<tr>
<td>AT₁</td>
<td>Angiotensin receptor type 1</td>
</tr>
<tr>
<td>AQP2</td>
<td>Aquaporin 2</td>
</tr>
<tr>
<td>CHF</td>
<td>Chronic heart failure</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>CRS</td>
<td>Cardiorenal syndrome</td>
</tr>
<tr>
<td>E/A</td>
<td>Ejection to acceleration</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
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<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HF</td>
<td>Heart failure</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxidase</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>KDIGO</td>
<td>Kidney disease: improving global outcome</td>
</tr>
<tr>
<td>LVEDP</td>
<td>Left ventricular end diastolic pressure</td>
</tr>
<tr>
<td>LVEDV</td>
<td>Left ventricular end diastolic pressure</td>
</tr>
<tr>
<td>LVESP</td>
<td>Left ventricular end systolic pressure</td>
</tr>
<tr>
<td>LVESV</td>
<td>Left ventricular end systolic volume</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>NAc</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>OH$^\cdot$</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>O$_2$</td>
<td>Oxygen supply</td>
</tr>
<tr>
<td>O$_2$$^\cdot$</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>PO$_2$</td>
<td>Tissue oxygen tension</td>
</tr>
<tr>
<td>PV catheter</td>
<td>Pressure volume catheter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>QO$_2$</td>
<td>Oxygen consumption</td>
</tr>
<tr>
<td>RBF</td>
<td>Renal blood flow</td>
</tr>
<tr>
<td>RIFLE</td>
<td>Risk, injury, failure, loss of function, end stage kidney disease</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>SV</td>
<td>Stroke volume</td>
</tr>
<tr>
<td>TGF</td>
<td>Tubular glomerular feedback</td>
</tr>
<tr>
<td>TPR</td>
<td>Total peripheral resistance</td>
</tr>
<tr>
<td>UO</td>
<td>Urinary output</td>
</tr>
<tr>
<td>V$_2$</td>
<td>Vasopressin receptor 2</td>
</tr>
</tbody>
</table>
Introduction

What follows is a general introduction that starts with the kidneys and how they filter blood to produce urine. Then blood pressure regulation is introduced with the important hormone system, renin-angiotensin-aldosterone-system (RAAS) and how it affects kidney structure and blood pressure through regulating vascular resistance and fluid balance. Thereafter the heart is introduced and finally the diseases in focus for the present thesis, heart failure and acute kidney injury. Finally, oxidative stress is addressed since that is something that we found to be interesting in two of the articles in this thesis.

The kidneys

The human body is a complex physiological system where several organs are interacting in order to function properly. Two of these organs, the kidneys, are functionally important when regulating sodium and fluid balance, blood pressure, maintaining acid-base balance and excreting waste products. The functioning units in the kidneys enabling these tasks are called nephrons, and can be divided into different sections. The first section is called glomerulus, and it is where the blood is filtered and the primary urine is produced. The glomerulus is connected to the tubule, where important metabolites can be reabsorbed and waste products be excreted, resulting in the production of secondary urine in the collecting duct.\(^1\) The ability of the nephrons to produce hyperosmotic urine is important since it enables excretion of solutes and waste products with a minimal loss of water.\(^2\)

Due to the physiological function of the kidneys they are well perfused, and the volume blood, approximately 1L/min, that passes through the kidneys per time unit is called renal blood flow (RBF). RBF is auto regulated which means that despite fluctuations in blood pressure levels, the renal blood flow is constant through the kidneys.\(^3\) When the blood pass through the kidneys, it is filtered through glomerulus. The speed of this filtration process called glomerular filtration rate (GFR), is defined as the amount of plasma filtered per time unit (ml/min). The filtration through glomerulus enables excretion of waste products from the body. It is important that GFR is kept constant, and it is therefore regulated through autoregulation. Autoregulation is implicated through resistance regulation of the afferent and the efferent arteriole called
the myogenic response. \(^4\) GFR can also be regulated through tubular glomerular feedback (TGF). TGF is a mechanism that works through the juxtaglomerular apparatus that sense the tubular load to the distal tubule. This is sensed by the macula densa cells in the juxtaglomerular apparatus, \(^5\) which sends signals to the rest of the juxtaglomerular apparatus in order to adjust the tonus in the afferent arteriole which regulates the GFR. \(^6\)

Anatomically, the kidneys are divided in two layers, cortex and medulla, which both have different functions. The cortex is the well-perfused outer layer where the glomeruli are located and the metabolically active proximal and distal tubule run. The medulla is less well perfused, and the inner layer of the kidneys, through which loop of Henle stretches. \(^1\) The oxygen consumption is high in the kidneys, mainly in the cortex, due to the high work of reabsorption. \(^7\) Since the medulla is less perfused with only 10-15% of the blood flow it is also less oxygenated. \(^8\) Renal oxygenation is dependent on two things, oxygen supply (O\(_2\)) to the kidneys as well as oxygen consumption in the kidneys. \(^9\)

**Blood pressure regulation**

Mean arterial pressure (MAP) is the pressure within the major arterial system of the body. MAP is dependent on two parameters, cardiac output (CO) and total peripheral resistance (TPR) and it can be calculated through MAP = CO*TPR. \(^10\) There are different ways for the body to regulate blood pressure where Angiotensin II (AngII) and fluid balance will be further discussed.

**Regulation through Angiotensin II**

The body regulates blood pressure through different mechanisms. One mechanism is through the hormone AngII, which is produced in the kidneys via RAAS. If the blood pressure is low, RAAS stimulates the kidneys to reabsorb more water. This leads to an increased blood volume as well as increased blood pressure through restored cardiac output. \(^11\) Via the macula densa cells in the juxtaglomerular apparatus, the kidneys sense both reduced and elevated blood pressure levels. \(^12\) In response to a reduced pressure, the kidneys release Renin. Renin converts angiotensinogen into angiotensin I (AngI). Through angiotensin converting enzyme (ACE), AngI is converted into AngII. \(^13\) Within the kidneys AngII stimulate reabsorption through angiotensin receptor type 1 (AT1). \(^13\) Systemically, AngII causes vasoconstriction which increases peripheral resistance leading to increased blood pressure. Peripheral noradrenergic activity and central activity of sympathetic nervous system is increased by AngII, which also stimulates the release of aldosterone and vasopressin. \(^14\)

Even though AngII help compensating for reduced CO and through that blood pressure, elevated AngII can be harmful, especially over time. If there
is no hypovolemic or hypotensive state, it is well known that AngII causes hypertension through elevated sodium and water reabsorption and by stimulation of aldosterone and intra renal mechanisms. AngII can also directly cause damage to the heart and kidneys, both through causing hypertrophy of the heart but also as fibrosis in the heart and kidneys. Vasconstriction can be caused by AngII itself, but AngII can also cause proliferation of smooth muscle cells and through that increase vascular resistance further. Something that is well established is that high dietary salt, over time, causes hypertension. AngII in combination with high salt diet, might accelerate the development of hypertension and exacerbate the renal vascular lesions.

Blood pressure regulation through fluid balance regulation

Another mechanism, except from RAAS, though which the systemic blood pressure can be controlled is through fluid balance regulation. Depending on the fluid balance in the body, it can be regulated through excretion or reabsorption of water in the collecting duct. For example, a too low blood pressure causes reabsorption of water, which increases the blood volume and therefore also the pressure. Hypothalamus sense the osmolality in plasma, a high osmolality leads excretion of vasopressin (ADH, anti-diuretic hormone). ADH bind to the vasopressin receptor 2 (V2) on principal cells in the late distal tubule as well as collecting duct of the kidneys. In the principal cell, ADH triggers a phosphorylation cascade that activates Aquaporin (AQP2) storage vesicles, which becomes inserted to the apical membrane. AQP2 are water channels that will reabsorb water into the body.

The heart

Another important organ is the heart, which constantly interacts with the kidneys in order for the body to function properly. The heart is responsible for the circulation of blood, enabling blood flow to different organs in order to meet their metabolic needs. The heart is divided into right and left atria, together with right and left ventricle. Blood flows into the right atria via vena cava, from where it is pumped into the right ventricle. From the right ventricle, it flows through the circulatory system of the lungs, where the blood is oxygenated. The oxygenated blood is then pumped back into the heart via the left atria, and into the left ventricle. From the left ventricle the blood flows out to the rest of the body. In order for the blood to flow in one direction there are valves between each atria and chamber called the mitral valve and the tricuspid valve. There are also two valves, the pulmonary and the aortic, that goes to the arteries leaving the heart. The amount of blood that is pumped from the ventricle per minute is called CO. CO is dependent on two different parameters, stroke volume (SV) and heart rate (HR). SV is the volume that the
Heart is able to pump in one heart beat; while HR is the amount of beats that the heart takes per minute.\textsuperscript{31} SV is dependent on several different factors such as preload, which is the amount of blood that enters the left ventricle. SV is also dependent on the contractility of the ventricle, as well as the afterload, which is the impedance that the ventricle has to work against.\textsuperscript{32}

In order for the heart to keep up with the metabolic needs of the body, a certain blood flow and blood pressure levels need to be maintained. Both heart and kidneys are sensitive to blood pressure variations. A too high pressure can cause glomerular damage as well as renal vascular damage, but it can also cause damage to the heart through development of acute or chronic heart failure.\textsuperscript{33} A too low pressure can be harmful as well, due to the limitations of oxygen supply, which prevents organ function and cause tissue damage.\textsuperscript{34}

Due to the different tasks of the left and the right ventricle, they work differently. Due to the high systemic blood pressure, the left ventricle is good at working against a higher pressure. Normally the pressure in the circulatory system of the lungs is relatively low in comparison to the systemic blood pressure. Therefore the right ventricle is better at coping with increased volumes rather than increased pressure.\textsuperscript{35} If the pulmonary pressure increases, the right ventricle adapts by thickening its wall, resulting in a more forceful contraction.\textsuperscript{36} Eventually the right ventricle will not be able to keep up, and therefore increased pulmonary pressure as well as an increased resistance might lead to right ventricular failure.\textsuperscript{37}

**Heart failure**

Heart failure (HF) means that the heart is not able to pump enough blood to meet the metabolic needs of the body.\textsuperscript{34} The heart can to some extent, keep up with a higher pressure and increased workload, through compensatory mechanisms, but after a while it can lead to further dysfunction and in the end de-compensation.\textsuperscript{34} RAAS is one of the most important compensatory mechanisms, which reacts to a lower blood flow through the kidneys, and through that stimulating the kidneys to retain more sodium and water. But, together with a failing heart this can lead to fluid overload and therefore fluid congestion.\textsuperscript{38} Fluid overload is a quite common symptom among HF patients and is associated with higher risk of re-hospitalization and death.\textsuperscript{39} Another compensatory mechanism during HF is the activation of the sympathetic nervous system (SNS). Baroreceptors in the aorta will sense a lower blood flow, SNS is activated releasing neurotransmitters such as norepinephrine (NE), causing peripheral vasoconstriction leading to increase blood pressure. SNS activation also increases SV and HR, which will increase CO.\textsuperscript{40}
HF can be divided into different subgroups depending on what was causing the failure. One of the most common causes of HF is myocardial infarction.\textsuperscript{41,42} HF can also be caused by systemic hypertension, that over time can cause damage to the heart, by causing left ventricular hypertrophy and dysfunction.\textsuperscript{43,44} Hypertension in the pulmonary system would on the other hand cause dysfunction of the right ventricle.\textsuperscript{45} Due to the normally low pressure, around 15 mmHg, in the pulmonary circulatory system, a slight increase in pressure can cause damage relatively fast to the right ventricle.\textsuperscript{46} Previous study show that when treating Balb/CJ and C57BL/6J with the combination treatment of AngII and high salt diet, Balb/CJ present with early restriction of pulmonary flow, reduced left ventricular filling and reduced CO. This resulted in fluid retention and peripheral edema, signs of right ventricular failure.\textsuperscript{47}

Pathologies of the heart can occur during different parts of the cardiac cycle, which cause different problems leading up to HF. Patients can for example display with either diastolic dysfunction or systolic dysfunction. HF with preserved ejection fraction, also called diastolic dysfunction is where the relaxation period of the cardiac cycle is disturbed.\textsuperscript{48} HF could also be presented as systolic dysfunction which means reduced ejection fraction.\textsuperscript{49}

Compensatory mechanisms are not only activated during HF, but can also be activated when the heart muscle is in good shape, for example after haemorrhage, which cause decreased CO due to decreased preload.\textsuperscript{50} Haemorrhage might cause decreased blood flow through several vital organs such as the kidneys, activating compensatory mechanisms such as RAAS in order to increase AngII. Increased AngII leads to fluid retention which increases the pressure.\textsuperscript{51}

### Acute Kidney Injury

Acute kidney injury (AKI) is defined as sudden loss of, or decline in kidney function\textsuperscript{52}, causing decreased GFR together with decreased urinary output.\textsuperscript{53} The prevalence of AKI is increasing and it affects 2-7\% percent of all hospitalized patients.\textsuperscript{54,55} AKI is a disease with a high mortality\textsuperscript{56,57} and several different risk factors such as sepsis,\textsuperscript{58} fibrosis\textsuperscript{59} or hypoxia\textsuperscript{60}. Critical illness is a great risk factor where patients admitted to the critical care unit have an increased risk of developing AKI.\textsuperscript{61} Another major risk factor for AKI is HF, due to the insufficient amount of blood and decreased oxygen delivery to the kidneys.\textsuperscript{62,63} Major surgery is also a great risk, again because of decreased oxygen delivery to the kidneys through haemorrhage and hypotension.\textsuperscript{64–66} Haemorrhage can lead to decreased renal perfusion and therefore also decreased renal function, which may lead to increased oxygen consumption (\textit{QO}_{2}) and hypoxia in the kidney.\textsuperscript{67}

There are four different definitions in order to determine if a patient has AKI. One of those is the RIFLE criteria, which stands for Risk, Injury, Failure,
Loss of kidney function and End stage kidney disease (Figure 1) which classifies the renal disease based on serum creatinine values and urinary output (UO).  

![GFR Criteria](image)

<table>
<thead>
<tr>
<th>GFR Criteria</th>
<th>Urine output criteria</th>
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<tbody>
<tr>
<td><strong>Risk</strong></td>
<td></td>
</tr>
<tr>
<td>Increased serum creatinine x 1.5, GFR decrease &gt; 25%</td>
<td>UO &lt; 0.5 ml/kg/h * 6h</td>
</tr>
<tr>
<td><strong>Injury</strong></td>
<td></td>
</tr>
<tr>
<td>Increased serum creatinine x 2, GFR decrease &gt; 50%</td>
<td>UO &lt; 0.5 ml/kg/h * 12h</td>
</tr>
<tr>
<td><strong>Failure</strong></td>
<td></td>
</tr>
<tr>
<td>Increased serum creatinine x 3, GFR decrease &gt; 75%</td>
<td>UO &lt; 0.3 ml/kg/h * 24h or anuria * 12h</td>
</tr>
<tr>
<td>Or creatinine &gt; 4 mg/dl</td>
<td></td>
</tr>
<tr>
<td><strong>Loss</strong></td>
<td>Persistent ARF = Complete loss of renal function &gt; 4 weeks</td>
</tr>
<tr>
<td><strong>ESRD</strong></td>
<td>End stage renal disease &gt; 3 months</td>
</tr>
</tbody>
</table>

**Figure 1.** Acute kidney injury classification according to risk, injury, failure, loss of kidney function and end stage renal disease (RIFLE) criteria. The RIFLE criteria takes serum creatinine levels, a measurement of glomerular filtration rate (GFR), and urine output (UO) into account.

There is also the classification from the acute kidney injury network (AKIN) which is a more recent version of the RIFLE criteria, but it is only based on serum creatinine levels, and not related to the GFR. Kidney disease: improving global outcome (KDIGO) is another classification that is made on evaluation of both the RIFLE and the AKIN criteria. AKI is, in the KDIGO guidelines, defined as an abrupt decrease in kidney function over seven days or less. In addition, chronic kidney disease (CKD) is defined by the persistence of kidney disease for more than 90 days. According to the Acute Disease Quality Initiative (ADQI) AKI develops into acute kidney disease (AKD) after 7 days, and can be further developed into chronic kidney disease after 90 days. Due to the fact that AKI is just the starting point of kidney disease and that it might progress into CKD, it is a very severe disease with no good cures.

**Cardiorenal syndrome**

As talked about in the beginning of this thesis, the heart and the kidneys do work in collaboration with each other. Cardiorenal syndrome (CRS) occurs when either heart or kidneys starts failing which then effects the other organ negatively resulting in failure of that organ as well. It can be both an acute
or chronic disorder in one of the organ that causes and acute or chronic disorder in the other. CRS can be divided into five different subgroups (Type 1-5 CRS) depending on how and in which organ it started. Type 1 CRS are patients presenting with HF that results in AKI, while type 2 CRS starts with chronic HF that results in CKD. Type 3 CRS are patients that have AKI resulting in acute heat failure (AHF) and type 4 CRS are patients with CKD that develop chronic heart failure (CHF). The last subgroup is type 5, which is a secondary CRS, with patients who have a systemic process such as sepsis that leads to heart and kidney failure. These five definitions where established to make it easier for characterization of clinical presentation which enable a more proper diagnosis and treatment.

Reactive oxygen species

One system that study II identified as particularly important was reactive oxygen species (ROS). ROS are rest products that are produced when oxygen molecules are degraded. The production of ROS happens throughout cells, for example via the electron transport chain in the mitochondria, but also through oxidoreductase enzymes, or through metal catalysed oxidation. ROS are usually taken care of by the cells but elevated levels of ROS are referred to as oxidative stress. Oxidative stress can be harmful since it might cause damage to lipids, proteins and DNA. Although, ROS are not only harmful, they also serves as signalling molecules to regulate biological and physiological processes. There are different kinds of ROS such as hydrogen peroxide (H$_2$O$_2$), superoxide radical (O$_2^-$), and hydroxyl radical (OH$^-$).

Oxidative stress are known to be harmful in several different pathologies. In chronic heart failure oxidative stress play a detrimental role, as it does in hypertension. ROS has been linked to heart failure as well as disease progression in both humans and animal models. The detrimental role of oxidative stress is also worsened with the treatment of AngII and high dietary salt. Due to the high amount of mitochondria in the kidneys, it makes them exposed to high levels of ROS and therefor susceptible to the damage of ROS, causing development of AKI.

Since all aerobic organisms are exposed to oxidative stress as a result of the mitochondrial respiration, there are also regulatory mechanisms, such as antioxidants, protecting the cells from the free radicals. One of these mechanisms is Glutathione (GSH), which is an intracellular peptide that has diverse function in the body, such as antioxidant abilities. The glutathione system is an endogenous system that is one of the most important antioxidant system of the body. GSH works as a free radical scavenger but it is also a substrate for glutathione peroxidase which also works as an antioxidant.
Aims

The overall aim of this thesis was to investigate the effect of Angiotensin II blockage or treatment on renal and cardiac function. Each study had more specific aims as follows.

I  To investigate effects of the angiotensin II receptor blocker Losartan on renal oxygen metabolism after resuscitated haemorrhage and on the efficacy of Norepinephrine for blood pressure management.

II  To investigate the differences in renal function and fluid handling by the use of renal gene expression in Balb/CJ and C57BL/6J mice after treatment with Angiotensin II and high salt diet. To investigate differences in oxidative stress levels. Further, to establish the difference in susceptibility by measuring mortality.

III  To investigate if Balb/CJ have maladaptive cardiac response to Angiotensin II and salt, independently, or to a combination treatment that potentially could be tied to oxidative stress.

IV  Evaluate right and left ventricular function with cardiac catheterization after treatment with Angiotensin II and high salt diet, or after a bolus injection of Angiotensin II, in Balb/CJ and C57BL/6J mice.
Methods

Animal models

This thesis is based on experiments in three different animal models. Male Wistar rats from Taconic, Denmark, was used in study I in order to enable measurement of kidney function after haemorrhage. Since study I was a follow up study, the same rat model was used as in the previous study.87

Throughout study II-IV Balb/CJ and C57BL/6J mice, Taconic, Denmark was used. These are two commonly used mouse strains, throughout different types of research.88 Due to the fact that they are commonly used, it is important to evaluate these strains to display differences that might exist between them. Balb/CJ are known to be a more sensitive mouse strain.89 In C57BL/6J AngII treatment can induce hypertensive kidney damage.25 Balb/CJ and C57BL/6J also present different type of immune response, where Balb/CJ are Th1 responders and C57BL/6J are Th2 responders.90

In excess of these mice, in study II, female and male Balb/CJ and C57BL/6J from Janvier, France, was used. All studies were performed in accordance with the national institute of health (NIH) guidelines for use and care of laboratory animals, and approved by the local animal research ethics committee of Uppsala. The experiments of study II and III were also in accordance with the NIH guidelines for the treatment of experimental animals, and the committees for animal experiments at the University of Bergen, and Umeå University.

Study protocol and treatment of study I

Male Wistar rats (Taconic, Denmark), 7-8 weeks old, were divided into two groups, Losartan and Control. The Losartan group received Losartan (60 mg/kg/day, Sandoz, Denmark, n=8) in the drinking water, and the Control group (n=7) received ordinary tap water for seven days. Losartan is an angiotensin receptor blocker (ARB) that is used to treat hypertension, by blocking AT1 receptor.91 Through blockage of the AT1-receptor, the effects of AngII is blocked resulting in a decreased blood pressure.92 Due to the blood pressure lowering effects of Losartan, patients are recommended to stop their treatment before surgery, since there are beliefs that ARB treatment can interfere with blood pressure management during anaesthesia.93 As described earlier, AKI is a risk factor for CKD, but Losartan treatment have been shown to reduce the
risk of CKD development after AKI, and therefore also reduced mortality in mice.\textsuperscript{94} Throughout study I and the treatment period of one week, animals were fed standard rat pellet and kept on a 12h/12h light/dark cycle. In the end of the treatment period blood pressure was measured with a tail cuff system, on awake animals from both control and Losartan treated groups. After this, acute experiments were performed.

Study protocol and treatment of study II and III

Previously our group treated Balb/CJ and C57BL/6J with AngII (1.0 \( \mu \text{g/min/kg} \)) and salt (4\% NaCl), which resulted in very high mortality among Balb/CJ mice, already after a few days of treatment.\textsuperscript{47} Due to the high mortality, the dose of AngII and Salt was lower in the following studies.

![Figure 2. Schematic picture of the experimental design in study II and III displaying timeline and the different treatment groups Control, Angiotensin II (AngII, 0.5 \( \mu \text{g/min/kg} \)) Salt (3\% NaCl), AngII+Salt (0.5 \( \mu \text{g/min/kg} + 3\% \text{NaCl} \)) or AngII+Salt+N-acetylcysteine (0.5 \( \mu \text{g/min/kg} + 3\% \text{NaCl} + \text{NAc 150 mg/kg/24h} \)).](image)

In study II and III male Balb/CJ and C57BL/6J was used and divided into five different treatment groups Control, AngII (0.5\( \mu \text{g/min/kg} \), Sigma-Aldrich A9525), high salt diet (Salt, 3\% NaCl, Special Diet Services, Witham, UK), AngII+Salt (0.5\( \mu \text{g/min/kg} \) AngII + 3\% NaCl) and AngII+Salt+N-acetylcysteine (0.5\( \mu \text{g/min/kg} \) AngII + 3\% NaCl + 150 mg/kg/24h NAc). AngII was distributed with mini osmotic pump (1007D, Alzet, Cupertino, CA) placed subcutaneously on the neck of the mice, during isoflurane anaesthesia and a small surgery on 15 minutes. The mice were treated throughout four days (first 24 hours as Control, Figure 2), after which acute experiments or echocardiography measurements was performed. In these two studies, one of the treatment groups was treated with NAc. NAc works as a direct antioxidant and reduces ROS in both mice and humans,\textsuperscript{95,96} but it also works as a glutathione precursor.\textsuperscript{97}

Study protocol and treatment of study IV

In study IV, left and right ventricular function were evaluated on Balb/CJ and C57BL/6J mice. Left ventricular function was measured in Control, AngII
(0.5µg/min/kg), Salt (3% NaCl) and AngII+Salt (0.5µg/min/kg AngII + 3% NaCl) treatment groups. The animals were treated throughout four days and the cardiac catheterization was performed on day four. Right ventricular measurements were performed on animals that had not been treated prior to experiment, instead these animals got a bolus injection of AngII, 100 µl (0.75mg/ml), during the experiment.

Acute experiments on rats (Study I)

In study I acute experiments was performed on male Wistar rats. The rats were anesthetized with Inactin, i.p (140 mg/kg; Sigma-Aldrich, St. Louis, MO, USA). The rats were placed on a surgery table with a servo-controlled heating pad, keeping the body temperature at 37°C. Catheterization of the femoral artery enabled blood pressure measurements (DTX Plus Transducer; Becton Dickinson, Singapore), blood sampling and haemorrhage. Catheterization of the femoral vein enabled continuous infusion of Ringer’s Acetate (6ml/h/kg), and resuscitation after haemorrhage. A flank incision exposed the left kidney which was immobilized in a kidney cup. Catheters were placed in the bladder and the ureter for urine collection, and a flow probe (size 0.7, AD Instruments, Oxford, UK) was placed around the renal artery to measure blood flow. Oxygen consumption was measured with a Clark-type microelectrode (Unisense, Aarhus, Denmark). The electrode was inserted 1.5 mm into the left kidney, making it possible to measure cortex oxygenation. The electrode was further inserted into the medulla (3.5 mm) enabling measurement of medullary oxygenation.

![Figure 3. Schematic picture of the experimental design with both treatment and acute experiment of study I. Rats where treated seven days prior to surgery as Control or with Losartan, and then acute experiment was performed. During acute experiment animals were haemorrhaged 20% of their blood volume and resuscitated with the same amount. The rats were also treated with the blood pressure manager norepinephrine (NE).](image)
After the surgery and placement of all the catheters, the rats recovered for 60 minutes, followed by a baseline collection period. The baseline collection period was followed by a haemorrhage of 20% of their blood volume, and then the rats were resuscitated with the same volume Ringer’s Acetate. Resuscitation was followed by another resting period of 60 minutes followed by another data collection period. During the data collection periods, both before and after resuscitated haemorrhage, measurements of tissue oxygen tension (PO2), RBF and MAP were taken. Arterial and venous blood sample were obtained at the end of each data collection period, enabling blood gas analysis using an iStat point-of-care analysis machine (Abbot, USA). After the last data collection period a bolus dose of NE (0.32 µg/kg) was injected via the catheter in the femoral vein. At the same time MAP and RBF were continuously measured in order to record the maximal effect compared to the pre-bolus level. Upon completion of the experiment the rats were euthanized (Figure 3 show experimental design).

Calculations (Study I)
QO2 was measured indirectly both before and after resuscitation by subtracting the venous O2 content from the arterial O2 content multiplied with the RBF. Oxygen delivery was calculated by multiplying arterial O2 with the RBF. These calculations were performed on values both before haemorrhage and after resuscitated haemorrhage.

Metabolic cages (Study II)
Metabolic cages (MMC100, Hatteras Instruments, NC, USA) enable measurements of food and water consumption, as well as urine production over time, in this case 24 hours. Both food and water was weighed prior to the stay in metabolic cages, and after the 24h which made it possible to calculate the consumption. The mice were put in the cage with free access to food and water. The urine was collected via a funnel, making the urine go into a special tube while the feces end up on the side.

Acute experiments mice (Study II)
Acute experiments was performed on mice anaesthetized with isoflurane. This enabled measurements of sedated GFR as well as sedated MAP. After anaesthetization, the mice were placed on a servo-controlled heating pad, keeping the body temperature at 37 °C. A catheter was placed in the bladder through which urine was collected. Catheterization of jugular vein made it possible to infuse 3H-inulin (Bionuclear Scandinavia AB, Sweden) in Ringer’s acetate.
(infusion rate 0.35 ml/h). After the surgery mice rested for 45 min followed by 30 min collection period, during which urine was collected. After 15 minutes of the collection period, a plasma sample was taken. GFR was calculated as inulin clearance (([^3]H-inulin) in urine*urine flow)/ ([^3]H-inulin) in plasma) and the concentration of inulin in plasma and urine was analysed using liquid scintillation (Tri-Carb 2910TR, Perkin Elmer, Massachusetts, USA).

TBARS (Study II)
Measurements of urinary TBARS is an indirect measurement of reactive oxygen species. The urine was collected via metabolic cages, described previously, and mixed (1:1.25) with 0.67% thiobarbituric acid, after which the samples were incubated for one hour at 97 °C. After the incubation period the samples were cooled on ice and then mixed with 1 mM NaOH: Methanol (9:91), vortexed and centrifuged (3000 rpm, 5 min). The supernatant was transferred to a fluorescence plate and the fluorescence was measured at 532 nm excitation and 553 nm emission (Safire II, Teacn, Austria).

Measurements of sodium balance (Study II)
Sodium balance was measured in urine and plasma collected from either metabolic cages or acute experiments on mice. The sodium concentration was measured using flame photometry (Model IL 943, Instrumentation Laboratory, Massachusetts, USA).

Awake GFR (Study II)
GFR was measured on conscious mice with a single bolus injection of fluorescein isothiocyanate (FITC)-inulin (Sigma-Aldrich, MO, USA). 1.5% FITC-inulin was dissolved in phosphate buffered saline (PBS, Medicago AB, Sweden) and dialyzed in PBS at 4°C overnight, using a 1000 Da cut-off dialysis membrane (Spectra/Por® 6 Membrane, Spectrum Laboratories Inc, CA, USA). 0.2 ml FITC-inulin was injected in the tail-vein while the animals were restricted after which blood samples were collected at 1, 3, 5, 10, 15, 35, 55 and 75 minutes. Plasma was mixed with 500 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4), and the fluorescence was measured at 496 nm excitation and 520 nm emission (Safire II, Tecan Austria GmbH, Austria). Inulin clearance was calculated using a two-compartment pharmacokinetic model.98
Awake mean arterial pressure (Study I and II)

Awake MAP was measured on both mice and rats via tail-cuff system (CODA, Kent Scientific, CT, USA). The animals were kept restrained on a servo controlled heating pad, keeping their body temperature. The tail cuff was placed high up in the tail enabling awake MAP measurements.

RNA isolation (Study II and III)

Both kidneys and heart were flushed with ice cold PBS, via cannulated aorta, after which they were stabilized in RNA-later (Sigma-Aldrich). Using a RNeasy mini kit the RNA was extracted from the kidneys, and through and RNeasy fibrous tissue mini kit RNA was extracted from the hearts (Qiagen, West Sussex, UK). Samples that had an RNA integrity number (RIN) >7, when measured with Aglient 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) were accepted for microarray measurements.

Gene expression microarray (Study II and III)

Heart and kidney tissue were taken from all treatment groups with eight samples per group (Control, AngII, Salt, AngII+Salt) were used for microarray measurements. A total amount RNA of 250 ng was reverse-transcribed, amplified and labelled with Biotin-16-UTP using Illumina TotalPrep RNA Amplification Kit (Applied Biosystems/Ambion, USA). Using NanoDrop and Aglient 2100 Bionalyzer, the Biotin-labelled cRNA was controlled. After that the Biotin-labelled cRNA (750ng) was hybridized to Illumina MouseRef-8 v2.0 Expression BeadChip arrays (Illumina, San Diego, CA, USA). Using the Illumina iScan Reader the microarrays were scanned, and bead level data was provided for further analysis. The data is accessible at ArrayExpress (Illumina, San Diego, CA, USA).

SNP analysis (Study II and III)

Using published SNP data from the mouse genome database (dbSNP Build 128, downloaded 2011-11-24 from www.informatic.jax.org), the genomic variation between the two mouse strains was compared.
PCR validation (Study II and III)

Validation of gene expression was performed via custom-designed Taqman micro-fluidic Low Density Arrays (LDA, ABI, USA). It was validation of 47 genes with coding-non-synonymous SNPs, as well as differential expression with a ranks sum <1000 in either heart or kidney. The top ranked genes had a rank sum <100 in either heart or kidney. Normalization was performed with 18S RNA. Total RNA of 200 ng was transcribed to cDNA through the usage of RT core-kit (Qiagen) and RT-PCR was performed using an ABI Prism9700 (ABI, USA).

Echocardiography (Study III)

Echocardiography was measured on mice during light isoflurane anaesthesia using Vevo 1100 (Visual Sonics, Toronto, Canada). The echocardiography measurements was performed on mice before and after treatment with AngII+Salt or NAc, thus animals served as their own control. Echocardiography enable measurements of left ventricular end diastolic volume (LVEDV), left ventricular end systolic volume (LVESV), CO, SV, EF, and HR in parasternal long-axis view of the left ventricle (Figure 4). Mitral valve flow velocity was assessed with Pulsed wave Doppler, enabling measurements such as E/A ratio. E/A ratio stands for ejection to acceleration ratio, and is a measurement of diastolic function. Left ventricular posterior wall thickness was measured with M-mode, in parasternal long-axis view, with the probe positioned at the largest diameter.

![Figure 4](image_url)

*Figure 4.* Picture taken with echocardiography of the left ventricle in parasternal long axis view. The left picture show the left ventricle in diastole and the right picture show the left ventricle in systole. The markings enable measurements such as left ventricular end diastolic volume (LVEDV) and left ventricular end systolic volume (LVESV).
Catheterization of the left ventricle (Study IV)

Left ventricular catheterization was performed with a pressure volume catheter (PV-catheter, PV-1030, Millar Instruments, USA) connected to MPVS ultra system (Millar, USA) and Power lab 4/35 (AD instruments, Australia) during isoflurane anaesthesia. The PV-catheter was inserted via the right carotid artery into the left ventricle of the heart. Figure 5 show an echocardiography picture of the PV-catheter placed in the left ventricle.

Figure 5. Pressure volume catheter in the left ventricle of a mouse, picture was taken with echocardiography (Vevo 1100).

PV catheterization enables pressure and volume measurements of the left and right ventricle, such as left ventricular end systolic pressure (LVESP), LVESV, left ventricular end diastolic pressure (LVEDP) and LVEDV. Together with HR, these measurements can be used enabling other calculation of SV (SV=LVEDV-LVESV) CO (CO=SV*HR), and EF (EF=SV/LVEDV). These measurements were performed on Control and AngII+Salt treated Balb/CJ and C57BL/6J mice. Figure 6 represents an example of a pressure volume loop in a Control treated Balb/CJ.

Figure 6. Pressure volume loop in a Control treated Balb/CJ mouse. The width of the loop represent stroke volume. In the top left corner of the curve, left ventricular end systolic volume (LVESV) and left ventricular end systolic pressure (LVESP) can be measured. In the bottom right corner left ventricular end diastolic volume (LVEDV) and left ventricular end diastolic pressure (LVEDP) can be analysed.

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Separately the pressure and volume curves look like Figure 7, where the measurements represents four seconds of recording time in a Balb/CJ control-mouse. Even though this measurement is more invasive that for example echocardiography, it is a great advantage that it can measure pressure as well.

Catheterization of the right ventricle (Study IV)

Right ventricular measurements were performed during isoflurane anaesthesia with the same PV-catheter as for the left ventricle. The right ventricle was entered via the right jugular vein. In order for the animals to physiologically stable they were tracheotomised and mechanically ventilated (Physio Suite, Kent Scientific, USA). Catheters was also placed in the femoral vein for intravenous injections, femoral artery for measurements of systemic blood pressure. During the experiment Ringer’s Acetate was infused via the femoral vein to maintain fluid balance.

Statistics

In study I, the data were reported as mean ± standard error (SEM). Differences between control and losartan groups we assessed using Student’s T-test. Comparisons across groups before and after haemorrhage and resuscitation were tested using two-way ANOVA. The change after resuscitated haemorrhage was tested using paired T-tests. P value was accepted as significant when p<0.05.
In Study II, the results are shown as mean values ± SEM. Since the animals served as their own controls, data obtained were analysed with T-tests for single comparisons and a linear mixed effects model for multiple group comparisons, and individual contrasts of least-squares mean were adjusted using Tukey’s method. Data that was collected from multiple groups were analysed with a two-way ANOVA together with Tukey’s post-hoc test, and $p < 0.05$ was accepted as significant. The microarrays were analysed with empirical Bayes (eBayes) together with false discovery rate (FDR). Using delta-delta-CT normalized to 18S the real-time PCR was analysed. The analysis of gene ontology enrichment was performed with Gene Ontology Enrichment Analysis Toolkit for genes with FDR < 0.05. Survival meta-analysis done using Mantel-Haenzsel statistics, taking preliminary study, microarray study, physiological studies into account. They were taken into account as separate studies since there were different follow up times and different treatments.

The results in study III were also shown as mean ± SEM. Echocardiography results were analysed with linear mixed effects model, and individual contrasts of least-square means were adjusted using Tukey’s method. The results that were from multiple independent groups were analysed using two-way ANOVA and Tukey’s post-hoc test. The significant level was set to $p < 0.05$. The microarrays were as in study II, analysed with empirical Bayes (eBayes) and false discovery rate (FDR). Real-time PCR was analysed using delta-delta-CT and it was normalized to 18s. Gene ontology enrichment was assessed using gene ontology enrichment analysis Toolkit for genes with an FDR of $< 0.05$ for each group compared to sham and when comparing strains at baseline.

In study IV the data is shown as mean ± SEM. The statistical analysis of the left ventricular pressure measurement was analysed using a two-way ANOVA with Bonferroni post-hoc test. Statistical significance was accepted at $p < 0.05$. Analysis were performed in GraphPad Prism 5.
Results

Losartan effects on kidney function after resuscitated haemorrhage (Study I)

Study I investigated kidney function in Wistar rats, after seven days of treatment with the ARB Losartan. Blockage of AngII with Losartan during haemorrhage did not worsen the effects of the haemorrhage on the kidneys. No difference were detected in RBF after resuscitated haemorrhage when comparing Control and Losartan treated animals, despite that anaesthetized MAP was as expected lower in the Losartan group (Figure 8).

*Figure 8. Mean arterial pressure (MAP) and renal blood flow (RBF) before and after resuscitated haemorrhage in Control (n=7) and Losartan (n=8) treated animals. The values are represented as mean ± SEM.* indicates p-value <0.05 compared to before haemorrhage within same treatment group. # indicated p-value <0.05 comparing in between treatment groups after resuscitated haemorrhage.

When looking into the oxygenation of the kidneys, neither cortical nor medullary PO2 were lower in the Losartan group compared to the control group after haemorrhage (Figure 9). At the same time, the animals did not produce enough urine to enable GFR calculations, which probably is a result of the haemorrhage.
Figure 9. Cortical and medullary tissue oxygenation (PO$_2$) before and after resuscitated haemorrhage in Control (n=7) and Losartan (n=8) treated animals. The values are represented as mean ± SEM and * indicates p-value <0.05 compared to before haemorrhage within same treatment group.

Blood pressure management after Losartan treatment (Study I)

Study I also investigated blood pressure managing effects after resuscitated haemorrhage while treated with Losartan. When administering a bolus dose of NE to Control or Losartan treated rats, the blood pressure managing effects of NE was not worsened in rats treated with Losartan (Figure 10). We did not see any difference on RBF after resuscitation and the bolus dose of NE.

Figure 10. Effects on mean arterial pressure (MAP) in Control and Losartan treated animals after resuscitated haemorrhage and a bolus dose of norepinephrine (NE). * indicates p-value <0.05 when comparing MAP after resuscitated haemorrhage and after NE treatment.
AngII+Salt treatment cause high mortality in Balb/CJ (Study II)

In study II renal function and renal gene expression was evaluated. When performing a survival meta-analysis (Figure 11) comparing the different study groups used within this project, it revealed a higher mortality in Balb/CJ mice with a relative risk of 0.15. (95% CI=0.05-0.48) and with a power of 99.7% for detecting $p<0.05$. When adding the antioxidant NAc, the significant difference in mortality between Balb/CJ and C57BL/6J where abolished.

**Figure 11.** Forrest plot displaying mortality in Balb/CJ and C57BL/6J after meta-analysis where different time points of experiments were taken into account. The meta-analysis was performed on Balb/CJ and C57BL/6J treated with Angiotensin II (AngII) and high salt diet (Salt), or with AngII+Salt and N-Acetylcysteine (NAc). The combined mortality show a relative risk of 0.15 (95% CI=0.05-0.48) favouring C57BL/6J, power of 99.7% detecting $< 0.05$. Total n: number of animals, 59 C57BL/6J and 62 Balb/CJ treated with AngII+Salt, and 39 C57BL/6J and 36 Balb7CJ treated with AngII+Salt+NAc. Events: number premature deaths or sacrifices because of edema or reduced general condition.

**Effects of AngII+Salt treatment on renal function (Study II)**

When further investigating what AngII and salt treatment might do to different animal models, differences in physiology were evaluated in Study II and III. Investigations of renal function in both Balb/CJ and C57BL/6J showed that
Balb/CJ treated with combination treatment of AngII+Salt, retain more sodium and produce less urine in comparison to control and when comparing to C57BL/6J with the same treatment. Although we did not see any differences on net water balance (Figure 12). Balb/CJ also presented with edema.

Figure 12. Display water consumption, sodium intake, urine excretion, sodium excretion, net water balance and net sodium balance in Balb/CJ and C57BL/6J divided in five different treatment groups Control, AngII (0.5 µg/min/kg), Salt (3% NaCl), AngII+Salt (0.5µg/min/kg AngII, 3% NaCl) and NAc (0.5µg/min/kg AngII, 3% NaCl, 150 mg/kg/24h NAc). * indicate p-value <0.05 comparing treatment to control. # indicate p-value <0.05 comparing strains within the same treatment group. ¤ indicate p-value <0.05 between AngII+Salt and AngII+Salt+NAc treated animals within same strain.

Despite a lower urine excretion in Balb/CJ mice compared to C57BL/6J, no differences in GFR was detected (Figure 13) suggesting no major kidney
damage displayed in these animals. No major difference was seen on MAP, neither when comparing between groups nor between strains, but Balb/CJ mice show higher MAP after Salt treatment in comparison to Control.

Figure 13. Show anaesthetised mean arterial pressure (MAP, A) and glomerular filtration rate (GFR, B) in Balb/CJ and C57BL/6J after treatment, Control, AngII (0.5 µg/min/kg), Salt (3% NaCl), AngII+Salt (0.5µg/min/kg AngII, 3% NaCl). * indicate p-value <0.05

Microarray analysis of gene expression identified oxidative stress as an important, differentially regulated system after AngII+Salt treatment, and gene ontology enrichment analysis show an enrichment of glutathione transferase system. Interestingly, evaluations of oxidative stress through measurement of urinary TBARS show lower oxidative stress in Balb/CJ compared to C57BL/6J (Figure 14). When treating the animals with NAc, it reduced the oxidative stress in C57BL/6J, at the same time it increased the mortality in the same strain (Figure 11).

Figure 14. Urinary TBARS as an indirect measurement of oxidative stress, in Balb/CJ and C57BL/6J after treatment with Control, Angiotensin II (AngII, 0.5 µg/min/kg), Salt (3% NaCl), AngII+Salt (0.5µg/min/kg AngII, 3% NaCl) and AngII+Salt+N-acetylcysteine (0.5µg/min/kg AngII, 3% NaCl, 150 mg/kg/24h NAc). * indicate p-value <0.05 comparing treatment to control. # indicate p-value <0.05 comparing strains within the same treatment group. ¤ indicate p-value <0.05 between animals treated with AngII+Salt and NAc within same strain.
When investigating gene expression in the kidneys, microarray analysis show different gene expression in Balb/CJ and C57BL/6J mice. After AngII+Salt treatment, 571 genes were changed in Balb/CJ mice while the same treatment changed 760 genes in C57BL/6J. Hierarchical clustering using differentially expressed genes after AngII+Salt indicated cluster by strain.

AngII+Salt treatment on cardiac function (Study III)
Cardiac physiology was evaluated with several different methods throughout this thesis. In study III, cardiac function was evaluated with echocardiography in Balb/CJ and C57BL/6J mice after treatment with AngII+Salt. These measurements reviled a lower SV in Balb/CJ mice after AngII+Salt treatment compared to Control animals. No difference was seen in CO when comparing the treatment groups, but Balb/CJ had lower CO than C57BL/6J both as control and after AngII+Salt treatment (Figure 15).

![Figure 15. Evaluation of cardiac function with echocardiography in Balb/CJ and C57BL/6J treated as Control or with Angiotensin II (0.5 µg/min/kg) + Salt (3% NaCl). Cardiac output, stroke volume, ejection fraction and heart rate was evaluated. * indicate p-value <0.05 comparing treatment to control. # indicate p-value <0.05 comparing strains within the same treatment group.](image)

AngII+Salt treatment did induce cardiac hypertrophy in C57Bl/6J compared to control, and a tendency to a thicker LV posterior wall in Balb/CJ
mice. It also resulted in a reduced end diastolic volume in both strains treated with AngII+Salt compared to Control (Figure 16).

Figure 16. Evaluation of end-diastolic volume and left ventricular posterior wall thickness in Balb/CJ and C57BL/6J treated as Control or with AngII+Salt treatment. * indicate p-value <0.05 comparing treatment to control. # indicate p-value <0.05 comparing strains within the same treatment group.

Due to the enriched glutathione transferase activity seen in the microarray analysis in study II, cardiac function after NAc treatment was evaluated. NAc worsened cardiac function in both Balb/CJ and C57BL/6J by reducing CO and SV, but no effect was seen on EF and HR (Figure 17).

Figure 17. Cardiac function measured with echocardiography in Balb/CJ and C57BL/6J mice treated as Control or with Angiotensin II (AngII, 0.5µg/min/kg) + high salt diet (Salt, 3% NaCl) + N-acetylcysteine (NAc, 150 mg/kg/24h). * indicate p-value <0.05 comparing treatment to control. # indicate p-value <0.05 comparing strains within the same treatment group.
Diastolic dysfunction was induced in both Balb/CJ and C57BL/6J after treatment with NAc, stated by a reduced E/A ratio. End diastolic volume was reduced in both strains after treatment with NAc. NAc treatment also resulted in increased left posterior wall thickness in both strains compared to Control (Figure 18).

Microarray analysis show different gene expression in Balb/CJ and C57BL/6J mice after AngII+Salt treatment. In Balb/CJ AngII+Salt treatment changed 667 genes while the same treatment changed 359 genes in C57BL/6J. Gene expression in Balb/CJ mice also seemed to be driven by AngII, seen with unsupervised hierarchical clustering of significantly expressed genes. Gene ontology enrichment analysis performed on differently expressed genes in the two strains after AngII+Salt treatment, displayed enrichment of structural and inflammatory categories. In Balb/CJ, enrichment was seen in antigen related processes and ribosomal genes in heart. AngII also caused enrichment in extracellular and contractile proteins in Balb/CJ. In Balb/CJ mice, cardiac beta-myosin heavy chain (Myh7), as well as skeletal muscle actin (Acta1) was higher at baseline and increased even more after AngII+Salt treatment.
Catheterization of left and right ventricle in Balb/CJ and C57BL/6J mice (IV)

In study IV left and right ventricular function was measured in Balb/CJ and C57BL/6J. Investigations of left ventricular end systolic pressure and volume, and left ventricular end diastolic pressure and volume did not display any differences among strains (Figure 19). There was a tendency to a lower end diastolic volume in Balb/CJ.

Figure 19. Display left ventricular end systolic pressure (LVESP, A), Left ventricular end diastolic pressure (LVEDP, B) left ventricular end systolic volume (LVESV, C) and left ventricular end diastolic volume (LVEDV, D) measured with pressure volume catheter, in Balb/CJ and C57BL/6J mice after treatment as Control or with Angiotensin II (AngII, 0.5µg/min/kg) + highs salt diet (Salt, 3% NaCl).

In the animals evaluated with cardiac catheterization, the same tendency was detected, as when investigating cardiac function with echocardiography. No effects on CO was seen, but Balb/CJ show a decreased SV after AngII+Salt treatment (Figure 20).
Figure 20. Display left ventricular cardiac output, stroke volume, ejection fraction and heart rate measured with pressure-volume catheter, in Balb/CJ and C57BL/6J mice treated as Control or with Angiotensin (AngII, 0.5µg/min/kg) + high salt diet (Salt, 3% NaCl). * indicate p-value <0.05 comparing treatment to control. # indicate p-value <0.05 comparing strains within the same treatment group.

Right ventricular function was evaluated with the PV-catheter during baseline and after a bolus injection of AngII (Figure 21), due to the low amount of animals, no statistics has been performed on these parameters. The same parameters was measured as for left ventricular function such as CO, SV, EF and HR. RVESV, RVEDV, RVESP and RVEDP were calculated as well (Figure 22), but no differences were detected there either.
Figure 21. Right ventricular (RV) cardiac output (CO, A), stroke volume (SV, B), ejection (EF, C) fraction and heart rate (HR, D) in Balb/CJ and C57BL/6J mice at baseline and after a bolus dose of Angiotensin II.

Figure 22. Right ventricular end systolic volume (RVESV, A), right ventricular end diastolic volume (RVEDV, B), right ventricular end systolic pressure (RVESP, C) and right ventricular end diastolic pressure (RVEDP, D) in Balb/CJ and C57BL/6J mice at baseline and after a bolus injection of AngII.
This thesis investigates how AngII affect renal and cardiac function in Wistar rats and in the two mouse strains Balb/CJ and C57BL/6J.

Previously, our group concluded that Losartan does not affect blood pressure negatively after haemorrhage, but that study was carried out without resuscitation after the haemorrhage. In a clinical setting, haemorrhage is followed by resuscitation, therefore study I was carried out investigating blood pressure managing effects in Losartan treated animals after resuscitation. In study I, RBF was lower in both control animals and Losartan treated animals, suggesting it being an effect of the haemorrhage rather than Losartan itself. When administering the blood pressure manager NE, MAP increased in both Losartan treated animals as well as the control group indicating that blood pressure can still be controlled after haemorrhage, despite Losartan treatment. There is an ongoing discussion on whether patient on Losartan treatment should stop their ARB treatment prior to surgery due to its hypotensive effects. At the same time there are other results suggesting that Losartan does not seem to affect blood pressure negatively during anaesthesia.

While investigating kidney oxygenation it was slightly lower in cortex of control rats after resuscitated haemorrhage but unchanged in Losartan treated animals in both cortex and medulla. It is well known that cortical and medullary oxygenation is regulated differently, where the medulla is said to be more sensitive to changes in blood flow due to it normally being poorly perfused. When looking at the present data, medullary oxygen tension is only slightly lower than the cortical even at baseline, which might be explained by the measuring techniques and the depth of the measurements. The measurements of the medullary oxygenation was carried out at a depth of 3.5 mm. That might be deep enough to penetrate the metabolically active outer medulla, and reach the less active inner part, where the oxygen tension might be closer to cortical values.

Cortical oxygenation was lower in the control animals after resuscitated haemorrhage, but not in the Losartan treated animals. This difference might depend on the acute effect of AngII causing vasoconstriction and increased tubular metabolism due to increased proximal tubular reabsorption of sodium.

Fluid resuscitation did not normalized MAP or RBF, at the same time that is not suggested by current guidelines. When dealing with resuscitation after haemorrhage it is important to resuscitate carefully. Actually, the lower
pressure in Losartan treated animals should amplify any detrimental effect on renal oxygenation, which strengthens the argument that Losartan does not harm the kidneys.

Previously we have shown that NE may increase the effects of AngII on the afferent arteriole. However, in study I Losartan did not affect RBF after NE, which might suggest an alternative buffering interaction between AT₁ and α-adrenergic receptors through an additional mechanism in vivo.

Study II investigated differences in renal function between Balb/CJ and C57BL/6J after AngII+SALT treatment. The main finding of Study II was that regulation of renal oxidative stress seem to be different between the two mouse strains, and it seems to be important for fluid balance regulation in Balb/CJ, making them more prone to decompensation. Due to a higher mortality rate in Balb/CJ compared to C57BL/6J after AngII+SALT, the lower levels of oxidative stress in Balb/CJ mice, was an interesting finding. Oxidative stress is known to sometimes be harmful, and it has been connected with several different disease states, such as heart failure, but also in hypertension. Due to the higher mortality rate of Balb/CJ mice in our study, the assumption would be that the levels of oxidative stress would be higher in Balb/CJ than in C57BL/6J, which we do not see. A previous study show that C57BL/6J had higher levels of oxidative stress, associated with lower glutathione-S-transferase micro-1 expression, although, that was when comparing to the mouse strain 129S6. In diabetic mice, Balb/CJ show elevated oxidative stress compared to control, while C57BL/6J did not show the same increase. Due to the increased fluid reabsorption in Balb/CJ as well as the increased levels of oxidative stress, these results might point towards the potential importance of oxidative stress in fluid volume regulation. This is in line with earlier findings that antioxidant treatment in spontaneously hypertensive rats, increase proximal tubular reabsorption.

Study III continued with analysing the differences in Balb/CJ and C57BL/6J mice after AngII+SALT treatment, but with focus on cardiac function, through microarray studies as well as echocardiography. The main finding was that the treatment of AngII+SALT cause a greater response in gene expression in Balb/CJ mice than in C57BL/6J mice.

The study also contained measurements of cardiac function which validated our previous findings of a decompensated cardiac physiology in these mice. Interestingly, when treating Balb/CJ and C57BL/6J with NAc it worsened the cardiac function in both strains. Cardiomyopathy and congestive heart failure have been linked to elevated glutathione and reductive stress, but that was seen in mutant alpha B-crystallin transgenic mice. Previously C57BL/6J mice presented with increased HR in after AngII+SALT treatment, which is presented in these mice as well. The increased HR could potentially work as a compensatory mechanism to the hypertrophy we detect and its possible effect on SV. The worsening in cardiac function and the fact that NAc
reduces cardiac output in C57BL/6J might partially explain the increased mortality after AngII+Salt+NAc treatment seen in study II.

Connected to the increased left ventricular wall thickness seen in study III, it is also interesting that Myh7 and Acta1 where elevated in Balb/CJ both at baseline but even further after AngII+Salt treatment. Myh7 and Acta1 are both hypertrophy markers that are elevated in heart failure. Overexpression of Myh7 in mice has under cardiovascular stress, been affecting negatively, which could be a contributing reason for the higher sensitivity in Balb/CJ mice after AngII+Salt treatment.

The focus of study IV was on cardiac function in Balb/CJ and C57BL/6J mice, as in study III, but evaluated with the technique cardiac catheterization. This enabled measurement of both the left and the right ventricle. End diastolic volume was reduced in study III this is not something that we could validate with study IV. However left ventricular stroke volume was reduced in both measurements. Reduced stroke volume of the left ventricle as seen in both study III and study IV, might be signs of reduced filling of the left ventricle, which can be a sign of Balb/CJ presenting with an end diastolic dysfunction. Although, there were no difference in LVEDV or LVEDP, which would be expected to be reduced if the filling of the left ventricle was impaired.

Due to the high mortality in these mice after a short period of time, and due to the fact that they do not present with obvious left ventricular dysfunction, at least not obvious enough to explain the high mortality, investigations of the right ventricle was performed. RV measurements could give us indications of an increased pressure in the pulmonary circulatory system. Although, in the few animals measured we cannot draw any conclusions of increased pulmonary pressure. It becomes important to fill the groups and do right ventricular measurements on more mice.

Differences in Balb/CJ and C57BL/6J are important to address since they are two commonly used mouse strains that are marked healthy and used within different type of research. Patients with heart failure, that also present with fluid congestion and renal failure are common in the ICU department and it is important to find an animal model that is easy to use within research and that present the symptoms that are in line with the symptoms of patients.
Conclusions

The conclusion of this thesis is that Angiotensin II affects both renal and cardiac physiology differently depending on strain and treatment setting.

I Losartan did not decrease renal oxygenation after resuscitated haemorrhage and it did not decrease the efficiency of norepinephrine as a blood pressure manager in normotensive rats.

II Balb/CJ retain more sodium and fluid than C57BL/6J, which might be connected to the higher mortality in Balb/CJ mice. Interestingly C57BL/6J present with higher levels of oxidative stress than Balb/CJ.

III Balb/CJ and C57BL/6J have different gene expression in response to the treatment of AngII+Salt. C57BL/6J are less sensitive to AngII+Salt treatment compared to Balb/CJ, but when reducing the levels of oxidative stress, it worsens cardiac function and increases mortality in C57BL/6J.

IV The decompensation of Balb/CJ mice treated with AngII+Salt does not seem to be caused by cardiac or pulmonary vascular pathology.
Populärvetenskaplig sammanfattning

Hjärtat och njurarna är livsviktiga organ för att människokroppen, och för att den ska fungera normalt. Hjärtat hjälper till att pumpa runt blodet i koppen och upprätthåller därmed cirkulationen, så att den mängd syre och näring som behövs når de olika organen. Njurarna hjälper samtidigt till att hålla en vätskebalans i kroppen samt utsöndra restprodukter som kroppen inte behöver. Njurarna bidrar också till att upprätthålla blodtrycket, både genom utsöndring av hormonet Angiotensin II (AngII) men också genom vätskeregleringen. AngII kan vara direkt blodtryckshöjande men kan också indirekt få njurarna att spara på mer vatten vilket ökar den totala volymen blod som finns i kroppen och därmed ökar blodtrycket.


I artikel I har vi använt oss av läkemedlet Losartan som är ett relativt vanligt blodtryckssänkande läkemedel som ges till patienter med högt blodtryck. Losartan blockerar AngII och leder därmed till lägre blodtryck. Det pågår dock en diskussion kring om patienter ska sluta åta Losartan innan man genomgår operation. Detta för att man är orolig att Losartan försämrrar andra blodtrycksreglerande substanser som man kan behöva använda vid operation om det exempelvis sker en större blödning. Därför har vi i artikel I undersökt vad som händer i Wistar-råttor efter en större blödning under operation, om råttorna har varit behandlade med Losartan fram tills operationen. Vi ville undersöka effekten på blodtryck samt på blodflöde till njurarna, och vi ville undersöka om man kunde höja blodtrycket med noradrenalinstrot Losartanbehandlingen. Viktigt att poängtera är att dessa råttor efter blödningen fick tillbaka den volym vätska som de förlorat under blödningen. Det vi kan se är att råttorna får ett lägre blodtryck efter blödning samt återställt blodvolym med vätskeersättning, vilket är ett väntat resultat eftersom de förlorat en stor mängd

I studie II-IV har vi istället för råttor använt oss av musstammarna Balb/CJ och C57BL/6J. Dessa två stammar är ganska vanliga inom forskning, men vad vi har sett är att de reagerar väldigt olika på behandling med AngII och mat av hög salt halt. AngII som vi pratade om tidigare har en blodtryckshöjande effekt, vilket även mat med hög salt halt har. I de här två musstammarna klarar den ena stammen (C57BL/6J) behandlingen mycket bättre än den andra (Balb/CJ). Balb/CJ kommer redan efter fyra dagar att må så pass dåligt att flera avlider, samtidigt som C57BL/6J klarar sig bra. I studie II-IV har vi därför undersökt vad det är som händer i de här två olika musstammarna som kanske skulle kunna förklara varför Balb/CJ mår så mycket sämre av den här behandlingen. Kan det vara så att de eventuellt utvecklar hjärt- eller njursvikt som gör att de mår så dåligt?

I artikel II har vi fokuserat på njurfunktion samt skillnader i genuttryck i Balb/CJ och C57BL/6J. Vi delade in dem i fem olika grupper men jag kommer främst att fokusera på gruppen som vi behandlade med både AngII och hög salt mat. Efter de här försöken kunde vi se att det var flera skillnader i genuttryck, i både Balb/CJ och C57BL/6J efter behandling i jämförelse med kontroll. Vi kunde också se att Balb/CJ sparan på mer vätska i kroppen och utsöndrar mindre urin än vad C57BL/6J mössen gör. Vi kunde dock inte se någon skillnad på njurfunktionsparametern GFR (glomerular filtration rate), eller på blodtrycket efter AngII och salt behandling vilket tyder på att deras njurar inte är jättessjuka.

I artikel III undersökte vi hjärtfunktionen genom ultraljudsmätningar på båda musstammarna. Vi undersökte också genuttryck i hjärtat som även här blev förändrat efter AngII och salt behandling. Ultraljudsmätningarna visade att Balb/CJ verkar ha lite sämre hjärtfunktion än vad C57BL/6J har efter behandling med AngII och salt. Dock verkar de inte ha så dålig hjärtfunktion att endast det skulle kunna förklara den höga dödligheten.

I studie IV undersökte vi även hur hjärtfunktionen av Balb/CJ och C57BL/6J, fast med en annan teknik, nämligen hjärtkateterisering. Den går ut på att man opererar in en kateter i antingen vänster eller höger kammare av hjärtat. Med den här kateteren kan man mäta både blodtrycket inne i hjärtat men också den blodvolymen som hjärtat klarar av att pumpa vid varje hjärt slag. Med dessa olika mått kan man sedan göra en bedömning av hjärtfunkt-
ionen. När vi mätte funktionen i den vänstra kammaren gav det liknande re-
sultat som med ultraljudsbilderna, alltså lägre slagvolym i Balb/CJ möss efter
AngII och salt behandling. Dock visade inte dessa undersökningar på sämre
funktion av den vänstra kammaren på samma sätt som ultraljudet visade det.
Vi mätte också funktionen av den högra kammaren med hjärtkateteriseringen,
men där är de undersökta djuren för få för att man ska kunna dra några slut-
satser.

I den här avhandlingen har jag alltså försökt presentera samspelet mellan
hjärta och njurar samt vad hormonet AngII kan göra med både hjärt- och njur-
funktion, genom att antingen tillsätta AngII eller blockera det.
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References

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)