Functional Aspects of the Juxtaglomerular Apparatus

Control of Glomerular Filtration and Renin Release

JOHAN SÄLLSTRÖM
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Abstract

The juxtaglomerular apparatus (JGA) is a control unit of the kidney, that regulates glomerular filtration rate (GFR) and renin release, and hence extracellular volume and blood pressure. The tubuloglomerular feedback (TGF) mechanism is a negative feedback loop that regulates GFR. Neuronal nitric oxide synthase (nNOS) is highly expressed in the macula densa cells of the JGA, and regulates the sensitivity of the TGF mechanism. Hypertension has been proposed to be caused by an increased sensitivity of the TGF due to nNOS deficiency. In diabetes, reduced TGF activity due to increased sodium-glucose reabsorption is suggested to cause hyperfiltration. Glomerular hyperfiltration has clinical significance, since it correlates with the risk of developing nephropathy.

In this thesis, the role of nNOS in the control of blood pressure and renin release was investigated in nNOS knockout mice (nNOS⁻⁻) treated with low- and high sodium diets. The nNOS⁻⁻ were normotensive, but displayed an impaired renin regulation, and failed to increase renin in response to a low sodium diet. A significantly larger renin increase during phosphodiesterase 3 (PDE3) inhibition was found in nNOS⁻⁻ compared to the wild types, resulting in similar renin levels.

Furthermore, the role of TGF and proximal glucose reabsorption in diabetes-induced hyperfiltration was investigated in adenosine A₁-receptor knockout mice (A₁AR⁻⁻) that are known to lack a functional TGF mechanism. Diabetes was induced in A₁AR⁻⁻ and wild types by injection of alloxan. The diabetic A₁AR⁻⁻ displayed a similar degree of hyperfiltration as their wild-type controls. Inhibition of renal sodium-glucose transporters reduced GFR in both genotypes, but the reduction was even more pronounced in the A₁AR⁻⁻.

In conclusion, the results indicate that renin secretion during low sodium conditions is mediated by nNOS-derived nitric oxide via cGMP-mediated inhibition of PDE3, whereas deletion of the nNOS gene does not cause hypertension. Diabetes-induced hyperfiltration is not mediated by TGF, but appears to be dependent on increased renal glucose reabsorption.

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

I Neuronal nitric oxide synthase-deficient mice have impaired renin release but normal blood pressure.  
Sällström J, Carlström M, Jensen BL, Skøtt O, Brown RD, Persson AEG.  

II Neuronal nitric oxide synthase supports renin release during sodium restriction through inhibition of phosphodiesterase 3.  
Sällström J, Jensen BL, Skøtt O, Xiang G, Persson AEG.  
*Manuscript.*

III Diabetes-induced hyperfiltration in adenosine A1-receptor deficient mice lacking the tubuloglomerular feedback mechanism.  
Sällström J, Carlsson PO, Fredholm BB, Larsson E, Persson AEG, Palm F.  

IV Inhibition of sodium-linked glucose reabsorption in the kidney normalizes diabetes-induced glomerular hyperfiltration in conscious adenosine A1-receptor-deficient mice.  
Sällström J, Engström T, Fredholm BB, Persson AEG, Palm F.  
*Manuscript.*

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### Abbreviations

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<td>7-NI</td>
<td>7-nitroindazole</td>
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<td>adenosine A1-receptor knockout mice</td>
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<tr>
<td>AC</td>
<td>adenylate cyclase</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>bw</td>
<td>body weight</td>
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<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>GFR</td>
<td>glomerular filtration rate</td>
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<td>GU</td>
<td>Goldblatt units</td>
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<td>JG</td>
<td>juxtaglomerular</td>
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<td>JGA</td>
<td>juxtaglomerular apparatus</td>
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<td>Kf</td>
<td>filtration coefficient</td>
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<td>nNOS-/-</td>
<td>neuronal nitric oxide synthase knockout mice</td>
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<td>PAH</td>
<td>para-amino hippuric acid</td>
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<td>P&lt;sub&gt;BS&lt;/sub&gt;</td>
<td>hydrostatic pressure in Bowman's space</td>
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<td>P&lt;sub&gt;GC&lt;/sub&gt;</td>
<td>hydrostatic pressure in the glomerular capillaries</td>
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<tr>
<td>P&lt;sub&gt;prox&lt;/sub&gt;</td>
<td>hydrostatic pressure in the proximal tubule</td>
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<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PRC</td>
<td>plasma renin concentration</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SGLT</td>
<td>sodium-glucose-linked transporter</td>
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<td>SNGFR&lt;sub&gt;prox&lt;/sub&gt;</td>
<td>single nephron GFR measured in the proximal tubule</td>
</tr>
<tr>
<td>SNGFR&lt;sub&gt;dist&lt;/sub&gt;</td>
<td>single nephron GFR measured in the distal tubule</td>
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<tr>
<td>TGF</td>
<td>tubuloglomerular feedback</td>
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<tr>
<td>π&lt;sub&gt;BS&lt;/sub&gt;</td>
<td>oncotic pressure in Bowman's space</td>
</tr>
<tr>
<td>π&lt;sub&gt;GC&lt;/sub&gt;</td>
<td>oncotic pressure in the glomerular capillaries</td>
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Introduction

The kidneys regulate the body’s fluid balance and are therefore important for blood pressure regulation. This is achieved by controlling the amount of water and solutes excreted, and by the release of hormones. The tubuloglomerular feedback (TGF) mechanism is a negative feedback loop located in the juxtaglomerular apparatus (JGA) of each nephron, that regulates the glomerular filtration rate (GFR) to match tubular transport capacity. Nitric oxide locally generated in the macula densa of the JGA by neuronal nitric oxide synthase (nNOS), has been shown to have an important effect on the sensitivity of the TGF mechanism. Earlier data from several models of experimental hypertension shows that an increased TGF sensitivity is observed during the development of hypertension\textsuperscript{1,2}, suggesting that TGF can play a causal role in the development of the disease.

Renin is a proteolytic hormone that is secreted from the granulated juxtaglomerular (JG) cells in the wall of the afferent arterioles of the kidney. In the circulation, renin is the rate-limiting step in the formation of the sodium-retaining and vasoconstrictive peptide angiotensin II, thus constituting an important regulator of blood pressure and sodium balance. The close proximity of the JG cells and the nNOS-rich macula densa, suggests a possible role of NO in renin regulation. However, studies on this issue have provided conflicting results.

Nephropathy as a consequence of long-term diabetes is a significant health problem\textsuperscript{3}. The mechanisms causing nephropathy in diabetes have however not yet been elucidated. Early in the disease, diabetic patients are commonly found to exhibit glomerular hyperfiltration\textsuperscript{4,5}. However, in one third of the diabetic patients, GFR starts to decline after some time, as a result of a progressive diabetic nephropathy. It has been demonstrated that diabetic patients that hyperfiltrate early in the disease, have an increased risk of developing microalbuminuria and nephropathy\textsuperscript{6-8}. According to one hypothesis, hyperfiltration is caused by a reduced activity of the TGF mechanism, caused by increased proximal sodium-glucose reabsorption that will reduce the NaCl concentration at the macula densa\textsuperscript{9}.

In the studies presented in this thesis, knockout mice were used to investigate the influence of different aspects of the JGA on the regulation of GFR, blood pressure, and renin release. Effort has been made to improve the experimental techniques and, whenever it was possible, to perform measurements in conscious animals to avoid the confounding effects of anaesthesia.
Regulation of glomerular filtration

GFR is tightly regulated to match tubular reabsorption in order to maintain fluid and electrolyte homeostasis. Without such careful control, fluctuations in arterial blood pressure would either lead to fluid loss or retention. The autoregulation of GFR is performed by two different mechanisms; the myogenic response and TGF. All nephrons work as single autoregulating units and possess both mechanisms.

Like the filtration in other vessels, the filtration process in the glomeruli is dependent on two factors; the net filtration pressure and the filtration coefficient (Kf). The relationship between these factors and GFR can be expressed by the Starling equation (Figure 1). The filtration pressure, which is the sum of the factors favouring filtration and those opposing filtration, can be affected by several parameters. The glomerular capillary hydrostatic pressure (PGC) is higher than the hydrostatic pressure in Bowman’s space (PBS), and this pressure difference (PGC - PBS) favours filtration. Since the glomerular membrane does not filter molecules larger than 50 Å, the ultrafiltrate is virtually protein-free and the oncotic pressure in Bowman’s space (πBS) is therefore close to zero. Accordingly, the oncotic pressure difference between the glomerular capillaries and Bowman’s space (πGC-πBS) opposes filtration.

Figure 1. Illustration of the factors affecting net filtration pressure and GFR.

The oncotic pressure difference will increase from the afferent to the efferent side of the glomerular capillaries when πGC increases due to continuous filtration. Since the hydrostatic pressure difference will be essentially un-
changed, the filtration will reduce along the capillary network when approaching the efferent side.\textsuperscript{10} Munich-Wistar rats have been extensively used for micropuncture, due to the frequent occurrence of superficial glomeruli. In this strain, filtration equilibrium eventually occurs when the factors favouring and opposing filtration balance each other\textsuperscript{11}. However, the more commonly used Sprague-Dawley rats do not reach filtration equilibrium due to the higher $P_{GC}$\textsuperscript{12}, while mice, however, have not yet been investigated.

Filtration pressure is directly influenced by renal plasma flow, the tonus of the afferent and efferent arterioles, and the rate of reabsorption in the proximal tubule. An increase in renal plasma flow will slow down the increase of plasma proteins in the glomerular capillaries, thus reducing $\pi_{GC}$, which consequently will increase filtration pressure and GFR. An increased contraction of the afferent arteriole will reduce GFR by decreasing plasma flow and $P_{GC}$. The effects of the tonus of the efferent arteriole are more difficult to predict. An increased contraction will reduce glomerular plasma flow, which will act to reduce GFR, but will also increase $P_{GC}$, which in turn will counteract the decreased GFR.\textsuperscript{10} The rate of reabsorption in the proximal tubule affects filtration pressure by changing $P_{BS}$, i.e. an increased reabsorption will decrease $P_{BS}$ thus favouring filtration\textsuperscript{15}.

The afferent and efferent arterioles are innervated by sympathetic adrenergic nerves. Nerve stimulation will cause a contraction of both arterioles, which in turn will decrease GFR and renal plasma flow\textsuperscript{14}. The contractile effect of norepinephrine is mediated by adrenergic $\alpha_1$ receptors\textsuperscript{15}. Adrenergic $\beta_1$ receptors are also present, and apart from their role as a trigger of renin release, they also appear to dampen arteriolar contraction induced by $\alpha_1$ receptors\textsuperscript{16}. Glomerular ultrafiltration is also affected by many hormones and other substances. Angiotensin II acts on angiotensin-II AT\textsubscript{1} receptors in both arterioles, which will reduce renal blood flow. However, since the contractile effects are larger on the efferent side, GFR will not change to a large extent during high plasma levels of angiotensin II.\textsuperscript{17} Adenosine is formed in the kidney by ATP metabolism, and can constrict the afferent arterioles by activation of adenosine $A_1$ receptors ($A_1$AR)\textsuperscript{18}. Adenosine plays an important role in the TGF mechanism which will be discussed in detail below.

The myogenic response

The myogenic response is the ability of the afferent arteriole to contract in response to increased arterial pressure, which will reduce blood flow to the glomerulus. This is probably mediated by stretch-sensitive cation channels in the vascular smooth muscle of the arteriole, that increase $Ca^{2+}$ influx\textsuperscript{19}. Interestingly, the myogenic response of the afferent arteriole has a special ability to respond to fast systolic blood pressure oscillations, most likely in order to protect the glomeruli from damage\textsuperscript{20}. 
The TGF mechanism

The TGF mechanism is a negative feedback loop located in the JGA of each nephron that controls GFR. If the macula densa detects increased tubular NaCl concentration, TGF is activated, constricting the afferent arteriole\textsuperscript{21}, thus matching the tubular sodium load to the reabsorption capacity. The relation between the luminal perfusion rate and the feedback response is non-linear\textsuperscript{22} and the normal tubular flow rate is located on the steep portion of the curve, which makes the system sensitive to small changes in flow rate\textsuperscript{23}. In 1980, it was suggested that adenosine was the mediator of the TGF response\textsuperscript{24}, which provided a link between the metabolic demand from the NKCC2 transporters in the macula densa cells and vasoconstriction. The afferent arterioles have a predominant expression of A\textsubscript{1}ARs\textsuperscript{25,26} which mediates vasoconstriction\textsuperscript{27} by G\textsubscript{i} protein-mediated activation of phospholipase C\textsuperscript{28}, that will increase [Ca\textsuperscript{2+}]\textsubscript{i}\textsuperscript{29}. Further studies in A\textsubscript{1}AR\textsuperscript{-/-} confirmed that the TGF mechanism is dependent on A\textsubscript{1}ARs, since these mice completely lack the TGF response\textsuperscript{30,31}.

Proximal reabsorption

The rate of proximal reabsorption influences GFR, as demonstrated mainly in studies where proximal reabsorption has been inhibited by carbonic anhydrase inhibitors. These inhibitors reduce GFR by direct effects on proximal intratubular pressure as well as via TGF activation as a consequence of the increased distal NaCl load. Based on the different effects of carbonic anhydrase inhibition on SNGFR measured proximally and distally, TGF effects accounted for approximately 50\% of the GFR decrease\textsuperscript{32}. Further studies demonstrated that removal of the influence of TGF by co-administration of dopamine that dilates the afferent arteriole, was only able to partially restore GFR after carbonic anhydrase inhibition, despite a higher glomerular capillary pressure than animals only receiving the inhibitor\textsuperscript{33}. Moreover, carbonic anhydrase inhibition was recently found to decrease GFR in A\textsubscript{1}AR\textsuperscript{-/-} lacking a functional TGF mechanism\textsuperscript{34}. In hyperfiltering diabetic rats, several studies have demonstrated a reduced proximal intratubular pressure\textsuperscript{35-38}, which might be secondary to the increased glucose reabsorption, but also due to lower hydraulic flow resistance in the distal nephron segments during high tubular flow rates\textsuperscript{13}. Consequently, it is possible that the low \(P_{BS}\) contributes to the elevated GFR found during diabetes.
Nitric oxide

Nitric oxide is a gaseous, short-lived, potent vasodilator that affects vascular smooth musculature via a cyclic guanosine monophosphate (cGMP) dependant pathway. The substance was first shown to be produced from vascular endothelium and was known as the endothelium-derived relaxing factor until it was identified as NO.

Nitric oxide synthases

Nitric oxide is produced enzymatically when L-arginine is cleaved into citrulline at a wide range of places in the body by three types of nitric oxide synthases; endothelial (eNOS), inducible (iNOS) and neuronal (nNOS) nitric oxide synthase. Nitric oxide deficiency has been shown to have a role in hypertension, both in animal experiments and in studies of human genomics. eNOS expressed in blood vessels is important for maintaining normal endothelial function, and mice with targeted deletion of the gene for eNOS, develop hypertension, whereas polymorphism of the gene in humans is associated with an increased incidence of hypertension. nNOS was first detected in the brain, but has later been found in several other tissues such as the heart and kidneys. In the kidneys, nNOS is abundant in the macula densa cells of the JGA, where it regulates the tonus of the afferent arteriole and modulates the TGF mechanism.

Modulation of TGF response by nitric oxide

The focused expression of nNOS at the macula densa indicates a functional role of the enzyme in the JGA. Tubular infusion of an unspecific NOS antagonist decreased the glomerular capillary pressure, whereas an NO donor increased the pressure, an effect that was abolished by the presence of an NO scavenger. Similar observations were made in isolated JGAs. Consequent studies demonstrated that NOS inhibition increased the sensitivity of the TGF response, shifting the TGF curve to the left and also demonstrated that NOS inhibition reduced the SNGFR at a given perfusion rate. More evidence of a specific role of nNOS was provided by experiments using the relatively selective nNOS inhibitor 7-nitroindazole (7-NI), which caused a similar sensitization as unspecific NOS blockade. In nNOS−/−, SNGFR was reduced and the proximal-distal SNGFR difference was increased, supporting the notion that nNOS-derived NO sensitizes TGF. However, the maximum stop-flow response was not found different between the strains. The effects of NO appears dependent on cGMP, and can be mediated either through a direct vasodilatory action on the smooth muscle cells in the afferent arteriole, or alternatively, via inhibition of the NKCC2 transporters in the macula densa cells, thus reducing NaCl influx. Physiologically, nNOS-
derived NO might be important when there is a need to excrete excess volume, e.g. during volume expansion\textsuperscript{58}.

**Nitric oxide and hypertension**

Intravenous infusion of an unspecific NOS inhibitor leads to an elevation of the systemic blood pressure, due to an increase in peripheral vascular resistance caused by inhibition of eNOS in the vessels. There are some new findings indicating that peripheral resistance also might be affected by nNOS\textsuperscript{59}, but this isoform is not generally considered involved in the short-term blood pressure regulation, and acute nNOS inhibition does not increase blood pressure\textsuperscript{54}. However, long-term nNOS inhibition by 7-NI caused hypertension in rats, which was characterized by increased TGF sensitivity and reduced GFR in the initial phase. After the hypertension had developed, these parameters were normalized.\textsuperscript{60} Interestingly, this development has similarities to that seen in two hypertensive strains of rats; the spontaneously hypertensive rat\textsuperscript{1,61} and the Milan hypertensive strain\textsuperscript{2,62}, which exhibited volume retention and an increased TGF sensitivity during the development phase of hypertension. The cause of hypertension in those strains has further been localized to the kidney, due to the fact that transplantation of a kidney from a hypertensive rat to a normotensive control rat also transferred the hypertension\textsuperscript{63,64}. In light of these findings, it appears interesting to study mice with targeted deletion of nNOS. However, blood pressure in mice with nNOS deficiency has not been found to be different from that in wild-type mice\textsuperscript{65,66}, but the blood pressure has only been measured during anaesthesia, which is known to influence cardiovascular parameters and might mask differences.

**The renin angiotensin aldosterone system**

The renin angiotensin aldosterone system is important for the normal regulation of blood pressure and electrolyte homeostasis, and is stimulated in situations of low blood pressure or low sodium intake. Renin is a proteolytic enzyme that is produced from the granulated JG cells in the wall of the afferent arteriole. Already back in 1898, Robert Tigerstedt at the Karolinska Institute discovered that a crude renal extract was able to increase blood pressure when it was injected\textsuperscript{67}. They realized that the extract contained an active compound and called it renin, but the general interest of the finding was low, and it took almost 40 years before the full renin angiotensin system was described.\textsuperscript{68} Renin is the central player in the system, and converts the physiological inactive protein angiotensinogen to angiotensin I. Angiotensin I itself displays no vasoactive properties, but is rapidly transformed into the highly active peptide angiotensin II by the action of the angiotensin converting enzyme (ACE). Angiotensinogen is continuously released from the liver, and
ACE is mainly localized to the vascular endothelium, particularly in the lungs. The classical physiological effects of angiotensin II include general vasoconstriction, increased renal electrolyte reabsorption, and thirst. Angiotensin II will increase aldosterone release from the adrenal gland, which, in turn, will promote sodium retention. All these effects are mediated through angiotensin AT$_1$ receptors and will act to prevent a fall in blood pressure.$^{69}$ Angiotensin II also acts on angiotensin AT$_2$ receptors, which appear to have opposite effects as the AT$_1$ receptors. This is illustrated by studies in knockout mice, where deletion of the gene encoding AT$_{1A}$ receptors*, decreased blood pressure and abolished the pressor response to angiotensin II infusion$^{70}$. Conversely, deletion of the gene encoding AT$_2$ receptors has been shown to increase blood pressure slightly$^{71,72}$, and augmented the pressor response to angiotensin II infusion$^{72}$. Angiotensin II has a short plasma half-life and is further degraded by different peptidases to shorter peptides (angiotensin III-IV) that are less vasoactive but have other properties that are still not fully described. Humans and most mice strains only have one renin gene (Ren-1), however some mice strains contain a duplicated copy of the gene (Ren-2) that mainly expresses renin in the submandibular glands$^{73}$. The 129 strain, which many genetically altered mice are derived from, expresses this extra copy. If the targeted gene is close to the Ren-2 gene in the genome, the extra renin gene might follow the mutated gene even when the strain is inbred to another strain for several generations, which could complicate the interpretation of renin data. However, a recent study could not find a clear influence of the Ren-2 gene on the circulating renin levels$^{74}$.

Renin release

The pathways regulating renin release can be assigned to four categories; renal baroreceptors, macula densa, renal nerves, and hormones. Intracellularly, an increased cAMP concentration promotes renin exocytosis from the JG cells, whereas an increased Ca$^{2+}$ concentration inhibits exocytosis.

The renal perfusion pressure affects renin release via renal baroreceptors$^{75,76}$, although no direct evidence of those receptors has been presented so far. Experiments in whole animals suggest that the mechanism is independent from the macula densa pathway, since it is preserved in the non-filtering kidney and during NKCC transport inhibition$^{77,78}$. However, experiments using isolated afferent arterioles have yielded conflicting results. One study demonstrated a clear pressure dependency$^{79}$, while a study from another group failed to show this dependency.$^{80}$ The latter experimental protocol was however slightly different, utilizing a model without perfusion. Mechanistically, increased perfusion pressure could reduce renin release via increased

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*Mice express two types of AT$_1$-receptors; AT$_{1A}$ and AT$_{1B}$, respectively, of which the AT$_{1A}$ subtype appears most important and best mimics the human AT$_1$-receptor.
intracellular Ca\(^{2+}\) concentration in the JG cells. The pressure could either be sensed directly by the JG cells, or by the endothelial cells. The presence of gap junctions between the endothelial cells and the JG cells\(^{81}\) suggests that Ca\(^{2+}\) might be transferred between those cells. Connexin 40 knockout mice do not reduce their plasma renin concentration (PRC) in response to an increased perfusion pressure, which suggests a specific role of gap junctions composed by this connexin in endothelial-JG cell communication.\(^{82}\)

The macula densa cells in the distal tubule regulate renin release (as well as GFR) by sensing the NaCl concentration of the filtrate\(^{83}\). Increased NaCl concentration at the macula densa will increase salt transport across the NKCC2 carriers, which will increase adenosine exposure of the smooth muscle and JG cells in the afferent arteriole. The activated A\(_1\)ARs increases [Ca\(^{2+}\)] in the smooth muscle cells\(^{29}\), but also in the JG cells', probably due to the presence of gap junctions.\(^{84}\) Furthermore, direct activation of A\(_1\)ARs in the JG cells reduces renin release\(^{85,86}\), possibly via [Ca\(^{2+}\)] increase\(^{87}\), or as a consequence of the receptor’s coupling to the G\(_i\) protein which will reduce the intracellular cyclic adenosine monophosphate (cAMP) concentration\(^{88}\). The macula densa pathway’s role is supported by recent data from knockout mice, showing that deletion of the NKCC2A gene causes an abolished renin reduction after an acute sodium load\(^{89}\). Furthermore, the basal PRC has been reported to be increased in A\(_1\)AR\(^{-/-}\) mice\(^{30,90,91}\), and the acute response to a saline load is abolished\(^{92}\), however the basal PRC was unaltered in the latter study. Interestingly, the macula densa pathway also seems to be important for the renin-suppressing effects of an increased renal perfusion pressure, since this response is impaired in A\(_1\)AR\(^{-/-}\) mice\(^{91}\).

The macula densa cells have a high expression of COX-2 and nNOS, which may act stimulatory on renin release, as indicated by the increased expression of both enzymes during conditions with a high PRC\(^{93-95}\). During a low luminal sodium chloride concentration at the macula densa, the [Ca\(^{2+}\)] has been shown to increase in the macula densa cells\(^{96}\). PGE2 is a powerful stimulant of renin release\(^{97}\), which acts on EP2 and EP4 receptors on the JG cells\(^{98}\). A high Ca\(^{2+}\) concentration will increase the activity of cytosolic phospholipase A2, which would increase the availability of arachidonic acid\(^{99}\) that is the rate-limiting step in prostaglandin production. This mechanism is supported by a recent study where release of PGE2 from macula densa cells correlated negatively with the luminal sodium chloride concentration\(^{100}\). For long-term adaptation, evidence indicates that p38 and p44/42 mitogen-activated protein kinases could be activated by a low extracellular [Cl\(^{-}\)], which would promote COX-2 expression\(^{101}\). NO derived from nNOS could also be involved in prostaglandin signalling, since it may increase the activity of COX-2\(^{102}\). NO also directly affects renin release via its second messenger cGMP, and the main effect seems to be stimulatory, even though

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NO during some circumstances could have an inhibitory role, which will be discussed in detail below.

The JGA is innervated by sympathetic nerve fibres\textsuperscript{103}, and renal nerve stimulation increases renin release\textsuperscript{104}, while bilateral denervation reduces PRC\textsuperscript{94,105}. Furthermore, administration of isoproterenol increases renin release, while propranolol decreases the release\textsuperscript{106}. Adrenergic $\beta_1$ receptors on the JG cells will activate adenylate cyclase (AC) via the $G_s$ protein, which will increase renin release\textsuperscript{107}. Sympathetic nerve stimulation of the JG cells also appears to stimulate renin synthesis, which is demonstrated by the low renin mRNA levels in denervated kidneys\textsuperscript{105} or kidneys from animals deficient of $\beta_1/\beta_2$ receptors\textsuperscript{108}. The degree of renal sympathetic nerve activity is affected by baroreceptors in the circulatory system. High-pressure baroreceptors in the carotid sinus and low-pressure baroreceptors in the atria, will increase renal sympathetic nerve activity and renin release during a fall in systemic blood pressure\textsuperscript{107}. The physiological mechanism that appears to rely most on renal nerves for a normal PRC response, is loss of blood volume, since this response is reduced by renal denervation\textsuperscript{109}, and abolished if the pressure fall in the atria is prevented\textsuperscript{110}. The response to alteration in dietary sodium intake is relatively well-preserved after renal denervation\textsuperscript{94,105}, or in animals deficient of $\beta_1/\beta_2$ receptors\textsuperscript{108}, indicating that other pathways are more important for the adaptation to salt intake.

Renin release is affected by several hormones, of which angiotensin II and atrial natriuretic peptide (ANP) probably are the most important. Angiotensin II serves as a negative feedback loop, limiting the renin secretion by binding to AT$_1$ receptors on the JG cells that increase $[Ca^{2+}]_i$\textsuperscript{87,111}. Conversely, AT$_1$ receptor antagonists increase renin secretion\textsuperscript{112}. An increased distension of the atrium, secretes ANP\textsuperscript{113,114}, which reduces renin release\textsuperscript{115} by acting on the guanyl cyclase-coupled natriuretic peptide receptor-A\textsuperscript{116}. Accordingly, inhibition of renin release by ANP in isolated JG cells, is associated with an increased intracellular cGMP concentration\textsuperscript{117}.

**Intracellular regulation of renin release**

Renin is predominantly formed and released from the JG cells, which are mainly located in the distal end of the afferent arteriole. JG cells are modified smooth muscle cells, but they have a sparse content of myofibrils and a low degree of myosin expression\textsuperscript{118}. Renin is stored in vesicles in the cytoplasm, that are secreted by exocytosis. The main intracellular factors regulating renin release are the concentrations of cAMP and $Ca^{2+}$. Cyclic AMP stimulates exocytosis, and the production is regulated by G protein-coupled receptors that control AC activity. JG cells express various receptors that stimulate AC activity through the $G_s$ protein, e.g. $\beta_1$, EP2/EP4 and IP-receptors. The $[cAMP]_i$ is also regulated by the degree of degradation by phosphodiesterases, and data indicates that the most important are
Cyclic GMP acts as a competitive inhibitor of PDE3, since this isoform also degrades cGMP, but at a much lower catalytic rate. Cyclic AMP mediates exocytosis through a protein kinase A-dependent pathway, but the exact mechanisms are not yet understood. Ca$^{2+}$ acts inhibitorily on renin release, in opposition with the role in most other secretory cells. This might be explained by the presence of the Ca$^{2+}$ inhibitable AC5 and 6 isoforms in the JG cells. In the long-term, renin release can also be affected by the number of JG cells. This is achieved by metaplasia, i.e. smooth muscle cells are replaced by JG cells. For example, this occurs in mice with a deficient angiotensinogen production in order to increase the production of Angiotensin II.

Nitric oxide and renin release

The mRNA levels of nNOS and renin in the renal cortex correlate directly, which suggests a causal association. However, the role of NO in renin regulation has been a matter of controversy, and over the last years, in vivo evidence has been presented for both a stimulatory and an inhibitory role of the substance. The disparate results may have been caused by the use of different NOS inhibitors as well as different doses. In vitro data has shed some light on the issue. In cortical kidney slices, NO was shown to inhibit renin release, while in isolated JG cells, NO initially had an inhibitory role, but after some hours had a stimulatory role. A stimulatory role of NO was also observed in isolated perfused rat kidneys. In isolated perfused JGAs, NO at the macula densa appears to support renin release, whereas NO in the arterioles appears to have an inhibitory role, which illustrates that the effects of NO vary to a large extent with the place of action. The effects can be different in various anatomical structures, and also when different NOS isoforms are blocked. In 1994, it was suggested that NO from the macula densa could increase renin secretion by inducing production of cGMP, that competitively inhibits PDE3 in the JG cells, thus increasing the cAMP concentration and the renin release in response to diverse stimuli. Conversely, the inhibitory effect might be caused by cGMP-mediated activation of G kinase, that directly inhibits renin exocytosis.

Glomerular hyperfiltration and diabetic nephropathy

Diabetes is the leading cause of end-stage renal disease worldwide. During end-stage renal disease, GFR has reached such low levels that death will occur without dialysis. A strong predictor of future nephropathy and increased mortality is microalbuminuria, which is defined as the excretion of 30-300 mg albumin/24 hours. The source of albumin is leakage from the glomerulus which might be caused by a decrease in negatively charged...
heparan sulphate proteoglycans, that are important for charge-based permeability of the glomerular basement membrane, in particular since albumin is negatively charged. Early in the disease, before the development of microalbuminuria and nephropathy, glomerular hyperfiltration is commonly found. The increase is largest in newly diagnosed, untreated diabetes, and GFR is returned towards normal values by intensive insulin treatment. Hyperfiltration has clinical importance since the presence of early hyperfiltration correlates with an increased risk of developing microalbuminuria and nephropathy later on in the disease.

Renal glucose handling

Glucose is reabsorbed in the proximal tubule by secondary active transport. At the apical membrane, glucose and sodium are transported into the cell by sodium-glucose-linked transporters (SGLT), driven by the low $[Na^+]_i$ caused by the basolateral Na-K pump. The accumulated glucose leaves the cell through the basolateral membrane by facilitated diffusion through GLUT proteins. In the early proximal tubule, glucose is reabsorbed by the high-capacity/low-affinity SGLT2 protein ($1Na^+/glucose$), while the low-capacity/high-affinity SGLT1 protein ($2Na^+/glucose$) reabsorbs glucose in the late proximal tubule. When the plasma glucose reaches approximately 11 mM, the SGLTs starts to saturate and glucosuria will occur if the concentration exceeds this level. During diabetes, the chronically high glucose levels will increase the proximal glucose reabsorption capacity, probably due to proximal hypertrophy and upregulation of SGLT1/2. Since glucose is reabsorbed by cotransport, the reabsorption of sodium will increase as well. This has been demonstrated in perfused proximal tubules, where increased tubular glucose concentration increased sodium uptake significantly. Furthermore, data obtained by lithium clearance in diabetic patients, indicates an increased proximal reabsorption of sodium.

Mechanisms causing diabetes-induced hyperfiltration

The diabetic kidney is characterized by growth, and increased GFR and blood flow. The renal enlargement can be caused by several factors, but the most central is probably the increased filtration of glucose which will upregulate glucose transporters and cause growth of the proximal tubule. The relation to the glucose load is demonstrated by the finding that there is a positive correlation between kidney size and insufficient glycaemic control.

Increased renal blood flow is generally found during diabetes and could be the factor responsible for hyperfiltration, but the mechanisms are not fully understood. Renal blood flow can increase either in response to a primary vascular, or to a primary tubular event. A primary vascular event is a direct
relaxation of the afferent arteriole (e.g. due to increased production of a vasodilator), which consequently will increase blood flow. A primary tubular event will instead dilate the afferent arteriole indirectly, via the tubuloglomerular feedback mechanism.

A proposed mechanism for a tubular cause for diabetes-induced hyperfiltration is the tubular hypothesis of glomerular filtration\(^9\). According to this theory, glomerular hyperfiltration is secondary to increased proximal reabsorption. The resulting reduction in the NaCl load to the macula densa will reduce the TGF response, consequently increasing the GFR. Several investigators have found an increased proximal reabsorption in diabetes, both in patients and in models of experimental diabetes\(^{38,150,152}\). Mechanisms that can cause this include increased expression of SGLTs\(^{147,148}\), and hypertrophy of the proximal tubule, increasing the reabsorption area\(^{153}\). The dependence on renal growth was confirmed by blocking ornithine decarboxylase, which prevented the development of hyperfiltration in diabetic rats\(^9\). Furthermore, by micropuncture experiments, it has been confirmed that the NaCl concentration close to the macula densa is reduced\(^{38,154}\).

As discussed above, the degree of proximal reabsorption affects GFR directly by affecting the \(P_{\text{prox}}\) and the net filtration pressure. Consequently, it has been proposed that hyperfiltration could directly result from the increased proximal reabsorption found during diabetes\(^{155}\), which is supported by the findings that \(P_{\text{prox}}\) actually is reduced during the disease\(^{35-38}\).
Aims of the investigation

This thesis deals with functional aspects of the JGA. The first two studies focus on the role of nNOS in blood pressure and renin regulation, whereas the two following studies investigate mechanisms for the development of diabetes-induced glomerular hyperfiltration.

Specifically, the aims were to:

- Investigate if nNOS deficiency can cause hypertension. (Study I)
- Investigate the role of nNOS in the regulation of renin release. (Study I & II)
- Determine the roles of TGF and renal glucose reabsorption in diabetes-induced hyperfiltration. (Study III & IV)
Methods

Animals

All experiments were approved by the animal ethics committee of Uppsala.

nNOS knockout mice

The nNOS-deficient mice used in Study I & II were bred at the BMC animal department by using heterozygous breeding pairs (B6.129S4-Nos1tm1Plh, The Jackson Laboratory, Bar Harbor, ME). Their genotype was determined by taking a small biopsy specimen from their tail or ear at the age of three weeks. DNA was extracted from the tissue using a preparation kit (DNeasy Tissue Kit, Qiagen, Hilden, Germany) whereby their genotype was determined using PCR. The primers used were as follows: 5’-tca gat ctg atc cga gga gg-3’; 5’-ttc cag agc gct gtc ata gc-3’; 5’-ctt ggg tgg aga ggc tat tc-3’; 5’-agg tga gat gac agg aga tc-3’. Amplification was carried out for 37 cycles; denaturation at 94°C for 35 s, annealing initially at 64°C for 45 s with a decrease in temperature of 0.5°C per cycle until 58°C, extension at 72°C for 45 s per cycle with a final extension time of 2 min. The animals in study I were given two diets (TD90228 & TD92034, Harlan Scandinavia, Denmark) containing different sodium concentrations; low sodium (0.01% NaCl) and high sodium (4% NaCl), whereas the animals in study II were only given low sodium diet (0.01% NaCl; Lantmännen, Kimstad, Sweden).

A1AR knockout mice

The A1AR knockout mice developed by Johansson and co-workers156 used in Study III & IV were bred and genotyped at The Karolinska Institute. The strain has been inbred on a C57BL/6J background (Jackson Laboratories, Bar Harbor, MA). The animals in study III & IV were given standard rodent chow (0.7% NaCl; Lantmännen, Kimstad, Sweden). Diabetes was induced by a single bolus injection of alloxan (75 mg/kg bw) in the tail vein. Animals with a plasma glucose concentration ≥20 mM were considered diabetic.
Experimental protocols
Protocol for study I

Series 1 – Telemetric blood pressure measurements
- Low sodium diet (10 days)
- Telemetry recording (2-4 days)
- High sodium diet (10 days)
- Telemetry recording (2-4 days)

Series 2 – Plasma renin concentration
- Low- or high sodium diet (10 days)
- Blood sample

Series 3 – Plasma aldosterone, potassium and renin mRNA
- Low- or high sodium diet (10 days)
- Blood sample
- Renal cortex harvested

Series 4 – Renal excretion
- Low- or high sodium diet (10 days)
- Metabolic cage (24 h)
- Normal cage (1-2 days)

Repeated 4 times
**Protocol for study II**

<table>
<thead>
<tr>
<th>Series 1 – Plasma renin concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low sodium diet (10 days)</td>
</tr>
<tr>
<td>Milrinone/Vehicle or Zaprinast/Vehicle injection</td>
</tr>
<tr>
<td>Blood sample</td>
</tr>
<tr>
<td>Normal sodium diet (14 days)</td>
</tr>
</tbody>
</table>

Repeated once (cross-over design)

<table>
<thead>
<tr>
<th>Series 2 – Glomerular filtration rate and renal plasma flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low sodium diet (10 days)</td>
</tr>
<tr>
<td>Milrinone/Vehicle or Zaprinast/Vehicle injection</td>
</tr>
<tr>
<td>PAH/Inulin clearance</td>
</tr>
<tr>
<td>Normal sodium diet (14 days)</td>
</tr>
</tbody>
</table>

Repeated once (cross-over design)

<table>
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<tr>
<th>Series 3 – Telemetric blood pressure measurements</th>
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</thead>
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<tr>
<td>Low sodium diet (10 days)</td>
</tr>
<tr>
<td>Telemetry recording (30 min)</td>
</tr>
<tr>
<td>Milrinone/Zaprinast/Vehicle injection</td>
</tr>
<tr>
<td>Telemetry recording (30 min)</td>
</tr>
<tr>
<td>Washout (2 days)</td>
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</table>

Repeated

<table>
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<tr>
<th>Series 4 – Acid dissection and tissue cAMP concentration</th>
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</thead>
<tbody>
<tr>
<td>Low sodium diet (10 days)</td>
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<tr>
<td>Renal cortex harvested from one kidney</td>
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<tr>
<td>Other kidney placed in acid</td>
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</table>

<table>
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<tr>
<th>Series 5 – Cell culture</th>
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</thead>
<tbody>
<tr>
<td>Cells seeded and cultured (24 h)</td>
</tr>
<tr>
<td>New medium with either vehicle or milrinone</td>
</tr>
<tr>
<td>Incubation (2 h)</td>
</tr>
<tr>
<td>Cells harvested</td>
</tr>
</tbody>
</table>
Protocol for study III

Series 1 – Glomerular filtration rate and histology
- Alloxan injection
- Diabetes 2-3 weeks
- Surgery
- Inulin bolus and infusion (40 min)
- Urine collection and blood sampling (60 min)
- Left kidney formalin fixed

Series 2 – Renal excretion
- Alloxan injection
- Diabetes 2-3 weeks
- Metabolic cage (24 h)
- Normal cage (1-2 days)

Repeated once

The normoglycemic controls were treated in the same manner, except for the alloxan injection.

Protocol for study IV

Series 1 – TGF and diabetes-induced hyperfiltration
- GFR measurement
- Alloxan injection
- Diabetes 4-5 weeks
- GFR measurement

Series 2 – Glucose reabsorption and diabetes-induced hyperfiltration
- Alloxan injection
- Diabetes 4-5 weeks
- Phlorizin/Vehicle injection + GFR measurement
- Recovery (4 days)
- Phlorizin/Vehicle injection + GFR measurement

The normoglycemic controls were treated in the same manner, except for the alloxan injection.

Telemetric blood pressure measurements

Implantation of transmitters (Study I&II)

All surgical equipment was sterilized. Animals were anaesthetized with ~2% Isoflurane (Abbott, Solna, Sweden) in 40% O₂ and 60% N₂, and a midline incision was made from the lower mandible to the sternum. A telemetric blood pressure transmitter (TA11PA-C10, Data Sciences International, St.
Paul, MN) was introduced into the left carotid artery and advanced to the aortic arch. The catheter was secured with a 5-0 silk ligature and a drop of tissue glue (Vetbond; 3M Animal Care Products, St Paul, MN). The body of the transmitter was placed subcutaneously on the right flank and the incision was closed using 5-0 sutures. The animals were allowed to recover for ten days before experiments commenced.

Data processing of chronic data (Study I)

During every experimental period, data for blood pressure and heart rate was sampled for 5 s every second minute during a period of at least 48 hours, using a computer program (PC-Lab 5.0, AstraZeneca, Mölndal, Sweden). Physical activity was measured by the telemetric system during 30 s every second minute, by analyzing variations in the received radio signal during animal movement. To minimize the impact of animal movement on blood pressure and heart rate measurements, new mean values were calculated only from measurements sampled when the animals had been physically inactive for at least 10 minutes. Sampling was continued until the animal moved once again. Figure 2 shows a plot of a heart rate recording demonstrating how the selection was performed. To study the heart rate and blood pressure during physical activity, mean values were calculated in the same manner but instead using values sampled when concomitant activity was being recorded.

![Figure 2](image-url)

*Figure 2.* The green-dashed peaks show activity, the red/blue line heart rate. The line turns blue (—, round dots) and data is saved when the animal has been inactive for at least 10 minutes. When the animal moves again, data is discarded and the line turns red (—, square dots).
Acute blood pressure response (Study II)

The telemetric system was connected to an analog-to-digital converter (MacLab; ADInstruments Pty Ltd, Bella Vista, NSW, Australia), whereby blood pressure was measured for 30 minutes in undisturbed nNOS\(^{+/+}\) and nNOS\(^{--}\) mice maintained on a low sodium diet, whereupon they were administered an i.p. injection of milrinone (0.5 or 3 mg/kg bw), zaprinast (10 mg/kg bw) or their corresponding vehicles. The pressure was followed for another 30 minutes, whereupon 5-minute mean values were calculated.

Measurements of renin and aldosterone

Plasma renin concentration (Study I&II)

In study I, nNOS\(^{+/+}\) and nNOS\(^{--}\) were placed on either a low or high sodium diet for ten days after which they were briefly anaesthetized with isoflurane. A blood sample (~100 μl) was taken from the retro-orbital plexus and immediately spun down using a cooled centrifuge, whereby plasma was isolated and frozen in liquid nitrogen.

In study II, animals were given a low sodium diet for ten days, whereupon either a PDE inhibitor (Sigma-Aldrich Sweden AB, Stockholm, Sweden) or vehicle was given as an i.p. injection. After 15-20 minutes, a blood sample was taken as described above. Animals were then returned to a normal sodium diet for two weeks, whereafter they once again were given low sodium diet for ten days. The experiment was repeated, but animals that had been given milrinone, were instead given vehicle, and vice versa, allowing us to calculate the effects of milrinone on PRC in every subject. Three different series were performed where the animals were given the PDE3 inhibitor milrinone (0.5 & 3 mg/kg), the PDE5 inhibitor zaprinast (10 mg/kg) or their corresponding vehicles.

The PRC was measured by radioimmunoassay, using the antibody trapping technique\(^{157}\). In brief, 10 μl plasma from each sample was serially diluted (25, 50, 100 and 200X). Five microlitres of each dilution of plasma was incubated in duplicate for 24 hours together with a mixture of rabbit angiotensin I antibody and renin substrate (angiotensinogen equivalent to 1200 ng angiotensin I/ml) from rats. After the incubation step, the reaction was stopped by addition of 1 ml cold barbital buffer, an angiotensin I tracer was added, and radioimmunoassay was performed. Renin values were standardized by reference to renin standards obtained from The Institute for Medical Research (Holly Hill, London, UK), and the values are expressed in standard Goldblatt units (GU). One Goldblatt unit is defined as the amount of renin needed to increase blood pressure in a dog by 30 mmHg in 3 minutes and was formerly called “dog unit”\(^{158}\).
Plasma aldosterone concentration (Study I)

The large plasma volume needed (~500μl) for the aldosterone analysis required a different blood sampling technique. nNOS+/+ and nNOS-/- maintained on a low sodium diet for ten days were anaesthetized by Isoflurane, whereafter the abdomen was opened by a midline incision. Using a needle, a blood sample was quickly taken from the vena cava to a syringe containing EGTA as anticoagulant. Plasma aldosterone concentration was measured with a commercial radioimmunoassay (COAT-A-COUNT, Diagnostic Products, Los Angeles, CA).

Renin mRNA levels in the renal cortex (Study I)

The renal cortex from nNOS+/+ and nNOS-/- maintained on a low sodium diet for ten days was dissected, snap frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated with mini RNeasy columns (Qiagen, Albertslund, Denmark), quantified by spectrophotometry, and stored at -80°C. cDNA for amplification was obtained by reverse transcription of 1 μg total RNA with a reverse transcriptase kit (iScript cDNA synthesis kit, BioRad). Primer sequences were: Renin sense 5´gct-atg-tga-aga-agg-ctg-3´ antisense 5´ttc-tct-tct-cct-tgg-ctc-3´, 113 bp, covering bases 858-970 (GenBank BC061053); Ribosomal 18S subunit sense 5´ctg-tgg-taa-ttc-tag-agc-3´ antisense 5´-agg-tta-tct-aga-gtc-acc-3´,158 bp covering bases 151-308 of rat sequence (GenBank V01270). For qPCR, 50 ng cDNA in duplicate was used as a template and mixed with primers and iQ-SYBR Green Supermix (BioRad) in a final volume of 25 μl. The mixture was denatured for 3 min at 95°C and 44 cycles were run on an iQ-Thermocycler (BioRad): denaturation 30 s at 95°C, annealing and extension 45 s at 60°C. Standard was linearized plasmid containing a fragment of mouse renin cDNA. The standard curve was constructed by plotting threshold cycle (Ct-values) against serial dilutions of plasmid standard. Specificity was established post-run for each plate setup by melting curve analysis. Negative controls were water instead of cDNA and reverse transcriptase-negative samples. Random samples from each plate were loaded on agarose gels to confirm the amplification product.

Renin genes (Study I)

The nNOS-/- are originally derived from the 129 mouse strain and later inbred on the C57Bl/6J strain for at least ten generations. To exclude the possibility that the mice used in the present study carry a second renin gene that could confound the results, PCR was performed on genomic DNA using primers for the Ren-1 and Ren-2 genes, published by Hansen and co-workers74. DNA from A1AR-/-, known to carry both genes, was used as a positive control.
Renal excretion and plasma potassium concentration

Urine collection in metabolic cages (Study I&III)

For the renal excretion measurements, mice were placed in metabolic cages for 24 hours with access to food and water ad libitum (Figure 3). This procedure was repeated on the same animal; four times in study I, and two times in study III. Between the measurements, the animals were put back into their normal cage for 1-2 days. The urine volumes were measured gravimetrically and urine osmolality was determined with an osmometer (Fiske 210 Micro-Sample Osmometer, Fiske Associates, Norwood, MA), whereas sodium and potassium concentrations were determined by flame photometry (IL943, Instrumentation laboratory, Milan, Italy). The water consumption was calculated from the weights of the water bottle and water spillage collection chamber. In study III, urinary protein excretion was measured by a standard laboratory assay based on the Lowry method (Bio-Rad DC Protein Assay, Bio-Rad Laboratories, Hercules, CA).

![Figure 3. Schematic illustration of a metabolic cage used for urine collections; a) water bottle b) water spillage collection chamber c) feed container d) feed spillage collection chamber e) urine collection chamber f) faeces collection chamber.]

Measurement of plasma potassium concentration (Study I)

The potassium concentration in plasma was measured using flame photometry (IL943, Instrumentation Laboratory, Lexington, MA) from the samples used for aldosterone measurement.

Measurements of cAMP (Study II)

The concentration of cAMP was determined in cortical tissue from nNOS\(^{+/+}\) and nNOS\(^{-/-}\) maintained on a low sodium diet for ten days. Also, the milrinone response was investigated in cultured As4.1 cells, a renin producing cell line.
cAMP concentration in the renal cortex

The renal cortex from kidneys from nNOS^{+/+} and nNOS^{-/-} was separated and mortared in liquid nitrogen and transferred to an Eppendorf tube with 200 μl 5% trichloroacetic acid in H_{2}O. The tube was vortexed and sonicated for 5 minutes, whereupon it was centrifuged at 10000 rpm for 10 minutes. The supernatant was moved to a new tube where the trichloroacetic acid was removed by ether extraction. The cAMP concentration was measured in acetylated samples using an ELISA (Cayman Chemicals, Ann Arbor, MI). The tube with the pellet was dried and the weight of the tissue was calculated by subtracting the weight of the empty tube. The cAMP concentration was related to the dry weight of the tissue.

cAMP concentration in cultured As4.1 cells

The renin-producing cell line As4.1 was acquired from ATCC (Manassas, VA) and cultured in Dulbecco’s Modified Eagle’s Medium with 10% fetal bovine serum, penicillin (178 units/ml) and streptomycin (0.178 mg/ml) according to the manufacturers’ instructions. For the experiments, 240 000 cells/well were seeded in a 24-well plate and cultured for 24 hours, whereupon the medium was exchanged with new medium with added forskolin (5 μM) and either milrinone (100 μM) or vehicle (DMSO). The total DMSO concentration in all wells was 0.15%. Cells were incubated for two hours, whereafter the medium was discarded and 100 μl 0.1 M HCl added. After 20 minutes, the cells were harvested using a cell scraper and transferred to Eppendorf tubes. After 5 minutes of sonication, the tubes were spun at 10000 rpm for ten minutes, whereupon the supernatant was collected. The cAMP concentration was measured in acetylated samples using an ELISA as described above and related to the protein concentration which was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Acid dissection of renal tissue (Study II)

The acid dissection technique was used to visualize the degree of renin activation. By this technique, the JG cells can easily be identified without using any staining, due to the different optical properties of JG cells and smooth muscle cells. The kidneys from nNOS^{+/+} and nNOS^{-/-}, maintained on a low sodium diet for ten days, were removed and placed into 5M HCl at 37°C for one hour. The tissue was thereafter placed in distilled water at 4°C for at least 24 hours before the dissections were performed. Using a dark field stereo microscope, the afferent arteriolar branches were classified into three categories by a blinded observer; JG cells in juxtaglomerular position, midafferent JG cell recruitment, or no visible JG cells. The ratio between...
each category and the total number of visible arterioles was calculated for every dissected branch. The arterioles from 3-10 branches from each kidney were counted twice, whereby a mean value from each kidney was calculated for the statistics.

Measurements of GFR and renal plasma flow

GFR was measured by two different techniques. In the first experiments (Study III), we measured inulin clearance in anaesthetized animals as the relation between the plasma concentration and the excreted amount. When we performed the study, we realized that it would be an advantage to be able to perform repeated measurements in the same animal, before and after an intervention, and also avoid anaesthesia. We evaluated different techniques, and found that the single bolus injection method was most appropriate. By this technique, inulin clearance in study IV was measured in conscious mice using only the plasma disappearance curve after the injection. In study II, we injected both inulin and para-amino hippuric acid (PAH), which also allows estimation of the renal plasma flow.

GFR during anaesthesia (Study III)

The mice were anaesthetized with Isoflurane (~2 % in 40 % oxygen; Univentor 400; AgnThos, Stockholm, Sweden) and placed on a servo-controlled heating pad maintaining body temperature at 37.5 °C. Polyethylene catheters were placed in the carotid artery for blood pressure measurements, and in the jugular vein for the infusion of Ringer’s solution. An additional catheter was placed in the bladder for urine collection.

A bolus dose of [³H]methoxy-inulin (26 kBq in 0.1 ml saline; American Radiolabeled Chemicals, St. Louis, MO) was given followed by the continuous infusion of fluid containing 260 kBq/ml. The infusion rate was set to 0.3 ml/h in control animals and 0.6 ml/h in diabetic animals, in order to compensate for the increased urinary flow rate in the diabetic animals. Clearance measurements were started after an initial 40-min recovery period. Urine was collected during two consecutive 30-min periods. Blood samples (20 μl) were taken from the carotid artery in heparinized glass capillary tubes in the middle of each period. The ³H-activities in urine and plasma samples were determined by a liquid scintillation counter (Wallac 1409, Wallac Oy, Turku, Finland) and the GFR calculated as the clearance of inulin (CL_{inulin}) from the urine flow rate (UF) and the activities of inulin in urine and plasma, respectively:

\[ CL_{inulin} = \frac{(UF \times [inulin]_{urine})}{[inulin]_{plasma}} \]
GFR in conscious mice (Study IV)

GFR in conscious mice was estimated by measurements of inulin clearance with a technique modified from the method described by Qi and co-workers. \[^{160}\] \[^{3}\text{H}\]methoxy-inulin was dissolved in saline and thereafter dialyzed in 2000 ml of saline at +4°C overnight using a 1000 Da cut-off dialysis membrane (Spectra/Por® 6 Membrane; Spectrum Laboratories Inc, Rancho Dominguez, CA). The dialyzed inulin was filtered through a 0.22 μm syringe filter before use, and injected (~85 kBq in 200 μl) into the tail vein of the conscious mice. The syringe was weighed before and after the injection to calculate the exact dose. Approximately 5 μl blood was collected from a small cut at the tip of the tail at 1, 3, 7, 10, 15 and approximately 35, 55 and 75 minutes after the bolus injection, and the plasma concentration of inulin was determined by liquid scintigraphy. GFR was calculated as the plasma clearance of inulin using standard formulas \[^{161}\] by dividing the given i.v. dose by the total area under the plasma inulin time curve (AUC\(_{0-\infty}\)):

\[
CL_{\text{inulin}} = \frac{\text{Dose}_{\text{i.v}}}{\text{AUC}_{0-\infty}}
\]

The AUC\(_{0-\infty}\) was estimated using non-compartmental pharmacokinetic data analysis \[^{161}\] from summation of the trapezoid areas that are formed by connecting the data points (Σtrapezoids), a procedure referred to as the linear trapezoidal rule. The area of the terminal phase (AUC\(_{\lambda}\)), i.e. from 15 minutes to infinity (including the residual area after the last measurement), was calculated based on the equation for the slope. The area before the first measurement (AUC\(_{\text{back-extr}}\)) was calculated using linear back-extrapolation.

\[
\text{AUC}_{0-\infty} = \text{AUC}_{\text{back-extr}} + \Sigma\text{trapezoids} + \text{AUC}_{\lambda}
\]

A plot demonstrating the disappearance of inulin and PAH in plasma and the areas used for the calculation is shown in figure 4.

GFR and renal plasma flow in conscious mice (Study II)

The effect of milrinone (0.5 mg/kg) on GFR and renal plasma flow was estimated by simultaneous measurements of inulin- and PAH clearances, respectively. Similar to the technique described above, animals were given a bolus injection of \[^{3}\text{H}\]methoxy-inulin (~110 kBq) and of \[^{14}\text{C}\]PAH (~40 kBq; PerkinElmer Sverige AB, Upplands Väsby, Sweden) dissolved in 200 μl saline into the tail vein, whereupon blood samples were taken from the tail tip, as described above. The \(^3\text{H}\) and \(^{14}\text{C}\) activities in each sample were determined by liquid scintigraphy. Inulin- and PAH clearances were calculated using non-compartmental pharmacokinetic data analysis as described
above. Each animal served as its own control using a similar experiment design as in the renin series.

![Figure 4](image.png)

**Figure 4.** Representative semi-logarithmic plot of plasma activity of $[^3\text{H}]$Methoxy-Inulin and $[^{14}\text{C}]$Para-Amino Hippuric Acid after a bolus injection of the substances. The solid lines represent values taken during the actual experiments, whereas the dashed lines represent the interpolated residual data for the end of the terminal phase.

**Histology (Study III)**

The left kidney was fixed by the infusion of 4% phosphate-buffered formalin via the carotid artery while the renal vein was cut open. The infusion was continued for 10 minutes, whereafter the kidneys were dissected and weighted. The middle part of the left kidney was placed in formalin and embedded in paraffin. Sections (5 μm) were stained with haematoxylin-eosin, periodic acid-Schiff and Picro-Sirius. Different compartments of the sections (glomeruli, tubular system, medulla and papilla) were investigated for morphological changes by a blinded observer. The parameters scored were; fibrosis/sclerosis, inflammation, and tubular dilatation. A score of 0–3 was given, with 0 being no alterations and 3 given for severe alterations. Special attention was given to the papillary–medullary transition which is especially susceptible to develop fibrosis.
The entire renal cortical area of one section from the first five animals in each group was photographed. Computer software (Scion Image; Scion Corporation, MD) was then used to manually determine the area of all visible glomeruli (40-98 glomeruli/section) and the surrounding capsule in the cross-section, respectively. Mean values for the area of the glomeruli and capsules were calculated for every section. Assuming that the glomeruli are circular, the mean diameters (d) were calculated from the areas. From the calculated mean diameters, an approximation of the diameter around the waist (D) was calculated using the following equation:\(^{162}\):

\[
D = \frac{4}{\pi} \times d
\]

The glomerular and capsular volumes were then calculated using standard formulas. The volume of the urinary space was calculated as the difference between the volume of the capsule and the glomerulus.

**Statistics**

All values are expressed as mean±SEM, and for all comparisons P<0.05 was considered statistically significant. The calculations were performed in GraphPad Prism 4 (GraphPad Software, La Jolla, CA), MINITAB Release 14 (Minitab, State College, PA) and Microsoft Office Excel 2003 (Microsoft, Redmond, WA).

In study I, the cardiovascular response to an increased sodium intake was examined using a paired t-test, whereas comparisons between the two genotypes were performed using an unpaired t-test. Excretion, PRC and aldosterone data were examined using the unpaired t-test, whereas the renin mRNA data was evaluated by a one-way ANOVA followed by the Tukey–Kramer multiple comparison post hoc test.

In study II, the renin, blood pressure, GFR, and blood flow responses to PDE inhibition were examined using a paired t-test, whereas comparisons between the two genotypes were performed using an unpaired t-test. Differences in cAMP concentration and the degree of JG cell activation between the different groups were tested using an unpaired t-test.

In study III, GFR, excretion, body weight, and stereological data were examined using one-way ANOVA and, when appropriate, followed by Fisher’s post hoc test. The results from the histological evaluation were examined with the Kruskal-Wallis test.

In study IV, GFR and blood glucose data was analyzed using one-way ANOVA and, when appropriate, followed by Fisher’s post hoc test.
Results

nNOS in renin and blood pressure regulation (Study I&II)

Blood pressure and heart rate regulation

No difference in blood pressure between nNOS\(^{-/-}\) and nNOS\(^{+/+}\) mice was found on any of the sodium diets or in any experimental period (Figure 5). When animals were transferred from a low to a high sodium diet, the blood pressure increased slightly in both nNOS\(^{-/-}\) and nNOS\(^{+/+}\) mice, but no difference in the magnitude of change was found between the genotypes. During the day, but not the night, the heart rate in nNOS\(^{-/-}\) mice was reduced on both diets. However, when the animals were inactive (i.e. when data associated with physical activity was removed), the heart rate in nNOS\(^{-/-}\) was found to be reduced both during the day and night (Figure 6A).
Figure 6. 24-hour heart rate (A) during different sodium diets. For the inactive parts, measurements associated with activity have been removed. Panel B shows the mean activity level during the day and night. * denotes P<0.05 compared to inactive data, # denotes P<0.05 compared to inactive nNOS\(^{++}\), same diet, † denotes P<0.05 compared to the daytime, same genotype and diet.

Renin and aldosterone regulation

Analysis of genomic DNA demonstrated that nNOS\(^{++}\) and nNOS\(^{-/-}\) mice used in the present study only carry the Ren-1 gene. Figure 7C shows a representative gel with all investigated genotypes. During high sodium conditions, PRC was similar between the genotypes but under low sodium conditions, nNOS\(^{-/-}\) mice had a reduced PRC compared to nNOS\(^{++}\) mice (Figure 7A). During the low sodium diet, PRC in nNOS\(^{++}\) mice was elevated compared to that under high sodium conditions, while PRC in nNOS\(^{-/-}\) mice was unaffected. Renin mRNA level in the renal cortex was significantly stimulated by a low salt intake in nNOS\(^{++}\) mice, whereas the mRNA levels were unaffected in the nNOS\(^{-/-}\) mice (Figure 7B). Plasma aldosterone concentration was increased by low salt intake in nNOS\(^{-/-}\) and nNOS\(^{++}\) mice (Figure 7D) but there were no significant differences between the strains of mice. Also, the plasma potassium concentration was similar between the genotypes on both low- (nNOS\(^{++}\) 4.3±0.2 vs. nNOS\(^{-/-}\) 4.1±0.1 mM) and high sodium diets (nNOS\(^{++}\) 4.0±0.1 vs. nNOS\(^{-/-}\) 4.1±0.1 mM).

Renal excretion

During low sodium diet, the urine flow rate, osmolar and potassium excretion were found to be increased in nNOS\(^{-/-}\) compared to nNOS\(^{++}\) (Table 1). However, when animals were given a high sodium diet, no significant changes were found.
Table 1. 24-hour urinary excretion data from metabolic cages.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sodium diet</th>
<th>nNOS(^{+/+})</th>
<th>nNOS(^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine flow rate (μl/24 h/g bw)</td>
<td>Low</td>
<td>44 ±4</td>
<td>78 ±8*</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>90 ±12</td>
<td>118 ±9</td>
</tr>
<tr>
<td>Osmolar excretion (μOsm/24 h/g bw)</td>
<td>Low</td>
<td>55 ±5</td>
<td>75 ±5*</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>97 ±14</td>
<td>128 ±10</td>
</tr>
<tr>
<td>Sodium excretion (μmol/24 h/g bw)</td>
<td>Low</td>
<td>0.46 ±0.13</td>
<td>0.45 ±0.18</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>25 ±4.6</td>
<td>33 ±2.2</td>
</tr>
<tr>
<td>Potassium excretion (μmol/24 h/g bw)</td>
<td>Low</td>
<td>7.8 ±1</td>
<td>11 ±0.69*</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>8.3 ±1.3</td>
<td>10 ±0.62</td>
</tr>
</tbody>
</table>

* denotes P<0.05 compared to nNOS\(^{+/+}\), same diet.

Figure 7. Plasma renin (A), kidney renin mRNA (B) and plasma aldosterone (D) in relation to different sodium loads. Panel C shows an agarose gel from the genomic DNA analysis of the renin genes in these mice. DNA derived from A\(_{1}\)AR\(^{-/-}\) mice was used as a positive control for the Ren-2 gene. * denotes P<0.05.

PDE3 and renin regulation

During vehicle treatment, PRC in the nNOS\(^{-/-}\) was reduced compared to wild-type littermates (Figure 8A). Administration of milrinone (0.5 mg/kg) increased PRC significantly in both genotypes, but the increase was significantly larger in the nNOS\(^{-/-}\) mice (Figure 8B), and resulted in similar renin levels.
Figure 8. Paired measurements of plasma renin concentration after milrinone or vehicle administration, respectively (A). Mean values of the renin increase caused by milrinone (B). * denotes $P<0.05$ compared to vehicle, whereas # denotes $p<0.05$ compared to nNOS$^{+/+}$.

The blood pressure responses to milrinone and vehicle injections are shown in figure 9A. No differences were found in the basal blood pressure between the genotypes. The low milrinone dose (0.5 mg/kg) and vehicle injection, increased blood pressure similarly in both strains of mice. In contrast, a higher dose of milrinone (3 mg/kg) caused a marked and significant blood pressure reduction. Administration of the higher dose of milrinone increased PRC well above the levels found after 0.5 mg/kg, demonstrating that a maximum PRC was not reached during the lower dose (Figure 9B).

Figure 9. Blood pressure responses to milrinone and vehicle injections (A). Demonstration of the renin responses to the depressor and the non-depressor doses of milrinone (B). * denotes $P<0.05$ compared to vehicle, same genotype and dose, whereas # denotes $P<0.05$ compared to milrinone 0.5 mg, same genotype.
Low-dose milrinone (0.5 mg/kg) reduced GFR and PAH clearance similarly in both genotypes (Figure 10). During milrinone treatment, the nNOS\(^{-/-}\) had a reduced GFR compared to the nNOS\(^{+/+}\), but this difference was not significant during the vehicle period (P=0.17).

**cAMP measurements**

Administration of milrinone (100 μM) to the As4.1 cells caused approximately a 4-fold increase in intracellular cAMP concentration (Figure 11A). When cAMP concentration in cortical tissue from nNOS\(^{-/-}\) and wild-type mice was analyzed, there was no difference between the genotypes (Figure 11B).

**Figure 10.** Paired measurements of glomerular filtration rate and PAH clearance after administration of milrinone or vehicle, respectively. * denotes P<0.05 compared to vehicle, same genotype, whereas # denotes P<0.05 compared to nNOS\(^{+/+}\).

**Figure 11.** cAMP concentration in As4.1 cells treated with milrinone compared to cells only given forskolin (A). cAMP concentration in renal cortex (B). * denotes P<0.05 compared to cells only given forskolin.
PDE5 and renin regulation

In a separate experimental series, blood pressure and PRC were determined in response to a PDE5 inhibitor. Zaprinast had no effect on blood pressure compared to vehicle treatment. Also in this series, PRC in sodium-restricted wild-type mice injected with vehicle was significantly higher compared to nNOS\(^{-/-}\). However, zaprinast injection did not change PRC in wild-type or in nNOS\(^{-/-}\) mice.

Effect of nNOS deletion on JG cell index

Approximately 3500 arterioles from microdissected renal microvasculature were evaluated from each genotype (Figure 12). The microvessels from nNOS\(^{-/-}\) exhibited an increased percentage of renin-positive arterioles (Total activated). Furthermore, an elevated percentage of JG cells in juxtaglomerular position was observed, while no difference was found in the degree of recruitment.

*Figure 12. Visualization of renin cells by acid dissection. * denotes P<0.05 compared with nNOS\(^{++/+}\).*
Diabetes-induced hyperfiltration (Study III&IV)

TGF and diabetes-induced hyperfiltration

The baseline GFR was similar in both genotypes, and diabetic animals of both genotypes developed a pronounced and similar glomerular hyperfiltration (Figure 13A).

The glomerular volume was similar in all investigated groups (Figure 13B), while a tendency to increased fibrosis was observed in the diabetic groups compared to normoglycaemic animals, however no statistically significant differences were found between the groups.

In both genotypes, the induction of diabetes resulted in a similar increase in water intake, urinary flow rate, and protein and osmolar excretion, compared to normoglycemic animals (Table 2). During diabetes, the excretion of sodium and potassium was lower in the A₁AR⁻/⁻ compared to the A₁AR⁺/⁺.

Table 2. 24-hour urinary excretion data from metabolic cages.

<table>
<thead>
<tr>
<th></th>
<th>A₁AR⁺/⁺</th>
<th>A₁AR⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetes</td>
</tr>
<tr>
<td>Water intake (μl/24 h/g bw)</td>
<td>108 ±14</td>
<td>500 ±114*</td>
</tr>
<tr>
<td>Urinary flow rate (μl/24 h/g bw)</td>
<td>63 ±7</td>
<td>479 ±84*</td>
</tr>
<tr>
<td>Sodium excretion (μmol/24 h/g bw)</td>
<td>7.1 ±0.4</td>
<td>15.8 ±1.3*</td>
</tr>
<tr>
<td>Potassium excretion (μmol/24 h/g bw)</td>
<td>11.3 ±0.8</td>
<td>25.4 ±2.3*</td>
</tr>
<tr>
<td>Osmolal excretion (μOsm/24 h/g bw)</td>
<td>99 ±6</td>
<td>531 ±74*</td>
</tr>
<tr>
<td>Protein excretion (mg/24 h/g bw)</td>
<td>0.80 ±0.05</td>
<td>2.01 ±0.27*</td>
</tr>
</tbody>
</table>

* denotes P<0.05 vs. corresponding normoglycemic control group, whereas † denotes P<0.05 vs. corresponding A₁AR⁺/⁺ group.
Glucose reabsorption and diabetes-induced hyperfiltration

In study 4, we investigated the role of TGF and sodium-linked glucose transport on diabetes-induced hyperfiltration. GFR was measured by the single-bolus technique in conscious mice. The diabetic A1AR⁻/⁻ demonstrated a similar degree of hyperfiltration as their wild-type controls, thus confirming the results obtained in anaesthetized animals.

Administration of phlorizin reduced GFR in the diabetic groups, but not in the normoglycemic groups (Figure 14). Notably, the decrease in GFR was more pronounced in A1AR⁻/⁻ compared to the A1AR⁺/⁺. Phlorizin lowered the plasma glucose levels in all groups, although the reduction did not reach statistical significance in the normoglycemic A1AR⁻/⁻ group (Figure 14).

**Figure 14.** Effect of phlorizin on glomerular filtration rate and plasma glucose concentration. * denotes P<0.05 compared to corresponding normoglycemic group, # denotes P<0.05 compared to vehicle within the same genotype and plasma glucose status, and † denotes P<0.05 compared to phlorizin-treated A1AR⁺/⁺ group.
Discussion

The role of nNOS in renin regulation

The studies in this thesis demonstrate that nNOS-deficient animals have an impaired renin regulation that prevents the normal renin increase during sodium restriction. Since blood pressure and GFR were similar in nNOS\(^{-/-}\) and nNOS\(^{+/+}\) mice, we can rule out the possibility that the absent renin response is caused by changes in these parameters. The presence of the possibly confounding Ren-2 gene was also excluded by genotyping the animals. Consequently, it appears that the regulation of renin by sodium restriction depends critically on local generation of NO by nNOS in the JGA.

The stimulatory effects of NO on renin release have been proposed to be caused by cGMP-mediated competitive inhibition of PDE3, which hydrolyze cAMP. As a result, the cAMP levels will increase, and renin secretion will thus be stimulated\(^{135}\). A schematic illustration of the proposed hypothesis is shown in figure 15. In order to test whether this model explains the inability of appropriate renin regulation in the nNOS\(^{-/-}\), we examined the renin response to PDE3 inhibition in these genotypes. Supporting the hypothesis, the nNOS\(^{-/-}\), compared to the nNOS\(^{+/+}\), were found to exhibit a more pronounced renin increase after administration of the PDE3 inhibitor, suggesting that the PDE3 isoform in JG cells is more active due to the absence of nNOS-derived NO. Consequently, during PDE3 inhibition, the PRC levels were similar in both genotypes. This is supported by earlier findings, where milrinone blocked the renin-suppressing effects of NOS inhibition\(^{119}\). In patch clamp experiments on single JG cells, administration of cGMP increased renin exocytosis, and this was not further increased during PDE3 inhibition, supporting the notion of a common target\(^{163}\). Furthermore, data from the perfused isolated kidney suggests that the stimulatory effect of NO on renin secretion is mediated through cAMP, since inhibition of protein kinase A attenuates the stimulatory effect of NO\(^{133}\).

Earlier studies have suggested that PDE5 inhibitors could increase PRC by increasing the cGMP levels in JG cells\(^{164,165}\). Since the NO-cGMP levels in JG cells from nNOS\(^{-/-}\) are expected to be reduced, inhibition of PDE5 in these mice is predicted to have no or minor effects on renin. However, even administering the highest sub-depressor dose possible in mice, PRC did not increase in any of the groups, suggesting that PDE5 does not play a major role in renin release. One possible explanation might be that previous ex-
periments	extsuperscript{164,165} were performed during anesthesia, whereas the present series was done without. This view is supported by data from another study, using conscious rats, where PDE5 inhibition did not affect the PRC levels	extsuperscript{166}.

The renin mRNA levels were also reduced in the nNOS\textsuperscript{-/-} during sodium restriction, which indicates that nNOS-derived NO might have a stimulatory role in renin transcription. Earlier findings from cultured JG cells indicate that cAMP stimulates renin transcription\textsuperscript{167}. Following the discussion above, the cAMP concentration in JG cells of nNOS\textsuperscript{-/-} is assumed to be reduced. Accordingly, it appears plausible that the low cAMP concentration might contribute to the observed low renin mRNA levels.

Figure 15. Schematic demonstration of the mechanisms that couples nitric oxide production to increased renin exocytosis\textsuperscript{135}. The alternative pathway by which nitric oxide could inhibit renin release is also denoted. The solid lines denote stimulatory effects, whereas the dashed lines denote inhibitory effects. Denotations; GPCRs, G protein-coupled receptors, PKA, protein kinase A, PKG, protein kinase G, GC, guanyl cyclase.

The number of JG cells could also contribute to the regulation of PRC. Since a reduced number of JG cells hypothetically could have contributed to the deficient renin regulation in the nNOS\textsuperscript{-/-}, we measured the JG cell index. Interestingly, the nNOS\textsuperscript{-/-} mice, despite their inability to regulate renin, had a higher degree of renin-positive JG cells. This suggests that nNOS\textsuperscript{-/-} have increased the number of JG cells in order to compensate for their reduced ability to secrete renin. Importantly, this shows that the inability of
nNOS\textsuperscript{−/−} to increase PRC during sodium restriction is not caused by a reduced number of JG cells.

Taken together, the experiments support the notion that nNOS-derived NO increases the cAMP levels in JG cells via cGMP-mediated inhibition of PDE3, which would increase renin synthesis and also directly stimulate renin exocytosis.

**Aldosterone regulation in nNOS\textsuperscript{−/−} with impaired renin regulation**

Despite the impaired regulation of renin release in the nNOS\textsuperscript{−/−} during sodium restriction, the plasma aldosterone concentration was interestingly elevated to a similar degree as the nNOS\textsuperscript{+/+}. This was surprising, since normally, the renin concentration is considered as the rate-limiting step in the formation of angiotensin II, which regulates aldosterone secretion. Furthermore, the plasma potassium concentration was similar in both genotypes, indicating that this parameter had not affected the aldosterone concentration. ACTH was not measured, but this factor is only considered important in short-term regulation of aldosterone. These findings demonstrate that the aldosterone concentration, under certain circumstances, could be regulated independently of renin. It is possible that the adrenal expression of angiotensin II-AT\textsubscript{1} receptors has an active role in the aldosterone response, since the receptor concentration is normally upregulated during sodium restriction\textsuperscript{168}.

**Cardiovascular consequences of nNOS deficiency**

The telemetric system used in this study, enabled us to monitor blood pressure, heart rate, and physical activity simultaneously in conscious animals kept in their normal environment. It is essential that physical activity is monitored, as it is an important determinant of the measured blood pressure and heart rate in conscious mice\textsuperscript{44}. As expected, the activity was higher during the night than in the daytime, but did not differ between the genotypes. As all parameters were sampled simultaneously, the data could be analyzed for investigations of cardiovascular parameters during the day and night as well as in periods of rest and physical activity. By doing this, we were also able to rule out the possibility that minor differences in activity between genotypes could confound the results. However, we found no differences in blood pressure between the genotypes either during activity or inactivity, during night or day, or during different sodium diets. These results are in accordance with measurements performed on anaesthetized nNOS\textsuperscript{−/−}.
mice\textsuperscript{65,66}, but in contrast with two studies using pharmacological nNOS inhibition in rats, where blood pressure was found to increase\textsuperscript{60,128}. The blood pressure increase was associated with a sensitized TGF mechanism that was normalized when hypertension had developed\textsuperscript{60}. However, in another study, using a similar experimental protocol in rats, blood pressure was unaffected\textsuperscript{130}.

When comparing the present results obtained in genetically altered mice to the former data from 7-NI-treated rats, it is important to point out that there are obvious differences between the models. In rats, the hypertension was accompanied by an increase in renin release, while the nNOS\textsuperscript{−/−} mice instead displayed a reduced renin concentration. The main difference between the nNOS\textsuperscript{−/−} mice and the pharmacological approach used in the rats, is the degree of blockade. The dose of 7-NI administered to the rats gives a very low degree of blockade\textsuperscript{169}, while the nNOS\textsuperscript{−/−} have an almost complete inhibition. Interestingly, in another study, when a substantially higher concentration of 7-NI was used, the renin response to sodium restriction was abolished\textsuperscript{124}, resembling the present results obtained in nNOS\textsuperscript{−/−} mice. Furthermore, the exposure of an antagonist to different cells might differ, depending on the properties of the substance. Also, 7-NI is only a relatively selective nNOS inhibitor, and might inhibit other NOS isoforms to some degree\textsuperscript{43}.

The heart rate was found to be decreased in nNOS\textsuperscript{−/−} mice during the day and, when data associated with activity was removed from the analyses, the decrease was also significant during the night. Earlier studies have shown that isolated cardiac myocytes from nNOS\textsuperscript{−/−} mice have increased contractility, but hearts from such mice also display left ventricular hypertrophy, which increases with age\textsuperscript{66}. Under resting conditions, it is possible that in the nNOS\textsuperscript{−/−} mice, as a result of increased cardiac contractility with a slower heart rate, the same cardiac output is reached as in nNOS\textsuperscript{+/+}. Consequently, the reduced heart rate does not appear to affect blood pressure levels.

In view of the deficient renin regulation during the low sodium diet, it is interesting that the nNOS\textsuperscript{−/−} are able to maintain a normal blood pressure. In general, inhibition of the renin-angiotensin system lowers the blood pressure during sodium restriction. However, the normal aldosterone levels in the nNOS\textsuperscript{−/−} must clearly contribute to the conserved blood pressure regulation. Furthermore, the more sensitive TGF mechanism in the nNOS\textsuperscript{−/−} could help maintain blood pressure when renin levels are low\textsuperscript{55}. Taken together, the mechanisms that maintain blood pressure during sodium restriction in these mice are not clear and need further investigation.

\textsuperscript{*} The nNOS\textsuperscript{−/−} have some residual nNOS activity in the brain, due to expression of two alternative splice variants of the gene, nNOS\textsubscript{β} \& nNOS\textsubscript{γ}. Importantly, the macula densa cells in the nNOS\textsuperscript{−/−} have been shown to lack nNOS expression.
In conclusion, nNOS-derived NO appears to have a stimulatory role in renin release via cGMP-mediated inhibition of PDE3. Consequently, the normal renin response to sodium restriction is abolished in nNOS−/− mice. Furthermore, the presented data indicates that genetic deletion of nNOS does not cause hypertension.

**TGF and diabetes-induced hyperfiltration**

Several publications have presented arguments explaining diabetes-induced hyperfiltration as a TGF-mediated event. The theory presented, “The tubular hypothesis of glomerular hyperfiltration,” stipulates that hyperfiltration is caused by a series of events starting with increased sodium-glucose reabsorption in the proximal tubule. This will consequently reduce the NaCl load to the macula densa, resulting in a reduced TGF response and an elevated GFR.

In Study III, we investigated the effects of 2-3 weeks of alloxan-induced diabetes in A1AR−/− mice, known to lack a functional TGF mechanism. As a general characterization of the model, we measured urinary excretion and performed a histological evaluation of the kidneys. As expected, the diabetic animals were characterized by an increased diuresis and electrolyte excretion. Kidneys from the diabetic animals displayed only a tendency to increased fibrosis, which is in line with earlier studies demonstrating that the C57BL/6J background is relatively resistant to development of diabetic nephropathy.

Our main finding is that A1AR−/− mice develop a similar degree of hyperfiltration as wild-type animals with a functional TGF mechanism, which demonstrates that diabetes-induced hyperfiltration is an event independent of TGF. The glomerular volumes were not altered by diabetes, indicating that the hyperfiltration was independent from an increased filtration area. Two other groups have also studied hyperfiltration in A1AR−/− mice. In the study by Faulhaber-Walter *et al*, A1AR−/− mice were made diabetic by cross-breeding the animals with a hyperglycemic mouse strain, which caused an even more pronounced hyperfiltration than their wild-type controls. The other study by Vallon *et al* used A1AR−/− that were made diabetic with multiple injections of streptozotocin. Interestingly, those mice did not develop hyperfiltration. However, the hyperfiltration in the wild types was very small, which indicates that this experimental model differs from the one used in the present studies.

Furthermore, when SNGFRprox is measured without perfusion of the macula densa, the TGF mechanism is inactivated. Consequently, when SNGFR was assessed in this way in diabetic rats, hyperfiltration was still observed, which supports the TGF independency of diabetes-induced hyperfiltration.
Glucose reabsorption and diabetes-induced hyperfiltration

Since the TGF mechanism does not appear to mediate the development of diabetes-induced hyperfiltration, the explanation for hyperfiltration must be sought elsewhere. In Study IV, we investigated whether the increased proximal reabsorption during diabetes can contribute directly to hyperfiltration. Hypothetically, the increased reabsorption could reduce the pressure in the proximal tubule and Bowman’s space which in turn can increase the net filtration pressure. We therefore found it of interest to investigate the effects of SGLT inhibition. If hyperfiltration in diabetic mice depends on an elevated degree of sodium-glucose transport, they should reduce their GFR more than normoglycemic animals. In fact, diabetic animals are more sensitive to transport inhibition\textsuperscript{38,174}, but the GFR reduction has been ascribed to the TGF mechanism, as a consequence of increased distal NaCl delivery\textsuperscript{38}. In order to study the direct effects of transport inhibition, without the autoregulatory influence of TGF, it was necessary to perform the experiments in A\textsubscript{1}AR\textsuperscript{−/−}. To eliminate the possible influence of anesthesia, a new method for GFR assessments was used, which allowed measurements in conscious mice kept in their usual environment.

Wild-type and A\textsubscript{1}AR\textsuperscript{+/+} mice developed a similar degree of hyperfiltration, which confirms the results previously found in study III. Administration of phlorizin had no effect on normoglycemic mice, but reduced GFR in both A\textsubscript{1}AR\textsuperscript{+/+} and A\textsubscript{1}AR\textsuperscript{−/−}, demonstrating that the reduction is TGF-independent. The reduction could instead be a direct consequence of the inhibited proximal reabsorption, which would increase proximal intratubular pressure, thus reducing net filtration pressure. This is supported by earlier findings demonstrating that intratubular perfusion by phlorizin is associated with an increased pressure in Bowman’s space in diabetic rats, while control rats were unaffected\textsuperscript{38}. The fact that diabetic animals are more sensitive to SGLT1/2 inhibition, supports the idea that the upregulated proximal reabsorption directly causes hyperfiltration. The proposed mechanism is depicted in figure 16.

Interestingly, phlorizin reduced GFR to a larger extent in diabetic A\textsubscript{1}AR\textsuperscript{−/−} mice than diabetic wild types. The reduced GFR could decrease tubular NaCl concentration sensed by the macula densa. A functional TGF mechanism will partially preserve GFR via a dilation of the afferent arteriole. Thus, the reduction will be greater in the A\textsubscript{1}AR\textsuperscript{−/−} animals, since they lack this compensatory component. Consequently, TGF does not appear to have a causative role in the development of hyperfiltration, but rather functions to stabilize the high GFR.
Figure 16. Schematic illustration demonstrating the proposed hypothesis of hyperfiltration occurring without a functional TGF mechanism.

In conclusion, despite that the mechanisms are not fully understood, the experiments show that tubular glucose reabsorption plays a pivotal role in maintaining elevated GFR during diabetes, at least in the short-term perspective. These findings suggest that the observed increased reabsorption during diabetes, directly increases GFR by lowering proximal intratubular pressure.
Summary and conclusions

- Deficiency of nNOS causes an inability to increase plasma renin concentration during low sodium conditions, probably due to a reduced degree of PDE3 inhibition.

- Genetic deletion of the nNOS gene in mice does not cause hypertension.

- The TGF mechanism is not involved in the development of diabetes-induced hyperfiltration.

- Sodium-linked glucose reabsorption appears to directly contribute to the development of diabetes-induced hyperfiltration.
Future perspectives

The methods used in this thesis were shown to be useful in studying vascular and renal function in genetically modified mice. Especially, the methods for the assessment of GFR and renin in conscious or briefly anaesthetized mice, could be used to study other problems in renal physiology.

The present studies indicate an important role of renal glucose reabsorption in the development of diabetes-induced hyperfiltration. Several pharmaceutical companies are currently developing selective SGLT2 inhibitors as a new solution to the reduction of plasma glucose concentration in type 2 diabetes. Given the GFR-lowering effects of phlorizin described in the present paper, long-term administration of SGLT2 inhibitors might also be effective as a renoprotective agent in type 1 diabetes. Consequently, it would be interesting to investigate the effects of long-term SGLT inhibition on GFR and also renal damage. The newly developed SGLT2 knockout mice could also prove useful for this experiment.
Syftet med undersökningarna som presenteras i denna avhandling har varit att öka förståelsen kring njurens roll i blodtrycksregleringen samt att utforska mekanismer som orsakar njursvikt vid diabetes.


På grund av de stora mängderna som filtreras varje dag, räcker det med små fel för att vår vätskebalans ska bli felaktig. Om njuren reabsorberar för mycket, kan blodtrycket stiga okontrollerat och på motsvarande sätt leder minskad reabsorption till blodtrycksfall. Eftersom båda dessa tillstånd är potentiellt livshotande har däggdjuren utvecklat sofistikerade reglermekanismer samt att njurens blodflöde, filtration och reabsorption precis motsvarar kroppens vätskebehov. En viktig reglermekanism är den tubuloglomerulära återkopplingsmekanismen (TGF) och en förutsättning för dess funktion finns i njurens anatomi. Varje tubulus återvänder till sin egen glomerulus och bildar där den s.k. juxtaglomerulära apparaten. I tubulussävgen vid den juxtaglomerulära apparaten finns macula densa, som består av speciella celler som känner av natriumkloridkoncentrationen i tubulussävgen. Om koncentrationen stiger är det ett tecken på att filtrationen i glomerulus ökat, vilket aktiverar en process som begränsar blodflödet till glomerulus. Filtrationen återgår på detta sätt till den normala.

Njuren kan även påverka blodtrycket genom frisättning av det blodtryckshöjande hormonen renin. Renin är ett enzym och verkar i cirkulationen indirekt blodtryckshöjande genom att klyva peptiden angiotensin I från proteinet angiotensinogen. Angiotensin I omvandlas snabbt till den aktiva formen angiotensin II som höjer blodtrycket genom kontraktion av kärl samt ökning av njurens återupptag av natrium.
Kväveoxid, hypertension och renin (Arbete I&II)


I njurens juxtaglomerulära apparat produceras kväveoxid av neuronalt kväveoxidsyntas (nNOS) vilket har visats kunna påverka TGF-mekanismens känslighet. Vid situationer när kroppen vill göra sig av med mycket elektrolyter, kan kväveoxidproduktionen öka vilket främjar utsöndring av vätskeöverskottet. En felaktig kväveoxidproduktion skulle därmed kunna leda till bristande blodtrycksskondrött. I försök på råttor gavs en nNOS-blockerare under fyra veckor. Intressant nog fann forskarna att TGF-mekanismen hade en ökad känslighet, samtidigt som blodtrycket gradvis steg. Denna utveckling påminner om den som har setts i särskilda rättstammar med en ärftlig benägenhet att utveckla högt blodtryck. På samma sätt skulle vissa former av högt blodtryck hos människa kunna förklaras.

Kväveoxid har också visats vara inblandat i reninfrisättningen, vilket skulle kunna bidra till enzymets påverkan på blodtrycket. Detta har studerats på många sätt, bland annat genom att man gett olika typer av kväveoxidsyntashämmare till råttor. Experimenten har dock inte gett någon entydig bild av kväveoxids funktion vid reninfrisättning. I vissa arbeten har kväveoxid visat sig verka stimulerande, medan andra har påvisat en hämmande roll.

I denna avhandling studerades den specifika rollen av nNOS i regleringen av blodtryck och reninfrisättning med hjälp av en genetiskt modifierad musstam som saknar nNOS.


För att utvärdera reninkoncentrationen i mössen togs ett blodprov vid båda saltdieterna. nNOS-knockarna visade sig då ha en felaktig reninfrisättning, och saknade den normala ökningen man ser vid en lågsaltdiet. Eftersom detta ansågs intressant, gjordes ytterligare en studie för att försöka förstå hur nNOS kan påverka reninfrisättningen. Mössen behandlades med en hämmare som blockerar fosfodiesteras 3 (PDE3), vilket tidigare visat sig öka reninfrisättningen. Djuren som saknar nNOS visade sig då vara känsligare för denna hämmare, och uppvisade en större ökning än mössen med intakt
nNOS-gen. Kväveoxids verkan sker genom bildning av signalsubstansen cGMP. Tidigare försök har visat att cGMP fungerar som en naturlig hämmare av PDE3. Det är välkänt att reninfrisättning till stor del är beroende av signalsubstansen cAMP. PDE3 fungerar genom att bryta ned cAMP, och minskar därmed reninfrisättningen. Kväveoxid kan på detta sätt stimulera reninfrisättning, genom att blockera det nedbrytande enzymet PDE3.

Sammanfattningsvis tyder resultaten på att den normala reninfrisättningen under saltfattig kost orsakas av en ökning av enzymet nNOS. Den ökade mängden kväveoxid kommer att bilda cGMP, vilket hämmar PDE3 i de reninproducerande cellerna. Eftersom PDE3 bryter ned cAMP, som är viktigt för reninfrisättning, kommer njuren att frisätta mer renin och koncentrationen i cirkulationen kommer att stiga vilket förhindrar att blodtrycket faller vid saltbrist.

**Diabetes, hyperfiltration och nefropati (Arbete III&IV)**

En vanlig komplikation vid diabetes är njursvikt, d.v.s. en bristande glomerulär filtration. Eftersom njuren är viktig för blodtrycksreglering och utsöndring av slaggprodukter, är njursvikt ett livshotande tillstånd som antingen kräver livslång dialys eller en ny njure genom transplantation.

I studier har man funnit att många diabetiker på ett tidigt stadium av sjukdomen uppvisar en förhöjd glomerulär filtration, s.k. hyperfiltration. Dessa patienter löper en ökad risk att senare i livet drabbas av njursvikt. Mekanismerna för detta är oklara, men ökad kunskap skulle kunna möjliggöra utvecklandet av nya läkemedel som i sin tur skulle kunna förhindra hyperfiltrationen och den framtida risken att utveckla nefropati.


I avhandlingen testades denna hypotes i en djurmodell för diabetes. Djuren gjordes diabetiska genom att de fick en substans som förstör de insulinproducerande β-cellerna i bukspottkörteln. För att undersöka betydelsen av TGF, användes förutom vanliga möss adenosin A₁-receptor knockar, vilka saknar TGF-mekanismen. När den glomerulära filtrationen mättes före och

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References


66. Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, Hobai IA, Lennion CA, Burnett AL, O'Rourke B, Rodriguez ER,


123. Kim HS, Maeda N, Oh GT, Fernandez LG, Gomez RA, Smithies O. Homeostasis in mice with genetically decreased angiotensinogen is primarily by an increased number of renin-producing cells. *J Biol Chem.* 1999;274:14210-14217.


130. Wangenstein R, Sainz J, Rodriguez-Gomez I, Moreno JM, Osuna A, Vargas F. Chronic blockade of neuronal nitric oxide synthase does not affect long-


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