Aspects of Regulation of GFR and Tubular Function in the Diabetic Kidney

Roles of Adenosine, Nitric Oxide and Oxidative Stress

PATRIK PERSSON
Abstract

Diabetic nephropathy is the main cause for initiation of renal replacement therapy and early symptoms in patients include increased glomerular filtration rate (GFR), decreased oxygen tension and albuminuria, followed by a progressive decline in GFR and loss of kidney function. Experimental models of diabetes display increased GFR, decreased tissue oxygenation and nitric oxide bioavailability. These findings are likely to be intertwined in a mechanistic pathway to kidney damage and this thesis investigated their roles in the development of diabetic nephropathy. In vivo, diabetes-induced oxidative stress stimulates renal tubular Na⁺ transport and in vitro, proximal tubular cells from diabetic rats display increased transport-dependent oxygen consumption, demonstrating mechanisms contributing to decreased kidney oxygenation. In control animals, endogenous adenosine reduces vascular resistance of the efferent arteriole via adenosine A₂-receptors resulting in reduced filtration fraction. However, in diabetes, adenosine A₂-signalling is dysfunctional resulting in increased GFR via increased filtration fraction. This is caused by reduced adenosine A₂₅ receptor-mediated vasodilation of efferent arterioles. The lack of adenosine-signaling in diabetes is likely due to reduced local adenosine concentration since adenosine A₂₅ receptor activation reduced GFR only in diabetic animals by efferent arteriolar vasodilation. Furthermore, sub-optimal insulin treatment also alleviates increased filtration pressure in diabetes. However, this does not affect GFR due to a simultaneously induction of renal-blood flow dependent regulation of GFR by increasing the filtration coefficient. In diabetes, there is decreased bioavailability of nitric oxide, resulting in alterations that may contribute to diabetes-induced hyperfiltration and decreased oxygenation. Interestingly, increased plasma concentration of l-arginine, the substrate for nitric oxide production, prevents the development of increased GFR and proteinuria, but not increased oxygen consumption leading to sustained intra-renal hypoxia in diabetes. This thesis concludes that antioxidant treatment directed towards the NADPH oxidase as well maneuvers to promote nitric oxide production is beneficial in diabetic kidneys but is targeting different pathways i.e. transport-dependent oxygen consumption in the proximal tubule by NADPH oxidase inhibition and intra-renal hemodynamics after increased plasma l-arginine. Also, the involvement and importance of efferent arteriolar resistance in the development of diabetes-induced hyperfiltration via reduced adenosine A₂₅ signaling is highlighted.

Keywords: diabetes, diabetic nephropathy, glomerular filtration rate, renal blood flow, insulin, renal hemodynamics, micropuncture, oxygen, NADPH-oxidase, apocynin, streptozotocin, l-arginine, CGS21680, rats, mice

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*Equal contribution*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>( \pi_{GC} )</td>
<td>colloid osmotic pressure in glomerular capillaries</td>
</tr>
<tr>
<td>A&lt;sub&gt;1&lt;/sub&gt;AR</td>
<td>adenosine A&lt;sub&gt;1&lt;/sub&gt; receptors</td>
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<tr>
<td>A&lt;sub&gt;2&lt;/sub&gt;AR</td>
<td>adenosine A&lt;sub&gt;2&lt;/sub&gt; receptors</td>
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<tr>
<td>ADH</td>
<td>antidiuretic hormone</td>
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<tr>
<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>ASL</td>
<td>argininosuccinate lyase</td>
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<tr>
<td>ASS</td>
<td>argininosuccinate synthase</td>
</tr>
<tr>
<td>BH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>CAT1</td>
<td>cationic amino acid transporter 1</td>
</tr>
<tr>
<td>DMA</td>
<td>dimethylamiloride</td>
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<tr>
<td>FF</td>
<td>filtration fraction</td>
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<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
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<tr>
<td>K&lt;sub&gt;f&lt;/sub&gt;</td>
<td>filtration coefficient</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N-ω-Nitro-L-arginine methyl ester hydrochloride</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
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<tr>
<td>MD</td>
<td>macula densa</td>
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<tr>
<td>mTAL</td>
<td>medullary thick ascending limb</td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NHE</td>
<td>sodium/hydrogen-exchanger</td>
</tr>
<tr>
<td>NKCC</td>
<td>sodium-potassium-2-chloride transporter</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;(^{-})</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;ct</td>
<td>oxygen content</td>
</tr>
<tr>
<td>ONOO(^{-})</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>( P_{Bow} )</td>
<td>hydrostatic pressure in Bowman’s space</td>
</tr>
<tr>
<td>( P_{FF} )</td>
<td>tubular free flow pressure</td>
</tr>
<tr>
<td>( P_{GC} )</td>
<td>hydrostatic pressure in glomerular capillaries</td>
</tr>
<tr>
<td>( P_{NET} )</td>
<td>net filtration pressure</td>
</tr>
<tr>
<td>( P_{SF} )</td>
<td>tubular stop flow pressure</td>
</tr>
<tr>
<td>PTC</td>
<td>proximal tubular cells</td>
</tr>
<tr>
<td>( Q_{O_2} )</td>
<td>oxygen consumption</td>
</tr>
<tr>
<td>RAAS</td>
<td>renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>RBF</td>
<td>renal blood flow</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SGLT</td>
<td>sodium/glucose-linked transporter</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<tr>
<td>TBARS</td>
<td>thiobarbituric acid reactive substances</td>
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<tr>
<td>TGF</td>
<td>tubuloglomerular feedback</td>
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Introduction

The Kidney

The kidneys maintain a stable internal environment by regulating plasma volume, adjusting blood pH and excreting metabolic waste products. To efficiently fulfill these functions the kidneys receive a high blood flow equaling around 25% of cardiac output during rest. This results in a high glomerular filtration rate (GFR), around 125 ml/min in the human kidneys resulting in a production of 180 L of primary urine each day. The primary urine is basically plasma, except large plasma proteins, that is handled along the nephron by active and passive reabsorption of electrolytes, valuable molecules like glucose, amino acids and small proteins and water but also active and passive secretion of molecules that needs to be excreted in the final urine. This processing results in a production of a final urine of 1-2 L each day with varying osmolality depending on salt and fluid intake. Considering a blood volume of around 5 L in the human and a production of 180 L primary urine each day it is easy to imagine that only a very small mismatch in this system will either result in a complete depletion of body fluid and drop in blood pressure or an extreme accumulation in plasma volume and increase in blood pressure. Several systems cooperate to achieve a perfect match so that blood pressure is maintained, both locally in the kidney and systemic, including the renin-angiotensin-aldosterone system (RAAS), central nervous system innervation, neuro-endocrine regulation and endocrine hormones.

Anatomically the basic structure of the kidney is the nephron, which is derived from the Greek word for just kidney. Each nephron is a functional unit that works to a great extent independently of each other and the anatomy and function is conserved between mammals. The human kidney consists of around 0.8 to 1.5 million nephrons whereas the rat kidney consist of around 30 000 nephrons. Each kidney is supplied by blood by one renal artery that is further divided to segmental arteries, interlobar arteries, arcuate arteries, interlobular arteries and finally afferent arterioles. The afferent arteriole supplies the renal glomeruli with blood in which the filtration occurs. The glomeruli consists of a tuft of fenestrated capillaries surrounded by mesangial cells which is a modified smooth muscle cell with the ability to regulate contraction and thereby hydraulic conductivity and effective surface area for filtration which will be discussed in forthcoming sections. Mesangial cells are under influence of several hormones including angiotensin II (Ang II)
and nitric oxide (NO). A specialized epithelial cell, the podocyte, covers the glomerular capillaries; possessing processes that cover the capillaries. The processes are kept separated at a distance of 25 nm forming filtration slits. The glomerular capillary tuft including the podocytes are completely surrounded by a second epithelial layer forming a urinary space known as Bowman’s capsule, which is in direct connection to the tubular system. Glomerular capillaries are united into one efferent arteriole leaving the glomeruli, which tonus, and thereby pressure in glomerular capillaries is under influence of several factors and in this thesis are the influence of adenosine and NO investigated. A second capillary network of either peritubular capillaries or vasa recta is formed after the efferent arteriole, supplying the renal cells with oxygen (O$_2$), before the blood enters a venous system ending in the renal vein.

The tubular system consists of several different parts that are both anatomically and functionally different. The first part of the tubular system is proximal tubule, performing a major part of the active reabsorption in the kidney, 2/3rds of the filtered electrolytes are reabsorbed and the same amount of water is passively diffused by the osmotic gradient. Filtered glucose and amino acids are fully reabsorbed in the proximal tubule as long as maximal transport capacity is not exceeded as will happen in uncontrolled diabetes. The driving force for reabsorption is the basolateral Na$^+$/K$^+$-ATPase creating a Na$^+$ gradient that is used for secondary active transport by luminal transporters. Important transporters that are discussed in this thesis are the sodium/hydrogen-exchanger (NHE) isoform 3 and sodium/glucose-linked transporter (SGLT). The nephron proceeds into the medullary region in a U-shaped structure, the loop of Henle, that can be further divided into a thick descending, thin descending, thin ascending and finally a thick ascending part (mTAL). The renal medulla has high interstitial osmolality that enables reabsorption of water and concentration of the urine. The high osmolality is maintained by medullary countercurrent exchange of urea in vasa recta together with a low blood flow to allow for diffusion. The low medullary blood flow results in low oxygenation of this part of the kidney as will be discussed in forthcoming sections. The descending part of loop of Henle is water permeable leading to reabsorption of water as the filtrate passes, whereas the ascending part is water impermeable but has abundant expression of the furosemide-sensitive sodium-potassium-2-chloride (NKCC) transporter, which contributes to approximately 10-15% of total electrolyte reabsorption. The nephron returns to cortex and forms the distal convoluted tubules, with high expression of thiazide-sensitive Na$^+$-Cl$^-$ co-transporters which consist of two cell types, principal cells and intercalated cells where fine-tuning of Na$^+$ and H$^+$ reabsorption occurs to maintain blood pressure and acid-base balance, respectively. The last part of the nephron is the cortical and medullary collecting duct where the tubular system one more time will go through the hyperosmotic medulla to be able to reabsorb water. The
water reabsorption is regulated by plasma osmolality and therefore hydration status via osmo-sensitive receptors in hypothalamus. High plasma osmolality that occurs during dehydration will result in secretion of antidiuretic hormone (vasopressin, ADH) from the posterior pituitary gland. ADH will signal via G-protein coupled V_2 receptors in the distal tubule and collecting duct, stimulating insertion of aquaporins in the apical membrane promoting water reabsorption.

Regulation of GFR

GFR is under strict autoregulation maintaining GFR during fluctuations in blood pressure, matching GFR with the capacity for tubular reabsorption. Normal regulation to match oxygen supply with demand aims to increase organ blood flow and oxygen delivery during increased metabolism. However, this regulation is significantly more complicated in the kidney since renal blood flow (RBF) and therefore O₂ supply is correlated to GFR and subsequently O₂ demand, if maintaining Na⁺ balance. Two independent mechanisms, the myogenic mechanism and tubuloglomerular feedback (TGF), cooperate to regulate diameter of the afferent arteriole and thereby the driving forces for filtration in form of RBF and hydrostatic pressure in glomerular capillaries (P_{GC}). The myogenic mechanism constricts the afferent arteriole in response to increased arterial blood pressure whereas TGF works from the tubular side by specialized cells in macula densa (MD) in the early distal nephron, a region in each nephron where the distal tubule makes contact with its own glomeruli. MD cells sense the tubular flow of electrolytes by NKCC, specifically Cl⁻, and interpret electrolyte flow as a function of GFR. Increased reabsorption by MD NKCC results in a depolarization of the cell which triggers release of ATP which is further enzymatically degraded to adenosine in the interstitium in close proximity to the afferent arteriole where it elicit a vasoconstriction by binding to adenosine A₁ receptors, reducing RBF and P_{GC} and subsequently GFR. Decreased GFR will reduce electrolyte flow passing MD and therefore cease the ATP release (1).

Determinants of GFR

As mentioned in the previous section the driving force for filtration is P_{GC}. This driving force is opposed by a hydrostatic pressure in Bowmans’space (P_{Bow}) and colloid osmotic force in glomerular capillaries (π_{GC}). P_{GC} is naturally derived from the arterial pressure together with the vascular resistance of the afferent arteriole, but also by post-glomerular resistance in the efferent arteriole. In contrast to the afferent arteriole, the efferent arteriole can regulate GFR with minimal impact on total RBF. A constriction of the afferent arteriole will reduce P_{GC} and RBF, but a constriction of the efferent arteriole
will increase $P_{GC}$ and also GFR without influencing RBF. This leads to an increased filtration fraction (FF), a common observation in diabetes that can pose a problem when it comes to match $O_2$ supply with demand, but this will be discussed in detail in paper III and IV. Normal values for $P_{GC}$ are in the range of 44-50 mmHg (2-4). $P_{Bow}$ is mainly derived from flow resistance in the tubular system and can be modulated by the degree of proximal tubular reabsorption. A normal range is between 12-15 mmHg (2; 3; 5-9). The filtration barrier is normally impermeable for proteins leaving them in the glomerular capillaries where they will exert an osmotic pressure opposing filtration. $\pi_{GC}$ will thereby increase along the capillaries as filtration occurs which will reduce net filtration pressure ($P_{NET}$), and eventually even stop filtration. However, considering a mean $\pi_{GC}$ around 25 mmHg (10; 11), leads to the conclusion that $P_{NET}$ is between 4-13 mmHg. Certain studies suggest that $\pi_{GC}$ will rise to a level that will oppose $P_{GC}$ and stop filtration before the end of the glomerular capillaries, a concept named filtration equilibrium, which appears to occur in a special rat strain named the Munich-Wistar rat, due to a very low $P_{GC}$. This phenomenon would result in regulation of GFR solely dependent on the afferent arteriole and RBF and completely rule out the importance of the efferent arteriole in regulation of GFR. However, in other rat strains studied and also other species $P_{GC}$ is higher and filtration equilibrium does not occur, opening for an important role for the efferent arteriole as discussed in paper III and paper IV. The filtration barrier consists of fenestrated capillary endothelial cells, a glomerular basement membrane and podocytes, which is permeable for ions, small organic molecules and water, but rather impermeable for proteins. However, a debate exists to which extent small proteins are filtered and subsequently reabsorbed in the proximal tubule, and it was recently demonstrated in vivo that Ang II directly regulates permeability of the filtration barrier for macromolecules, by inducing the number of large pores (12). The collective permeability of the filtration barrier is usually summarized into a filtration coefficient ($K_f$), defined as volume filtered per mmHg of $P_{NET}$, yielding the formula that determines GFR.

\[
GFR = K_f (P_{GC} - P_{Bow}) - (\pi_{GC} - \pi_{Bow}) \quad (Equation \ 1)
\]

In the above Equation 1 forces determining net filtration pressure and thereby GFR are displayed. Glomerular capillary pressure ($P_{GC}$) originating from the interplay between diameter of the afferent and efferent arteriole is the driving force for filtration. Factors opposing filtration are pressure in Bowman’s space ($P_{Bow}$) originating from tubular reabsorption and hydraulic resistance of the nephron, and plasma colloid osmotic pressure $\pi_{GC}$ determined by plasma protein concentration and filtration fraction. Filtration of proteins is usually very low and therefore is colloid osmotic pressure in Bowman’s space ($\pi_{Bow}$) considered to be zero. Filtration coefficient ($K_f$) is determined by the permeability and surface area of the filtration barrier.
Adenosine in the kidney

Factors regulating afferent and efferent arteriole diameter, determining $P_{\text{NET}}$, includes Ang II, adenosine, endothelin-1, prostaglandins, NO and other factors. This thesis however, will focus on adenosine and mainly its effects via the adenosine A$_2$ receptors. Adenosine mediates its effects by activating P1 purinoceptors that are G protein-coupled. Four subtypes are identified, adenosine A$_1$, A$_{2a}$, A$_{2b}$ and A$_3$ receptor. Adenosine A$_1$ activation inhibits adenylate cyclase and increases intracellular $[\text{Ca}^{2+}]$ in afferent arterioles (13), leading to vasoconstriction (14), which is a component of the renal autoregulation of GFR through the TGF-mechanism (15; 16). Hence, infusion of adenosine leads to vasoconstriction of the afferent arteriole of superficial nephrons, resulting in a quick reduction in RBF (17). This is opposite to the effect in most other organs where adenosine elicits a vasodilation to increase blood flow at increased metabolic demands (18). In addition to expression on the afferent arteriole, adenosine A$_1$ receptors are found in mesangial cells, vasa recta and throughout the tubular system (19-21). Functionally, the main effects of adenosine in regulation of tubular reabsorption are in the proximal tubule and medullary thick ascending limb (mTAL). Adenosine A$_1$ receptor stimulation increases proximal tubule reabsorption of $\text{Na}^+$, $\text{HCO}_3^-$ and fluid (22). More specifically, activation of the adenosine A$_1$ receptor in isolated renal cells regulates NHE$_3$ in biphasic manner, where low concentration of adenosine stimulates and high concentration inhibits NHE$_3$ (23). In mTAL however, adenosine A$_1$ activation inhibits reabsorption (24). The renal medulla is capable of adenosine release that increases secondary to hypoxia (25). Physiologically, these disparate effects of adenosine A$_1$ receptor activation in regulation of tubular transport will shift site of tubular reabsorption to the well-oxygenated renal cortex during increased workload. Adenosine A$_{2a}$ and A$_{2b}$ receptor activation increases adenylate cyclase activity and causes vasodilation (26; 27) and adenosine has higher affinity to A$_{2a}$ compared to A$_{2b}$ receptors (28). Adenosine A$_{2b}$ is expressed on pre-glomerular vessels (29) in rat but found on both afferent and efferent arteriole in mouse (30). The efferent arteriole appears to express both A$_{2a}$ and A$_{2b}$ receptor. A$_{2a}$ is also found in outer medullary descending vasa recta. Indeed, medullary blood flow increases after adenosine infusion whereas cortical blood flow decreases (31; 32).
Diabetic nephropathy

Diabetes mellitus is associated with several complications and organs that possesses insulin-independent glucose uptake are extra susceptible. This includes the kidneys, which reabsorb glucose from the tubular lumen via SGLT leading to high intracellular concentrations of glucose. Indeed, diabetes is the main cause to end-stage renal disease requiring renal replacement therapy affecting up to 30% of all type-1 diabetes and increasing numbers of type-2 diabetes patients (33). The earliest sign of altered kidney function in diabetes is increased GFR in some patients, although this is not defined as renal disease. Clinically the degree of renal disease is based on presence of albumin in the urine, categorized in microalbuminuria (30-300 mg/24 h) and macroalbuminuria (>300 mg/24 h) and the actual GFR divided into 5 intervals where a GFR of less than 15 ml/min/1.73 m² is classified as chronic kidney disease stage 5 or end stage renal disease (34).

Diabetes-induced hyperfiltration

The mechanisms behind the diabetes-induced hyperfiltration are under debate, and also whether presence of hyperfiltration predicts development of renal disease is not fully clarified. Recent studies suggest that there is no correlation between hyperfiltration and progression to proteinuria but it is associated with a faster decline in GFR in later stages of the disease (35; 36). Increased GFR seems to be initiated by an increased proximal tubular reabsorption driven by the hyperglycemia. Glucose is freely filtered in the kidney

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*Figure 1. Simplified graph of beginning of a nephron with the factors determining GFR. See also Equation 1.*
leading to high proximal tubular glucose concentration. Glucose is normally completely reabsorbed in the proximal tubule by secondary active transport by the SGLT, driven by the low intracellular Na$^+$ concentration caused by the basolateral Na$^+$/K$^+$-ATPase. Two isoforms of SGLT are expressed in proximal tubule, SGLT2 in the early S1 segment of proximal tubule which possesses low affinity but high capacity for glucose reabsorption and transports glucose and Na$^+$ in a 1:1 ratio (37), mediating reabsorption of the bulk of tubular glucose (38), whereas high affinity low capacity SGLT1 in the later S3 segment of proximal tubule transports glucose and Na$^+$ in a 1:2 ratio, cleaning up any remaining glucose (39; 40). Together they are able to reabsorb all filtered glucose up to a blood glucose concentration of 15 mM. Indeed, progressive increase in tubular glucose load results in net Na$^+$ and fluid reabsorption (41). With more severe hyperglycemia, glucosuria will occur causing an osmotic diuresis.

Increased proximal tubular reabsorption will have two consequences for GFR. First, it will initiate an error signal to MD leading to a reduced TGF signal. Luminal Na$^+$, Cl$^-$ and K$^+$ concentration is reduced in the early distal nephron (4). This will be interpreted by MD as a low GFR, reducing adenosine release and subsequently A$_1$AR signaling, increasing RBF and GFR. However, A$_1$AR knock-out mice lacking a functional TGF mechanism (15; 16) still display diabetes-induced hyperfiltration (42; 43) and increased RBF is not a requirement for diabetes-induced glomerular hyperfiltration (9; 44; 45), although it is observed in some studies (46-48). Second, proximal tubular pressure is reduced in diabetes increasing $P_{\text{NET}}$, secondary to increased proximal tubular reabsorption through SGLT but also tubular hypertrophy reducing flow resistance in the distal nephron, buffering the increased tubular flow rate due to osmotic diuresis (49). Inhibition of SGLT increases $P_{\text{Bow}}$ and reduces GFR exclusively in diabetes, and knockout of SGLT2 in mice attenuates glomerular hyperfiltration but not tubular hypertrophy (50). $P_{\text{GC}}$ in diabetic animals can be increased, decreased or unchanged compared to control animals (7; 47; 51). Increased $P_{\text{GC}}$ can be mediated by an afferent arteriole dilation, which would result in a concomitant increase in RBF. As already mentioned, RBF can be increased or unchanged in the hyperfiltration phase of diabetes but this will be discussed in detail in the discussion of paper II. Increased $P_{\text{GC}}$ with unchanged RBF that is also observed in experimental diabetes is compatible with a constriction of the efferent arteriole, partly mediated by increased renal Ang II concentration, binding to efferent arteriolar AT$_1$ receptors. Indeed, AT$_1$ receptor inhibition decreases FF and low dose Ang II infusion increases GFR and decreases RBF resulting in increased FF in early type 1 diabetic patients (52). Furthermore, ACE-inhibition reduce, although not normalize GFR exclusively in hyperfiltering type 1 diabetics (53) as well as in animal models of type 1 diabetes (51). More recently, attention has been directed toward afferent and efferent arteriolar adenosine A$_2$ receptors (A$_2$AR) and its involvement in renal autoregul-
lation (54), and the aim of paper III and IV was to investigate A2AR signaling in the involvement of diabetes-induced hyperfiltration.

Reactive oxygen species

Diabetes is associated with increased load of oxidative stress, defined as an imbalance between production of reactive oxygen species (ROS) and antioxidant defense resulting in oxidative damage. In health, when ROS producing systems and antioxidant defense is in balance, the basal ROS production is important for normal redox signaling, for instance oxidation of cysteine residues, known to regulate activity of several enzymes (55). Also a massive but well regulated oxidative burst in neutrophils is important for the immune system to be able to kill pathogens (56). However, a generalized increased ROS production, as occur in diabetes, is an important component in the disease progression. However, important to mention though is that clinical trials with antioxidant treatment have shown on increased mortality (57; 58), stressing the fact that ROS are involved in normal physiological processes and a disruption might be harmful. Sources of increased ROS in diabetes include the mitochondria (59), uncoupled nitric oxide synthase (NOS) (60), xanthine oxidase (61; 62) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (63).

The most common ROS molecules are the superoxide anion (O$_2^-$), formed by one electron donation to molecular oxygen, the hydroxyl radical (OH$^-$), hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$). ONOO$^-$ is the reaction product between O$_2^-$ and NO thereby linking increased ROS production to reduced bioavailability of NO. Redox status is balanced by enzymatic antioxidant defense systems degrading, and molecular antioxidants scavenging ROS. A key system is the superoxide dismutases (SOD) catalyzing the reaction from O$_2^-$ to H$_2$O$_2$. Three isoforms are present in the human: SOD1 in the cytoplasm, SOD2 in the mitochondria and SOD3 in the extracellular space. H$_2$O$_2$ is further catalyzed to water and O$_2$ by catalase (64). Reduced SOD1 activity accelerates diabetic nephropathy in mice (65) and total antioxidant capacity is decreased in diabetic patients (66), and correlated to the degree of complications (67), linking the development of diabetic nephropathy to both increased ROS production but also reduced antioxidant defense. Of special interest for this thesis is the NADPH oxidase, the only enzyme with the sole purpose of producing O$_2^-$, Different isoforms of the NADPH oxidase is expressed in phagocytes, smooth muscle cells, endothelial cells and renal tubules to create huge amounts of O$_2^-$ in phagocytes during oxidative burst and a low basal O$_2^-$ production in the other tissues contributing to regulation of vascular tone, and thereby blood pressure, and tubular reabsorption, respectively (68-70). The originally discovered phagocytic NADPH oxidase consists of membrane bound enzymatic component, gp91$^{phox}$ or NOX-2, and regulatory cytosolic subunits, p47$^{phox}$, p67$^{phox}$,
p40\textsuperscript{phox} and Rac 1 (71). Today however, at least seven different homologues has been discovered with different tissue expression where Nox-1, Nox-2 and Nox-4 is detected in the microvasculature and tubule of the kidney (72). Diabetes results in up regulation of this enzyme system at different levels observed as increased phosphorylation, indicating activation, of the regulatory subunits p47\textsuperscript{phox} and p67\textsuperscript{phox} as well as increased protein expression of Nox-2 and Nox-4 (73-76). Therefore was the outcome of acute NADPH oxidase inhibition in diabetes investigated in study I.

Nitric oxide

NO determines vascular tone and thereby RBF (77) and tubular electrolyte transport, partly via direct interference with tubular transporters (78) as well as inhibition of mitochondria respiration (79). Thereby, NO is involved in determining renal oxygenation (80; 81). NO is produced by three different NOS, endothelial, inducible and neuronal, from the substrate L-arginine and several co-factors, including tetrahydrobiopterin (BH\textsubscript{4}), NADPH and O\textsubscript{2}. Production is regulated by availability of both substrate in form of L-arginine (82) and co-factors, mainly BH\textsubscript{4} (83) but also directly regulated depending on NO needs. Most important are phosphorylation at serine 1177, increasing NOS activity, mediated by Akt/protein kinase B, protein kinase A, 5\textsuperscript{'}-AMP-activated protein kinase (AMPK) and calmodulin-dependent kinase II, and phosphorylation at threonine 495, decreasing NOS activity, mediated by protein kinase C (84). NO bioavailability is reduced in diabetes and is linked to intra-renal hypoxia (80). Uptake of L-arginine is mediated by the amino acid transport system y\textsuperscript{+} or system y\textsuperscript{+}L where y\textsuperscript{+} is Na\textsuperscript{+}-independent and transports cationic amino acids and y\textsuperscript{+}L is Na\textsuperscript{+}-dependent and transports both neutral and cationic amino acids (85). System y\textsuperscript{+} is represented in the kidney by cationic amino acid transporter 1 (CAT1) (82). This result in a direct competition between several amino acids for the same transporter and the individual concentration of each will determine its transport. Importantly, NO production rate is limited by L-arginine transport by CAT1 and L-arginine concentration is reduced in diabetes and therefore an interesting intervention that is discussed in paper V.
Aims

The overall aim of this thesis was to investigate changes occurring in the kidney very early in the disease progression, usually before the classical clinical sign of diabetic nephropathy. Focus was directed towards events initiating the diabetes-induced increase in GFR but also changes in the tubular system, with emphasis on electrolyte reabsorption and oxygen metabolism.

Specific aims for the papers included in this thesis:

**Paper I**
To determine the role of NADPH oxidase derived ROS on tubular Na⁺ transport *in vivo* and its relation to kidney oxygenation. In isolated proximal tubular cells localize exaggerated ROS-dependent tubular reabsorption in diabetes to renal cortex.

**Paper II**
To elucidate the role of insulin in regulation of factors determining GFR in diabetes.

**Paper III**
To determine the role of adenosine A₂ receptors in regulation of GFR in diabetes.

**Paper IV**
To determine if reduced adenosine A₂a receptor signaling in diabetes is contributing to increased FF and GFR.

**Paper V**
To determine if restored plasma L-arginine concentration in diabetes prevents diabetes-induced kidney dysfunction.
Materials and Methods

Animals and chemicals

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) if not otherwise stated. Male Sprague-Dawley rats (Charles River, Sulzfeldt, Germany) were used in study I, II, IV and V. Male C57/BL6 mice (Charles River, Sulzfeldt, Germany) were used in study III. Animals had free access to tap water and standard rat (Ewos, Södertälje, Sweden) or mouse chow (LAB-FOR Lantmännen, Sweden). Animals were housed in groups in a temperature and light controlled environment and received daily care.

Animals were divided into the following groups:

- Study I  Control and diabetes.
- Study II Control, diabetes and diabetes+insulin.
- Study III Control and diabetes.
- Study IV Control and diabetes.
- Study V Control and diabetes with and without chronic L-arginine or L-citrulline supplementation in the drinking water.

Animal procedures

All animal procedures were performed in accordance with national guidelines of animal care and use and approved by the Uppsala animal ethics committee.

*Induction of diabetes (Study I, II, III, IV and V)*

Diabetes was induced by a single injection of either 55 mg/kg bw streptozotocin dissolved in 0.2 ml saline in the tail vein (Study I, II, IV and V) or 70 mg/kg bw alloxan dissolved in 0.2 ml saline in the tail vein (Study III). Animals were considered diabetic if blood glucose concentration was increased $>15$ mmol/L within 48 hours and remained elevated. Blood glucose concentration was determined with test reagent strips (FreeStyle, Abbott
Laboratories, Abbott Park, IL, USA) on blood samples obtained from the cut tip of the tail. Diabetes duration was 14-28 days.

**Insulin treatment (Study II) and supplementation of L-arginine and L-citrulline (Study V)**

Insulin treatment (10 IU/kg, subcutaneous) was given once a day and started 24 hours after diabetes was induced and carried out throughout the course of diabetes. L-arginine and L-citrulline supplementation was given in the drinking water and treatment started the same day as diabetes induction.

**Experimental protocols**

In study I untreated control (n=11) and diabetic (n=13) rats were used with a total diabetes-duration of 14±4 days until acute experiments. Additional control (n=7) and diabetic (n=8) rats with similar diabetes-duration were allocated for isolation of proximal tubular cells (PTC).

In study II untreated control (n=17), untreated diabetic (n=16) and insulin-treated diabetic (n=18) rats were used with a total diabetes-duration of 14±2 days until acute experiments. Additional untreated control (n=4), untreated diabetic (n=4) and insulin-treated diabetic (n=4) rats were allocated to micropuncture studies. Insulin-treated diabetic rats were given one daily subcutaneous injection of insulin (9 IU/kg/day) (Lantus, Sanofi Aventis, Frankfurt am Main, Germany).

In study III untreated control (n=11) and diabetic mice (n=10) were used with a total diabetes-duration 25±4 days until acute experiments.

In study IV untreated control (n=8) and diabetic (n=11) rats were used with a diabetes-duration of 14±4 days until acute experiments for investigation of whole kidney function. Additional control (n=5) and diabetic (n=8) rats with similar diabetes-duration were used for micropuncture.

In study V untreated control (n=12) and diabetic rats (n=9), L-arginine treated control (n=10) and diabetic (n=10) rats and L-citrulline treated control (n=11) and diabetic (n=10) rats were used with a total diabetes-duration of 21±5 days until acute experiments. L-arginine (1.25% to controls and 0.35% to diabetics) or L-citrulline (1.20% to controls and 0.20% to diabetics) treatment was administered in the drinking water throughout the course of diabetes.
In vivo kidney function in rat (Study I, II, IV and V) and mouse (Study III)

Surgery

Rats were anesthetized with thiobutabarbital (Inactin, 120 mg/kg bw for non-diabetic and 80 mg/kg bw for diabetic animals, intraperitoneal injection) and placed on a servo-controlled heating pad to maintain body temperature at 37.5°C. A tracheotomy was performed to assure unobstructed spontaneous breathing. A polyethylene catheter was placed in a femoral artery for monitoring of blood pressure and blood sampling and in a femoral vein for infusion of saline (5 ml/kg bw/h to non-diabetic animals and 10 ml/kg bw/h to diabetic animals). The bladder was catheterized for urinary drainage followed by a subcostal flank incision exposing the left kidney that then was immobilized in a plastic cup. The left ureter was catheterized for timed urine collections. An ultrasound flow probe (Transonic Systems Inc., Ithaca, NY, USA) was placed around the left renal artery to measure RBF. After surgery, all animals were allowed to recover for 40 minutes before the experiments were commenced.

Mice were anesthetized with isoflurane 1.5-2.0% in 100% O₂ (Abbott), placed on a servo-controlled heating pad maintaining body temperature at 37.5°C. Catheters were placed in carotid artery and jugular vein for determination of blood pressure, blood sampling and infusion of saline (0.35 ml/h to non-diabetic and 0.7 ml/h to diabetic mice). The bladder was catheterized for urine collection and the left kidney was exposed through a sub-costal flank incision. An ultrasound flow-probe was placed around the renal artery.

GFR (Study I, II, III, IV and V)

GFR was estimated by inulin-clearance. ³H-inulin (American Radiolabeled Chemicals, St. Louis, MO, USA) was administered as a bolus dose (185 kBq/kg) followed by a continuous infusion in Ringer solution (185 kBq/kg bw/h). ³H-inulin activity in urine from timed collections and in plasma was measured with standard liquid scintillation technique. Urine flow was measured gravimetrically and GFR calculated according to clearance equation, GFR=[inulin]₀*urine flow/[inulin]₀.

Renal oxygen tension (PO₂) (Study I and V)

Renal tissue oxygen tension was determined using modified Clark-type microelectrodes with an outer diameter of the tip of 10 µm (Unisense, Aarhus, Denmark). Electrodes were two-point calibrated in water saturated with either Na₂S₂O₅ to set zero or air to set 147 mmHg PO₂. Microelectrodes were adjusted by a micromanipulator to measure PO₂ at either 1 mm depth from
kidney surface for cortical PO$_2$ or at 5 mm depth from kidney surface for determining medullary PO$_2$.

**In vivo oxygen consumption (QO$_2$) (Study V)**

In vivo QO$_2$ was determined from the arterio-venous difference in oxygen content (O$_2$ct) multiplied by the RBF according to the formula: 

$$O_2\text{ct} = \left(\left[\text{hemoglobin}\right]\times\text{oxygen saturation}\times 1.34\right) + \left(\text{oxygen tension}\times 0.003\right).$$

**Electrolyte excretion and Li$^+$ clearance (Study I and III)**

Na$^+$ and Li$^+$ concentrations in urine and plasma samples were determined by flame spectrophotometry (model IL543, Instrumentation Lab, Milan, Italy). Li$^+$ clearance, as a marker of proximal tubular reabsorption, was measured in study I. Li$^+$ was administered as a 4 mg intraperitoneal injection of LiCl at the end of surgery followed by a continuous infusion of 2.1 mg/h. This resulted in plasma Li$^+$ concentration of 0.5-1.0 mM.

**Thiobarbituric acid reactive substances (Study I and V)**

Thiobarbituric acid reactive substances (TBARS) to assess oxidative stress level were measured in urine samples and determined fluorometrically. 100 µl of diluted urine sample were mixed with 125 µl thiobarbituric acid (Merck, Darmstadt, Germany) and heated to 97°C for 60 minutes. Standards were prepared from malondialdehyde-bis-(diethylacetate) (Merck-Schuchart, Schuchart, Germany). Samples were cooled on ice and 150 µl of 1 M NaOH and methanol (91:9) were added. Samples were centrifuged (3000 rpm for five minutes) and fluorescence was measured in the supernatant (ex. 532 nm, em. 553 nm, Safire 2, TECAN, Männedorf, Switzerland).

**Micropuncture (Study II and IV)**

Intratubular pressure was measured with a servo-controlled pressure system (World Precision Instruments, New Haven, CT, USA). Proximal tubular free flow pressure (P$_{FF}$) and stop flow pressure (P$_{SF}$) were measured. P$_{FF}$ in the unobstructed nephron of an early loop of a proximal tubule whereas P$_{SF}$ is the pressure recorded after which injecting mineral oil distal of the pressure pipette stops the tubular flow. P$_{SF}$ is a surrogate marker for P$_{GC}$ minus the oncotic pressure. Accordingly, the driving pressure for filtration, P$_{NET}$ was calculated from P$_{SF}$-P$_{FF}$.
**In vitro** oxygen consumption (QO₂)(Study I)

Isolation of proximal tubular cells and QO₂ measurement

PTCs were isolated from control and diabetic rats. Rats were anesthetized with thiobutabarbital and both kidneys were immediately removed and placed in ice-cold buffer. Renal cortex pooled from both kidneys were minced through a metallic mesh strainer and incubated with buffer containing collagenase (0.05% wt/vol) at 37°C for 90 minutes. Incubation was constantly bubbled with a gas-mixture of 95% O₂ and 5% CO₂. Suspension was then cooled on ice for 10 minutes followed by filtration through graded filters with pore sizes of 180, 75, 53 and 38 µm. Cells were centrifuged (200 g, 2 min) and the pellet was suspended in collagenase-free buffer. This process was repeated three times and cells were kept on ice until QO₂ was measured. Isolation procedure and subsequent QO₂ measurements were conducted in a buffer solution containing in mM, 113.0 NaCl, 4.0 KCl, 27.2 NaHCO₃, 1.0 KH₂PO₄, 1.2 MgCl₂, 1.0 CaCl₂, 10.0 HEPES, 0.5 Ca-lactate, 2.0 glutamine and 50 U/ml streptomycin. Osmolality and pH was adjusted to 300 mOsm/kg and 7.4, respectively. Glucose concentration in the medium was 5.8 mM for cells from normoglycemic control rats and 23.2 mM for cells from diabetic rats.

QO₂ was measured in an Oxygraph 2k (OROBOROS Instruments, Innsbrück, Austria). PTCs were incubated with vehicle, dimethylamiloride (DMA; 1 mM), apocynin (1 mM), ouabain (2 mM) and apocynin in combination with either DMA or ouabain for 10 minutes at 37°C. DMA was used to inhibit NHE₃, apocynin to inhibit the NADPH oxidase and ouabain to inhibit the Na⁺K⁺-ATPase. After incubation 50 µl of PTC suspension was injected into the oxygraph, and the rate of O₂ disappearance was recorded. At the end of each recording, 1 ml was removed to determine protein concentration. To avoid interference with the protein assay samples were centrifuged (15,000 g, 10 minutes) and resuspended in 200 µl dH₂O. Protein concentration was determined according to the Lowry method with DC Protein Assay (Bio-Rad, Hercules, CA, USA) and QO₂ was adjusted for protein concentration.

**Statistical analysis**

All statistical analysis were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA) or SAS (SAS Institute, Cary, NC, USA) and for all analysis a P<0.05 was considered significant. Descriptive statistics are presented as means ± SEM.
In study I all data were analyzed by 2-way repeated measure ANOVA followed by Bonferroni’s multiple comparisons test. In addition, data presented in Figure 5 was analyzed by one-way ANOVA followed by Bonferroni’s multiple comparisons test.

In study II correlation analysis were performed using least-squares linear regression to test whether regression slopes were significantly different from zero. All other analysis used a mixed model approach to account for the study design.

In study III and IV all data were analyzed by two-way ANOVA followed by Bonferroni’s multiple comparisons test.

In study V in vivo data were grouped in untreated, L-arginine treated and L-citrulline treated and analyzed with two-way ANOVA followed by Bonferroni’s multiple comparisons test. Plasma concentration of amino acids and related compounds were analyzed with one-way ANOVA followed by Fisher’s Least Significant Difference (LSD) test.
Results

NAPDH oxidase a determinant of kidney oxygenation and proximal tubular Na⁺ transport in diabetes (Study I)

In vivo kidney function
Diabetic rats displayed increased GFR, but similar RBF, resulting in increased calculated FF compared to normoglycemic controls. NADPH oxidase inhibition did not affect any of these parameters but increased both absolute and fractional excretion of Na⁺ (Figure 2), as well as fractional excretion of Li⁺ (Figure 3). Cortical and medullary PO₂ were reduced in diabetics and PO₂ was increased by NADPH oxidase inhibition only in diabetics (Figure 4). Urinary excretion of TBARS was increased in diabetics, reflecting increased oxidative stress, and acute NAPDH oxidase inhibition reduced it, confirming the effect of apocynin.

Figure 2. Absolute (A) and fractional (B) excretion of Na⁺ in control and diabetic rats during baseline and after NADPH oxidase inhibition with apocynin.
**Figure 3.** Fractional excretion of Li$^+$ in control and diabetic rats during baseline and after NADPH oxidase inhibition using apocynin.

**Figure 4.** Cortical (A) and medullary (B) oxygen tension in control and diabetic rats during baseline and after NADPH oxidase inhibition using apocynin.

**In vitro QO$_2$**

QO$_2$ in isolated PTC was increased in cells isolated from diabetic rats compared to cells from normoglycemic control rats. QO$_2$ was decreased in both groups after inhibition of the NADPH oxidase with apocynin, NHE$_3$ by DMA, and the Na$^+$/K$^+$-ATPase by ouabain. Combination of apocynin with either DMA or ouabain decreased QO$_2$ as well, but did not result in additive decrease in QO$_2$ compared to incubation with DMA or ouabain alone (Figure 5).
Insulin affects filtration hemodynamics and the mechanism causing diabetes-induced hyperfiltration (Study II)

GFR was increased in both untreated and insulin-treated diabetics compared to controls (Figure 6). Increased GFR was accompanied by increased RBF (Figure 7A) in diabetes+insulin and increased FF (Figure 7B) in untreated diabetes. A correlation between RBF and GFR was observed in diabetics+insulin, and the regression line was significantly different from both controls and untreated diabetes (Figure 8). $P_{\text{NET}}$ was increased in untreated diabetes, but not significantly different from control in diabetes+insulin (Figure 9).
Figure 6. Glomerular filtration rate in control, untreated diabetes and diabetes+insulin treated rats during baseline and after unselective nitric oxide synthase inhibition using L-NAME. * denotes P<0.05 compared to corresponding control.

Figure 7. Renal blood flow (A) and filtration fraction (B) in control, untreated diabetes and diabetes+insulin treated rats during baseline and after unselective nitric oxide synthase inhibition using L-NAME. * denotes P<0.05 compared to corresponding control.
Figure 8. Correlation between glomerular filtration rate and renal blood flow in control, untreated diabetes and diabetes+insulin treated rats. The slope of the regression line is significantly different between diabetes+insulin compared to both other groups.

![Correlation between glomerular filtration rate and renal blood flow](image)

Figure 9. Free flow (left), stop flow (middle) and calculated net filtration pressure (right) in control, untreated diabetes and diabetes+insulin treated rats.

Adenosine A₂-signaling reduces filtration fraction in controls but not in diabetics (Study III)

The adenosine A₂ antagonist DMPX increased GFR but decreased RBF in normoglycemic control but was without effect in diabetics (Figures 10 and 11), resulting in increased FF in controls.
Reduced adenosine A$_{2a}$ signaling mediates increased filtration fraction in diabetes (Study IV)

Mean arterial pressure (MAP) was lower in diabetics during baseline but both groups decreased MAP in response to adenosine A$_{2a}$ activation (Figure 12). RBF was not significantly different between the groups and unaffected by adenosine A$_{2a}$ activation in controls and in the low dose in diabetics, but decreased during the high dose (Figure 12). GFR was higher in diabetics and adenosine A$_{2a}$ activation decreased it, but had no effect in controls (Figure 13), resulting in reduced calculated FF only in diabetics (Figure 13).
Figure 12. Mean arterial pressure (left) and renal blood flow (right) in controls and diabetics during baseline and after infusion of two doses of the adenosine A2a-agonist CGS21680 into the renal artery. * denotes P<0.05 compared to baseline analyzed by Bonferroni’s multiple comparisons test. Result from 2-way ANOVA in left panel: interaction: ns, diabetes: P<0.05, CGS21680: P<0.05 and right panel: interaction: P<0.05, diabetes: ns, CGS21680: ns.

Figure 13. Glomerular filtration rate (left) and filtration fraction (right) in controls and diabetics during baseline and after infusion of two doses of the adenosine A2a-agonist CGS21680 into the renal artery. * denotes P<0.05 compared to baseline analyzed by Bonferroni’s multiple comparisons test. Result from 2-way ANOVA left panel: interaction: P<0.05, diabetes: P<0.05, CGS21680: P<0.05 and right panel: interaction: P<0.05, diabetes: ns, CGS21680: ns.

Micropuncture
P_FF was lower in diabetics compared to controls and not affected by adenosine A2a activation (Figure 14). P_SF was not significantly different between the groups during baseline, but was decreased by adenosine A2a activation in diabetics (Figure 14). Accordingly, calculated P_NET was decreased by adenosine A2a activation only in diabetics (Figure 15).
Figure 14. Free flow pressure (left) and stop flow pressure (right) in controls and diabetics during baseline and after infusion of adenosine A2a-agonist CGS21680 into the renal artery. * denotes P<0.05 compared to baseline analyzed by Bonferroni’s multiple comparisons test. Result from 2-way ANOVA. Left panel: interaction: ns, diabetes: P<0.05, CGS21680: ns. Right panel: interaction: P<0.05, diabetes: P<0.05, CGS21680: P<0.05.

Figure 15. Net filtration pressure in controls and diabetics during baseline and after infusion of the adenosine A2a-agonist CGS21680 into the renal artery. Calculated from P_{SF}-P_{FF}. * denotes P<0.05 compared to baseline analyzed by Bonferroni’s multiple comparisons test. Result from 2-way ANOVA: interaction: P<0.05, diabetes: ns, CGS21680: P<0.05.

L-citrulline improves plasma L-arginine in diabetes and prevents diabetes-induced glomerular hyperfiltration and proteinuria (Study V)

Baseline GFR was higher in untreated diabetics and diabetics treated with L-arginine compared to corresponding controls, whereas GFR in diabetics treated with L-citrulline was not (Figure 16). RBF was higher in untreated diabetics, but similar to corresponding controls in diabetics treated with either L-arginine or L-citrulline. Indeed, FF was increased in untreated diabetics but not significantly different from corresponding controls in L-arginine and L-citrulline treated diabetics (Figure 17). Untreated and L-arginine treat-
ed diabetics had elevated urinary protein excretion but L-citrulline treatment prevented this increase (Figure 18).

Figure 16. Glomerular filtration rate in untreated controls and diabetics (A), L-arginine treated controls and diabetics (B) and L-citrulline treated controls and diabetics (C), during baseline and after unselective nitric oxide synthase inhibition using L-NAME. Result from 2-way ANOVA (A) type: P<0.05 treatment: ns interaction: ns (B) type: P<0.05 treatment: ns interaction: ns (C) type: ns treatment: ns interaction: ns.

Figure 17. Filtration fraction in untreated controls and diabetics (A), L-arginine treated controls and diabetics (B) and L-citrulline treated controls and diabetics (C), during baseline and after unselective nitric oxide synthase inhibition using L-NAME. Result from 2-way ANOVA (A) type: P<0.05 treatment: P<0.05 interaction: ns (B) type: ns treatment: P<0.05 interaction: ns (C) type: ns treatment: P<0.05 interaction: ns.

Figure 18. Urinary protein excretion in untreated controls and diabetics (A), L-arginine treated controls and diabetics (B) and L-citrulline treated controls and diabetics (C), during baseline and after unselective nitric oxide synthase inhibition using L-NAME. Result from 2-way ANOVA (A) type: P<0.05 treatment: ns interaction: ns (B) type: P<0.05 treatment: ns interaction: ns (C) type: P<0.05 treatment: P<0.05 interaction: P<0.05
Plasma concentration of L-arginine was decreased in untreated diabetics and not significantly increased by L-arginine supplementation. However, L-citrulline treatment increased plasma L-arginine in both controls and diabetics (Figure 19). L-arginine transport by CAT1 is competitively inhibited by L-ornithine and L-lysine. Calculating a ratio between L-arginine and L-ornithine together with L-lysine reveals that this ratio is 1:9 and 1:13 in untreated controls and diabetics, respectively. This is improved to 1:5 in diabetics treated with L-arginine and further improved to 1:2 controls and diabetics treated with L-citrulline (Figure 19).

*Figure 19.* Plasma concentration of L-arginine (A) and ratio (L-Lys+L-Orn/L-Arg) (B) in untreated controls and diabetics, L-arginine treated controls and diabetics and L-citrulline treated controls and diabetics. * denotes P<0.05 compared to corresponding control, † denotes P<0.05 compared to untreated control and ‡ denotes P<0.05 compared to untreated diabetes.
Discussion

The main findings from the studies presented in this thesis are that the diabetes-induced increase in NADPH oxidase activity increases transport-dependent QO$_2$ in the proximal tubule and contributes to intra-renal hypoxia, but does not affect diabetes-induced hyperfiltration. Insulin on the other hand affects intra-renal hemodynamics, inducing a RBF-dependent regulation of GFR and alleviates the diabetes-induced increased P$_{\text{NET}}$ and subsequently FF. Adenosine A$_2$ receptor inhibition increase GFR, despite decreased RBF in control animals but lacks effect in diabetics. Indeed, adenosine A$_{2a}$ activation reduces GFR with maintained RBF, decreasing FF in diabetics by a preferential dilatation of the efferent arteriole decreasing P$_{\text{NET}}$. Furthermore, L-citrulline supplementation in diabetes increases plasma L-arginine concentration, in contrary to L-arginine supplementation, and prevents diabetes-induced GFR and proteinuria.

In study I was the correlation between NADPH oxidase activity and tubular reabsorption of Na$^+$ and kidney oxygenation investigated. O$_2^-$ stimulates tubular reabsorption of electrolytes in isolated mTAL by activating the transporters NKCC and NHE$_3$ (70; 86; 87). NADPH oxidase activity is increased in diabetes resulting in increased O$_2^-$ production (73; 74; 76) and diabetes-induced transport-dependent QO$_2$ in cells isolated from mTAL has been correlated to NADPH oxidase activity. However, in study I it was shown that diabetes-induced NADPH oxidase activity stimulates Na$^+$ reabsorption $\textit{in vivo}$. Absolute and fractional Na$^+$ excretion was increased secondary to NADPH oxidase inhibition only in diabetics and some parts of this increased reabsorption was localized to the proximal tubule using clearance of Li$^+$ as a marker (88). Though it has been shown that smaller amounts of Li$^+$ can be reabsorbed in mTAL, and under situations of Na$^+$ restriction in cortical collecting duct, increased transport-dependent QO$_2$ from the proximal tubule was confirmed using isolated PTC. Inhibition of either NADPH oxidase or Na$^+$/K$^+$-ATPase reduced QO$_2$ in isolated PTC but their effects were not additive suggesting that NADPH oxidase inhibition directly interferes with transport-dependent QO$_2$ in the proximal tubule. NHE$_3$ has previously been shown to be stimulated by O$_2^-$ in isolated mTAL (86), and cortical NHE$_3$ activity is increased in diabetes (89), probably via up regulation of Ang II AT$_1$ receptors by the increased oxidative stress, leading to an over activation of NHE$_3$ (90). Since the vast majority of filtered Na$^+$ is reabsorbed by NHE$_3$ it is likely that diabetes-induced increase in NADPH oxidase activ-
ity can stimulate proximal tubular transport by this mechanism. Indeed, it was no additive effect on \( \text{QO}_2 \) by NADPH oxidase inhibition when NHE3 was inhibited, providing further support for its involvement. Tissue \( \text{PO}_2 \) was decreased in diabetics in study I confirming previous reports from both animal models and patients (91-93). Decreased \( \text{PO}_2 \) in diabetes could be secondary to increased GFR increasing tubular load of electrolytes that needs to be reabsorbed to maintain \( \text{Na}^+ \) balance. However, this is not likely since NADPH oxidase inhibition increased both cortical and medullary \( \text{PO}_2 \) without affecting GFR. Furthermore, chronic treatment with the antioxidant DL-alpha-tocopherol prevented the diabetes-induced decrease in \( \text{PO}_2 \) but not glomerular hyperfiltration (91). Instead oxidative stress seems to reduce tubular transport efficiency and thereby increase basal \( \text{QO}_2 \). This is supported by the higher \( \text{QO}_2 \) in PTC isolated from diabetics compared to controls after \( \text{Na}^+/	ext{K}^-\text{-ATPase} \) inhibition. This is mechanistically explained by increased mitochondria uncoupling in diabetes and its sensitivity to redox status (94-96).

In study II was the influence of sub-optimal insulin treatment on regulation of GFR investigated. Diabetes-induced glomerular hyperfiltration is observed in both animal models of diabetes and in patients (4; 45; 97; 98). Increased GFR in diabetes has been suggested to be either RBF-dependent or pressure-driven. Hyperglycemia results in increased proximal tubular \( \text{Na}^+ \) reabsorption mediated by SGLT, resulting in decreased \( \text{Na}^+ \) delivery to the early distal nephron (4; 99). This will exert an error signal to MD resulting in TGF-inactivation and afferent arteriole dilation increasing RBF and GFR. However, mice lacking a functional TGF-mechanism still develop glomerular hyperfiltration (42; 43), but inhibition of SGLT or knockout of SGLT2 in diabetics normalizes and prevents the diabetes-induced increase in GFR, respectively (4; 50; 99). This is proof for proximal tubular reabsorption as crucial for development of glomerular hyperfiltration. Indeed, \( \text{P}_{\text{bow}} \) is decreased in diabetes, contributing to increase \( \text{P}_{\text{NET}} \), and is directly influenced by proximal tubular reabsorption (4; 5; 99) and a correlation between fractional \( \text{Na}^+ \) reabsorption and GFR exists in hyperfiltering type 1 diabetic patients (100). Indeed, increased GFR can be accompanied by increased RBF (46-48; 101), but it is not a pre-requisite for development of glomerular hyperfiltration (9; 45; 97; 102-104) suggesting two different mechanisms that are able to maintain elevated GFR during early diabetes; insulin seems to be an important factor regulating this. Insulin stimulates NO production and induces vasodilation of both afferent and efferent arteriole (105-107). Sub-optimal insulin treatment induced a correlation between RBF and GFR in diabetes, an observation not present in either controls or untreated diabetics. Furthermore, insulin induced a correlation between blood glucose and RBF, and although sub-optimal insulin treatment did not normalize blood glucose it was significantly lower compared to untreated diabetics indicating that both RBF and GFR should be considerably higher in insulin treated diabetics.
if blood glucose levels were comparable. Micropuncture data revealed that untreated diabetics had lower P_FF compared to both controls and insulin treated diabetics. Although P_SF was not significantly different between the three groups calculated P_NET was increased in untreated diabetics but not significantly different from controls in insulin treated diabetics. This is in line with previous a study where acute administration of insulin reduced P_NET with maintained glomerular hyperfiltration in diabetics (108). This clearly demonstrates that untreated diabetics have a pressure driven glomerular hyperfiltration and insulin treated diabetics, presenting normal P_NET, have a RBF-dependent glomerular hyperfiltration. Interestingly, RBF was similar between the two diabetic groups suggesting that insulin mechanistically affects K_f, since GFR was maintained despite reduced P_NET. Intra-renal Ang II is increased in diabetes and contributes to increased FF by constriction of the efferent arteriole (51; 109; 110), which can be off-set by NO-mediated vasodilation (111), eventually induced by insulin, explaining the normal P_NET. Vasoactive hormones, including Ang II also modulates mesangial cell tone and subsequently GFR, and local renal inhibition of NOS reduces K_f implicating a direct tonic control of K_f by NO (112-114). NOS3 and insulin receptors are expressed in glomeruli mediating glomerular NO production (115-117). Insulin treatment could therefore have resulted in increased glomerular NO production inducing mesangial cell relaxation, thereby increasing K_f. Higher K_f will drive filtration dynamics towards filtration equilibrium known to result in RBF-dependent regulation of GFR (11; 118) whereas during a higher K_f P_NET will be more important (11; 119; 120).

In study III and IV was the influence of adenosine A_2a receptor signaling in the glomerular hyperfiltration investigated. Adenosine A_2 inhibition increased GFR and reduced RBF in controls but was without effect in diabetics. As mentioned in previous sections renal autoregulation through the TGF-mechanism is mediated by adenosine A_1 receptors, but the response is dampened by simultaneous activation of adenosine A_2 receptors (54; 121) and expression studies reveal that adenosine A_2 receptors are expressed on both the afferent and efferent arteriole (30; 122). However, the main physiological effect of endogenous adenosine signaling is a vasodilation of the efferent arteriole to reduce FF. The physiological effect after blocking ambient adenosine A_2 signaling can be explained by differences in sub-type distribution where low-affinity adenosine A_2b mediates afferent arteriole dilation (122) and efferent arteriole dilation is mediated by combined adenosine A_2a and A_2b activation (30). Adenosine A_1 and A_2 receptors are upregulated in diabetes, especially adenosine A_2a receptors in renal cortex (123) and diabetics have increased vascular reactivity to infusion of adenosine (124). This would implicate that lack of effect in diabetics of adenosine A_2 inhibition is due to reduced endogenous ligand-activation. Therefore the hypothesis was tested that reduced adenosine A_2a signaling in diabetes mediates glomerular hyperfiltration by increasing FF. Indeed, infusion of the adenosine A_2a-
agonist CGS21680 was able to reduce FF and GFR in diabetics but was without effect in controls. This would suggest that adenosine $A_2a$ receptors are able to reduce FF and that diabetes is associated with reduced ligand activation and not deranged down-stream signaling. NO bioavailability is reduced in diabetes (80) and adenosine $A_2a$ receptor activation increases eNOS activity stimulating NO production (125). Furthermore, eNOS expression is inversely correlated to GFR and antioxidant treatment can prevent diabetes-induced glomerular hyperfiltration (95), indicating that reduced NO bioavailability is initiating increased GFR, eventually due to reduced adenosine $A_2a$ activation.

Figure 20. Mechanisms investigated in this thesis contributing to increased GFR in diabetes. Increased GFR is caused by increased filtration fraction mediated by reduced adenosine $A_2a$ receptor activation, as shown in study III and IV, most likely due to reduced concentration of adenosine locally around the arterioles leading to a vasoconstriction preferentially of the efferent arteriole. Decreased pressure in Bowman’s space, increasing net filtration pressure was observed in study II and IV, also known to be able to affect filtration fraction.

In study V was the outcome of normalized plasma concentration of L-arginine to improve NO production in diabetes investigated. L-citrulline but not L-arginine supplementation was effective in improving plasma concentration of L-arginine. This prevented the glomerular hyperfiltration and increased urinary protein excretion but not diabetes-induced $QO_2$. Plasma L-arginine is reduced in diabetes (126), and this might be crucial for disease progression. Despite high intracellular concentration above the $K_m$-value for NOS (127), NO production is highly dependent on extracellular L-arginine (128), and subsequently L-arginine transport across the plasma membrane via CAT1 which is a rate-limiting step in the NO production due to a caveolar complex between CAT1 and eNOS (129-131). This suggests a direct
presentation of L-arginine by CAT1. The superior effect of L-citrulline compared to L-arginine in improving plasma L-arginine concentration is explained by induction of de novo synthesis of L-arginine by L-citrulline. L-citrulline is normally synthesized from L-glutamine in enterocytes and released to the venous blood (132), filtered by the kidneys and reabsorbed in the proximal tubule. Proximal tubules express argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL), and are therefore able to synthesize L-arginine from L-citrulline (133-135). Furthermore, ASL is an adaptor protein binding CAT1 and NOS together keeping de novo synthesis of L-arginine in close proximity to the NOS (136). It has previously been shown that NO regulates kidney QO2 and PO2 (80; 81), but the outcome of study V with normalized GFR and proteinuria after increased plasma L-arginine concentration, but not QO2 and PO2, indicates that the potential NO-mediated effect occurred primarily in the vasculature and not in the proximal tubule.
Summary and conclusions

The main findings of this thesis are that increased NADPH oxidase activity increases transport-dependent QO$_2$ in the proximal tubule and contributes to intra-renal hypoxia in diabetes, but does not affect the hyperfiltration. Insulin on the other hand affect the mechanisms causing diabetes-induced hyperfiltration by altering intra-renal hemodynamics, inducing a RBF-dependent regulation of GFR but reduces P$_{NET}$ and subsequently FF. Adenosine A$_2$ receptor inhibition increase GFR, despite decreased RBF in control animals but lacks effect in diabetics. Indeed, adenosine A$_{2a}$ activation reduces GFR with maintained RBF, decreasing FF in diabetics by a preferential dilatation of the efferent arteriole decreasing P$_{NET}$. Furthermore, L-citrulline supplementation in diabetes increases plasma L-arginine concentration, in opposite to L-arginine supplementation, which subsequently prevents diabetes-induced hyperfiltration and proteinuria.

- NADPH oxidase activity determines transport-dependent QO$_2$ and intra-renal PO$_2$ in diabetes.
- Insulin induces RBF-dependent regulation of GFR but alleviates increased P$_{NET}$ in diabetes.
- Adenosine A$_2$ receptor activity decreases FF in controls but not in diabetics.
- Reduced adenosine A$_{2a}$ receptor activation causes diabetes-induced hyperfiltration by increasing FF.
- L-citrulline increases plasma L-arginine in diabetics and prevents diabetes-induced hyperfiltration and proteinuria.
Syftet med undersökningarna som ingår i denna avhandling har varit att klargöra vilka mekanismer som bidrar till en förändrad filtrationshastighet och syrgasmetabolism i njuren vid diabetes. Bakgrunden till genomförandet bygger på att diabetes är den vanligaste orsaken till en sviktande njurfunktion, så kallad diabetesnefropati, som kräver dialysbehandling eller organtransplantation. Den tidigaste förändringen i njuren vid diabetes är att glomerulerå filtrationshastigheten (GFR) stiger, dock ej hos alla individer, vilket har lett till att undersökningar initierats med mål att utvärdera om ett ökat GFR i det tidiga förloppet kan sammankopplas med en mer progressiv försmärning av njurfunktionen. Kliniskt definieras graden av njursjukdom efter utsöndringen av albumin i urinen, graderat som normoalbuminuri <30 mg/24 h, mikroalbuminuri 30-300 mg/24 h och slutligen makroalbuminuri >300 mg/24 h. Med ökad albuminuri avtar GFR succesivt tills uremi uppstår och dialysbehandling krävs. I nyligen publicerade meta-analyser har diabetisk hyperfiltration inte kunnat kopplas samman med utveckligen av albuminuri men med ett GFR som sjunker snabbare. Tidiga förändringar, observerade framförallt i djurmodeller, som föregår kliniska tecken på njursjukdom som albuminuri och reducerat GFR inkluderar ökad produktion av fria syrgasradikaler och minskad syrgastension i vävnaden. Diabetes via hyperglykemin stimulerar radikalproduktion från flera källor, bland annat från mitokondrien, överaktiverat NADPH-oxidas och frikopplat kväveoxidsyntas. I studie I visas att fria syrgasradikaler producerade från ett överaktiverat NADPH-oxidas stimulerar transportberoende syrgaskonsumtion i isolerade proximala tubulusceller och Na⁺-transport in vivo, då både absolut och fraktionell utsöndring av Na⁺ ökade efter akut inhibition av NADPH-oxidaset i diabetiska råttor men ej i friska kontroller. Detta resulterade också i en förbättrad syrgastension i både cortex och medulla. I studie II undersöktes mekanismerna bakom diabetesinducerad hyperfiltration och effekterna av insulin. Tre grupper studerades, kontroller, diabetiker och diabetiker med suboptimal insulinbehandling. Huvudfynden visar att obehandlade diabetiker har ett ökat nettofiltrationstryck och således en tryckberoende hyperfiltration medan suboptimal insulinbehandling normaliserar nettofiltrationstrycket men orsakar en blodflödesmedierad hyperfiltration. I studie III utvärderades om en förändrad adenosin A2-receptormedierad signalering vid diabetes påverkade GFR. Två grupper studerades, kontroll och diabetes, dels basalt, dels efter akut adenosin A2-inhibition. Huvudfynden visar att kontroller har en endogen A2-

Sammanfattningsvis visar denna avhandling att glomerulär hyperfiltration vid diabetes orsakas av minskad adeonsinsignalering via A₂a-receptorn och minskad plasmakoncentration av L-arginin, vilket även bidrar till utvecklandet av proteinuri. Vidare, fria syrgasradikaler från NADPH-oxidaset ökar transportberoende syrgaskonsumtion i njuren vilket samtidigt bidrar till minskad renal syrgastension.
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