The role of hypoxia for the development of diabetic nephropathy

**Temporal relationship and involvement of endothelin
receptor signaling**

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Public Perception of Science

Preface

This thesis is the reflection of me, my work and my experiences. It has mostly been frustration but apparently I managed to stay alive the whole way through.

For some it might have seemed easy but I am convinced that a big part has been extreme luck. I don't consider it my skill that it has sometimes run smoothly, because mostly it has been Murphy all over the place. Research is very much about luck. The luck to get the "right" animal batch, the luck to have equipment that isn't broken, the luck to have reviewers who like your paper, the luck that no one interferes with your settings, the luck to actually get results, etc etc. I could go on forever...

Anyway, here it is. My work. To be understood or not.

Stephanie

Science in Reality

Abstract

Diabetic nephropathy is one of the most common causes of end stage renal disease and develops in approximately one third of all diabetes patients. Disease progression is characterized by deteriorating glomerular filtration rate and escalating urinary albumin/protein excretion; both are used as clinical markers for disease progression. Recently, it has been proposed that intrarenal hypoxia is a unifying mechanism for chronic kidney disease, including diabetic nephropathy. Several mechanistic pathways have been linked to the development of intrarenal hypoxia and diabetic nephropathy including increased angiotensin II signaling, oxidative stress and hyperglycemia *per se*. Furthermore, pathological endothelin signaling has recently emerged as a possible contributing factor for chronic kidney disease and diabetic nephropathy. The overall aims of this thesis were therefore to determine the temporal relationship between development of intrarenal hypoxia and kidney disease as well as elucidate the potential link between endothelin signaling, intrarenal hypoxia and kidney disease in experimental insulinopenic diabetes.

It is well established that different mouse strains have different susceptibility for kidney and cardiovascular disease. The first step was therefore to compare four commonly used mouse strains with regards to development of kidney disease after onset of insulinopenic diabetes. From the results of this study, we concluded that the NMRI mouse strain has a disease progression closest to the human disease and this strain was chosen in the subsequent studies in mice.

The next step was to adapt and optimize a suitable method for repetitive measurements of intrarenal oxygen tension during the course of disease development. Electron paramagnetic resonance (EPR) oximetry had previously been used in tumor biology and was now adapted and optimized for measurements of kidney oxygenation in our diabetic mouse model. EPR oximetry in normoglycemic control mice recorded cortical oxygen tension values similar to previous reports using invasive techniques. Surprisingly, intrarenal hypoxia developed already within the first 72h after induction of hyperglycemia and persisted throughout the two-week study period. Importantly, this was well before albuminuria developed.

The final part of this thesis was to investigate the role of endothelin signaling for the intrarenal hypoxia in a diabetic rat model. Endothelin-1 signals via two distinctly different receptor-mediated pathways. In normal physiology, endothelin-1 binding to endothelin receptor type A (ETA) induces vasoconstriction, which can be blocked by the specific ETA antagonist BQ123, whereas endothelin-1 binding to endothelin receptor type B (ETB) induces nitric oxide-dependent vasodilation. ETB receptors can be selectively activated by Sarafotoxin 6c. The results from blocking ETA and activating ETB receptors demonstrated that endothelin-1 signaling via ETA receptors contributes to intrarenal hypoxia in the rat diabetic kidney, and that ETB stimulation significantly reduces the diabetes-induced intrarenal hypoxia. The beneficial effects on kidney oxygen availability in diabetes by ETA blockade or ETB stimulation were mainly linked to hemodynamic improvements rather than direct effects on kidney oxygen consumption or oxidative stress status.

In conclusion, by applying EPR oximetry in a mouse model of insulinopenic diabetes mimicking the human disease, we demonstrated intrarenal hypoxia already within the first couple of days after the onset of hyperglycemia, which is well before detectable signs of kidney disease development. Furthermore, blockade of ETA or activation of ETB receptors significantly reduced intrarenal hypoxia in the diabetic kidney. These results demonstrate involvement of ETA receptor signaling in diabetes-induced intrarenal hypoxia and ETA blockade or ETB activation might provide new therapeutical targets to reduce kidney hypoxia and disease progression in diabetes.

List of papers

This thesis is based on the following studies, which are referred to in the text with the Roman numerals:

- I Differences in susceptibility to develop parameters of diabetic nephropathy in four mouse strains with type 1 diabetes**
S Franzén, M Friederich-Persson, A Fasching, M Nangaku, P Hansell and F Palm. *Am J Physiol Renal* 2015, 309:F227–234.
- II Repetitive measurements of intrarenal tissue oxygenation in vivo using L band Electron Paramagnetic Resonance**
S Franzén, L Pihl, N Khan, F Palm and H Gustafsson. *Adv Exp Med Biol* 2014, 812:135-141.
- III Pronounced kidney hypoxia precedes albuminuria in type-1 diabetic mice**
S Franzén, L Pihl, N Khan, H Gustafsson and F Palm. (*In press*).
- IV Endothelin receptor A inhibition improves intrarenal tissue oxygenation by increased oxygen delivery in type-1 diabetic rats**
S Franzén and F Palm. *Diabetologia* 2015, 58(10):2435-2442.
- V Intrarenal ETB stimulation improves hypoxia in diabetic nephropathy in type-1 diabetic rats**
S Franzén, A Fasching and F Palm. (*Submitted*).

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Abbreviations

ANOVA – analysis of variance
ATP – adenosine triphosphate
BOLD MRI – blood-oxygen level dependent magnetic resonance imaging
DNA – deoxyribonucleic acid
DO₂ – delivered oxygen
EPR – electron paramagnetic resonance
ET-1 – endothelin-1
ETA – endothelin receptor type A
ETB – endothelin receptor type B
FENa – fractional sodium excretion
FF – filtration fraction
FITC – fluorescein isothiocyanate
GFR – glomerular filtration rate
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF – hypoxia inducible factor
LiPc – lithium phthalocyanine
MAP – mean arterial pressure
mTAL – medullary thick ascending limb
NADPH – nicotinamide adenine dinucleotide phosphate
Na/K-ATPase – sodium potassium adenosine triphosphatase
NO – nitric oxide
PAS – periodic acid-Schiff
PBS – phosphate buffered saline
pO₂ – oxygen tension
QO₂ – oxygen consumption
RBF – renal blood flow
S6c – Sarafotoxin 6c
TBARS – thiobarbituric acid reactive substances
TGF – tubuloglomerular feedback
TNa – transported sodium
TNa/QO₂ – transported sodium per oxygen consumed
UNa – urinary sodium excretion

Populärvetenskaplig sammanfattning

Diabetes är en av västvärldens vanligaste och snabbast ökande folksjukdomar. Ungefär 30% av alla diabetespatienter drabbas förr eller senare av njurskador. Det är fortfarande inte känt hur dessa njurskador uppkommer, men njurens syrgashantering har fått mer och mer fokus och tycks spela en betydande roll i utvecklingen av de tidiga skadorna. Nyligen har också en hel del fokus riktats på inblandningen av det så kallade endotelinsystemet, men länken mellan förändrad syrgashantering i den diabetiska njuren och endotelinsystemet är i dagsläget inte klarlagt.

Målet med den här avhandlingen var att undersöka just hur syrgashanteringen i njuren är relaterad till utvecklingen av njurskador vid diabetes, samt att klargöra vilken roll endotelinsystemet har i detta.

Studie I

Fyra olika musstammar (C57Bl/6, NMRI, BALB/c, 129Sv) undersöktes för att jämföra deras benägenhet att utveckla njurskador vid diabetes och därmed identifiera den mest lämpliga musstammen för de fortsatta studierna. Diabetiska möss jämfördes mot friska kontroller. Njurfunktionen utvärderades genom att mäta filtrationshastighet, proteinutsöndring i urinen och oxidativ stress, vilket gjordes innan diabetes inducerades samt fem och tio veckor därefter. Efter tio veckor utvärderades även tecken på strukturella njurskador. Alla fyra stammar utvecklade liknande grad av diabetes men skiljde sig avsevärt i utvecklingen av njurskador. NMRI möss uppvisade tidiga funktionella skador medan BALB/c möss var den enda som visar tecken på strukturella skador efter tio veckor. Den vanligtvis mest använda musstammen för att studera njurfunktionsstörningar, C57Bl/6, framstod som mest motståndskraftig mot njurskador och därmed minst lämplig för våra efterföljande studier.

Studie II

Metodiken för att mäta syrgastrick med elektron paramagnetisk resonans (EPR)-oximetri utvärderades och optimerades för att i senare studier möjliggöra kontinuerliga mätningar under en längre studieperiod. Under sövning placerades prober i både höger och vänster njure i friska NMRI möss, den stam som visat sig mest benägen att utveckla tidig funktionell skada i studie I. Från och med dag 9 efter implantation mättes syrgastrick med EPR-oximetri var fjärde dag upp till dag 25, följt av en sista mätning dag 45. Mätningar gjordes även efter manipulation av syrgashalten i den luft som mössen andades för att verifiera att metoden var pålitlig. Slutsatsen från dessa mätningar var att EPR-oximetri kan detektera fysiologiskt relevanta skillnader i njurens lokala syrgashalt över tid och att denna teknik kom att användas i studie III.

Studie III

Det är väl känt att diabetiska njurar arbetar under syrgasbrist, men när syrebristen uppstår är inte fastställt. NMRI-möss gjordes diabetiska och jämfördes mot friska kontroller. Syrgasmätningar med EPR-oximetri genomfördes innan diabetes startade

samt vid upprepade tillfällen under en två-veckors period därefter. I slutet av försöksperioden undersöktes även njurfunktion genom att mäta njurens filtrationshastighet och albuminläckage. Redan efter tre dagar visade de diabetiska mössen tydlig syrgasbrist i njurarna, vilken kvarstod under hela studieperioden. Anmärkningsvärt var att dessa möss inte uppvisade några begynnande tecken på njurskada i slutet av studien, vilket leder till slutsatsen att syrgasbrist i den diabetiska njuren uppkommer innan tecken på njurskada uppkommer. Således finns förutsättningar för att syrgasbrist skulle kunna vara mekanismen för att njurskador uppkommer vid diabetes.

Studie IV och V

Endotelin-1 binder två olika receptorer, ETA och ETB, vilka ger motsatta fysiologiska effekter. ETA har visats påskynda utvecklingen av njurskador vid diabetes men även vid andra sjukdomstillstånd, medan ETB har rapporterats vara skyddande. För att undersöka rollen av endotelinsystemet specifikt i njuren blockerades ETA (studie IV) eller stimulerades ETB (studie V) i råttor med diabetes som sedan jämfördes mot friska kontroller. Efter två veckor med diabetes mättes njurfunktion och syrgashantering i njurarna i sövda djur före och efter blockad av respektive receptor. Blockad av ETA eller stimulering av ETB resulterade båda i ökade syrgasnivåer i den diabetiska njuren. Dessa effekter visade sig bero på att både ETA blockad och ETB stimulering ökar blodflödet till njuren.

Sammanfattningsvis visar resultaten från de studier som ingår i denna avhandling att syrgasbrist uppträder före även de tidigaste tecknen på njurskada vid diabetes och därmed finns förutsättningar för att syrgasbrist faktiskt kan vara orsaken till njurskador vid diabetes. Signalering via endotelinsystemet kan i hög utsträckning påverka syrgastillgången i den diabetiska njuren, främst genom att reglera blodflödet. Dessa resultat visar att monitorering av njurens syrgastillgång skulle kunna ge tidig indikation på att skador är på väg att utvecklas samt att behandlingsstrategier för att manipulera endotelinsignalering skulle kunna skydda mot njurskador vid diabetes genom att förbättra syrgastillgången.

Introduction

The Kidney

The human kidney is composed of more or less 1,000,000 functional units also referred to as nephrons. Each nephron consists of a tubular system with the primary function to sustain body homeostasis by regulating blood pressure, electrolyte concentration, acid-base balance and waste product excretion. The kidneys receive approximately 25% of cardiac output, resulting in high renal blood flow (RBF). The cortical area of the kidney is the superficial part with high blood supply and oxygen availability. The inner kidney medulla on the other hand, is reached by only 10% of the total RBF and thus has considerably lower oxygen availability.

Initially, the blood is filtrated in the glomeruli, which is a capillary net. The glomerular filtration rate (GFR) is regulated by several forces depending on hydrostatic and oncotic pressures in the capillaries as well as the downstream tubule. The high RBF provides the basis for a high GFR of about 125 ml/min/1.73m². This equals a production of 180 L of primary urine every day. Further downstream, the proximal tubule is the segment of the nephron with the main reabsorption of sodium and volume (2/3 of all filtered load). All filtered glucose is normally also reabsorbed in the proximal tubule. The proximal tubular cells have a high density of mitochondria due to the high energy demand for active electrolyte transport. Concomitantly, proximal tubular cells account for a large portion of the kidney oxygen consumption (QO₂). The proximal tubules are aligned in the cortical area of the kidney. Following the proximal tubules, the loop of Henle descends into the less oxygenized kidney medulla. The loop of Henle can be divided into three parts, namely the medullary descending limb, the medullary ascending thin limb and the medullary thick ascending limb (mTAL). The function of the latter is to reabsorb electrolytes and create the hyperosmotic medullary interstitium used to create concentrated urine if needed. The distal tubule, mainly located in the kidney cortex, is involved in fine tuning electrolyte balance, and hence arterial blood pressure, as well as acid-base balance. Finally, the distal tubule is connected to the collecting ducts descending into the renal medulla, which are under hormonal control to regulate urine volume and electrolyte excretion.

From the primary urine volume of about 180 L per day that is produced by the filtrating glomerular capillary, the final urine volume of 1-2 L is achieved by reabsorption of electrolytes and water as well as by active secretion of waste products along the nephron. Going from these 180 L of primary urine to the final volume of 1-2 L, about 1.5 kg of sodium chloride is actively transported across the tubular cells along the nephron resulting in an impressive demand for energy. The kidney accounts for approximately 10% of total body QO₂ at rest, of which 80% is due to active transport of electrolytes along the nephron [1]. The renin angiotensin aldosterone system is a major hormonal system regulating electrolyte transport in most nephron segments. However, endothelin (ET)-1 has also been shown to significantly regulate active electrolyte transport in the kidney.

At least two distinctly different mechanisms maintain a constant GFR, namely the myogenic response inherited in most resistance vessels and the tubuloglomerular feedback (TGF). Macula densa cells located in the juxtaglomerular apparatus sense the sodium chloride load in the passing filtrate of the distal nephron and regulates afferent arteriolar tonus. The resulting effect is a close match between GFR and tubular transport capacity resulting in a constant GFR. Importantly, the TGF signal also influences renin release from the granular cells of the distal afferent arteriole, and thus, TGF further influence on arterial blood pressure.

Oxygen Metabolism

Even though the kidney receives a high blood supply a proportionally low portion of what is delivered is being consumed. Oxygen tension (pO_2) in the renal artery is approximately 90-100 mmHg and surprisingly high also in the renal vein (approximately 80 mmHg), indicating that the kidney receives a substantial amount of oxygen without consuming it. This has been suggested to be the result of an intrarenal shunting mechanism. The shunting of oxygen is proposed to be the effect of close alignment of arterial and venous vessels within the kidney, resulting in an arterial-to-venous diffusion of oxygen [2-4]. This shunting mechanism may also contribute to the heterogeneous distribution of oxygen within the kidney, with a highly oxygenated kidney cortex (50-55 mmHg) and a poorly oxygenized inner medulla (15-20 mmHg). Interestingly, RBF is not under metabolic control like most other tissues and the oxygen availability is therefore heavily influenced by QO_2 in that particular region of the kidney (Fig. 1).

Figure 1. Summary of factors influencing kidney oxygen availability.

Oxidative Stress

Production of reactive oxygen species (ROS) occurs naturally via several cellular pathways. The most common ROS is the superoxide radical. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the electron transport chain are major

sources of superoxide radicals. Both produce superoxide radicals during pathological conditions such as diabetes [5-7]. Oxidative stress is a state where high levels of ROS cause cellular damage to DNA, lipids and proteins and several reports have indeed indicated that antioxidant treatments improve organ function [8-10]. However, ROS are necessary for normal cell function, and have been reported to protect cell function during some circumstances [11, 12].

Electron Paramagnetic Resonance

Electron paramagnetic resonance (EPR) is a method used to detect unpaired electrons both *in vivo* and *in vitro*. EPR uses a magnetic field that excites the unpaired electron to a higher energy level. When the electron returns to its original state, the magnetic movement from the unpaired electron is detected. The sensitivity of the method depends on the radiofrequency, which possesses a problem when measuring large samples, e.g. a whole animal *in vivo*, since the tissue penetration depth decreases with increasing frequencies. Movements and background noise further complicate *in vivo* detection. However, the charcoal-like crystallite lithium phthalocyanine (LiPc), with a peak-to-peak signal line-width that is proportional to the surrounding oxygen concentration, has an impressive signal-to-noise ratio allowing for detection of regional tissue oxygen availability *in vivo* [13, 14]. This technique is frequently and successfully being used in tumor biology, and there are some prior reports also using this technique for detection of kidney oxygenation [15, 16].

Diabetes

Diabetes is one of the world's most common diseases. According to the International Diabetes Federation, 1 in 11 people have diabetes (415 million cases), and by 2040, the prevalence is predicted to have increased to 1 in 10 (642 million cases) [17]. Furthermore, about 5 million deaths worldwide are caused by diabetes every year. In Sweden, the prevalence for diabetes is progressing rapidly. According to the Swedish Diabetes Foundation, almost 50,000 patients suffer from type 1 and whereas 450,000 suffers from type 2 diabetes in Sweden today [18].

Type 1 diabetes, also known as juvenile diabetes or insulinopenic diabetes, is a chronic autoimmune disease in which the pancreatic β -cells are destroyed and therefore cannot produce sufficient amounts of insulin. The result is a defective blood glucose regulation. Type 1 diabetes can be treated, not cured, with insulin and it is imperative to keep blood glucose levels under control. Type 2 diabetes, also known as non-insulin-dependent diabetes or adult-onset diabetes, is characterized by insulin resistance. This type of diabetes is often related to obesity and may sometimes be overcome by improved diet and exercise. The insulin resistance is the result of high insulin stimuli, such as glucose, demanding a high workload for the β -cells to produce considerable amounts of insulin. Gradually, more and more insulin will be required to elicit the same blood glucose regulation and eventually, the β -cells will not be able to meet the insulin demand needed to control the blood glucose concentration. The chronically elevated demand for insulin by β -cells may eventually result in β -cell death and develop into a more diabetes type 1-like condition. Diabetes type 1 and 2 are both

severe diseases associated with numerous secondary complications, including neuropathy, retinopathy, vasculopathy and nephropathy. The risk for secondary complications and premature death is negatively correlated to the degree of blood glucose control.

The Diabetic Kidney

Diabetic nephropathy develops in approximately 30% of diabetes patients [19, 20] and is the single most common cause of end stage renal disease [21, 22]. Furthermore, most patients develop kidney damage within the first 20 years after diagnosis [19]. Initially, GFR increases and albuminuria is less than 30 mg/day. As diabetic nephropathy progresses, GFR starts to decline (<90 ml/min/ 1.73m^2) and microalbuminuria develops (30-300 mg/day). As the disease progresses alterations such as glomerular mesangial matrix expansion, thickening of glomerular basement membranes, podocyte loss and hypertrophy, inflammation and infiltration of immune cells, glomerulosclerosis and tubulointerstitial fibrosis are common findings and microalbuminuria progresses to macroalbuminuria (>300 mg/day) and GFR is further reduced (end stage renal disease defined as <15 ml/min/ 1.73m^2) [23-30]. Even though there is vast knowledge of how diabetic nephropathy presents in humans, more information is needed to completely elucidate the mechanistic pathways of diabetic nephropathy. NO production, hypoxia, oxidative stress, inflammatory pathways and mitochondria uncoupling are some of the mechanisms that have recently received increasing attention since all of these seem to play important roles in the initial onset and further development of diabetic nephropathy.

Oxygen Metabolism in Diabetes

Since the kidneys have a high oxygen demand combined with regions with very low tissue pO_2 , they are highly sensitive to changes in oxygen availability. In diabetes, the high glucose load results in increased sodium and glucose co-transport in the proximal tubule that concomitantly increases workload and kidney QO_2 resulting in intrarenal hypoxia [31]. Furthermore, the increased mitochondria leak respiration in diabetic kidneys also contributes to increase total QO_2 [32, 33]. Normally, the acute and most efficient way to compensate for hypoxia would be to increase blood flow. However, the RBF is not under metabolic control since it is regulated to maintain a high and steady GFR.

The cellular defense against chronic hypoxia is hypoxia inducible factor (HIF), which regulates more than 200 hundred genes involved in regulating the cellular metabolism, angiogenesis and oxidative stress defense. The HIF α -subunit is degraded by prolyl hydroxylases in a process dependent on oxygen. In the absence of oxygen, i.e. during hypoxia, the HIF α -subunit accumulates and dimerizes with the β -subunit and binds to hypoxia-responsive elements in the DNA which results in increased gene expression of genes related to regulation of metabolism and oxygen availability [34, 35]. Although the diabetic kidney develops chronic hypoxia, HIF activation does not seem to occur. The reason for this seemingly contradiction is presently not clear, but

it has been shown that pharmacological HIF activation prevents kidney disease in a rat model of insulinopenic diabetes [36].

Animal Models of Diabetic Nephropathy

The most commonly used animal models of diabetic nephropathy are chemically induced insulinopenic diabetes in rats and mice. A single bolus dose injection of a β -cell toxic compound, usually streptozotocin or alloxan, results in a rapid destruction of the β -cells and hyperglycemia usually develops with 24-48h. These animals often present with blood glucose levels >20 mmol/L, but without developing ketoacidosis. A potential disadvantage using these rodent models of diabetic nephropathy is the discrepancy in disease development with the human disease and that different strains have different susceptibility to kidney disease. The alternative to chemically-induced diabetes in rodents are the transgenic models in which either insulin production or insulin sensitivity have been altered due to naturally occurring DNA mutations. These animals are considerably more expensive and the onset of hyperglycemia can occur over an interval of several weeks to months. Some more frequently used transgenic mouse models for insulinopenic diabetes include the OVE26 mouse [37-39] and the Insulin² Akita mouse [40-42]. The former mouse model has been reported to develop most of the symptoms associated with the disease progression in humans, whereas the latter model has some significant differences compared to the human disease. New transgenic animal models are constantly under development with the ultimate aim to provide further mechanistic insights in to the progression of diabetic nephropathy.

The Endothelin System

Endothelin-1 (ET-1) is an endogenously produced 21 amino acids peptide with potent vasoactive properties. ET-1 is produced in many different cells in the body, including tubular cells. The main production of ET-1 in the tubule occurs in the collecting duct, followed by smaller production in the mTAL, proximal tubule and the glomeruli. It is still not completely known how the remaining parts of the loop of Henle and the distal tubule contribute to ET-1 production. ET-1 is proposed to work mainly in a paracrine or autocrine fashion on the G-protein coupled receptors endothelin receptor type A (ETA) and endothelin receptor type B (ETB). These receptors are expressed in almost every cell in the body. The main and more obvious actions of ETA and ETB in the vasculature system are to regulate vascular tone and thus arterial blood pressure. This regulation is maintained during normal physiology by an equal distribution of ETA and ETB throughout the vasculature. ETA is located on the vascular smooth muscle cells inducing constriction by increasing intracellular calcium, whereas ETB are mainly expressed on endothelial cells in the vasculature inducing NO-dependent vasodilation. However, ETB receptors are also expressed in vascular smooth muscle cells and here they induce vasoconstriction in a similar way as ETA [43, 44]. Interestingly, the ETB pathway is regarded a mechanism to clear ET-1 from the circulation, since the binding of ET-1 to ETB is irreversible and result in ET-1 being destined for lysosome degradation after internalization [45]. However, ETA can

maintain the signaling activity for several hours after internalization, allowing a more potent vascular response for ETA compared to that of ETB signaling.

In the kidney, there is a high concentration of both ETA and ETB receptors along the entire nephron although ETB have been reported to have higher expression [46]. ETA and ETB receptors expressed in tubular cells contribute to the regulation of electrolyte and water homeostasis, which have profound implications for regulation of arterial blood pressure. There is conflicting data regarding the presence and function of ETA and ETB in specific nephron segments, but the main conclusion is that ETA serves as vasoconstrictor in the vascular beds effecting RBF, GFR and mainly cortical RBF whereas ETB are more involved in the regulation of water and sodium homeostasis by regulating medullary RBF and directly inhibiting tubular transport [47, 48]. ETB-mediated natriuresis and diuresis are reported to involve several different cellular signaling pathways, including protein kinase C, mitogen activated protein kinase and cyclooxygenase. However ETB-mediated NO release seems to be most important. Taken together, all of these ETB signaling pathways ultimately result in the inhibition of tubular sodium transport [49]. In diabetes, ET-1 levels are increased indicating a potential involvement for the alterations in tubular function commonly occurring in the diabetic kidney [50].

Aims

Intrarenal hypoxia has been proposed as a unifying mechanism for the progression of chronic kidney damage, including diabetic nephropathy, and the focus of this thesis was to further elucidate its role and potential mechanism causing the hypoxia in the diabetic kidney. Using rodent models of insulinopenic diabetes, the temporal relationship between the onset of intrarenal hypoxia and development of diabetic nephropathy was investigated and the specific involvements of ETA and ETB receptors for the diabetes-induced intrarenal hypoxia were studied. This thesis includes the following studies with the specific aims:

- Study I:** Investigate the susceptibility to kidney disease in four different commercially available mouse strains after chemical induction of insulinopenic diabetes.
- Study II:** Develop, optimize and evaluate a method for repetitive measurements of regional renal tissue pO_2 in mice.
- Study III:** Investigate the temporal relationship between onset of intrarenal hypoxia and development of kidney disease in a mouse model of insulinopenic diabetes.
- Study IV:** Investigate the effect of acute *in vivo* intrarenal blockade of ETA receptor in a rat model of insulinopenic diabetes.
- Study V:** Investigate the effect of acute *in vivo* intrarenal activation of ETB receptor in a rat model of insulinopenic diabetes.

Material & Methods

All experimental protocols were approved by the local ethical committee and performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985). All animal experiments were conducted either at the Department of Medical Cell Biology, Division of Integrative Physiology, Uppsala University, or at the Department of Medical and Health Sciences, Divisions of Radiological Sciences or Drug Research, Linköping University.

Chemicals and animals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Male 8 weeks old mice (C57Bl/6Tac, BomTac:NMRI, BALB/cAnNTac, 129S6/SvEv) were purchased from Taconic (Ry, Denmark) and housed separately or in pairs with standard mouse chow and water *ad libitum*. Male 8 weeks old rats (Sprague Dawley) were purchased from Charles River (Sulzfeld, Germany) and were housed in pairs with standard rat chow and water *ad libitum*.

Induction of diabetes (Study I and III-V)

Diabetes was induced in mice by an intravenous single bolus injection (200 µl) of alloxan monohydrate dissolved in sterile saline (in mg/kg: C57Bl/6 75, NMRI 75, BALB/c 67, 129Sv 75) in the tail vein. In rats diabetes was induced by an intravenous single bolus injection (200 µl) of streptozotocin dissolved in sterile saline (55 mg/kg) in the tail vein. Blood glucose levels ≥ 15 mmol/L were considered diabetic. Blood glucose levels were monitored with a Free Style Lite kit (Abbott Laboratories, Abbott Park, IL, USA) by a blood sample from a minimal cut at the tip of the tail or a small needle stick on the proximal tail vein.

Measurements of glomerular filtration rate (Study I and III-V)

Conscious GFR was measured in mice (Study I and III) by injecting fluorescein isothiocyanate (FITC)-inulin dissolved in phosphate buffered saline (PBS) (2 % weight/volume). After being dialyzed overnight (Spectra/Por® 6 Membrane 1:1000 Da, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) in PBS protected from light under constant stirring at 4°C FITC-inulin was filtered (Acrodisc® 0.45 µm 25 mm, SA) and a single bolus (200 µl) was injected intravenously in the tail vein. Blood samples were collected from the tip of the tail in heparinized capillary tubes (Drummond Microcaps, SA) at 1, 3, 5, 9, 14, 35, 55 and 75 min after the injection. Syringe pre- to post-weight was used to calculate the exact dose administered. FITC fluorescence in plasma was analyzed in a black 384-well plate (Greiner Bio-One GmbH, Kremsmuenster, Austria) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (500 mmol/L, pH 7.4) using a Tecan Safire II (Greiner Bio-One GmbH, Kremsmuenster, Austria) at 496/520 nm excitation/emission. GFR was calculated from the area under the curve using a non-compartmental pharmacokinetic data analysis [51-53]. In Study VI and V,

radioisotopes were infused during the acute procedures and blood samples from the blood gas analysis was recollected, centrifuged and transferred to Eppendorf tubes® (3010X). Standard liquid scintillation technique was then used to analyze plasma and urine concentrations of the radioisotopes for estimations of the GFR [54].

Urinary excretion of protein, albumin, electrolytes and creatinine (Study I and III-V)

Metabolic cages were used for 24-hour urine collection. Urinary protein concentrations were measured using the colorimetric DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. Urinary sodium and potassium concentrations were measured by flame photometry (IL 943, Block Scientific Inc., Bohemia, NY, USA). Urinary creatinine concentrations were analyzed by an enzymatic kit (Abbott Laboratories, Abbott Park, IL, USA,) and urinary excretion of albumin was analyzed with a mouse ELISA kit (Bethyl Laboratories Inc., Montgomery, TX, USA) according to manufacturer's instructions. Excretion rates were calculated by multiplying urinary concentrations by urine flow. In Study I and III, urinary excretion parameters were corrected for urinary creatinine concentrations in order to allow for relevant comparisons between the groups.

Oxidative stress (Study I, IV and V)

Urinary excretion of thiobarbituric acid reactive substances (TBARS) and protein carbonyls in the kidney cortex were used as markers for oxidative stress levels. For TBARS (Study I and IV-V), urine samples were mixed with 50 mmol/L HCl and thiobarbituric acid (0.67%). Samples were incubated for 30 min at 95°C and then cooled in water to room temperature. Thereafter, methanol:butanol (3:17) was added and the samples were centrifuged at 2500 rpm for 20 min. The supernatant was transferred to a transparent 384-well plate and the absorbance at 535 nm measured using the Tecan Safire II. Protein carbonyls in kidney cortex (Study I) was analyzed using a commercial kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instruction. Urinary excretions of TBARS and tissue protein carbonyls were normalized to creatinine and protein concentrations, respectively (Study I).

Histology (Study I)

Kidney cortex, medulla and whole transverse slices were fixed in methyl Carnoy's solution. Sections (3 µm) were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin. Injury was scored in a blinded fashion according to a scale from 0 to 4 as described previously [55].

Lithium phthalocyanine (Study II and III)

LiPc (Clin-EPR, NH, USA) oximetry probes were loaded into 23 G needles as previously described [56]. Anaesthetized NMRI mice (1.5-2% Isoflurane, Florene, Apoteket AB, Sweden) were placed with a 10-15 mm vertical incision below the diaphragm. A 4 mm superficial vertical injection was done in the left kidney for placement of the cortical probe (Study II and III) and 4 mm deep transversal injection in the right kidney for placement of the medullary probe (Study II). The peritoneum and skin was closed separately (6.0 Vicryl, AgnTho's AB, Lidingö, Sweden) and 5

mg/kg/24 h of Carprofen (Rimadyl Bovin, Apoteket, Sweden) was administered subcutaneously in order to provide pain relief.

***In vivo* measurements using Electron Paramagnetic Resonance (Study II and III)**

All measurements were performed using an L band Elexsys II E540 (Bruker Bio-Spin GmbH, Rheinstetten, Germany). Anaesthetized mice (1.5-2% Isoflurane) were placed on the back (Study II) or on the right side (Study III) inside the EPR resonator. Measurements were performed using a L band EPR spectrometer equipped with an E540 GCL Triple axis coil set (gradient field strength up to 40 G/cm) and an E540 R36 L band Resonator (36 mm sample access) connected to an EPR 066L-AMC L band Microwave Bridge with settings accordingly; 36 mW applied microwave power, 0.2 G modulation amplitude, 20 ms time constant, 5 s sweep time, 256 measurement points, 3 G sweep width and 40 sweeps added together for each measurement. The EPR signals from the two probes in each mouse were separated with 1 G/cm, gradient angle: $\Phi=0$, $\theta=0$ along B_0 (Study II). No EPR signal could be detected for the empty resonator. The recorded EPR spectra were imported into MATLAB and peak-to-peak linewidth was analyzed using an in-house developed MATLAB script. Kidney pO_2 was calculated by comparison of the EPR linewidth of the LiPc probes with spectra obtained from a calibration probe made from the same batch and measured at different pO_2 . For Study II, mice were allowed to breathe 21% oxygen (room air) for normal physiology and 10% air (room air nitrogen mixture 1:1) for acute hypoxia during 5 min. Measurements were performed at day 9, 13, 17, 21 and 45 post LiPc implantation. For Study III, mice were breathing 21% oxygen during EPR measurements and measurements were conducted before and 3, 11 and 15 days after the diabetes induction.

Surgical preparation (Study IV and V)

Rats were anaesthetized with an intraperitoneal injection of the thiobutabarbital Inactin (120 mg/kg for controls and 80 mg/kg for diabetics) and body temperature was maintained at 37°C using a servo-controlled heating pad. By a vertical incision, a breathing tube was placed in the trachea for spontaneous ventilation. Catheters were inserted into the carotid artery and the jugular vein in order to monitor mean arterial pressure (MAP) and for continuous infusion of 3H -inulin (185 kBq/h/kg; controls 5 ml/h/kg and diabetics 10 ml/h/kg; American Radiolabeled Chemicals, St. Louis, MO, USA), respectively. A midline incision was then used to catheterize the bladder to allow drainage. By a vertical left sided incision, the left kidney was exposed and immobilized in a plastic cup followed by catheterization of the left ureter for collection of urine. A catheter was thereafter placed into the lumbar artery and advanced into the left renal artery in order to allow for continuous intrarenal drug delivery. A flow probe (Transonic Systems, Ithaca, NY) was placed on the left renal artery and measurements started after a 30-40 min recovery period.

Dose-response (Study IV and V)

For dose-response relationships a separate series of control rats were surgically prepared, with the exceptions of no ureter catheter and the jugular infusion was

standard saline, in order to accurately determine the optimal dose for the subsequent experiments. After the surgical preparation and recovery period, the intrarenal infusion rates were stepwise increased from 0-64 $\mu\text{l}/\text{min}$ with 5 min measurements of MAP and RBF for each increase. For Study IV, the selective ETA antagonist BQ123 was infused at rates to provide blood concentrations of 0, 0.25, 0.5, 1, 2, 4, 8, and 16 nmol/L. For Study V, the selective ETB agonist Sarafotoxin 6c (S6c) was administered at concentrations of 0, 1.5625, 3.125, 6.25 and 12.5 pmol/h. The doses selected for the subsequent full studies were selected based on maximal effect on RBF without systemic effects on arterial blood pressure.

Acute *in vivo* procedures (Study IV and V)

After surgical preparation and the recovery period a baseline period (intrarenal saline infusion) was started with urine collection and recording of RBF and MAP. Halfway through the period, pO_2 measurements were performed (Clark type oxygen microelectrode, $\sim 10 \mu\text{m}$ OD, Unisense A/S, Aarhus, Denmark) at three different sites in the kidney to determine both cortical (1 mm) and medullary (3-4 mm) pO_2 . At the end of that period, an arterial blood sample from the carotid catheter and a renal vein sample collected in a heparinized syringe were taken for analysis of blood gases and electrolytes status (iSTAT® System, Abbott Laboratories, Abbott Park, IL, USA) in order to calculate kidney QO_2 and electrolyte transport efficiency.

After the experimental period, rats were euthanized with an intravenous injection of saturated KCl and kidneys were weighed and examined for any changes in gross morphology.

RBF, MAP, pO_2 , urine output, arterial and venous pH, pO_2 , O_2 saturation (SO_2), sodium, hematocrit and hemoglobin were determined from direct measurements during the experiments, whereas renal vascular resistance (RVR), urine flow, GFR, filtration fraction (FF), O_2 content, QO_2 , delivered oxygen (DO_2) and urinary sodium excretion (UNa), fractional sodium excretion (FENa), transported sodium (TNa) and TNa/QO_2 were calculated after the experiments.

Calculations:

$$\text{RVR} = \text{MAP} / \text{RBF}$$

$$\text{Urine flow} = \text{urine volume} / \text{time}$$

$$\text{GFR} = (\text{urine flow} * \text{urinary } ^3\text{H content}) / \text{arterial } ^3\text{H content}$$

$$\text{FF} = \text{GFR} / (\text{RBF} * (1 - \text{hematocrit}))$$

$$\text{Arterial/venous } \text{O}_2 \text{ content} = (\text{hemoglobin} * \text{SO}_2 * 1.34) + (\text{pO}_2 * 0.003)$$

$$\text{QO}_2 = \text{arterial } \text{O}_2 \text{ content} - \text{venous } \text{O}_2 \text{ content}) * \text{RBF}$$

$$\text{DO}_2 = \text{arterial } \text{O}_2 \text{ content} * \text{RBF}$$

$$\text{UNa} = \text{urinary sodium content} * \text{urine flow}$$

$$\text{FENa} = \text{UNa} / (\text{GFR} * \text{arterial sodium concentration})$$

$$\text{TNa} = (\text{arterial sodium concentration} * \text{GFR}) - (\text{UNa})$$

$$\text{TNa}/\text{QO}_2 = \text{transported sodium} / \text{QO}_2$$

Statistical analysis

All statistical analyzes were performed using GraphPad Prism 6.1 (GraphPad Software, La Jolla, CA, USA). All data are presented as mean $\pm$ SEM and $p < 0.05$ was considered significant.

Study I: Repeated measurements one-way ANOVA followed by Tukey's post hoc test was used to analyze the effect over time in each group. Unpaired Student's t-tests were used for comparison between control and diabetic groups within strains at a defined time point. Correlation analysis was made using a least-squares linear regression.

Study II: Student's t-tests were used to compare cortical versus medullary pO_2 and 21% oxygen vs. 10% oxygen. Repeated measurements one-way ANOVA was used to analyze cortex and medulla separately as a function of time.

Study III: Repeated measurements 2-by-2 ANOVA followed by Bonferroni's post hoc test was used to analyze pO_2 in controls versus diabetics as a function of time and unpaired Student's t-test was used to compare kidney parameters in controls versus diabetics.

Study IV and V: For comparison between controls and diabetics during baseline and experimental periods the 2-by-2 repeated measurements ANOVA followed by Bonferroni's post hoc test was used. Repeated measures one-way ANOVA followed by Fisher's LSD test was used to analyze the dose-response relationship to S6c. Unpaired Student's t-tests were used for comparison animal characteristics between controls and diabetics.

Results

Susceptibility to develop kidney damage in mice with alloxan-induced insulinopenic diabetes (Study I)

All mice developed severe hyperglycemia, *i.e.* blood glucose levels >20 mmol/L. Glomerular hyperfiltration was demonstrated in C57Bl/6 and NMRI (Fig. 2), whereas proteinuria and oxidative stress levels were increased in all strains except C57Bl/6 (Figs. 3-5). Interestingly, the only strain developing structural alterations at the end of the 10-week study period was BALB/c (Fig. 6).

Figure 2. Glomerular filtration rates in C57Bl/6 (a), NMRI (b), BALB/c (c) and 129Sv (d) at baseline and at five and ten weeks after the induction of diabetes. * $P < 0.05$ versus baseline within the group. † $P < 0.05$ versus corresponding time in the control group.

Figures 3 & 4. Proteinuria (upper) and oxidative stress levels by urinary TBARS (lower) in C57Bl/6 (a), NMRI (b), BALB/c (c) and 129Sv (d) at baseline and at five and ten weeks after the induction of diabetes. * $P < 0.05$ versus baseline within the group. † $P < 0.05$ versus corresponding time in the control group.

Figure 5. Oxidative stress levels by protein carbonyls in C57Bl/6, NMRI, BALB/c and 129Sv ten weeks after the induction of diabetes.

Figure 6. Histology in C57Bl/6, NMRI, BALB/c and 129Sv ten weeks after the induction of diabetes.

Repeatability and sensitivity of EPR oximetry with LiPc probes to monitor kidney oxygenation (Study II)

EPR oximetry allowed for simultaneous detection of pO_2 in both kidney cortex and medulla (Fig. 7), and LiPc probes placed in both cortex and medulla could be used to continuously and repetitively record kidney pO_2 over the entire 45-day study period (Fig. 8). Acute reduction of the oxygen content in the inspired air (from 21% to 10%) rapidly altered the kidney tissue pO_2 in both cortex and medulla (Fig. 9).

Figure 7. Original tracing of the linewidth of the EPR spectra of implanted LiPc probes in kidney cortex and medulla during normal conditions (21% O_2) in the same mouse.

Figure 8. Kidney oxygen tension in cortex and medulla over a 45-day study period.

Figure 9. Kidney oxygen tension in cortex (left) and medulla (right) during normal conditions (21% O₂) and acute reduction of the oxygen content in the inspired air (10% O₂).

Hypoxia precedes albuminuria in mice with insulinopenic diabetes (Study III)

Mice developed sustained hyperglycemia (>15 mmol/L) within the first 48 h which persisted during the entire study period. Already three days after diabetes induction, kidney cortex displayed significant hypoxia which was sustained throughout the study (Fig. 10). Fifteen days after the induction of diabetes, these mice had developed glomerular hyperfiltration without any indication of increased urinary albumin leakage (Fig. 11).

Figure 10. Kidney oxygen tension in healthy control and diabetic mice at baseline and early on after the onset of diabetes.

Figure 11. Glomerular filtration rates (left) and urinary albumin excretions (right) in control and diabetic mice at baseline and early on after the induction of diabetes.

ETA inhibition improves intrarenal oxygenation by improving oxygen delivery in rats with insulinopenic diabetes (Study IV)

Two weeks of diabetes resulted in significant intrarenal tissue hypoxia in both cortex and medulla (Fig. 12). Intrarenal oxygen availability was improved by ETA blockade using BQ123. Kidney QO_2 was unaffected by ETA blockade, whereas DO_2 was improved (Fig. 13). GFR and FF were decreased and RBF was increased (Fig. 14). Proteinuria and oxidative stress status were unaffected by ETA blockade (Fig. 15), whereas increased urinary sodium excretion increased (Fig. 16).

Figure 12. Kidney oxygen tension in cortex (upper) and medulla (lower) in control and diabetic rats during baseline and after blockade of ETA receptors.

Figure 13. Kidney oxygen consumption (upper) and oxygen delivery (lower) in control and diabetic rats during baseline and after blockade of ETA receptors.

Figure 14. Renal blood flow (upper), glomerular filtration rate (middle) and filtration fraction (lower) in control and diabetic rats during baseline and after blockade of ETA receptors.

Figure 15. Proteinuria (upper) and oxidative stress status (lower) in control and diabetic rats during baseline and after blockade of ETA receptors.

Figure 16. Urinary sodium excretion (a), fractional sodium excretion (b), transported sodium (c) and transported sodium per consumed oxygen (d) in control and diabetic rats during baseline and after blockade of ETA receptors.

Activation of ETB receptors improves kidney oxygenation by improving oxygen delivery in rats with insulinopenic diabetes (Study V)

Acute intrarenal activation of ETB improved both kidney cortical and medullary pO_2 in diabetic rats (Fig. 17), although it did not affect total kidney QO_2 (Fig. 18). The improved pO_2 after ETB activation was the results of increased DO_2 secondary to increased RBF (Fig. 18). Urinary sodium excretion increased whereas all other parameters of kidney function remained unaffected by activation of ETB receptors (Fig.19).

Figure 17. Kidney oxygen tension in cortex (upper) and medulla (lower) in control and diabetic rats during baseline and after activation of ETB receptors.

Figure 18. Kidney oxygen consumption (upper), oxygen delivery (middle) and renal blood flow (lower) in control and diabetic rats during baseline and after activation of ETB receptors.

Figure 19. Urinary sodium excretion (a), fractional sodium excretion (b), transported sodium (c) and transported sodium per consumed oxygen (d) in control and diabetic rats during baseline and after activation of ETB receptors.

Discussion

Three main results are presented in this thesis: a strain-dependent susceptibility to develop diabetic nephropathy in mice; intrarenal hypoxia occurs before the earliest sign of kidney disease; manipulation of endothelin receptor signaling can improve intrarenal tissue pO_2 in diabetes.

In study I, the susceptibility to develop functional and histological alterations in response to insulinopenic diabetes was investigated in four different commercially available mouse strains; C57Bl/6, NMRI, BALB/c and 129Sv. In agreement with other studies, C57Bl/6 was found to be highly resistant to develop kidney disease [42, 57, 58]. BALB/c and 129Sv mice developed proteinuria and oxidative stress, but no glomerular filtration. On the contrary, it was found that NMRI mice developed both substantial proteinuria and functional alterations closely resembling the human disease. Thus, this strain was chosen for the subsequent studies to determine the temporal relationship between tissue hypoxia and kidney disease in diabetes.

The underlying mechanisms for glomerular hyperfiltration in diabetes are still under debate. Sällström and colleagues demonstrated that glomerular hyperfiltration in diabetic mice is independent of the TGF mechanism, since adenosine A1-receptor deficient mice lacking a functional TGF response still develop diabetes-induced glomerular hyperfiltration [59, 60]. It has therefore been proposed that glomerular hyperfiltration is caused by direct effects on filtration pressures caused by the increased reabsorption of electrolyte and volume in the proximal tubule [61]. Since tubular glucose load increases in diabetes as a result of the increased plasma levels of glucose, sodium transport via sodium-glucose linked transporters located to the proximal tubule will increase resulting in increased volume reabsorption and decreased tubular free flow pressure. This will result in increased net filtration pressure and increased GFR. Indeed, acute blockade of these receptors do normalize GFR in hyperfiltrating diabetic rats [31]. Nevertheless, glomerular hyperfiltration has been questioned to play a role for diabetic nephropathy [62, 63], and using this parameter as the sole criteria for diabetic nephropathy should probably be avoided. Interestingly, GFR and urinary protein/albumin leakage are the clinically used biomarkers for progression of kidney disease [64-69]. The urinary excretion of proteins is considered to predict the progression of kidney disease. As presented in study I, there appears to be a correlation between proteinuria and oxidative stress in diabetic mice. Oxidative stress may originate from several sources and has been shown to contribute to the increased kidney QO_2 in diabetes via inducing mitochondrial leak respiration [32, 33]. Increased mitochondria leak respiration concomitantly increases kidney QO_2 by decreasing the efficiency to produce ATP. The leak respiration possibly is a mechanism to control mitochondrial membrane potential, and thus also control mitochondrial ROS production [70-72]. Thus, increased leak respiration can be considered an antioxidant defense mechanism.

In study II, a novel technique was evaluated to monitor tissue pO_2 repetitively over time in the same subjects. As a result from study I, NMRI mice were used and monitored over a 45 day period using implantable LiPc probes and L band EPR oximetry. Over the entire study period, the EPR technique demonstrated high sensitivity to rapid changes in oxygen availability.

Today there are several techniques available for monitoring intrarenal tissue pO_2 . Of these, blood oxygen level-dependent (BOLD) magnetic resonance imaging is suggested as a minimally invasive imaging technique that already is used in the clinic [73]. Indeed, intrarenal hypoxia in diabetic rats has been reported using the BOLD technique [74]. However, since the BOLD technique has several inherent problems, including being highly influenced by changes in hematocrit and perfusion rates, the use of the BOLD technique for determining kidney oxygenation has been challenged [75]. Experimentally, there are several techniques available for measuring intrarenal tissue pO_2 . The most commonly used is probably Clark type electrodes. However, this technique is invasive and only allows for measurements during the acute settings. Recently, Koeners and colleagues developed a novel technique for continuous measurements of tissue pO_2 in conscious rats. By implanting a telemetry device in the rat abdomen and placing carbon paste electrodes into the kidney tissue continuous recordings of intrarenal pO_2 were obtained [76]. Although this technique could detect differences in response to different interventions, the absolute values do not agree with what has been obtained with the gold-standard technique. However, this method shows promising features but requires further development before reliable absolute values of intrarenal pO_2 can be reported.

EPR oximetry is on the developing path towards clinical implementation. There are several studies demonstrating the usefulness of EPR oximetry in tumor and liver biology [77-81]. As demonstrated in Study II, intrarenal tissue pO_2 can be monitored for a prolonged time and EPR oximetry clearly has the potential to be implemented into clinic practice to monitor e.g. graft function and wound healing.

In study III, the NMRI mouse strain was used to investigate the temporal relationship between intrarenal hypoxia and development of kidney disease in diabetic mice. For this purpose, EPR oximetry was applied and pronounced hypoxia was detected three days after the induction of diabetes and persisted through the 15-day study period. Interestingly, these diabetic mice did not develop increased urinary albumin leakage within the study period, which leads to the conclusion that intrarenal hypoxia occurs before kidney disease in this animal model of insulinopenic diabetes. This result is of great importance since this finding provides the basis for intrarenal hypoxia as a unifying mechanism for chronic kidney disease, including diabetic nephropathy.

As reported previously, hypoxia is present early on after the induction of diabetes [10, 74, 82-86]. However, it has been difficult to elucidate the sequence of events, due to the lack of suitable techniques for repetitive detection of absolute kidney tissue pO_2 . Hypoxia is recognized as a unifying mechanism for chronic kidney disease, including

diabetic nephropathy [87-91]. Recently, Friederich-Persson and colleagues demonstrated that hypoxia *per se*, independently of confounding factors such as hyperglycemia, hypertension or oxidative stress, results in the onset and progression of kidney disease [92].

Several interventions have been successful in improving tissue oxygenation and prevent disease progression. Apocynin to inhibit the NADPH oxidase or 4-hydroxy tempo to directly scavenge ROS will prevent both the diabetes-induced intrarenal hypoxia and functional alterations, indicating oxidative stress is a pivotal mechanism for the onset and progression of diabetic nephropathy [9, 85]. Superoxide radicals *per se* have been reported to increase sodium reabsorption by stimulating the sodium potassium chloride co-transporter-2 and thus further contribute to the increased kidney QO_2 [93]. As reported by Nordquist and colleagues [36] pharmacological activation of the HIF system in diabetic rats prevented kidney hypoxia, glomerular hyperfiltration and urinary protein leakage. The mechanism for these beneficial effects of HIF activation for diabetic kidney function was reported to include induction of endogenous antioxidant defense systems to counteract the elevated oxidative stress. It is somewhat surprising that HIF is not activated in the hypoxic diabetic kidney, but hyperglycemia can result in covalent modification of the p300 coactivator [94]. The coactivator p300 is required for normal HIF-mediated gene activation and this modification results in a defective HIF signaling and lack of induction of genes to counteract tissue hypoxia in diabetes. Indeed, the HIF system has gained increasing interest in the fight against the growing burden of chronic kidney disease, including diabetic nephropathy [95-97]. Further studies are needed to elucidate the mechanism for why the kidneys are so susceptible to develop hypoxic injury even though blood supply and DO_2 remain remarkably high.

The last two studies included in this thesis (IV and V) investigated the role of endothelin signaling via ETA and ETB receptors for the hypoxia in the diabetic rat kidney. Both blockade of ETA receptors and activation of ETB receptors improved kidney tissue pO_2 in diabetes. The effects were not mediated by changes in kidney QO_2 or oxidative stress even, yet urinary sodium excretion increased substantially after these treatments. The improved kidney pO_2 was due to increased RBF, which subsequently improved DO_2 to the kidney tissue.

ET-1 participates in the development of hypertension as well as both diabetic and non-diabetic chronic kidney disease [98], and blockade of endothelin receptor signaling is beneficial for kidney function in diabetes [99, 100]. Systemic administration of endothelin receptor agonists or antagonists will have effects on renal hemodynamics but also on systemic circulation and arterial blood pressure. Therefore, we developed a technique to deliver these substances so that only renal circulation was affected without potential confounding systemic effects. For this purpose, a catheter was inserted into the left renal artery, via the lumbar artery, and pilot experiments were conducted to determine the optimal dose resulting in maximal effect on RBF, and calculated RVR, without altering systemic arterial blood pressure.

The main findings of study IV and V were that either blockade of ETA receptors or activation of ETB receptors improved pO_2 in the hypoxic kidney in diabetic rats. Interestingly, both cortical and medullary pO_2 were increased after blockade of ETA receptors or activation of ETB receptors. However, oxidative stress status was not altered by any of these interventions. Activation of ETA receptors induces production of superoxide radicals from the NADPH oxidase [101-103] and it is therefore somewhat surprising that blockade of ETA receptors increased RBF, but did not affect oxidative stress status. A possible explanation is that NADPH oxidase-derived radical formation indeed was inhibited by blocking ETA receptors, but that we were unable to detect this in the acute setting when the effect only was studied within the first hours after the interventions. It is possible that the effect would have been more prominent and detected if we studied the effects of chronic blockade of ETA receptors over days to weeks. It has also been reported that the beneficial effects of ETA receptor blockade for diabetic kidney function mainly is due to reduced inflammation [104]. Additionally, uncoupled NO synthase 3 can be a major source of superoxide radicals in diabetes [105]. NO availability is significantly reduced in the diabetic kidney at least partly due to reduced substrate availability [106-108]. ETB receptor activation results in NO synthase-derived NO production, which indeed would explain the increased RBF and reduced RVR. This would provide an additional mechanism for protecting long-term kidney function in diabetes, since endothelial dysfunction due to impaired endothelium derived relaxing factor/NO signaling has been implicated in both vasculopathy and nephropathy [109].

Expression of the mitochondrial protein uncoupling protein 2 is similar in wildtype and ETA knockout mice [110], indicating that ETA receptors do not directly regulate mitochondrial leak respiration. This may explain why kidney QO_2 and TNa/QO_2 were unaffected by blockade of ETA receptors in the diabetic rats. It should be noted that blockade of ETA receptors affected the diabetes-induced glomerular hyperfiltration, altered filtration fraction in addition to improving kidney pO_2 whereas activation of ETB receptors only affected kidney pO_2 . It should be noted that the beneficial effects of altering ETA and ETB receptor signaling do not seem to be additive [100]. It is not completely clear what the exact mechanism is for the beneficial effects of manipulating endothelin signaling for diabetic kidney function, but it has been suggested to involve reduced inflammation, macrophage infiltration and transforming growth factor- β signaling [104, 111, 112].

Antagonists for the ETA receptor or combination of ETA and ETB receptor antagonists are used clinically to treat pulmonary hypertension [113]. ETA receptor antagonists have been tested in patients with chronic kidney disease [98], with some promising results [114, 115]. However, as the doses of the ETA receptor antagonists are increased to maximize the beneficial effects on kidney outcome, these patients experienced escalating fluid retention resulting in increased heart failure-related mortality [116]. Current development of ETA receptor antagonists aims to minimize fluid retention by combining the ETA receptor antagonist with a natriuretic treatment such as full blockade of the renin-angiotensin system [117].

In conclusion, the results presented in this thesis demonstrate that hypoxia develops early after the start of hyperglycemia and therefore potentially can be an initial key event for the onset and progression of diabetic nephropathy. Furthermore, the intrarenal hypoxia in diabetes can be significantly improved by either blockade of ETA receptors or activation of ETB receptors, indicating that endothelin signaling may play a pivotal role for the development of intrarenal hypoxia in diabetes. The important roles of ETA and ETB receptors for the regulation of kidney pO_2 in the diabetic may provide new targets to fight the increasing burden of diabetic nephropathy.

Figure 20. Summary of the mechanisms by which endothelin-1 influences tissue hypoxia via ETA and ETB receptors in the diabetic kidney.

Conclusions

- NMRI mice are more susceptible to develop the functional alterations commonly observed in the human disease, whereas BALB/c is the only strain developing structural alterations.
- L band EPR oximetry using LiPc probes can repetitively and reliably monitor intrarenal pO_2 during at least a period of up to 45 days.
- Intrarenal tissue hypoxia develops already within the first three days after the onset of insulinopenic diabetes in mice, which is well before the earliest sign of diabetic nephropathy.
- Blockade of ETA receptors and activation of ETB receptors improve kidney pO_2 in insulinopenic diabetes, which is mainly the result of increased RBF and DO_2 .

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