The Role of Kidney Oxygen Homeostasis for the Development of Kidney Disease

CARLA CARVALHO
The relation between oxygen supply and demand determines tissue oxygen tension (PO₂). When intrarenal tissue PO₂ decreases, any compensatory increase in oxygen supply via increased renal blood flow is likely to increase glomerular filtration rate. The resulting increased tubular load of electrolytes destined for active transport increases oxygen consumption, thus affecting intrarenal tissue PO₂. Consequently, the kidney is particularly sensitive to alterations in oxygen homeostasis and kidney hypoxia is acknowledged as a common pathway to end stage renal disease. Different factors that can affect intrarenal oxygen homeostasis, including alterations in blood pressure and sodium intake dietary or pathologies such as diabetes mellitus, anemia or atherosclerosis. This thesis focuses on understanding how these factors influence kidney oxygen homeostasis.

Pronounced reduction in sodium intake caused tissue hypoxia in kidney cortex via activation of the renin-angiotensin-aldosterone leading to increased tubular sodium reabsorption. Angiotensin II and aldosterone affect kidney oxygen handling differently. Whereas angiotensin II mainly affects kidney oxygen delivery, aldosterone mainly affects oxygen consumption. The hypoxia-inducible factor (HIF) system is a cellular defense mechanism against prolonged hypoxia. Although diabetes causes intrarenal hypoxia, hyperglycemia per se also prevents HIF-activation. Therefore, the effects of type 1 diabetes were evaluated in genetically modified mice with chronic HIF-activation. Diabetic mice with globally increased HIF activity, due to heterozygote prolyl hydroxylase-2 deficiency, displayed reduced mitochondria leak respiration and preserved cortical PO₂. Diabetic mice with kidney-specific HIF activation, due to homozygous deficiency of von Hippel-Lindau, developed reduced mitochondria leak respiration and reduced urinary albumin excretion.

The normal age-related decline in kidney function has been proposed to be due to, at least in part, increased oxidative stress, which can induce mitochondrial leak respiration via activation of uncoupling proteins. Indeed, two-year old mice deficient of uncoupling protein-2 presented with improved mitochondria efficiency and reduced urinary protein excretion.

Summarizing, the data presented in this thesis provide clear support for potent influence of the renin-angiotensin-aldosterone system, HIF activation and mitochondria function on intrarenal oxygen availability. Maintaining kidney oxygen homeostasis may be a unifying strategy to protect kidney function.

Keywords: Oxygen, Kidney mitochondria, Hypoxia-inducible factors, Prolyl hydroxylase, von Hippel-Lindau, Diabetes, Aging, Kidney, Sodium, RAAS

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"If you dare nothing, then when the day is over, nothing is all you will have gained."  
List of Papers

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


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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide translocator</td>
</tr>
<tr>
<td>AT1</td>
<td>Angiotensin II receptor type 1</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>Bcl2</td>
<td>B-cell lymphoma 2</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CAP</td>
<td>Canrenoic acid potassium salt</td>
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<tr>
<td>CAT</td>
<td>Carboxyatractylocide</td>
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<tr>
<td>CoQ</td>
<td>Coenzyme Q</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid</td>
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<tr>
<td>ESRD</td>
<td>End stage renal disease</td>
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<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FADH2</td>
<td>1,3-dihydro-flavine adenine dinucleotide</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HIF</td>
<td>Hypoxia-inducible factors</td>
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<td>HRE</td>
<td>Hypoxia response elements</td>
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<tr>
<td>mQO2</td>
<td>Mitochondrial oxygen consumption</td>
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<tr>
<td>mTAL</td>
<td>Medullary thick ascending limb of the loop of Henle</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
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<tr>
<td>p300</td>
<td>E1A binding protein p300</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PGK1</td>
<td>Phosphoglycerate kinase 1</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase</td>
</tr>
<tr>
<td>PO2</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>QO2</td>
<td>Oxygen consumption</td>
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<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone system</td>
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<tr>
<td>RBF</td>
<td>Renal blood flow</td>
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<tr>
<td>RCR</td>
<td>Respiratory control ratio</td>
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<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TGF</td>
<td>Tubuloglomerular feedback</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
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<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
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Introduction

The Kidney

The kidneys are responsible for regulating extracellular volume, electrolyte concentration and pH. This is achieved by their ability to control urinary excretion of electrolytes, waste products, bicarbonate and hydrogen ions, as well as regulate the activity of the renin-angiotensin-aldosterone system (RAAS). The nephron is the functional unit of the kidney and it is divided in glomerulus, proximal tubule, loop of Henle, distal tubule and collecting duct. Nutrients and electrolytes are filtered in the glomerulus, forming the primary urine, while large proteins and cells remain in the plasma. In the proximal tubule, all the glucose and the majority of electrolytes are reabsorbed. About 65% of the sodium (Na\(^+\)) is reabsorbed in this segment, mainly driven by the concentration gradient created by the Na\(^+/K^+\)- ATPase localized to the basolateral membrane of the tubular cells. In the loop of Henle, the hyperosmotic milieu characteristic of the medulla is created. This is due to the combination of a water permeable descending limb and a water impermeable ascending limb, where the medullary thick ascending limb of the loop of Henle (mTAL), plays a major role in the reabsorption of circa 25% of the filtered Na\(^+\). Finally, the distal tubule and collecting duct have pivotal roles in the regulation of acid-base balance and the final regulation of water and electrolyte balance.

The kidneys receive about 25% of the cardiac output, to support a high glomerular filtration rate (GFR) of approximately 125 ml min\(^{-1}\). Despite ample renal blood flow (RBF), the renal tissue displays heterogeneous oxygenation. The cortico-medullary oxygen tension (PO\(_2\)) \((I, 2)\) is the result of heterogeneous oxygen consumption (QO2) in different nephron segments in combination with the close proximity of feeding arteries and draining veins resulting in the oxygen diffusion shunt \((3, 4)\). Indeed, the cortex has a tissue PO\(_2\) about 30-40% higher than the medulla \((5, 6)\). In the normal kidney, an attempt to increase intrarenal tissue PO\(_2\), by increasing RBF also increases GFR and, thus, tubular load of Na\(^+\), which also increases the metabolic demand to sustain the resulting increased active tubular transport. For this reason, it is important to keep RBF and GFR relatively constant, regardless of fluctuations in blood pressure, a concept known as autoregulation. Two main mechanisms are responsible for the autoregulation of GFR and RBF; The fast acting myogenic mechanism and the somewhat slower acting tubuloglomerular feedback.
mechanism (TGF). The myogenic mechanism is the ability of afferent arterioles to constrict in response to a sudden increase in perfusion pressure, resulting in a constant perfusion pressure in the distally located glomerular capillaries. The TGF mechanism involves sensing of the $\text{Na}^+$- and $\text{Cl}^-$-load to the macula densa and signaling to the afferent arteriole to control perfusion pressure in order to maintain GFR within normal range. Notably, TGF signaling is also involved in the regulation of renin release.

Renin-Angiotensin-Aldosterone System

The RAAS is depicted in Fig.1. Renin release from granular cells in the distal afferent arteriole in response to reduced blood pressure is caused by a direct effect of reduced renal perfusion pressure on the renin producing cells, reduced perfusion pressure on GFR, and via the increased sympathetic activity due to reduced arterial pressure. Once released into the blood, renin enzymatically converts angiotensinogen in angiotensin I, which is thereafter cleaved by the angiotensin converting enzyme (ACE) into angiotensin II. Angiotensin II signals via angiotensin type 1 receptors (AT$_1$) to promote tubular water and $\text{Na}^+$ retention and vasoconstriction of resistance vessels, but also cell proliferation, oxidative stress and inflammation (7, 8). Angiotensin also stimulates aldosterone secretion from the adrenal glands, which further increasing tubular $\text{Na}^+$ reabsorption via mineralocorticoid receptor signaling and endothelial $\text{Na}^+$ channels in the late distal tubule and collecting duct.
Figure 1. Schematic representation of activation of the renin-angiotensin-aldosterone system. Briefly, a reduction in blood pressure lowers renal perfusion pressure and NaCl load to macula densa, which together with increased sympathetic activation, stimulates renin release from granular cells in the distal part of the afferent arteriole. Renin cleaves angiotensinogen into angiotensin I, which is thereafter converted into angiotensin II by angiotensin converting enzyme. Angiotensin II exerts different effects through the angiotensin AT1 receptors leading to increased tubular Na⁺, Cl⁻ and water reabsorption, increased production of antidiuretic hormone (ADH) and aldosterone, as well as increased vascular resistance. All of these mechanisms contribute to normalize arterial blood pressure.

The Mitochondrion

The mitochondrion is often referred to as the “power plant” of the cell, producing ATP (adenosine triphosphate) from adenosine diphosphate (ADP) and inorganic phosphate (Pi), via oxidative phosphorylation (OXPHOS), summarized in Fig. 2. Briefly, the electron transport chain (ETC), located in the inner membrane of the mitochondrion, consists of complexes I-IV, ATP-synthase and adenine nucleotide translocase (ANT). The nicotinamide adenine dinucleotide (NADH) or 1,3-dihydro-flavine adenine dinucleotide (FADH₂), pro-
duced during glycolysis and the tricarboxylic acid (TCA) cycle, serve as electron donors to complexes I and II, respectively. These complexes share the same purpose, to transfer electrons to Coenzyme Q (CoQ). While complex I is responsible for the oxidation of NADH by CoQ, complex II, that contains succinate dehydrogenase (an enzyme from TCA cycle), catalyzes the oxidation of FADH$_2$ by CoQ. Complex III oxidizes CoQ through cytochrome c and, finally, complex IV promotes the oxidation of the reduced cytochrome c by molecular oxygen (O$_2$), which is the final electron acceptor. Through these oxidation processes, complexes I, III and IV shuttle protons (H$^+$) from the mitochondria matrix to the intermembrane space, creating the electrochemical gradient used by the ATP-synthase to create ATP from ADP and Pi. The produced ATP is thereafter exchanged from the mitochondria matrix into the intermembrane space for ADP by the ANT (9).

![Figure 2](image_url)  
*Figure 2.* Schematic representation the mitochondrial oxidative phosphorylation. Briefly, NADH and FADH$_2$, originating primarily from the Krebs cycle, donate electrons to complexes I and II, respectively. Electrons are transported to complex III and further on to complex IV, through sequential oxidations. During this process, protons are shuttled to the intermembrane space creating the electrochemical gradient used by the ATP synthase to produce ATP from ADP and Pi (inorganic phosphate). Adenine nucleotide translocator (ANT) transports ATP to the intermembrane space in exchange for ADP. Production of ATP through oxidative phosphorylation is coupled to oxygen consumption, since oxygen is the final electron acceptor in complex IV. Uncoupling proteins (UCP) can be activated by different factors, i.e. increased membrane potential, and allow the passage of protons to the intermembrane space without production of ATP.

Due to the role of oxygen as an electron acceptor, OXPHOS can only occur in the presence of oxygen. Hence, mitochondria ATP production is coupled to QO$_2$, i.e. coupled respiration. Mitochondrial uncoupling, or leak respiration, refers to the QO$_2$ occurring due to proton leak across the inner membrane via e.g. the ANT or uncoupling protein (UCP) (10-13). Uncoupling, or leak, does
not result in any production of ATP. In normal mitochondria, leak respiration accounts for about 15-20% of mitochondrial 

QO2. However, leak respiration can provide the mitochondrion some control over its own membrane potential. If the membrane potential is too high, i.e. if there is increased production of superoxide radicals, proton pumps are inhibited and there is a further increase in superoxide radicals production, initiating a vicious cycle that leads to ETC dysfunction (14-16). By increasing leak respiration, the mitochondrion can reduce the harmful membrane potential to levels that are optimal for electron transport through the different complexes. UCP-1, also known as thermogenin, was the first UCP described, in brown adipose tissue, and it was associated with non-shivering thermogenesis (17). Later, other isoforms were discovered, including UCP-2, which is the predominant isoform expressed in the kidney (18, 19).

Hypoxia-Inducible Factors

Cellular hypoxia triggers activation of hypoxia-inducible factors (HIF) to facilitate cell adaptation and survival (20). HIF is a heterodimer composed of HIF-α and HIF-β subunits, that binds to hypoxia-response elements (HRE) present in the DNA when activated (21). While HIF-β can be found in all cells, HIF-α subunit is continuously being degraded, under normoxic conditions (22). There are three known isoforms of HIF-α; HIF-1α, -2α and -3α (23) with different cellular and tissue expression patterns (21, 24, 25). Fig. 3 is a schematic representation of HIF-activation. There are three prolyl hydroxylase (PHD) isoforms (PHD1-3) (21) and all require oxygen, α-ketoglutarate and iron (II) for full catalytic activity. In the presence of oxygen, PHDs hydroxylate residues proline 402 and 564 of HIF-α subunit (26). The hydroxylated HIF-α is recognized by the on Hippel-Lindau (VHL) tumor suppressor gene, and targeted for proteasomal degradation (27-30). Under hypoxic conditions, and hence in the absence of oxygen, PHDs are not able to hydroxylate the HIF-α subunit, allowing the α- and β-subunits to form the active heterodimer that initiates gene activation via binding to HRE in the promotor sequence of hundreds of genes. HIF activation affects cells and tissue adaptation, including modulation of cellular energy metabolism, stimulation of angiogenesis and erythropoiesis (31-35). Due to this wide range of activity, HIF-activation can be both beneficial or detrimental, depending on the organ, the condition and the duration of the HIF-activation.
Figure 3. Schematic representation of oxygen-dependent degradation of hypoxia inducible factors (HIF). During normoxia, prolyl hydroxylases (PHD) hydroxylate proline residues of the HIF-α subunit. The products are then recognized by the von Hippel-Lindau protein (VHL) and targeted for proteasomal degradation. Since PHDs are oxygen dependent, during hypoxia they remain inactive, allowing HIF-α and –β subunits to for an active heterodimer with the CREB-binding protein/E1A binding protein p300. The active heterodimer binds to hypoxia responsive elements (HRE) in the promotor sequence of hundreds of genes responsible for maintaining cellular and tissue homeostasis and adaptation to an hypoxic environment.

Although hypoxia is the main mechanism for activation of HIF, non-hypoxic stimuli can also activate it (36-42). Different studies have shown that growth factors (36), cytokines (39), vascular hormones (40) and viral proteins (42) can induce HIF activation via increased HIF-α protein translation. This, in itself, appears to alter the equilibrium between synthesis and degradation, leading to increased HIF signaling (22). Nevertheless, this thesis will focus on HIF activation during tissue hypoxia.
Diabetes Mellitus and Diabetic Kidney Disease

Type 1 diabetes mellitus, or insulinopenic diabetes mellitus, is a metabolic disease caused by insufficient glucose-induced insulin secretion. It usually develops at an early age, as a result of the destruction of pancreatic β-cells, due to autoimmune disorders or viral infections. Type 2 diabetes, or non-insulin-dependent diabetes, is characterized by a reduced sensitivity to insulin normally, and usually has a later onset compared to type 1.

Hyperglycemia has serious detrimental effects, that in the long term, leads to vasculopathy, neuropathy and nephropathy, with increased risk of end-stage renal disease (ESRD) and cardiovascular disease and mortality.

In normal conditions, all filtered glucose is reabsorbed in the proximal tubule by the Na+/glucose-linked transporters. About 10-67% of type 1 diabetes patients and 6-73% of type 2 diabetic patients develop glomerular hyperfiltration in an early stage (43). This might be due to 1) a saturation the Na+/glucose-linked transporters causing glycosuria and increased osmotic diuresis, and 2) increased proximal tubule reabsorption of Na+ together with glucose, reducing distal load of electrolytes and inactivation of the TGF mechanism (44). Histological alterations in the diabetic kidney include accumulation of extracellular matrix, thickening of glomerular basement membrane, loss of endothelial fenestration, loss of podocyte structure and tubulo-interstitial changes (45-47). In experimental models of insulinopenic diabetes, such alterations have been linked to increased tubular workload and increased oxidative stress (45). Microalbuminuria is an early indication of diabetic kidney disease, and as the disease progresses GFR starts to decline, fibrosis develops and the initial microalbuminuria progresses to macroalbuminuria and later proteinuria. About 45% of patients with diabetic kidney disease progress to ESRD requiring renal replacement therapy (48).

Diabetes and Hypoxia

Diabetes leads to functional and morphological changes of the kidney that can cause intrarenal hypoxia (49, 50). The increased tubular Na+ load is one mechanism by which QO2 increases (51). Also, it has been shown that diabetes causes mitochondria dysfunction via activation of mitochondrial leak respiration (52), also leading to increased mitochondrial QO2 and tissue hypoxia (53). Hypoxia has been acknowledged as a final common pathway to ESRD (54-56). Despite causing intrarenal hypoxia, hyperglycemia also hinders HIF-activation (24, 57-59). It has been suggested that both hyperglycemia and oxidative stress cause posttranslational modification of E1A binding protein p300 (p300), which is an essential component of the active HIF heterodimer (59). It has also been reported that hyperosmolarity per se directly affects HIF activation in dermal fibroblasts and endothelial cells (58). However, the beneficial
effects of HIF activation in the kidney is somewhat of a controversial issue. Several studies have reported renoprotective effect of HIF activation (60, 61), whereas other studies report that increased HIF signaling causes fibrosis (62) and proliferation of podocytes (63). Thus the currently available knowledge indicates that the effects of increased HIF signaling depend on the duration of HIF activation, cells in which HIF signaling was increased and experimental model used.

Aging and Kidney Function

Aging is related to a slow decline in kidney function (64). Humans are estimated to have a gradual decrease in GFR of about 0.4-2.6 mL min\(^{-1}\) year\(^{-1}\) (65). Physiological alterations can include increased oxidative stress, alterations in the RAAS activity and loss of nephrons (65-69). Increased oxidative stress (70) increases the formation of advanced glycosylation end-products (AGE) and superoxide radicals. While the first can reduce HIF-1 activity (71), the latter can increase mitochondria leak respiration, altering the oxygen homeostasis in the kidney (53). Age-induced vascular dysfunction and increased renal vascular resistance impair RBF leading to decrease filtration pressure in glomerular capillaries and decreased GFR (72, 73). All of these alterations can negatively affect kidney oxygen homeostasis and increase the susceptibility of the aging kidney to irreversible damage.
Aim

The overall aim of this thesis was to advance our understanding about factors affecting kidney oxygen homeostasis in health and disease. For this purpose, different hypotheses were tested.

Study I
We hypothesized that reduced dietary Na\(^+\) intake activates the RAAS and causes intrarenal tissue hypoxia.

Study II
We hypothesized that increased HIF activation, by reducing PHD2 gene expression genetically, protects mitochondria function and intrarenal oxygen homeostasis in a mouse model of type 1 diabetes.

Study III
We hypothesized that kidney-specific activation of HIF, by genetic deletion of VHL, protects mitochondria function and intrarenal oxygen homeostasis in a mouse model of type 1 diabetes.

Study IV
We hypothesized that global genetic deletion of UCP-2 protects mitochondria function in the ageing kidney, thus also protecting kidney function.
Methods

Animal Models

All animals were housed in a controlled environment, with 12h light-dark cycles, controlled temperature and humidity. Rats and mice had water and chow ad libitum. All experiments were performed in accordance with the National Institutes Guidelines for Use and Care of Laboratory Animals and approved by the local Animal Care and Use Committee.

Study I

Sprague-Dawley rats (Charles River, Sulzfeldt, Germany), 8-9 weeks old, were divided into two groups. The normal Na⁺ group received normal Na⁺ (0.25%) diet and the low Na⁺ group received low Na⁺ (0.025%) diet for 14 days. Later, about half the rats in each group received either intrarenal candesartan, an AT₁ receptor blocker, or canrenoic acid potassium salt (CAP), a mineralocorticoid receptor blocker. Therefore, at the end-point of the experimental setting there were four groups: normal Na⁺ diet with candesartan (n=13) and CAP (n=11) and low Na⁺ diet with candesartan (n=12) and CAP (n=9).

Rats were anesthetized with an intraperitoneal administration of thiobutabarbital (120 mg kg⁻¹) and placed on a heating pad to keep the core body temperature stable at 37°C. Rats were tracheostomized, to facilitate spontaneous breathing and both left carotid artery and femoral vein were cannulated for continuous blood pressure measurement, blood collection and saline infusion (0.9% NaCl, 5 mL kg⁻¹ h⁻¹). Urine was drained through a catheter placed in the bladder. A subcostal flank incision was made in order to expose the left kidney and further immobilize it in a plastic cup. A catheter was placed in the left ureter for urine collection. A lumbar artery was then used to insert a catheter ~1-2 mm into the left renal artery, for precise intrarenal infusion. The placement of this catheter was confirmed with infusion of Lissamine green 10% solution. The rats were allowed to stabilize for 45 min before the start of the experimental period. Fig. 4 summarizes the experimental protocol. After the baseline measurement period, either candesartan (AstraZeneca, Mölndal, Sweden; 4.2 µg kg⁻¹ in 200µL) or CAP (bolus 20 mg kg⁻¹ in 200 µL) were
administrated. The first was slowly infused into the renal artery, for 10 min, while the latter was infused intravenously.

Study II
Global deletion of PHD2 is embryonically lethal (74) and a PHD2+/− model was therefore created by replacing exon 2 with a neomycin resistance cassette (75). This animal model was first developed and described Mazzone et al to study oxygen delivery, endothelial cell morphogenesis and vessel function in tumour biology (75). In our study, PHD2+/− and aged matched genetic controls were used and it has previously been shown that heterozygote PHD2 deletion causes a profound increased HIF-1α protein expression (76).

Four groups were used in this study: normoglycemic wild type (n=16), diabetic wild type (n=10), normoglycemic PHD2+/− (n=16) and diabetic PHD2+/− (n=11). Diabetes was induced by low-dose intraperitoneal administration of streptozotocin (50 mg kg−1), for five consecutive days. Streptozotocin is an antimicrobial agent, that causes destruction of pancreatic β-cells, leading to insulinopenic diabetes mellitus (77). Multiple low-dose administration of streptozotocin is believed to mimic the pathogenesis of type 1 diabetes mellitus, since it prompts an inflammatory response responsible for the destruction of the β-cells (78). Using this approach, mice presented with hyperglycemia about two weeks after the first injection of streptozotocin and studied about 5-6 weeks thereafter.

Study III
VHL−/− male mice and aged matched male C57Bl/6J (wild type) were used in this study. Kidney specific genetic deletion of VHL was achieved by breeding Ksp1.3/Cre mouse (79) with the 2-lox allele, VHLhfl mouse (80). The resulting VHL−/− mouse has shown lack of VHL mRNA and protein expression in

Figure 4. Summary of experimental design of Study I
the renal tubules, mainly in the loop of Henle, distal tubule and collecting ducts, but not in the glomeruli and peritubular capillaries (79). VHL−/− mice have been reported to have a normal phenotype and kidney function, albeit having increased frequency of non-obstructive hydronephrosis. Nevertheless, these mice had no other morphological abnormalities (79).

Four groups were used: normoglycemic wild type (n=13), diabetic wild type (n=18), normoglycemic VHL−/− (n=19) and diabetic VHL−/− (n=11). Animals were included in the study at 20-27 weeks of age and diabetes was induced similarly to study II.

**Study IV**

Male UCP-2−/− mice and C57BL/6 (wild type) littermates used in this study. UCP-2−/− mice (B6.129S4-Ucp2tmLowl/J, stock no.005934) were originally purchased from Jackson Laboratories and continuously bred at the animal facilities of Uppsala University (Uppsala, Sweden). This UCP-2−/− mouse model was created by inserting a PGK-NEO cassette to replace exons 3-7 of the gene, on a C57BL/6 background (81). Animals were kept for approximately two years, before the start of experiments.

**Evaluation of Kidney Function**

**General Parameters**

In Study I, a transducer (model P23dB; Statham Laboratories, Los Angeles, CA, USA) connected to the catheter in the left carotid artery was used to measure blood pressure, while RBF was measured with an ultrasound probe (Transonic Systems Inc., Ithaca, NY, USA) placed around the left renal artery. These parameters were recorded continuously with a Power Lab instrument (AD Instruments, Hastings, UK) connected a computer.

**Measurement of Glomerular Filtration Rate (GFR)**

In Study I, GFR was estimated, in anaesthetized rats, by inulin clearance (185 kBq kg⁻¹ h⁻¹ of ³H-Inulin, American Radiolabeled Chemicals, St. Louis, MO, USA).

In Studies II, III and IV, GFR was assessed in awake mice, by clearance of fluorescein isothiocyanate (FITC)-inulin (2%, dissolved in phosphate buffer saline (PBS)). Briefly, circa 200 µL were administered intravenously and blood samples were collected at specific time points after injection. Care was taken to weight the syringe used to administered FITC-inulin pre and post injection. In a 384-well black plate 2 µL sample/standard/blank were added to 68 µL 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer
(500 mmol L\(^{-1}\) in PBS at pH 7.4) and FITC fluorescence (496/520 nm excitation/emission) was measured in Tecan Safire 2 (Tecan Group, Männedorf, Switzerland). GFR was estimated using non-compartmental pharmacokinetic data analysis (82, 83).

**Urinary Excretion of Electrolytes, Protein and Albumin**

In all studies, urine volume was determined gravimetrically. The urine concentration of Na\(^+\) was measured by flame spectrophotometry (model IL543; Instrumentation Lab, Milan, Italy). In Study I, tubular transported of Na\(^+\) was calculated using the following equation: 

\[
T_{Na} = [P_{Na}] \times \text{GFR} - [U_{Na}] \times \text{Urine flow},
\]

where [P\(_{Na}\)] is the concentration of Na\(^+\) in the plasma and [U\(_{Na}\)] is the concentration of Na\(^+\) in the urine.

Urinary protein concentration was determined by the Lowry method, using the DC Protein Assay (BioRad Laboratories, Hercules, CA, USA). Albumin concentration in the urine was measured with an ELISA kit (R&D Systems, Minneapolis, MN, USA), according to manufacturer’s instructions.

Results are expressed per min (Study I) or per 24h (Studies II, III and IV).

**Measurement of Oxygen Handling in the Kidney**

In Studies I and II, kidney tissue PO\(_2\) was assessed both in cortex and medulla, with modified Clark-type oxygen microelectrodes (Unisense, Aarhus, Denmark). Air (PO\(_2\)=147 mmHg) and a water solution saturated with Na\(_2\)S\(_2\)O\(_5\) (PO\(_2\)=0 mmHg) where used to perform a two-point calibration. A micromanipulator was used to help keep the electrode stable and at the right depth for the regional measurement. Cortical PO\(_2\) was measured at 1 mm depth in both Study I and II and medullary PO\(_2\) was measured at 4 mm depth in Study I and 2 mm depth in Study II. Rats were under Inactin anesthesia (Study I) during this procedure, and mice were under isoflurane anesthesia (1-5% in medical air) (Study II).

Oxygen delivery to the kidney was calculated as arterial content of O\(_2\) multiplied by RBF and kidney QO\(_2\) was obtained by the product of the arteriovenous difference in oxygen content and RBF.

**Evaluation of Mitochondria Function**

**Mitochondrial Isolation**

Isolation of mitochondria was performed immediately after kidney removal. Cortex was dissected on ice, placed on a glass homogenizer (Potter Elvehjem)
and homogenized, at 800 rpm, in cold isolation buffer (250 mmol L\(^{-1}\) sucrose, 10 mmol L\(^{-1}\) HEPES, 1 mmol L\(^{-1}\) ethylene glycol-bis(β-aminoethyl ether)-N,N',N″,N‴-tetraacetic acid (EGTA), 1 g L\(^{-1}\) bovine serum albumin (BSA) with pH 7.4 adjusted with KOH. The homogenate was centrifuged for 10 min, at 700g (+4°C) and the supernatant divided between three 1.5 mL tubes before further centrifugation at 10,000g for 10 min (+4°C). The buffy coat was removed from the pellet, by gently washing with isolation buffer and, after resuspension, the samples were pooled and centrifuged one more time at 7000g, for 5 min (+4°C). Another washing step followed before resuspension of the pellet in 1 μL of preservation medium/mg initial tissue weight (84). The mitochondrial suspension was stabilized on ice for 30 min before the experiments.

**High Resolution Respirometry**

Mitochondria function was assessed by high-resolution respirometry, using a 2-channel respirometer (Oxygraph 2k; Oroboros, Innsbruck, Austria). The chambers were kept at a constant temperature of +37°C, and filled with respiration medium (0.5 mmol L\(^{-1}\) EGTA, 10 mmol L\(^{-1}\) KH\(_2\)PO\(_4\), 20 mmol L\(^{-1}\) HEPES, 110 mmol L\(^{-1}\) sucrose, 3 mmol L\(^{-1}\) MgCl\(_2\) 6H\(_2\)O, 60 mmol L\(^{-1}\) K-lactobionate, 20 mmol L\(^{-1}\) taurine, and 1 g L\(^{-1}\) BSA). The Oxygraph utilizes Clark-type electrodes to measure oxygen dissolved in the respiration medium, and a background correction was performed to control for the constant, but low, QO\(_2\) by the electrodes and any potential oxygen diffusion out of or into the respiration medium (85). Data was acquired with DatLab7 (Oroboros).

Complexes I+II mediated state 3 respiration was supported by the addition of pyruvate (5 mmol L\(^{-1}\)), malate (2 mmol L\(^{-1}\)), complex I electron donors, and succinate (5 mmol L\(^{-1}\)), complex II electron donor, in the presence of saturated levels of ADP. Mitochondria total leak respiration was measured in the presence of pyruvate, malate and oligomycin (25 μmol L\(^{-1}\) to block the ATP synthase). Respiratory control ratio (RCR) was calculated as the ratio between complex I mediated state 3 and state 2 respiration (measured in the presence of substrates, but lack of adenylates). Guanosine diphosphate (GDP; 2 mmol L\(^{-1}\), to inhibit UCP activity) was added in order to assess the UCP-mediated leak respiration and ANT-mediated leak respiration was measured after addition of carboxyatractylycocide (CAT; 5 μmol L\(^{-1}\), to inhibit ANT activity). Regulated leak respiration was defined as the sum of UCP- and ANT-mediated leak respiration and unregulated leak respiration was determined by subtracting mediated leak respiration to the total leak respiration. All values presented were normalized to mitochondrial protein as determined using the DC Protein Assay (BioRad Laboratories).
Gene Expression and Quantification of Angiotensin II

Tissue Collection

In Study I, additional control (n=10) and low Na+ (n=10) rats were anesthetized (Inactin, 120 mg kg\(^{-1}\) i.p.) and ice-cold saline solution was infused through the heart. To wash the kidneys before extraction, the renal vein was cut open. The left kidney was collected for angiotensin II extraction and quantification while the right kidney was dissected and stored in RNAlater (Ambion, ThermoFisher Scientific, Waltham, MA, USA) or snap frozen in liquid nitrogen.

In Studies II and III, the right kidney was extracted after the acute experiment and a section of the kidney cortex was stored in RNAlater.

Angiotensin II Extraction and Quantification (Study I)

Extraction of angiotensin II from renal tissue was performed as previously described (86). Briefly, the left kidney was weighed, homogenized in cold methanol (10% wt/vol) and stored at -80°C. Before extraction, the samples were thawed, centrifuged for 10 min (+4°C) and dried in a vacuum centrifuge overnight. PBS (50 mmol L\(^{-1}\), pH 7.4) was used to reconstitute the dried residue. A phenyl-bounded solid phase extraction column (Discovery® DSC-Ph SPE Tube, Sigma Aldrich) was conditioned with methanol and equilibrated with water before adding the reconstituted samples. After washing, a solution of 90% methanol in water was used to elute the angiotensin II from the column. The samples were dried under vacuum and angiotensin II was determined using the Angiotensin II EIA kit (Peninsula Laboratories Inc., San Carlos, CA, USA) in accordance with the manufacturer’s instructions.

RNA Extraction and Quantitative Polymerase Chain Reaction

In Study I, kidney cortex sections were thawed, RNAqueous®-4PCR (Ambion) was used for RNA extraction and iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA) was used to synthesizes cDNA. cDNA levels were then evaluated by real time quantitative polymerase chain reaction (PCR), using the iCycler PCR system (Hoffmann-La Roche, Basel, Switzerland). In short, amplification reactions contained 2 µL cDNA, 2 µL Mix (LightCycler® FastStart DNA Master SYBR Green I; Hoffmann-La Roche), 2.5 µL primers mix and 3.5 µL of water.

In studies II and III, 20-30 mg of tissue were used to extract RNA, with RNeasy® Mini Kit (Qiagen, Strasse, Germany), following manufacturer’s instructions. DNase I (Thermo Fisher Scientific, Vilnius, Lithuania) treatment followed, before reverse transcription to cDNA, with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK). SYBR®
Green PCR Master Mix was used for the amplification executed using QuantStudio 5 (Applied Biosystems). The β-actin (Studies I, II and III) was used as housekeeping gene. Results are presented as $2^{-\Delta\Delta Ct}$ relative to the control group. Primers are listed on table 1.

Table 1. List of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNIP3</td>
<td>AACAGCACTCTTGCTCTGAGG</td>
<td>CCGACTTTGACCAAATCCCA</td>
<td>II</td>
</tr>
<tr>
<td>NHE3</td>
<td>AGTGGTCCAATTGATAGG</td>
<td>GACCATTATTGTCCGTACTTG</td>
<td>I</td>
</tr>
<tr>
<td>PGK1</td>
<td>ATTCTGCTTGGACAATGGAGC</td>
<td>AGGCATGGGAACACCATCA</td>
<td>III</td>
</tr>
<tr>
<td>PHD2</td>
<td>GGGCAACTACAGGATAACGGG</td>
<td>CTCCACTTACCTTGGCGT</td>
<td>II</td>
</tr>
<tr>
<td>β-actin</td>
<td>AAGACCTCTATGCAACAC</td>
<td>TGATCTTCATGCTGCTAGG</td>
<td>I</td>
</tr>
<tr>
<td>β-actin</td>
<td>CAGCTTCTTTCGACTCCTT</td>
<td>AGGAGTCTTCGACCCATTC</td>
<td>II&amp;III</td>
</tr>
</tbody>
</table>

BNIP3 - BCL2 and adenovirus E1B 19-kDa-interacting protein 3; NHE3 - Na+/H+ exchanger 3 (Slc9a3); PGK1 - phosphoglycerate kinase 1; PHD2 – prolyl hydroxylase 2.

Statistics

Statistical analysis in all studies were performed using Graph Pad Prism (GraphPad Software, San Diego, CA, USA). Two-way ANOVA was used to determine differences between groups, followed by Fisher’s Least Significant Difference post hoc test, in Studies I, II and III. Unpaired student’s t-test was used in Study IV. A p-value less than 0.05 was considered statistically significant.
Results

The Effect of Low Na⁺ Diet on Kidney Oxygen Homeostasis (Study I)

Rats on a low Na⁺ diet for 14 days have similar MAP compared to rats on a normal Na⁺ diet (Fig. 5). However, reduction of Na⁺ intake caused increased QO₂ in the kidney (Fig. 6) and decreased T_{Na} efficiency, manifested as decreased T_{Na/QO₂} (Fig. 7).

![Figure 5. Mean arterial blood pressure during baseline in rats given a normal Na⁺ or low Na⁺ diet.](image1)

![Figure 6. Oxygen consumption during baseline in rats given a normal Na⁺ or low Na⁺ diet.](image2)

![Figure 7. Transport of Na⁺ per oxygen consumption (QO₂) during baseline in rats given a normal Na⁺ or low Na⁺ diet.](image3)

Rats in the low Na⁺ group had increased intrarenal tissue concentration of angiotensin II compared to the normal Na⁺ group (22.3 ±3.0 vs 32.8±3.6, respectively; p<0.05). However, relative mRNA expression of Na⁺/H⁺ exchanger isoform 3 (NHE3) were similar in both groups (1.05±0.10 vs 0.96±0.10; NS).
Rats in the low Na⁺ group had a shift in the normal cortico-medullary tissue PO₂ gradient, with reduced tissue PO₂ in the cortex and increased tissue PO₂ in the medulla (Fig. 8A and B).

**Figure 8.** Cortical (A) and medullary (B) partial pressure of oxygen during baseline in rats given a normal Na⁺ or low Na⁺ diet.

### The Differential Effect of Angiotensin II and Aldosterone on Kidney Oxygen Homeostasis (Study I)

Administration of candesartan, an AT₁ receptor inhibitor, increased RBF in both groups (Fig. 9). Similarly, oxygen delivery rate was also increased in all rats that received candesartan (Fig. 10). These changes appeared to be independent of the dietary Na⁺ intake. Candesartan administration increased QO₂ in the normal Na⁺ group only (Fig. 11), but increased cortical PO₂ in the low Na⁺ group to values similar to those observed for the baseline of the normal Na⁺ group (Fig. 12).

**Figure 9.** Renal blood flow in rats on normal or low Na⁺ diet, during baseline and after angiotensin II type 1 (AT₁) receptor blockade using candesartan.
Administration of CAP, a mineralocorticoid receptor blocker, did not cause any changes to RBF or oxygen delivery (Fig. 13A and B). However, it altered kidney QO2 of both rats receiving normal Na+ diet as well as those receiving a low Na+ diet. CAP increased QO2 in normal Na+ diet rats, but reduced QO2 of low Na+ diet rats to the same level as that seen in baseline of normal Na+ rats (Fig. 14).

Figure 10. Oxygen delivery in rats on normal or low Na+ diet, during baseline and after angiotensin II type 1 (AT1) receptor blockade using candesartan.

Figure 11. Oxygen consumption in rats on normal or low Na+ diet, during baseline and after angiotensin type 1 (AT1) receptor blockade using candesartan.

Figure 12. Cortical partial pressure of oxygen in rats on normal or low Na+ diet, during baseline and after angiotensin type 1 (AT1) receptor blockade using candesartan.

Figure 13. Renal blood flow (A) and oxygen delivery rate (B) in rats on normal or low Na+ diet, during baseline and after angiotensin II type 1 (AT1) receptor blockade using candesartan.
The Effects of Reduction in Prolyl Hydroxylase 2 on Mitochondria Function (Study II)

Normoglycemic PHD2+/− rats had a reduction of PHD2 in the kidney of almost 50%. Induction of diabetes further reduced expression of PHD2 both in normoglycemic as well as in PHD2+/− mice (Fig. 15).

Diabetic wild type mice also had increased mitochondria leak respiration, when compared to normoglycemic wild type mice (Fig. 16A). While regulated leak respiration was increased in diabetic PHD2+/− mice, compared to normoglycemic PHD2+/− mice (Fig. 16B), diabetic wild type mice had increased unregulated leak respiration, compared to normoglycemic wild type mice (Fig. 16C). Further, UCP-dependent leak respiration was similar in all four groups, however diabetic wild type and PHD2+/− mice had increased ANT-dependent leak respiration when compared to normoglycemic mice (Figs. 16D and E). Also, BCL2 and adenovirus E1B 19-kDa-interacting protein 3 (BNIP3) was increased in normoglycemic PHD2+/− mice compared to normoglycemic wild
type mice (1.2±0.1 vs 1.0±0.1, respectively; p<0.05), an indication of increased HIF-activation in the PHD2+/− mice.

Figure 16. Mitochondrial leak respiration from mitochondria extracted from kidney cortex of wild type and PHD2+/− mice with and without streptozotocin-induced diabetes. (A) Total leak respiration, (B) total regulated leak respiration, (C) total unregulated leak respiration, (D) Uncoupling protein (UCP) mediated leak respiration, (E) Adenine nucleotide translocator (ANT) mediated leak respiration total unregulated leak respiration.
The Effects of Reduction in Prolyl Hydroxylase 2 on Kidney Function (Study II)

Diabetic wild type mice had decreased cortical tissue PO2 compared to normoglycemic wild type mice, while diabetic PDH2+/− maintained a similar cortical tissue PO2 compared to normoglycemic PHD2+/− mice (Fig. 17). Albuminuria was increased in both diabetic wild type and diabetic PHD2+/− mice compared to the normoglycemic mice (Fig. 18).

The Effects of Kidney-Specific Deletion of von Hippel-Lindau on Mitochondria Function (Study III)

Diabetes caused a decrease in RCR only in diabetic wild type mice. On the other hand, normoglycemic VHL−/− mice presented reduced RCR compared to normoglycemic wild type mice (Fig. 19). In general, VHL−/− mice had reduced complexes I+II mediated state 3 respiration (Fig. 20). Also, PGK1 was increased in diabetic VHL−/− mice, compared to normoglycemic VHL−/− mice (1.54±0.14 vs 1.14±0.11, respectively; p<0.05).
Mitochondria leak respiration was in general lower in VHL^{-/-} mice than in wild type mice (Fig. 21A). Regulated leak respiration was decreased in diabetic VHL^{-/-} mice, compared to diabetic wild type mice (Fig. 21B). There were no changes in unregulated leak respiration (Fig. 21C). VHL^{-/-} mice have shown, in general, a lower UCP-dependent and ANT-dependent leak respiration (Fig. 21D and 21E) and diabetic VHL^{-/-} mice have a lower ANT-dependent leak respiration compared to diabetic wild type mice.

Figure 20. Complexes I+II mediated state 3 respiration of mitochondria from wild type and VHL^{-/-} mice with and without streptozotocin-induced diabetes.
Figure 21. Mitochondrial leak respiration in kidney cortex of wild type and VHL$^{-/-}$ mice with and without streptozotocin-induced diabetes. (A) Total leak respiration, (B) total regulated leak respiration, (C) total unregulated leak respiration, (D) Uncoupling protein (UCP) mediated leak respiration, and (E) Adenine nucleotide translocator (ANT) mediated leak respiration total unregulated leak respiration.

The Effects of Kidney-Specific Deletion of von Hippel-Lindau on Kidney Function (Study III)

Albuminuria was only increased in diabetic wild type mice compared to normoglycemic wild type mice (Fig.22).
The Effect of Aging and UCP-2 Deletion on Mitochondria and Kidney Function in Healthy Mice (Study IV)

Mitochondria from kidneys of UCP-2−/− mice had increased respiratory control ratio (Fig. 23). Although GFR was similar in the two groups (Fig. 24), UCP-2−/− mice had lower proteinuria when compared to wild type mice (Fig 25).

Figure 22. Urinary excretion of albumin in wild type and VHL−/− mice with and without streptozotocin-induced diabetes.

Figure 23. Respiratory control ratio of kidney mitochondria from 2-year old wild type and UCP-2−/− mice.

Figure 24. Glomerular filtration rate of 2-year old wild type and UCP-2−/− mice.
Figure 25. Urinary excretion of protein of 2-year old wild type and UCP-2⁻/⁻ mice.
Discussion

The main aim of this thesis was to advance our understanding regarding the mechanisms controlling renal oxygen homeostasis in health and disease. To do so, we studied the relationship between reduced Na\(^+\) intake and RAAS activation, two different experimental models of genetic HIF activation in diabetes, and the role of mitochondrial leak respiration for age-related decline in kidney function, utilizing global deletion of UCP-2.

Low Na\(^+\) intake is recommended to patients at a high risk of cardiovascular disease in order to control blood pressure and, thus, reduce the risk for incidence of cardiovascular disease and stroke. Although the negative effects of high Na\(^+\) diet on blood pressure and cardiovascular diseases are well known (87), it is still an ongoing debate regarding the beneficial effects of reduced dietary Na\(^+\) intake (87-90). In Study I, 90% reduction of dietary Na\(^+\) intake for two weeks did not reduce arterial blood pressure, but did alter the renal oxygen homeostasis. In order to preserve electrolyte and water balance, urinary excretion of electrolytes and volume should somewhat match intake. Rats receiving low Na\(^+\) diet had decreased T\(_{Na}/QO_2\) and increased kidney QO\(_2\) due to compensatory activation of RAAS, as evident from increased intrarenal tissue levels of angiotensin II. Frindt et al. recently reported that restricted Na\(^+\) intake results in increased tubular Na\(^+\) reabsorption in the proximal tubule and subsequently decreased Na\(^+\) load to the more distal parts of the nephron (91). Also, in a previous study by Riquier-Brison and colleagues, activation of angiotensin AT\(_1\) receptors resulted in recruitment of NHE3 and Na\(^+\)-Pi co-transporter 2, allowing for increased tubular transport of Na\(^+\) (92). This can explain why, in Study I, rats on low Na\(^+\) had increased tubular Na\(^+\) reabsorption despite unchanged gene expression of NHE3.

Rats on low Na\(^+\) diet had inverted cortico-medulary PO\(_2\) gradient. This was originally reported by Stillman et al., where chronically salt depleted rats developed cortical hypoxia and medullary hyperoxia (93). They also observed that salt depleted rats had reduced mTAL mass, which could partially explain the increased medullary PO\(_2\).

In order to better understand the role of RAAS activation, the possible involvement of angiotensin II acting on AT\(_1\) receptors was studied by administering candesartan, and the possible effects of increased aldosterone signaling was studied by administration of CAP to block mineralocorticoid receptors.
Activation of the angiotensin AT₁ receptor can influence Na⁺ reabsorption directly and indirectly. It can activate NHE₃ to stimulate Na⁺/H⁺ exchange in the proximal tubule (94), promote Na⁺/K⁺-ATPase activity (95), and increase Na⁺/HCO₃ co-transport (94). Angiotensin II signaling via AT₁ receptor causes vasoconstriction of the efferent arteriole, resulting in increased ne filtration pressure in glomerular capillaries and increased tubular Na⁺ load. The increased tubular Na⁺ load in combination with a direct stimulation of proximal reabsorption increase proximal tubular transport and, thus, also QO₂ in this region of the kidney. In Study I, inhibition of angiotensin AT₁ receptors increased RBF and oxygen delivery regardless of Na⁺ intake. Interestingly, QO₂ remained unchanged in the low Na⁺ group, but increased in the normal Na⁺ group. This effect on the normal Na⁺ group might be due to redirection of Na⁺ reabsorption to distal parts of the tubule, where TNa is less efficient. A similar observation was reported by Leong and colleagues, where administration of captopril, to inhibit angiotensin II production by ACE, decreased Na⁺ reabsorption in the proximal tubule, although GFR and blood pressure were unchanged. This was at least in part attributed to reduction of NHE₃ in the proximal tubule (96). Since there was already a shift of Na⁺ transport to less efficient parts of the tubule in the low Na⁺ group, inhibition of angiotensin signaling via AT₁ receptors did not have further effect on kidney QO₂ in our study. However, it is important to notice that the increased oxygen delivery in the low Na⁺ group restored cortical PO₂ values close to those of the control group, indicating a potent role of oxygen delivery to maintain oxygen homeostasis.

Inhibition of aldosterone signaling, by inhibiting mineral corticoid receptor activation, on the other hand did not affect RBF, or oxygen delivery to the kidney. It did, however, reduce kidney QO₂ during Na⁺ restriction. This is an indication that increased QO₂ in response to reduced dietary Na⁺ intake is caused by increased aldosterone signaling. Indeed, it has been reported that plasma aldosterone levels and ENaC activity increase in Na⁺ depleted rats (97). Furthermore, aldosterone mineralocorticoid receptor activation is important for Na⁺ conservation during low Na⁺ intake (98).

In summary, low Na⁺ diet increases QO₂, reduces TNa/QO₂, and consequently alters the normal cortico-medulary PO₂ gradient. Angiotensin II activation of AT₁ receptors is mainly affecting RBF and oxygen delivery to protect cortical PO₂ during dietary Na⁺ restriction. Aldosterone signaling via mineralocorticoid receptors mainly affects kidney QO₂ and the shift of Na⁺ reabsorption to more distal and less efficient parts of the nephron.

Diabetes alters kidney oxygen homeostasis via a combination of increased oxidative stress, increased tubular load due to the initial glomerular hyperfiltration and mitochondrial dysfunction (49, 99, 100). As a consequence, intrarenal tissue hypoxia develops early on after the onset of diabetes (101). Despite the hypoxic environment, hyperglycemia per se prevents effective HIF
activation (24, 57, 59). In Study II, a circa 50% reduction in kidney levels of PHD2 was associated with protected mitochondria function in a mouse model of insulinopenic diabetes. Interestingly, diabetes per se decreased PHD2 levels, both in wild type as in PHD2+/− mice, which may be a mechanism to counteract the direct negative effect of hyperglycemia on hypoxic HIF activation.

Diabetic wild type mice had increased total mitochondria leak respiration, which was prevented by the increased HIF signaling in the diabetic PHD2+/− mice. Interestingly, diabetic wild type mice had predominantly unregulated leak, whereas diabetic PHD2+/− mice had mainly regulated leak. Mitochondria leak respiration is an important defense mechanism to regulate mitochondria membrane potential and limit production of harmful superoxide radicals. It has been shown in previous studies that UCP-2 is upregulated in the kidney of diabetic mice (52) and UCP-2+/− mice were partially protected against diabetic kidney disease (102). UCPs are mainly activated by increased levels of superoxide radicals (11) and UCP-dependent leak respiration can be prevented by antioxidant treatment (103). In Study II, there were no difference between groups with regards to UCP-dependent leak respiration. However, ANT-dependent leak was increased in both diabetic groups independent of HIF activity. This might be explained by the prolonged hyperglycemia. A study by Cardoso and colleagues has shown that ANT-mediated mitochondria leak respiration is the predominant mechanism during prolonged hyperglycemia (104).

Mitochondria are highly sensitive to increased HIF activity. Zhang et al. reported that HIF activation induces mitochondria autophagy. The mechanism was identified to involve increased BNIP3 expression, which disrupted the interactions between B-cell lymphoma 2 (Bcl2) and Beclin-1 (105). Mitochondria autophagy is important to remove dysfunctional and damaged mitochondria in order to protect normal function. The increased gene expression of BNIP3 observed in Study II supports the hypothesis that diabetic wild type mice have a higher number of dysfunctional mitochondria, which would explain the increased unregulated mitochondrial leak respiration. Indeed, increased mitochondria leak respiration is associated with increased total kidney QO₂ (52) and intrarenal tissue hypoxia (53). Protected mitochondria function in diabetic PHD2+/− mice with increased HIF signaling can help explain the improved cortical tissue oxygenation in these animals. However, this does not appear sufficient to prevent the development of diabetic kidney disease, as seen by the increased albuminuria in both diabetic groups. Several studies have demonstrated the beneficial effects of pharmacological PHD inhibition for preserving kidney function in diabetes (60, 61). However, the magnitude of HIF activation was likely several fold higher in these studies compared to what was achieved using or genetic approach. Nevertheless, the results from Study II demonstrate that also a seemingly insignificant increase in HIF activity has positive effects on mitochondria function in the diabetic kidney.
In Study III, we used a mouse model of kidney-specific VHL−/− to selectively increase HIF activation in the kidney. Much like what was observed in Study II, mitochondria function in the diabetic kidney was protected by increased HIF signaling. RCR, as an indication of mitochondria efficiency, was significantly reduced in diabetic wild type mice and prevented by kidney-specific HIF activation. Another mechanism by which HIF activation can improve mitochondria function during hypoxia, is through promoting a shift from aerobic to anaerobic metabolism (106-108). In order to promote cell survival, HIF-1α regulates the shunting of glucose from the TCA cycle, thus reducing mitochondria workload and O2 (106). These previous reports together with the finding that inhibition of mitochondria O2 is dependent on HIF activation (107), highlight the possibility that increased HIF activity in the VHL+/+ mice inhibit complex I and II-mediated mitochondrial respiration regardless of the glycemic status. Furthermore, increased expression of phosphoglycerate kinase 1 (PGK1), a pyruvate dehydrogenase inhibitor, in diabetic VHL+/+ mice indicates that pyruvate is shunted away from the TCA cycle and the mitochondria. VHL+/+ mice had lower total mitochondria leak respiration, which was predominately due to lower regulated leak respiration. This may indicate a smaller fraction of dysfunctional mitochondria in these animals. Importantly, while diabetic wild type mice developed albuminuria, an early indication of kidney disease, diabetic VHL+/+ mice did not. Therefore, the results from Study III provide additional support for a beneficial effect of increased HIF activity in the diabetic kidney, and that the protective mechanism involves protected mitochondria function and maintained oxygen homeostasis.

Friederich-Persson and colleagues have previously demonstrated the importance of increased mitochondrial O2, due to increased mitochondria leak respiration, for the development of intrarenal hypoxia and development of kidney disease (14). During normal conditions, mitochondria leak respiration accounts for only for a fraction of total mitochondria O2. However, in pathological conditions such as diabetes, increased production of superoxide radicals directly affects kidney oxygen homeostasis. As previously mentioned, UCPs are proteins mediating proton leak across the inner mitochondria membrane. UCP-2 is the main isoform expressed in kidneys (19), and protein leak via UCP-2 is activated by superoxide radicals from the matrix side (11, 109). Aging is normally associated with a slow decline in kidney function (64, 110) and increase in oxidative stress (68, 111). In Study IV, two-year old UCP-2−/− mice had similar GFR to that of aged-matched wild type mice. However, UCP-2−/− mice had increased mitochondria efficiency, indicated by higher RCR, and reduced urinary protein excretion. The protective effects of UCP-2 deletion on mitochondria efficiency is likely to protect oxygen homeostasis, which provides further support for positive effects of maintaining oxygen metabolism also in otherwise healthy, by ageing kidneys. However, a limitation of this study is that C57BL/6 mice are known to be very resistant to
kidney disease, and it is possible that the effects of the genetic intervention might have been significantly larger if using a more damage-prone mouse strain. In a previous study, Franzén and colleagues evaluated four different mouse strains in regards to their susceptibility to develop diabetic kidney disease. It was demonstrated that Balb/C, NMRI and 129S mouse strains all develop more profound indications of diabetic kidney disease compared to the most commonly used C57BL/6 mouse strain (112). Nevertheless, our results suggest that improvement in mitochondria efficiency by preventing UCP-2-mediated mitochondrial inefficiency has positive effects on oxygen homeostasis and function in the ageing kidney.
Conclusions

**Study I**
Low dietary Na\(^+\) intake impairs kidneys oxygenation due to RAAS activation.

Angiotensin II signaling via AT\(_1\) receptors have major impact in oxygen delivery, while aldosterone signaling has a major impact on QO\(_2\).

**Study II**
Activation of HIF, through reduction of PHD2, protects mitochondria function in type 1 diabetic mice. This, in turn, protects cortical oxygen homeostasis in this disease model.

**Study III**
Kidney specific deletion of VHL protects mitochondria function in type 1 diabetes mouse model.

**Study IV**
Deletion of UCP-2 protects mitochondria efficiency and kidney function in aged mice.
Popular Scientific Summary

The kidneys are important organs with different main function, such as regulation of blood pressure, reabsorption of important electrolytes and molecules, excretion of waste products and drug, and regulation of acid-base balance. Usually, when tissue oxygen levels decrease (hypoxia) there is an increase of oxygen supply, i.e. increased blood flow. However, in the kidneys, an increased renal blood flow would increase the amount of plasma filtered per unit of time (glomerular filtration rate). Sodium transport along the tubule requires energy consumption. The main energy source in our cells are the mitochondria, which require oxygen to produce energy in the form of ATP. Therefore, if glomerular filtration increases, more sodium needs to be transported, more energy is needed and, consequently, more oxygen is utilized, worsening the tissue hypoxia. In healthy kidneys, a few mechanisms help to keep the glomerular filtration rate constant. Fast mechanism can act on the arterioles transporting blood into the glomeruli, protecting the glomeruli from fluctuations in mean arterial blood pressure, while slower acting mechanisms activate production and release of different hormones, through activation of the renin-angiotensin-aldosterone system, that can regulate blood pressure and sodium and electrolyte reabsorption. Since kidney tissue hypoxia can lead to kidney damage, it was the main aim of this thesis to improve our understanding regarding factors involved in the kidney oxygen homeostasis in disease and health.

In the first study, a low sodium diet was given to otherwise healthy rats. Usually patients with a high risk for cardiovascular diseases are advised to reduce their sodium consumption, in order to help reduce blood pressure. We observed that a drastic reduction of sodium intake, for two weeks, did not change blood pressure, but increased kidney tubular workload leading to cortical hypoxia. This is due to the action of the renin-angiotensin-aldosterone system. When specialized cells in the distal tubule sense a decrease of sodium, renin is released and angiotensin II is produced. This hormone can induce vasoconstriction, increase sympathetic activity, aldosterone production, water reabsorption and sodium and chloride reabsorption. All of these effects lead to an increase in blood pressure. In this study, angiotensin II activity through angiotensin II type 1 receptor mainly mediated renal oxygen delivery, while aldosterone mainly affected intrarenal oxygen consumption.
In Studies II and III, the effect of chronic activation of hypoxia-inducible factors (HIF) was studied, in type 1 diabetes mouse model. HIF are transcription factors, activated during hypoxia, that promote cell adaptation and survival. HIF is composed by two subunits, HIF-α and HIF-β. During normal conditions, HIF-α subunit is constantly being degraded, by undergoing hydroxylation by prolyl hydroxylases (PHDs) allowing HIF-α to be targeted by von Hippel-Lindau (VHL) protein for proteasomal degradation. Since PHDs require oxygen to function, during hypoxia the α- and β-subunit are able to bind, forming an active heterodimer. Diabetes can lead to intrarenal hypoxia, due to increased tubular workload and increased oxidative stress. However, it can also negatively impact on HIF-activation. In Study II and III, a prolyl hydroxylase heterozygote model and a kidney specific von Hippel-Lindau knockout model were used, respectively. Both studies shown that promoting HIF activation during diabetes increased mitochondria efficiency, thus reducing intrarenal oxygen consumption.

Aging is usually associated with a gradual decline in renal function, as seen by the progressive loss of glomerular filtration rate. Aging is also associated with increased oxidative stress. Oxidative stress can affect mitochondria, decreasing its efficiency (by affecting the membrane potential), thus increasing the amount of oxygen spent in energy production. As a protective mechanism, uncoupling proteins are activated. These proteins can help restore the membrane potential of the mitochondria, by allowing the passage of protons across the mitochondria inner membrane. Since this process still consumes oxygen, but does not lead to energy production, it is called mitochondria leak respiration. While defending the mitochondria from oxidative stress, mitochondria leak respiration also increases mitochondrial oxygen consumption. This can have a negative impact in intrarenal oxygen homeostasis. In Study IV, the effects of aging were studied in healthy UCP-2 knockout mice. Although there were no differences in functional changes, mitochondria in UCP-2 knockout mice was more efficient, probably leading to the reduced urinary protein excretion observed in these mice.

Overall, this thesis presents new data regarding the importance of maintaining oxygen homeostasis in the kidneys.
Sumário

Os rins exercem diferentes funções, como regulação da pressão arterial, reabsorção de electrólitos e moléculas importantes, excreção de substâncias e moléculas residiuais e fármacos, e regulação do equilíbrio ácido-base. Normalmente, quando há uma redução dos níveis de oxigénio num tecido (hipóxia), há um aumento do fluxo sanguíneo como resposta, que leva ao aumento da quantidade de plasma filtrada por unidade de tempo (taxa de filtração glomerular). O transporte de sódio, ao longo dos túbulos requer energia sob a forma de adenosina trifosfato (ATP). A principal fonte de energia das células são as mitocôndrias. Estas consomem oxigénio para produzir ATP. Desta forma, se a taxa de filtração glomerular aumenta, o transporte de sódio nos túbulos aumenta, sendo necessária a presença de mais ATP, havendo um maior consumo de oxigénio. Consequentemente, há um agravamento da hipóxia. Existem mecanismos diferentes para proteger o glomérulo e manter a taxa de filtração constante. Existe um mecanismo com uma rápida resposta de acção que protege o glomérulo contra flutuações da pressão arterial, ao regular a contração das artérias aferentes e eferentes. Já o sistema renina-angiotensina-aldosterona tem uma acção mais lenta. A ativação deste sistema leva ao aumento de angiotensina II, que regula a pressão arterial não só por causar vasoconstrição, mas também por regular a reabsorção de electrólitos ao longo dos túbulos e por estimular a produção de aldosterona. Tendo em conta que hipóxia renal pode levar à lesão renal, o objetivo desta tese foi o de melhorar o conhecimento relativo aos fatores envolvidos na manutenção da homeostasia de oxigénio nos rins, na saúde e na doença.

Doentes com alto risco de doenças cardiovasculares são aconselhados a reduzir a ingestão de sódio, de modo a ajudar a reduzir e controlar a pressão arterial. No primeiro estudo, uma dieta com baixo teor de sódio foi dada a ratos saudáveis durante duas semanas. Neste estudo, a pressão arterial não sofreu alterações, contudo houve um aumento da reabsorção de electrólitos nos túbulos gerando hipóxia no cortex renal. A ativação do sistema renina-angiotensina-aldosterona foi um dos contribuinte para resultados observados, sendo que a ativação do recetor tipo I da angiotensina II, pela angiotensina II, teve efeitos a nível do fluxo sanguíneo renal, enquanto que a aldosterona teve mais efeitos a nível do consumo de oxigénio.
Nos Estudos II e III foi estudado o efeito da ativação crónica de fatores induzidos pela hipóxia (HIF), em modelos animais de diabetes mellitus tipo 1. HIF são fatores de activação que, em situação de hipóxia, promovem a transcrição de genes que facilitam a adaptação e sobrevivência das células. HIF é composto por duas subunidades. A subunidade α é hidroxilada por prolil hidroxilases (PHD) e reconhecida pela proteína von Hippel-Lindau (VHL) que a marca para degradação. Como as PHDs requerem oxigénio para funcionarem, em caso de hipóxia, a subunidade α liga-se à β, formando um heterodímero funcional. A diabetes mellitus pode causar hipóxia intrarenal, devido ao aumento do consumo de energia associado ao transporte de eletrólitos nos túbulos e ao aumento de stress oxidativo. Contudo, esta patologia tem um impacto negativo na ativação de HIF. Nos Estudos II e III foram utilizados ratinhos heterozigóticos para a PHD e ratinhos com knockout renal de VHL, respectivamente. Ambos os estudos revelam que promover a ativação de HIF durante diabetes aumenta a eficácia da mitocôndria e, desta forma, reduz o consumo de oxigénio no tecido renal.

O envelhecimento está associado a um declínio gradual da função renal, visível pela redução lenta da taxa de filtração glomerular. O envelhecimento também está associado a um aumento dos níveis de stress oxidativo. Este, por sua vez, pode afectar a mitocôndria negativamente, aumentando o seu potencial de membrana e diminuindo a sua eficácia. As mitocôndrias tem na sua membrana interna “uncoupling proteins” (UCP), que podem ser ativadas pelo aumento de stress oxidativo. Estas proteínas permitem a passagem de protões pela membrana interna sem haver produção de ATP. A este mecanismo dá-se o nome de “leak respiration”. Contudo, se por um lado este mecanismo protege a mitocôndria, por outro lado aumenta o consumo de oxigénio, tendo um impacto negativo na homeostasia de oxigénio no tecido renal.

No Estudo IV, estudamos os efeitos do envelhecimento em ratinhos knockout para a UCP-2. Embora não houvessem diferenças a nível da função renal, estes ratinhos apresentam uma mitocôndria mais eficiente, o que provavelmente leva à redução de proteína excretada na urina observada nestes animais.

Em conclusão, esta tese apresenta novos resultados relativos à importância da manutenção do balanço dos níveis de oxigénio no tecido renal.
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