Chapter 1

General introduction

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Introduction

The kidneys have various functions, of which the excretion from the body of excess water and water-soluble waste products is one. To this end fluid from the blood is filtered and processed by the two kidneys. Each kidney contains a million microscopically sized functional units called nephrons, see figure 1.1. A nephron is composed of a filtering unit, the glomerulus, from where the filtered fluid flows into a tubule (see arrows), which contains different segments. In the regulation of glomerular filtration small blood vessels are important, which are positioned at the inflow and outflow sites of each filtering unit. They are called afferent and efferent arteriole, respectively (figure 1.1). The filtration function of the kidney can be harmed during the course of various illnesses, one of them being diabetes mellitus (45). This disease, which at present increases dramatically in the world is subdivided in two main forms (see below). The second designated type 2, is often preceded by overweight or obesity (13, 42).

In the present thesis obesity and in particular diabetes mellitus have been simulated in rats, in order to investigate microvascular determinants that are involved in damaging the small filter-units in each kidney. We were particularly interested in recognizing alterations in the molecular mechanisms possibly related to changes in functioning and reactivity of the small feeding and draining arterioles of the glomeruli (figure 1.1). We studied, among others, the reactions of these vessels to changes in local blood pressure, designated the
General introduction

myogenic response, to depolarization of the cell-membrane and to the circulating hormone angiotensin II, with special attention to the modulating influence of alterations in signaling molecules generated within the so-called cyclo-oxygenase (COX) pathway.

General aspects of diabetes mellitus

In the 2nd century AD, the word diabetes, meaning “siphon”, i.e. a bent tube through which liquid can flow away from a vessel, was first used by the Greek physician Aretaeus to describe patients with great thirst and excessive urination. In the 17th century it was noticed that the urine of many of these patients had a sweet smell and taste, so the word mellitus, meaning “like honey,” was added to the name of the disease (2). Diabetes mellitus is seen as one of the main threats to human health in the 21st century (35, 62). When untreated it is characterized by chronic hyperglycemia, i.e. high glucose levels in the blood (62). Increased concentrations of glucose in the blood and body fluids alter, among others, the structure and function of various proteins. There are two main forms of diabetes mellitus.

Type 1 diabetes, also called insulin-dependent diabetes mellitus, is due primarily to autoimmune-mediated destruction of beta cells in the pancreatic islets, resulting in absolute insulin deficiency (62). In the Netherlands 74,000 people are affected by type 1 diabetes (1). In addition, an increase of 23% was found between 1980-1990 in a study that compared diabetes incidence in the Netherlands among children younger than 20 years of age (46). This comparison suggests an increase in the number of type 1 diabetic patients also in the near future.

Type 2 diabetes is characterized by insulin resistance, which is defined by a relative insensitivity of the target tissues for insulin, resulting in a reduced glucose uptake (62). Of all diabetic patients 85-90% is affected by type 2 diabetes. This means that in The Netherlands alone 526,000 people, or 3.3% of the total population, suffer from this disease. It is predicted that in 20 years time the number of type 2 diabetic individuals will increase by 35% (1). A risk factor for its development is obesity. One out of every 3 people with obesity will develop type 2 diabetes mellitus (13, 42).

General aspects of obesity

The prevalence of obesity and of people with overweight in The Netherlands has been reported to be 1.6 million individuals, which is 10% of the population (3). Obesity occurs when the number of calories consumed exceeds the number that is metabolized, the remainder being stored in adipose (fat) tissue.
The kidney

In addition to the excretion of excess water, waste products and foreign substances, the kidneys regulate extracellular fluid volume and osmolarity, maintain ion balance, regulate pH, and participate in endocrine pathways. The various segments of the functional unit, i.e. the nephron (see figure 1.1, upper panel), execute these functions in collaboration with the surrounding blood vessels. Arterial blood arriving at a nephron has passed via successively smaller interlobular arteries, and finally the distal interlobular artery, into the afferent arteriole. From there it runs into the glomerulus, where plasma without proteins filters out from the capillary loops into the capsule of Bowman. From there the filtrate flows into the first and subsequent tubular segments of the nephron (arrows figure 1.1). Blood leaves the glomerulus via the efferent arteriole, and from there it flows first through microvessels surrounding the tubular segments of that same nephron before finally leaving the kidney via the renal vein.

The resistance of the pre- and postglomerular microvessels determines glomerular filtration rate and regulates glomerular capillary pressure. Determinants of filtration rate as measured in a single nephron are: single nephron plasma flow, glomerular capillary pressure, capillary ultrafiltration coefficient and systemic oncotic pressure (7). Increases in the first 3 and/or a decrease in the last one may increase filtration rate. Subsequent analyses

Figure 1.2. Current and expected prevalence of diabetes mellitus, type 1 and type 2 combined (dashed line), and the concomitant (predicted) rise in diabetic nephropathy (solid line). Adapted from King et al. (32).
revealed that glomerular capillary pressure was the main determinant of glomerular damage (7). Glomerular capillary pressure is mainly determined by the resistance of the immediate preglomerular arterioles and the efferent arteriole (40). When a glomerulus is damaged it can not properly filter anymore. When this happens to many glomeruli, nephropathy or kidney failure will develop.

**Diabetic nephropathy**

The four main complications of diabetes mellitus are: nephropathy, cardiovascular disease, retinopathy and neuropathy. The latter three are beyond the scope of the present thesis. Diabetic nephropathy is initially characterized by an increased loss of albumin in the urine. This is thought to be preceded by hyperfiltration, which is often found in diabetic patients with normoalbuminuria. Clinically, more albumin in the urine is divided into micro- and macroalbuminuria. A slow loss of glomerular filtration capacity is thought to start in the microalbuminuric phase, which is characterized as an urinary albumin excretion between 20 and 200 µg/min. Microalbuminuria usually proceeds into macroalbuminuria which is defined as a urinary albumin excretion above 200 µg/min. This phase is associated with a more prominent decline in glomerular filtration rate.

Figure 1.2 shows the expected number of patients with diabetic nephropathy in the near future (solid line) based on the estimated increase of both types of diabetes mellitus combined (dashed line). It is known that 20-40% of the patients with type 1 diabetes mellitus will develop diabetic nephropathy, despite an improved glycemic control (32). As mentioned above, diabetic nephropathy is often preceded by a phase of hyperfiltration (e.g. 14, 17, 18, 20, 57). In addition to possible alterations within the glomerulus itself, e.g. of the capillary wall, the pre- and postglomerular arterioles also may play a role in the development of hyperfiltration (4, 59). A decrease in preglomerular resistance is seen as an important determinant, while postglomerular resistance often is maintained (9, 61). This combination results in a heightened glomerular pressure causing hyperfiltration, and with longer duration, damage to the glomerular capillaries. A decreased preglomerular resistance can be caused by a decreased sensitivity for vasoactive stimuli inducing constriction, and/or an increased sensitivity to vasodilatory substances.

**Nephropathy in obese patients**

Not only diabetes, but also obesity can change normal renal physiology. Even without hyperglycemia it is already associated, among others, with an increase in glomerular filtration rate, increased renal plasma flow and an
increased urinary albumin excretion (12). The latter is considered a first sign of endothelial and/or podocyte dysfunction in the glomerulus (5), while hyperfiltration provides a link to glomerulosclerosis (21). The pathogenetic

![Diagram of molecular mechanisms in vascular smooth muscle cells leading to vasoconstriction, and of angiotensin II, pressure and membrane depolarization induced signaling.](image)

**Figure 1.3.** Schematic overview of molecular mechanisms in vascular smooth muscle cells leading to vasoconstriction, and of angiotensin II, pressure and membrane depolarization induced signaling. Membrane depolarization can be evoked, among others, by inhibition of potassium-efflux, as happens with various vasoconstrictors (see text). SR sarcoplasmic reticulum; MLC myosin light chain; MLCK MLC kinase; CaM calmodulin; PLC phospholipase C; PIP2 phosphatidylinositol 4,5-biphosphate; IP3 inositol 1,4,5-triphosphate; IP3R IP3 receptor; Ca2+ calcium; DAG 1,2-diacylglycerol and PKC protein kinase C.
mechanisms involved, however, probably differ between obesity and diabetes mellitus (12).

Renal microvascular constriction or vasodilatation, and alterations in diabetic nephropathy

Changes in resistance of the kidney and its glomeruli can be regulated by vasoconstriction or vasodilatation. This is brought about by the vascular smooth muscle cells in the wall of the renal arterioles, which interact with surrounding cells such as the endothelium, mesangial cells in the glomerulus and tubular epithelium. Depolarization of the vascular smooth muscle cell membrane, or the response of these cells to a change in transmural pressure (myogenic response), or their responsiveness to the circulating signaling molecule angiotensin II or to locally produced products of the COX pathway are all important determinants of their functioning. First, basic aspects of vascular smooth muscle cell contraction will be shortly described. Thereafter, the role of each stimulus mentioned above in maintaining glomerular pressure will be succinctly introduced, followed for each stimulus by a paragraph discussing its involvement in diabetic nephropathy.

Vascular smooth muscle cell contraction

Vasoconstriction is brought about by contraction of vascular smooth muscle cells (VSMCs). For the latter a rise in the intracellular free calcium-ion concentration is important, as is shown in figure 1.3 (start at \( \text{Ca}^{2+} \) in the black box). Calcium then binds to calmodulin, and the calcium-calmodulin complex binds in turn to MLC-kinase (MLCK) thereby stimulating phosphorylation of myosin (myosin-P). Myosin-P is able to interact with actin filaments resulting in VSMC-contraction and vasoconstriction. Alternatively, when rho-kinase is activated (see also figure 1.3, lower right), it sustains vasoconstriction by inhibiting the dephosphorylation of myosin-P. It does so by converting the active myosin light chain (MLC) phosphatase to its inactive form (53).

Membrane depolarization

Membrane depolarization of VSMCs is a physiological component of many vasoconstrictor pathways, as is also schematically shown in figure 1.3. It can be evoked, among others, by inhibition of potassium-efflux by closure of potassium channels. Signaling pathways which lead to such closure operate either via activation of protein kinase C (PKC) or rho-kinase, or via inhibition of cAMP or cGMP. Both pathways are used by vasoconstrictors (30, 41). Membrane depolarization is able to cause a rise in intracellular calcium due to opening of voltage-gated calcium channels in the outer VSMC membrane (34, 48, 47).
Interestingly, this may lead to rho-kinase activation via an as yet unknown PLA$_2$ peroxidase Cox1/2 Prostaglandin H$_2$ synthase PGD$_2$ synthase Prostacyclin synthase PGI$_2$ synthase Thromboxane synthase TxA$_2$ synthase PGF$_2$ synthase

Figure 1.4. Schematic overview of cyclo-oxygenase (COX) pathways in vascular smooth muscle cells, and how angiotensin II (AngII), mark numbered 1, or hyperglycemia, mark numbered 2, can influence this balance. Hyperglycemia has other effects as well which are not shown (e.g. glycosilation of proteins and generation of reactive oxygen species (51)). Two isoforms of COX, designated 1 or 2 (COX 1/2), are present in the kidney. PLC phospholipase C; PIP$_2$ phosphatidylinositol 4,5-biphosphate; IP$_3$ inositol 1,4,5-triphosphate; IP$_3$R IP$_3$ receptor; Ca$^{2+}$ calcium; DAG 1,2-diacylglycerol and PKC protein kinase C; PLA$_2$ phospholipase A$_2$; AA arachidonic acid; PG prostaglandin (for I, D and E).
Interestingly, this may lead to rho-kinase activation via an as yet unknown mechanism (see figure 1.3) (34, 47, 48).

In arterioles of diabetic rats the responses of potassium channels were found to be increased, as well as their functional availability, thereby promoting dilatation of these microvessels (27). The same investigators also found a decreased responsiveness in these rats to membrane depolarization, as evoked by increasing the extracellular potassium chloride concentration. This indicates that afferent arteriolar contractile and calcium responses are diminished in diabetes mellitus (10, 11).

**Myogenic response**

The myogenic response is the acute constriction of a blood vessel in reaction to an increase in transmural pressure, and vasodilatation when pressure decreases. In the kidney, it is an important mechanism to protect the glomerulus from changes in systemic blood pressure (36). As transmural pressure was increased, vascular smooth muscle cells of renal arteries exhibited a linear depolarization from an average resting potential of -57±2 mV at 20 mm Hg to -38±2.4 mV at 120 mm Hg (22). This indicates that VSMC membrane depolarization is involved in the myogenic response, as is also depicted in figure 1.3. In renal arterioles the subsequent rise in intracellular calcium could be inhibited by L-type calcium channel blockers, suggesting that voltage-gated calcium entry mechanisms are responsible for the rise in intracellular calcium with an elevated transmural pressure (22, 25). How the vascular wall translates changes in transmural pressure into changes in VSMC membrane potential is at present unknown (15).

In afferent arterioles of diabetic rats an impaired myogenic responsiveness was found, which could be restored by a high dose of the aselective COX-inhibitor ibuprofen (26). Therefore, an alteration in the synthesis of one or more products within the different COX-pathways (see below) might play a role in the reduced reactivity to pressure as found in diabetic rats (26).

**Angiotensin II**

Angiotensin II is a circulating hormone that also can be formed in the kidney. It is an important regulator of the diameters of pre- and postglomerular arterioles, and hence, of glomerular hemodynamics (40). It can elevate intracellular calcium-levels in VSMCs by stimulating calcium mobilization from intracellular stores, and secondly by activating calcium entry mechanisms. When angiotensin II binds to its AT1-receptor phospholipase C (PLC) is activated, as is shown in figure 1.3. Activated PLC hydrolyses phosphatidylinositol 4,5-
biphosphate (PIP\(_2\)) to inositol 1,4,5-triphosphate (IP\(_3\)) and 1,2-diacylglycerol (DAG). IP\(_3\) binds to receptors on the sarcoplasmic reticulum where calcium channels are opened and stored calcium is released into the cytoplasm. DAG in turn, activates PKC which, among others, is thought to increase the sensitivity of the contractile apparatus for calcium (56).

In diabetic pre- and postglomerular microvessels various changes in the effects of angiotensin II have been found: an increase in reactivity (10), no effect of the diabetes (10, 49) or a decreased reactivity (28, 29). The study that observed in afferent arterioles an increased responsiveness to angiotensin II due to diabetes mellitus did not investigate any underlying mechanisms (10); by contrast, in efferent arterioles no change was seen. One of the studies that reported a normal afferent arteriolar reaction to angiotensin II found that this could occur thanks to a modulation of superoxide dismutase that suppressed an altered influence of endogenous nitric oxide (NO) caused by the diabetes mellitus (49). A decreased reactivity to angiotensin II due to diabetes could be restored by feeding the rats a diet enriched with 1% myo-inositol (29). Adding myo-inositol to the diet resulted in a increased incorporation of myo-inositol into the membrane which restored the vasoconstrictor capacity to angiotensin II (29), myo-inositol is a precursor of IP\(_3\) (see figure 1.3). In another study the decreased reaction to angiotensin II could be partially restored by the aselective COX-inhibitor indomethacin (28), again indicating an alteration in the synthesis of one or more products within the COX pathway (see below) due to diabetes mellitus.

**COX-pathway**

<table>
<thead>
<tr>
<th>Prostanoid</th>
<th>Constrictive</th>
<th>Dilatory</th>
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<tbody>
<tr>
<td>Thromboxane A(_2)</td>
<td>TP</td>
<td></td>
</tr>
<tr>
<td>PGE(_2)</td>
<td>EP(_1), EP(_3), EP(_2), EP(_4)</td>
<td></td>
</tr>
<tr>
<td>PGF(_{2\alpha})</td>
<td>FP</td>
<td></td>
</tr>
<tr>
<td>PGD</td>
<td></td>
<td>DP</td>
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Partially adapted from (8). PG prostaglandin (for E\(_2\), I\(_2\), F\(_{2\alpha}\), and D)  

Products of the COX-pathway, called prostanoids, are important mediators of renal hemodynamics (8, 23, 24, 43). As is shown schematically in figure 1.4, they are formed when phospholipase A\(_2\) (PLA\(_2\)) is activated by a rise in intracellular calcium and/or by PKC. PLA\(_2\) then liberates arachidonic acid from membrane phospholipids. Arachidonic acid is then bound by a complex of one of the COX enzymes and peroxidase. It is known that there are two COX isoforms (1 and 2) present in the kidney (60), of which COX-1 is the most abundant. The
general introduction

complex of COX-1 or -2 with peroxidase converts arachidonic acid into PGG$_2$ and PGH$_2$, which in turn are rapidly transformed into more stable prostanoids by cell-specific synthases (60). Prostanoids can alter vessel tone depending on which type is produced, as is shown in figure 1.4. For example, production of thromboxane A$_2$ leads in the kidney to vasoconstriction (50) and of prostaglandin I$_2$ (PGI$_2$) to vasodilatation (19).

Table 1.1 gives a schematic overview of the different prostanoid receptors present in the kidney and their main vasoactive action. Only prostaglandin E$_2$ (PGE$_2$) has both vasoconstrictive and vasodilatory properties.

Angiotensin II can stimulate the synthesis and release of various prostanoids (39, 50). As is shown in figure 1.4 (see mark numbered as 1), binding of angiotensin II to its receptor activates PLC, which leads to a subsequent increase in cytosolic calcium levels via IP$_3$. This, and the activation of PKC can both activate PLA$_2$ (50). Via the latter pathway also hyperglycemia can stimulate PLA$_2$ activity (16), as is indicated in figure 1.4 by number 2. Hyperglycemia has also other effects which are not shown in the figure such as alterations in metabolism, glycosylation of proteins and generation of free oxygen radicals (51).

As mentioned already, in diabetic renal microvessels the aselective COX-inhibitor indomethacin was capable of partially restoring an attenuated reaction to angiotensin II (28). However, which of the two COX-isoforms is involved, and to which extent is presently unknown. In renal microvessels a possible relationship between diabetes and COX-2 has not been investigated so far.

**Diabetes and obesity models used**

Type 1 diabetes mellitus was induced in male Sprague-Dawley rats by injection of streptozotocin (STZ). This naturally occurring antibiotic, which is produced by the fungus Streptomyces achromogenes, is in sufficient dose toxic for the beta cells in the pancreatic islets. The glucose moiety of STZ is the essential component that specifically directs STZ to the beta cell. Once inside the beta cell it causes destruction of DNA and apoptosis. Subsequently, there are not enough beta cells left to produce insulin, which will result in hyperglycemia. The STZ-injection is often combined with subsequent partial insulin substitution, as was also done in most of our experiments, to prevent a too high level of hyperglycemia.

As a prediabetic obesity model the genetically obese Zucker rat was used (33). These rats develop obesity because of leptin resistance due to a missense mutation in its hypothalamic receptor (55, 58). Lean control rats are genetically identical except for this mutation. The obese rat displays many of the health
problems seen in human obesity such as hyperlipidemia, late-onset hypertension, hypercholesterolemia, and hyperinsulinemia (6, 31, 38, 44, 52). Moreover, with increasing age the obese Zucker rat develops proteinuria and focal segmental glomerulosclerosis, ultimately leading to kidney failure (31, 38).

Visualization of microvessels in the hydronephrotic kidney

The isolated perfused hydronephrotic kidney was used to directly visualize that part of the renal microvasculature that is predominantly important for the regulation of glomerular capillary pressure, i.e. the distal interlobular arteries, afferent and efferent arterioles. Chronic hydronephrosis was induced by ligating the left ureter 6-8 weeks before the experiment. This results in a complete atrophy of the tubular elements with minimal effects on the pre- and postglomerular microvasculature (54). Since there are no tubular structures present, the tubulo-glomerular feedback mechanism is absent in this model. This allows for direct assessment of vascular reactivity of the microvessels just mentioned, without the need to account for possible indirect effects mediated via the tubulo-glomerular feedback mechanism. On the day of an experiment hydronephrotic kidneys were isolated as is extensively described in the materials and methods sections of chapters 2-6. Figure 1.5 shows schematically the set-up used for *in vitro* perfusion of hydronephrotic rat kidneys, as well as a picture of a hydronephrotic kidney in the actual set up. Perfusion flow through the kidney was measured with a flow-probe in the catheter between the pressurized reservoir and the renal artery.

In this thesis we mainly studied the responsiveness to angiotensin II, added directly to the perfusion fluid, which is described in chapters 3, 4, 5 and 6. Simultaneous measurements of distal interlobular arteries, afferent and efferent arterioles could often be performed. Membrane depolarization was evoked not only by increasing the extracellular potassium concentration (chapters 4 and 6), but also with barium chloride (chapter 6). The latter selectively blocks the inward rectifier potassium channel and could be conveniently used to obtain concentration-response curves (41).

A unique property of the hydronephrotic kidney is the possibility to study myogenic reactivity, as was done in chapters 2 and 6. Since the individual vessels in this model can be visualized without exposure to surgical trauma, ischemia, hypoperfusion or hypoxia, the myogenic response is always present (54). However, since only preglomerular vessels are myogenically active (25), pressure-induced diameter changes were determined for distal interlobular arteries and afferent arterioles only. Two important characteristics of the myogenic response in the hydronephrotic kidney model are: a) it is quite rapid.
with the steady state reached within seconds, and b) the degree of vasoconstriction is proportional to the elevation in perfusion pressure for the range of arterial pressures seen \textit{in vivo} (37).

Figure 1.5. Set-up used for in vitro perfusion of hydronephrotic rat kidneys and microscopic observation of the periglomerular microvessels (upper panel, adapted from (37)). Lower panel shows an example of a hydronephrotic kidney placed in this set up.
Outline of this thesis

Part I: diabetes mellitus

Why 20-40% of the diabetic patients develop nephropathy, and the others do not is currently unknown. We addressed the hypothesis that a loss of responsiveness of pre- and/or postglomerular arterioles to certain stimuli might be involved. To this end, we combined the aforementioned STZ-model of diabetes mellitus type 1 with hydropnephrosis and measured, among others, changes in diameter of these microvessels.

In chapter 2, the responsiveness to pressure was investigated in control and diabetic rats coming from two different suppliers, as well as a possible difference in sensitivity to the vasodilator prostanoid PGE\textsubscript{2} of control rats.

In chapter 3, a study is presented on the effects in diabetes of a new class of drugs: selective COX-2 inhibitors. The responsiveness to angiotensin II was investigated and the involvement of thromboxane A\textsubscript{2} here-in was determined.

In chapter 4, we investigated whether aspecific inhibition of COX could restore a decreased responsiveness to angiotensin II in diabetic rats. In addition, a possible involvement of the vasodilatory prostanoid PGE\textsubscript{2} was studied.

Part II: Obesity

In chapter 5, the influence of obesity on renal microvascular reactivity was studied. Obesity in humans is associated with proteinuria and an increased glomerular filtration, possibly related to an increase in glomerular capillary pressure. We investigated in obese Zucker rats which exhibited proteinuria, and in their lean controls, whether this is related to a chronic alteration in the diameter of pre- and/or postglomerular microvessels. We also investigated a possible change in their reactivity to angiotensin II.

Part III: rho-kinase

It is clear from the above that vascular tone of the smallest microvessels is important for proper regulation of renal hemodynamics (40). An important determinant of vascular tone is the contraction of vascular smooth muscle. This is determined not only by levels of intracellular free calcium, but also by the sensitivity of its contractile apparatus (53). A potential modulator of the latter is rho-kinase. In chapter 6, we addressed the question of a possible central role for rho-kinase in the regulation of periglomerular microvascular tone.
General discussion and summary

Chapter 7 presents a general discussion about the findings described in the preceding chapters as well as some new supplemental data.

The thesis ends with a summary of the results obtained and the conclusions drawn.
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