Chapter 7

General discussion

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General discussion

Main findings

This thesis characterizes different aspects of renal microvascular reactivity in rat models of diabetes mellitus and obesity, with the concomitant controls, using isolated perfused hydronephrotic kidneys. We have demonstrated in experimental diabetes mellitus that the renal microvascular myogenic response is more easily impaired in offspring from breeders selected for large litter size as compared to those from randomly selected parents (chapter 2). Moreover, control rats from the two substrains did not differ in their renal microvascular responsiveness to pressure, but offspring from breeders selected for large litters had an increased sensitivity to PGE$_2$ (chapter 2). In chapter 3 and 4 the involvement of cyclo-oxygenase (COX) inhibition on angiotensin II induced constriction was studied in the same diabetes model. In chapter 3 it was demonstrated that an increased production of thromboxane A$_2$ via the COX-2 pathway contributes to normal renal microvascular responsiveness to angiotensin II in experimental diabetes. In chapter 4, we showed that aselective COX-inhibition restored an attenuated preglomerular reaction to angiotensin II in diabetic animals. Using a rat model for obesity, we observed a preglomerular vasodilatation, a postglomerular vasoconstriction and an augmented reactivity to angiotensin II in the kidney (chapter 5). Finally, we showed that the procontractile molecule rho-kinase plays a pivotal role in renal microvascular constriction to membrane depolarization and other stimuli (chapter 6).

Prevalence of diabetic hyperfiltration and the myogenic response

Table 7.1. Percentage of type 1 diabetic patients (pt) who have developed hyperfiltration

<table>
<thead>
<tr>
<th>% pt with hyperfiltration</th>
<th>Number of pt in study</th>
<th>Reference</th>
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<tbody>
<tr>
<td>86</td>
<td>7</td>
<td>(16)</td>
</tr>
<tr>
<td>78</td>
<td>9</td>
<td>(7)</td>
</tr>
<tr>
<td>59</td>
<td>54</td>
<td>(29)</td>
</tr>
<tr>
<td>56</td>
<td>117</td>
<td>(12)</td>
</tr>
<tr>
<td>35</td>
<td>91</td>
<td>(37)</td>
</tr>
<tr>
<td>25</td>
<td>unknown</td>
<td>(70)</td>
</tr>
<tr>
<td>0</td>
<td>12</td>
<td>(63)</td>
</tr>
</tbody>
</table>

Table 7.1 shows a great variation in the percentage of type 1 diabetic patients that will develop hyperfiltration. Hyperfiltration is known to occur in the first years of diabetes and this condition is prognostic for the development of diabetic nephropathy (4, 42, 43, 17). This great variation is in contrast to what is found in animal studies where the majority report a prevalence of hyperfiltration
of 100% (e.g. (2, 13, 30, 5)). We believe that the latter might be caused by a publication bias. An exception is Wilkes et al. who reported hyperfiltration in all diabetic rats studied at a duration of 1 week; however, in rats from 2-12 months glomerular filtration rate was elevated only in about half of the cases (69). Van den Born et al. (64) and O’Donnel et al. (48) report hyperfiltration only when the blood glucose concentration is below 23.6 mmol/L. In chapter 3 we showed that the diabetic rats used for that study did not exhibit hyperfiltration. However, parameters like anesthetics, level of hyperglycemia, partial insulin substitution, duration of diabetes or rat strain are not able to account for the difference why some studies report hyperfiltration and others do not. Their range varies widely in the literature, which suggests a multifactorial etiology.

One of the causes for hyperfiltration might be a loss of preglomerular reactivity to e.g. pressure in diabetic renal microvessels. The diagram in figure 7.1 shows all rats used for the experiments in chapter 2 and their distribution in groups based on blood glucose level and reaction to pressure. From the 42 diabetic rats used in this study, 36% (n=15) attained a blood glucose level below 23.6 mmol/L, i.e. with a chance to develop hyperfiltration as reported by the studies mentioned above. These 15 rats were further divided into having a normal response to pressure (n=6; 40%) and having an attenuated response (n=9; 60%). The reason that not all rats developed either a normal or attenuated response, can not be explained by a substrain difference as 3 out of the 9 rats showing the attenuated response were from the vendor Harlan who did not select breeders for littersize. The other 6 with an attenuated response were obtained from Charles-River who selected on large litters. Also partial insulin substitution can be ruled out as a cause for the attenuated response since the 3 rats that
were not selected for litter size, i.e. from Harlan, did not receive this. Their blood glucose levels within the moderate range (i.e. < 23.6 mmol/L) were obtained by injecting less STZ, which likely resulted in a decreased number of apoptotic beta cells in the pancreas. Ergo, more insulin producing cells were left. These rats will have had a part of their internal insulin signaling mechanism still present whereas the rats in this comparison selected on litter size, i.e. Charles-River, had most of their insulin signaling eliminated and needed partial insulin supplementation to obtain moderate blood glucose levels. Hence, an attenuated renal microvascular response to pressure could be evoked by using different experimental methods to induce diabetes. This might prove useful for future experiments to unravel the processes why some diabetic patients develop nephropathy and others do not.

High glucose in the perfusion medium and the myogenic response

![Diagram of myogenic response in diabetic rats](image)

Figure 7.2. Pilot experiment suggesting no effect of disrupting the generation of reactive oxygen species on pressure induced changes in the diameter of interlobular arteries and afferent arterioles in 4 week diabetic rats derived from the supplier not selecting on large litter size i.e. Harlan. First and third panel shows the basal myogenic response and in the presence of tempol (100 µmol/L and 1 mmol/L respectively) in a diabetic kidney (low STZ, no insulin, blood glucose level 30.4 mmol/L). Second and fourth panel shows the basal myogenic response and in the presence of vitamin C, (1 µmol/L and 100 µmol/L respectively) in 1 diabetic kidney (normal STZ, insulin, blood glucose level 20.8 mmol/L).

Our findings indicate that increasing the glucose concentration in the perfusion medium does not affect the myogenic response of the preglomerular microvessels (chapter 2, figure 2.4 middle panel). This differs from Cipolla et al.
who have found that high glucose concentrations dilate cerebral arteries and diminish myogenic tone through an endothelial mediated mechanism that involves nitric oxide and prostaglandins (8). However, Arima et al. discovered that high glucose in renal afferent arterioles augments angiotensin II activity by inhibiting NO synthesis (1). As yet it is unknown whether inhibition of NO-synthesis is involved in the response to pressure in our diabetic microvessels. Interestingly, Hayashi et al. have shown that prostanoid derangements plays a role in the impaired responsiveness to pressure of the afferent arteriole in diabetic rats (22).

**Reactive oxygen species and an unaltered myogenic response in diabetic animals**

![Diagram](https://via.placeholder.com/150)

*Figure 7.3. Schematic representation of how hyperglycemia can lead to an increased synthesis of prostanoids via reactive oxygen species (ROS) production.*

As stated in chapter 2, reactive oxygen species (ROS) are implicated in the processes leading to diabetic nephropathy (10, 54). We performed 2 pilot-experiments to explore a possible involvement of ROS generation in diabetic nephropathy. The underlying hypothesis is depicted in figure 7.3. Hyperglycemia can increase the production of ROS from the mitochondrial electron transport chain. ROS stimulate nfkb, which can activate via COX-2 the synthesis of prostanoids with a vasodilator or vasoconstrictor mechanism (31); this has been unraveled in bovine aortic endothelial cells. Furthermore, in arteriolar vascular smooth muscle cells, pressure generated constriction is also dependent upon ROS (47). However, figure 7.2 shows that the disruption of ROS generation using two different inhibitors did not lead to an altered myogenic response in diabetic renal
microvessels. This seems to indicate that an increased ROS production is not used to normalize a diabetes induced impairment of the responsiveness to pressure. Other factors than ROS that might increase COX-2 levels in diabetes are shown in figure 1.4, angiotensin II (marked 1 in figure 1.4) as well as high glucose levels (marked 2 in figure 1.4) can increase COX-2 levels in diabetic vascular smooth muscle cells without interfering in the pathway described in figure 7.3.

Diabetes mellitus and COX-balance

The experiments reported in this thesis support the notion that the COX-balance might be important in diabetes induced changes in renal hemodynamics. Chapter 3 shows an involvement of thromboxane A₂ via COX-2 in keeping the responsiveness to angiotensin II within the normal range, while in chapter 4 a role for a vasodilatory prostaglandin is revealed by the aselective inhibition of COX, both in diabetic rats. In diabetic patients reports about COX-inhibition show contradictory effects. An aselective COX-inhibitor, indomethacin, was found to reduce an increased glomerular filtration rate as did lysine acetyl salicylate (soluble aspirin) [16, 25]. Another study found that the aselective COX-inhibitor, piroxicam, inhibited the increased glomerular filtration rate, while another inhibitor, sulindac, did not [18]. Two other studies found no effect of indomethacin in type 1 diabetic patients [7, 28], while all studies reported hyperfiltration in these diabetic patients. Thus in diabetic patients the involvement of COX derived prostanoids in the processes leading to diabetic nephropathy might depend on other as yet unknown factors.

However, we think that an adequate regulation of the COX-balance could be a reason why some diabetic patients will develop nephropathy and why others will not. We propose that diabetic patients who do not suffer from diabetic nephropathy, they modulate their COX-production in such a way that glomerular filtration is kept within a normal range. On the other hand, patients that do suffer from diabetic nephropathy are
incapable of this regulation. Future research should be focused on unraveling these processes. The key question will be to identify diabetic patients at an early stage and to point out into which group they fit.

An interesting future experiment in this respect would be to inhibit COX-2 while performing pressure response curves in diabetic kidneys of the supplier not selecting on litter size (i.e. Harlan); these rats had a normal responsiveness to pressure in the diabetic model with partial insulin substitution. This is of interest with respect to chapter 3 where we have shown that COX-2 is involved in normalization of the responsiveness to angiotensin II. It may be postulated that in diabetic kidneys from the supplier not selecting on litter size (i.e. Harlan), the response to pressure of renal microvessels is also normalized by COX-2.

Carnosine might be one factor involved in modulating the susceptibility to diabetic nephropathy. Interestingly, a Dutch group found that carnosine protects against the adverse effects of high glucose levels (in human mesangial cells and podocytes) and that carnosine (or derivatives) acts in a renoprotective way in diabetic patients (type 1 and 2 (27), see also figure 7.4). Carnosine functions as a natural ACE inhibitor and as a natural radical oxygen species (ROS) scavenger (27). As indicated in this thesis (see figure 1.4 and 7.3), hyperglycemia may increase via ROS production and the transcription factor nfkb the levels of COX-2 (31). Carnosine would influence this pathway because it can act as a ROS scavenger and thereby would ultimately influence the COX-2 pathway. We showed that the latter pathway is important for renal vascular reactivity in diabetic rats with a normal renal reaction to angiotensin II, while in rats with an attenuated reaction to angiotensin II inhibition of both the COX-1 and –2 pathway has a normalizing effect.

As has been shown in chapter 3, COX-2 inhibition attenuates the reaction to angiotensin II in diabetic kidneys, but not in control. These experiments were performed to study short term effects of COX-2 inhibitors. However, it would also be interesting to chronically suppress COX-2 and thereby study whether these rats will develop hyperfiltration. It may be hypothesized that when COX-2 is removed, the downstream thromboxane A$_2$ synthesis is also abolished and the renal microvessels will dilate causing hyperfiltration.

**COX-2 and hydronephrosis**

Theoretically, the development of hydronephrosis could trigger processes that would lead to COX-2 upregulation, since COX-1 is constitutively expressed in most tissues while COX-2 operates as an inducible enzyme by a number of inflammatory stimuli. However, in the kidney COX-2 is also constitutively expressed. COX-2 is found in mesangial cells of glomeruli (20, 26), cortical thick
ascending limb, macula densa and medullary interstitial cells (21, 68). It has been reported that bilateral ureteral obstruction for 24 hours caused a significant 14-fold induction of inner medullary COX-2 expression, whereas COX-1 did not change (46). No studies exist, which investigated the effect of a longer duration (6-8 weeks) of hydronephrosis on COX-2 expression profiles. Our study did not find a clear effect of COX-2 inhibition by NS398 on basal flow in control and diabetic kidneys.

Figure 7.5 shows the flow induced changes by angiotensin II with and without COX-2 inhibition by NS398 in normal perfused control and diabetic kidneys. Like in hydronephrotic kidneys angiotensin II induced a similar flow decrease in control and diabetic kidneys (left panel). Selective COX-2 inhibition attenuated the vasoconstrictor effect of angiotensin II in diabetic kidneys (middle panel), which is also seen in hydronephrotic kidneys. However, in control kidneys (right panel), selective COX-2 inhibition also attenuated the vasoconstrictor effects of angiotensin II. This in contrast to our findings in hydronephrotic kidneys. In hydronephrotic kidneys no tubular structures and macula densa are present, while it is known that these structures express a lot of COX-2 (21, 68). Our findings in hydronephrotic kidneys therefore show the vascular effect of diabetes and the involvement of COX-2 in this. Importantly, they exclude confounding processes like the tubulo-glomerular feedback mechanism. Future studies should address a possible contribution of this feedback and of macula densa derived COX-2, to the vascular changes induced by diabetes mellitus.

**Diabetes mellitus and angiotensin II**

Studies performed in chapter 3 and 4 showed different results with regard to the responsiveness to angiotensin II in type 1 diabetic rats. One study found a decreased responsiveness (chapter 4), while another study found a normal responsiveness (chapter 3), although the general characteristics of the diabetic model were identical. The reason for the discrepancy is unclear. Since these two studies were performed at different moments in time and seemed not overtly related to season of the year, a change over time in the rats used is one possibility. Such a change might well have been related to alterations in the COX-balance. In the present thesis we also discuss that the reaction to pressure can have a different effect although a similar model is used. Another group found divergent effects in their diabetes model: in one study it was reported that NO inhibition did not influence afferent arteriolar reaction, while efferent arteriolar reaction was attenuated (55). In an earlier study the same group reported that
responses to NO inhibition were decreased also in diabetic afferent arterioles, as well as in efferent arterioles (49).

Table 2. Concentrations of angiotensin II in the blood (pg/ml) in control or diabetic subjects.

<table>
<thead>
<tr>
<th>C</th>
<th>DM</th>
<th>Species</th>
<th>ref</th>
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<tbody>
<tr>
<td>5.1±3.3</td>
<td>5.1±1.4</td>
<td>human</td>
<td>(3)</td>
</tr>
<tr>
<td>0.023±1.9</td>
<td>0.034±3.8</td>
<td>human</td>
<td>(24)</td>
</tr>
<tr>
<td>0.013±4</td>
<td>0.0115±3</td>
<td>rat</td>
<td>(30)</td>
</tr>
</tbody>
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Moreover, it would also be interesting to know the angiotensin II concentration in the blood, to study whether in diabetic rats this hormone is compensating for a loss of pre- and/or postglomerular reactivity. However, in doing so it should be kept in mind that the kidney has its own renin angiotensin system, and the local angiotensin II concentrations in the kidney can be much higher compared to what is found in the blood (30). Table 7.2 shows concentrations of angiotensin II in diabetic patients or diabetic rats as recalculated from the literature towards the same units. The available data indicate that no major indication exist for a difference in the concentration of angiotensin II between diabetic subjects and controls.

**Diabetes mellitus and PKC-α**

In cultured vascular smooth muscle cells it has been shown that high glucose, to mimic in this system diabetes mellitus, caused membrane translocation of conventional PKCs among which PKC-α (50). Interestingly, our study described in chapter 6 shows that under normal conditions PKC-α is involved in angiotensin II induced constriction of postglomerular arterioles, but
not in that of preglomerular microvessels (figure 6.6). Therefore, it would be of interest to unravel the role of PKC-α in angiotensin II induced vasoconstriction of these renal arterioles also in diabetes mellitus.

**Basal glomerular hemodynamics in control and diabetic rats**

Baseline diameters of interlobular arteries and afferent arterioles were correlated in control as well as in diabetic kidneys (all R>0.6), while they did not correlate with those of the postglomerular efferent arteriole (R<0.3). This was found for the baseline diameters in chapter 3 as well as 4. It is not surprising that preglomerular arteriolar diameters are correlated since they are directly connected to each other, and communication can take place along the vessel wall. However, it is somewhat surprising that the diameter of the efferent arteriole does not (inversely) correlate with that of one or both of the preglomerular arterioles. The glomerular capillary bed is between these vessel types with the afferent arteriole and interlobular artery controlling the inflow resistance whereas the efferent arteriole controls the outflow resistance. Both are supported to act for example during hyperfiltrating conditions in the body when arterial pressure tends to decrease and renal perfusion pressure might

![Graph showing baseline diameters of ILA, AA, and EA](image)

Figure 7.6. Experimental baseline diameters of the preglomerular distal interlobular arteries (ILA) and afferent arterioles (AA), and the postglomerular efferent arteriole (EA) of obese and lean Zucker rats, Sprague-Dawley rats obtained from Harlan (S-D Harlan), Sprague-Dawley rats obtained from Charles-River (S-D Ch-R) and Wistar Kyoto rats (W-K) at 80 mm Hg perfusion pressure. N refers to the number of kidneys analyzed and is mentioned above the graph. *, p<0.05. Values are means±sem and based on the means per animals for each vessel-type.
become compromised. Preglomerular arterioles will dilate due to the myogenic response. Efferent arterioles will vasoconstrict, elevating the glomerular outflow resistance to maintain an adequate filtration pressure (19).

**Obesity and difference in baseline diameter**

Chapter 5 describes our findings of a preglomerular vasodilated and postglomerular vasoconstricted state in obese rat kidneys. Explanations regarding molecular differences between pre- and postglomerular microvessels are also discussed in this chapter. Interestingly, when we look at findings in chapter 6, we can add another explanation. In chapter 6 we found that PKC-α is partially involved in angiotensin II induced constriction in postglomerular arterioles but not in preglomerular microvessels. Moreover, Cooper et al. found that levels of common PKC -among which is PKC-α isozyme, mRNA, protein, and enzyme activity in soleus muscle are decreased in obese Zucker rats (9). PKC is capable of modulating vessel tone (44) and, hence, can influence diameter. Therefore, PKC-α would also be a likely candidate to explain the preglomerular vasodilated and postglomerular vasoconstricted state in obese kidneys.

The baseline differences as observed in chapter 5 seem to be relatively small, but as Poiseuille’s law summarizes: when vessel radius decreases, there is a dramatic drop in flow, as flow is directly related to vessel radius to the fourth power. This illustrates how small changes in vessel radius can have dramatic effects on flow.

Figure 7.6 shows in addition to the baseline diameters of obese and lean Zucker rats, those of two Sprague-Dawley substrains and of Wistar/Kyoto rats. Interestingly, the diameter of the postglomerular efferent arteriole of the obese rat is significantly smaller compared to all other groups. Constriction of the efferent arteriole will increase glomerular pressure (19) and can thereby cause changes in renal hemodynamics. As the postglomerular diameter is smaller in the obese rat compared to all other groups, it is likely that in these animals the glomerular pressure is higher due to a higher outflow resistance. In contrast, preglomerular baseline diameters are significantly larger in Sprague-Dawley and Wistar/Kyoto rats compared to the lean rat, while the preglomerular obese vessels only differ from their lean control.

**Obesity and vessel tone**

In our obese animals fat patches were not only observed in the kidney, but around the aorta as well. In addition, these rats have a very large deposit of abdominal fat. As is already suggested by Yudkin et al., fat deposits around arterioles can have vasoregulatory roles (72) by producing cytokines. For
example, tumor necrosis factor alpha can influence vessel tone of skeletal muscle arterioles (15). Future experiments should unravel whether these fat deposits have a vasoregulatory function also in the kidney. Additionally, it would be interesting to study where exactly the fat deposits are located in the kidney and if these deposits determine other physiological function as well.

**Obesity and renal perfusion**

The left panel in figure 7.7 shows that the total renal perfusion in obese kidneys is equal to the perfusion flow in lean kidneys ($p=0.70$). As is shown in table 5.1, the weight of the hydronephrotic kidney is significantly increased in obese rats compared to lean animals. When renal perfusion flow is corrected for this (right panel figure 7.7), no significant difference is seen between lean and obese kidneys ($p=0.23$), although in obese kidneys the perfusion flow per gram tissue tended to be smaller. In obese patients it was found that the renal plasma flow was increased by 31% (6).

![Figure 7.7. Renal perfusion flow in ml/min (left panel) and ml/min/gr (right panel) in lean ($n=5$) and obese ($n=3$) kidneys of Zucker rats. Values are means±sem.](image)

**Obesity and COX**

One might also believe that the COX-pathway can play a role in obesity. Komers et al. have shown that in 12 week old obese Zucker rats COX-2 is increased while COX-1 is decreased. This increase is paralleled by enhanced excretion of prostanoids in the urine (32).

**Rho-kinase and ways to study its possible involvement in different diseases**

The pivotal role for rho-kinase in renal microvascular constriction, as found in chapter 6, could lead to a potential involvement of rho-kinase in different diseases. One of the pathways that is able to activate rho-kinase is via
the monomer rhoA that after translocation to the cell membrane, and bound to GTP, acts as a molecular switch in cell signaling (57). In this regard, Massey et al. have shown an increased translocation of rhoA to cell membranes in the renal cortex of diabetic rats, indicating an involvement of the rhoA- rho-kinase pathway in renal diabetic injury of the kidney (39). In hypertensive animals administration of the rho-kinase inhibitor Y-27632 significantly decreased the blood pressure (56).

Figure 7.8 shows the effect of rho-kinase inhibition with Y-27632 on angiotensin II-induced diameter changes of interlobular arteries, afferent arterioles and efferent arterioles in control and diabetic kidneys. Adding the rho-kinase inhibitor resulted in a complete dilation of the preglomerular microvessels, and to a lesser extent in the postglomerular microvessels. Furthermore, no difference between control and diabetes could be distinguished indicating that the rho-kinase function itself does not seem to be impaired under diabetic conditions.

![Graph showing effect of Y-27632 on angiotensin II-induced changes in diameters of interlobular arteries (ILA), afferent arterioles (AA), and efferent arterioles (EA) in control and diabetic kidneys.](image)

Figure 7.8. Pilot experiment suggesting no difference in control (square, n=4) as well as diabetic kidneys (round, n=2) regarding the reversal of vasoconstriction by the rho-kinase inhibitor Y-27632 of angiotensin II (AngII) induced vasoconstriction in interlobular arteries (ILA), afferent (AA) and efferent arterioles (EA). Values are means±sem.

Other methods than the one used in our study might also be valuable in studying the involvement of rho-kinase in type 1 diabetes mellitus. The first is a rhoA activity assay (45). In its rested state rhoA resides in the cytosol bound to GDP. When angiotensin II binds to its receptor, rhoA associates with the membrane, and is bound to GTP, where it interacts with rho-kinase to initiate signaling cascades (57). The ratio of membrane bound to cytosolic rhoA can be visualized by a rhoA assay and is an indicator for rhoA activity. A second approach could be to try to activate rho-kinase by lysophosphatidic acid (LPA),
instead of inhibiting it. This makes it possible to study whether the renal microvessels are more sensitive to vasoconstrictor stimuli in the presence of LPA. In the present thesis, we tried to stimulate rho-kinase with LPA in a pilot-experiment. Figure 7.9 illustrates that LPA did not seem to influence the renal vasoconstrictor response to angiotensin II; in the presence of LPA angiotensin II was equally effective in inducing vasoconstriction. However, it can at present not be excluded that a technical problem was in this experiment the cause of this finding. LPA is a lipid, and in surroundings with excess water it can easily stick to the wall of tubing. Our renal perfusion set-up consists of a considerable amount of tubing which could have caused the LPA to remain trapped in the perfusion set-up, instead of flowing into the kidney. A third alternative to study the involvement of rho-kinase in diabetic kidneys could be to use activity assays for myosin light chain (MLC) kinase or myosin phosphatase (51), but these assays prove difficult to set up. Not useful for the study of rho-kinase involvement is monitoring calcium transients with the help of FURA. In rat aortic cells it was found that the inhibition of rho-kinase did not change the increase in calcium concentration as evoked by high extracellular potassium. This is explained by the notion that the site of action of rho-kinase inhibition is not calcium signaling, but the MLC kinase/myosin phosphatase system (51).

Link between different vasoconstrictor stimuli and rho-kinase

Figure 1.3 shows that membrane depolarization leads to an increase in intracellular calcium via opening of L-type calcium channels. Studies in cultured smooth muscle cells have pointed to a role for both an increase in intracellular calcium and rho-kinase activation in membrane depolarization-induced constriction (35, 41, 51, 52, 60, 67), and have suggested phosphatidylinositol 3-kinase (PI3K) as a link between the two (36). The latter has also been indicated by experiments in rabbit basilar artery (40). In addition, it has been shown in intact
medial strips of swine carotid artery that membrane depolarization activates PI3K (60). Moreover, PI3K is involved in angiotensin II-induced vasoconstriction (33, 62), which is known to involve membrane depolarization (53). Membrane depolarization of renal microvessels also depends on activated rho-kinase, as has been shown in chapter 6 of this thesis. Hence, we hypothesized that PI3K can act as a link between an increase in intracellular calcium levels and activation of rho-kinase in renal microvessels. However, preliminary data show that Wortmannin, a PI3K inhibitor, is not capable of inhibiting vasoconstrictor responses due to barium chloride, which causes membrane depolarization, while addition of the rho-kinase inhibitor Y-27632 is still capable of dilating the renal microvascular bed (figure 7.10). From this data we have to conclude that for the renal microcirculation PI3K is probably not an upstream regulator of rho-kinase, as was inferred from the studies performed in cultured vascular smooth muscle cells and other vascular beds.

Methodological considerations

Hydronephrosis

The present study used the hydronephrotic rat kidney as a tool to assess microvascular reactivity. During the onset of hydronephrosis macrophages infiltrate the kidney; they increase renal thromboxane production eliciting vasoconstriction (14, 34). For our studies the hydronephrotic kidneys were used at a duration of at least 6 weeks when tubular atrophy is complete. At this stage, renal thromboxane production has returned to normal levels (61). This is supported by our finding that the thromboxane A2 receptor antagonist SQ29548 had no effect on basal tone (figure 3.5). Moreover, also in the literature...
this has been described for renal afferent arterioles (23). Another argument validating the hydronephrotic kidney for use in the present study is the finding that the overall reactivity to angiotensin II was similar in the hydronephrotic and normal kidney, the angiotensin II dose eliciting halve of the flow decrease (IC$_{50}$ in negative log) being the same in both kidneys ($10^{-10}$ mol/L). In addition, studies performed in our laboratory comparing the reaction to PGE$_2$ of large interlobular arteries from hydronephrotic kidneys and such arteries isolated from normal kidneys showed that the responses to PGE$_2$ were similar (66), excluding a major effect of hydronephrosis on vessel reactivity.

Other characteristics of the hydronephrotic rat kidney model are: a) a decrease in kidney blood flow, but pathological changes in the blood vessels are relatively minor; b) the electrical properties such as membrane depolarization and resting membrane potential appear normal, indicating that the integrity of the vessel wall is well preserved; c) the hydronephrotic kidney is not filtering (58, 59). Yet, despite the disadvantages of diminished flow and no filtering capabilities, various aspects justify its use for microvascular studies, in addition to those mentioned in the previous paragraph. This model offers the advantage of a) studying diameter changes in vitro without interference of systemic and hormonal factors; b) absence of most tubuli makes it possible to study direct effects of various stimuli on the renal vasculature without interference of the tubulo-glomerular feedback system; c) the possibility to perform diameter measurements in a renal vascular bed that is still intact; d) study of vasoreactivity and intracellular signaling in the same preparation. Therefore, for studies of changes in reactivity of the renal microvascular bed the model seems suitable, and, hence, to answer the questions formulated in chapter 1.

Type 1 diabetes mellitus model

It has been said that the streptozotocin (STZ) type 1 diabetes model is characterized by dehydration (345). Figure 3.1 and 4.1 show the water intake during the 4 weeks of diabetes in rats which had free access to water. It shows that after 10 days of partial insulin substitution the water intake decreases considerably and is only slightly (but still significantly) higher compared to the level in controls. Moreover, we found in diabetic rats a normal plasma sodium concentration (chapter 3). Therefore, we can exclude that the rats were dehydrated in our model. Moreover, it has been noted that increased COX-2 expression occurs after dehydration (676). Figure 3.7, shows that in our diabetic rats the COX-2 protein levels were not significantly increased.

STZ was used in our diabetic model to eliminate pancreatic beta-cells. The source of STZ is the fungus Streptomyces. Interestingly, BafA1, an agent
ubiquitously present in soil and some vegetables such as potatoes, also belongs to the streptomyces species. Hence, dietary exposure of humans to a Streptomyces toxin is also possible, and could theoretically cause repetitive pancreatic islet beta cell damage and thus act as a diabetogenic agent. To which extent this happens in practice and contributes to the rising incidence of diabetes mellitus is, however, unknown.

In this thesis possible causes for the glomerular hyperperfusion and hyperfiltration in diabetes mellitus and obesity were studied. The aim was to give further clues as to the development of diabetic nephropathy, with special attention to the renal microvessels involved in the regulation of glomerular hemodynamics. The kidney is, however, a very complex organ and the cause of the problems may not only be the renal microcirculation. Other structures or mechanisms might also play a role like those responsible for the prevention of protein loss in the urine. The glomerular wall acts as a filter which is size and charge-selective. The structures responsible for this comprise the capillary endothelium, glomerular basement membrane, podocytes and tubular cells. Diabetic nephropathy is characterized by an increased loss of albumin in the urine.

The podocyte is the principal cell responsible for prevention of urinary protein loss. When it is damaged, the slits between the pores will enlarge, which will cause not only small molecules but also large molecules like albumin to pass through in larger quantities. These will then be measured in the urine. One factor that can contribute to a damage of podocytes in diabetes mellitus is loss of nephrin, a protein component of the slit membrane. Diabetes mellitus leads to an increased formation of advanced glycation end products (AGEs), for which the kidney is the only elimination organ. AGEs lead to a loss of nephrin and thereby contribute to proteinuria. In addition, loss of podocytes themselves due to the diabetes mellitus may result in progression of proteinuria.

Another cell-type preventing urinary protein loss are proximal tubular epithelial cells. Protein molecules passing through the filtration barrier are reabsorbed in these cells by active transport. This transport has a maximum capacity and if the amount of proteins filtered exceeds this limit, proteinuria will develop. Excess absorption of protein in proximal tubular cells leads to cell damage, release of inflammatory mediators, tubular interstitial fibrosis and finally destruction of the nephron (38, 71). Finally, another contributor could be heparan sulphate. Heparan sulphate is thought to play an important role in the selective properties of the glomerular capillary wall (11). Van den Born et al. have shown a reduction of heparan sulphate-associated anionic sites in the glomerular basement membrane in rats with diabetic nephropathy (65).
Concluding remarks

The studies in this thesis have shown that components of the COX-pathway are important in diabetic dysregulation of renal hemodynamics. Our studies therefore, suggest that a difference in the COX-balance might be one of the possible factors involved in determining whether a diabetic individual will develop nephropathy or not.

Furthermore, the hyperfiltration as found in obese patients might depend among others on a vasoconstricted state of the postglomerular arteriole, which is paralleled by a vasodilated basal state of preglomerular microvessels. Moreover, our studies have shown that rho-kinase plays a pivotal role in renal microvascular constriction not only of preglomerular microvessels, but also of postglomerular microvessels.
References


