Chapter 4

Restoration of attenuated pregglomerular microvascular reactivity to angiotensin II in experimental type 1 diabetes mellitus by aselective COX-inhibition

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Submitted
Abstract

Of all diabetic patients 20-40% will develop nephropathy, which is preceded by glomerular hyperfiltration. An important regulator of preglomerular microvascular resistance is angiotensin II. Moreover, prostanoids are important modulators of renal hemodynamics. The hypothesis addressed in this study is that diabetes can induce a decreased preglomerular vasoconstriction due to an enhanced antagonism by vasodilatory cyclo-oxygenase (COX) derived products. Type 1 diabetic male Sprague-Dawley rats, partially supplemented with insulin, and controls were employed. Isolated hydronephrotic kidneys were used to visualize the interlobular arteries and afferent arterioles.

The vasoconstrictor response to membrane depolarization (induced by KCl) was not different, indicating that the ability to constrict was not impaired. In contrast, diabetes decreased the responsiveness to angiotensin II in the two preglomerular microvessels examined, which could be restored by aselective COX-inhibition with diclofenac. Sensitivity to the major COX-derived prostanoid PGE$_2$ was, however, unchanged. This indicates that an increased production of one or more vasodilatory prostanoids is a possible cause for an attenuated microvascular renal response to angiotensin II in diabetic kidneys.

In conclusion, we suggest that in kidneys of diabetic patients who are prone to develop nephropathy changes in the COX-pathway may occur leading to an increased production of a vasodilatory prostanoid.
Introduction

Despite improvement in the quality of metabolic control, 20-40% of the diabetic patients still develop nephropathy (20, 21). The latter is preceded by a period of hyperfiltration and hyperperfusion resulting from preglomerular vasodilatation. The origin of this preglomerular vasodilatation is not known. Two vascular processes can be involved: one is a decrease in reactivity to stimuli that induce vasoconstriction, e.g. angiotensin II (16), the other an increase in reactivity to dilatory substances, such as prostanoids like prostaglandin E₂ (PGE₂) (13).

Angiotensin II is an important regulator of the diameters of pre- and postglomerular arterioles, and hence, of glomerular hemodynamics (25). Involvement of angiotensin II in the origination of diabetic nephropathy is presumed firstly by the observation of an increased risk for its development in patients with certain angiotensin II type 1 receptor genotypes (8), and secondly by the clinical use of angiotensin converting enzyme inhibitors which delays the loss of kidney function in patients with diabetic nephropathy (22, 38).

Prostanoids are important mediators of renal hemodynamics (11, 12, 28). They are formed when phospholipase A₂ is activated which liberates membrane phospholipids that are converted into arachidonic acid. A complex of cyclooxygenase (COX) and peroxidase converts arachidonic acid into PGG₂ and PGH₂ which in turn is rapidly transformed into more stable prostanoids (36). It is known that there are two COX isoforms (1 and 2) present in the kidney. COX-1 is in this organ the abundant one (36). It produces mainly PGE₂ in the kidney (10). The involvement of products of the COX pathway like PGE₂ in the processes leading to hyperfiltration is contradictory, some studies reported an increase of PGE₂ in the urine of diabetic patients (14), albeit that other studies found decreased (3) or normal (35) PGE₂ levels in the urine of diabetic rats and patients, respectively.

The hypothesis we address in this study is that diabetes mellitus induces a decreased responsiveness of preglomerular microvessels to vasoconstrictor stimuli due to an enhanced antagonism by vasodilatory COX-derived products. The isolated perfused rat hydronephrotic kidney model was used to visualize the preglomerular interlobular arteries and afferent arterioles. Kidneys were harvested from animals made diabetic with streptozotocin, which subsequently received partial insulin substitution; as well as from controls. Two vasoconstrictor stimuli were initially compared: direct cell membrane depolarization, as induced by increasing the extracellular potassium chloride concentration, and angiotensin II. Subsequently, a reduced responsiveness to the
latter in diabetic animals was evaluated regarding a possible involvement of COX derived products and/or a change in reactivity to the COX-product PGE$_2$. 
Materials and/or Methods

Male Sprague-Dawley rats \((n=25, 236.2\pm7.2 \text{ g})\) were obtained from Charles-River (Crl:CD®(SD)BR, Sulzfeld, Germany) and were at random assigned to a control or diabetic group. The animals were housed and handled according to the guidelines of the Institutional Animal Care and Use Committee. Their weight and water intake were assessed daily, while food consumption was determined on a weekly basis. They had free access to water and chow (AM2, Hope Farms, Woerden, the Netherlands). All experiments were performed between day 27 and 31 after streptozotocin (STZ) or buffer injection (see below).

Diabetes mellitus type 1 model

Two weeks after ligating the left ureter (see below), diabetes was induced by a single i.v. injection of 60 mg/kg STZ dissolved in 0.02 mol/L sodium citrate buffer (pH=4.5); control animals received the buffer only. To obtain moderate hyperglycemia, blood glucose was kept on average below 23.6 mmol/L (27) with daily insulin injections (Insulatard, Novo Nordisk, The Netherlands). The amount of insulin given depended on measured blood glucose levels and on water intake, i.e. whether the latter declined to near normal levels. Control animals were daily subjected to the same accompanying handling. Non-fasting blood glucose was determined in samples taken from the tail tip on Monday-, Wednesday- and Friday morning with an OneTouch Ultra meter (LifeScan, The Netherlands).

The percentage glycated hemoglobins (Hba1c) was determined after their separation from non-glycated hemoglobins by boronate affinity chromatography. Subsequently, the non-glycated as well as the glycated fraction was analyzed by HPLC (Primus CLC 385) using the DCCT and IFCC secondary reference method. This was performed in the clinical chemistry laboratory of the Isala hospital (location Weezenlanden, Zwolle, The Netherlands).

Hydronephrotic kidney model and microvascular diameters

To induce unilateral hydronephrosis, the rats were anesthetized with a mixture of isoflurane (3%, Abott), \(\text{O}_2\) (0.76 L/min) en \(\text{N}_2\text{O}\) (1.2 L/min). For pain relief during and after surgery they were injected subcutaneously with Temgesic (0.03 mg/kg Buprenorphine base; Schering-Plough B.V. Amstelveen, The Netherlands). Subsequently, their left ureter was exposed through a small mid-abdominal incision and tied off with a suture. Six to 8 weeks later, tubular atrophy had advanced to a stage that allowed direct visualization of the individual pre- and postglomerular arterioles (23).
Isolation and *in vitro* perfusion of hydronephrotic kidneys have been described in detail elsewhere (30). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.; Sanofi Sante, Maassluis, The Netherlands) and ketamine (25 mg/kg i.m.; Kombivet, Etten-Leur, The Netherlands). First, blood was again taken from the tail tip. Second, the kidney was exposed through a wide abdominal incision. Its renal artery was cannulated via the abdominal aorta and perfusion was started *in vivo*. Perfusion medium consisted of Dulbecco’s Modified Eagle’s Medium Base (DMEM) supplemented with (in mmol/L) 23.8 bicarbonate, 5.5 D-glucose, 1 sodium pyruvate and 5.6 HEPES (Sigma). The medium was equilibrated with 95% air - 5% CO₂ at 37°C. Under continuous single-pass perfusion, the kidney was excised and moved to the stage of an inverted microscope (Axiovert 100; Zeiss, Weesp, The Netherlands) that was equipped with a thin glass viewing port on the bottom surface. Perfusion flow was measured with a transonic flowsensor (1 N, TS410, Transonic systems Inc, Maastricht, the Netherlands). After this kidney had been excised a urine sample was taken to determine urinary protein concentration with the U/CSF assay (Roche diagnostics, Mannheim, Germany), and heart, lung and right kidney were excised to determine wet organ weight.

To visualize renal microvessels, a small region of the transparent cortex of the hydronephrotic kidney was immobilized and transilluminated with a light rod. Distal interlobular arteries (ILA) and afferent arterioles (AA) were visualized by means of a CCD camera (7020/20; Philips, Eindhoven, The Netherlands) and recorded for off-line analysis using a VHS video recorder (RTV 825; Blaupunkt, Hildesheim, Germany). Images were digitized using a computer equipped with an acquisition board (model IVG-128; Datacube, Peabody, MA). Vessel diameters were determined with an automated custom designed program (34). In vessel segments of ~5 µm length the distance between vessel walls was scanned at ~1 Hz intervals for a period of 30 s. Measurements were obtained at each pixel and averaged. Each final value was derived from ~30 of these measurements. ILAs were determined just before the bifurcation of AAs and the latter were measured just after branching from ILAs.

After isolation, kidneys were allowed to equilibrate for at least 60 minutes. Perfusion pressure was monitored at the level of the renal artery and kept constant at 80 mm Hg in all experiments by adjusting the pressure inside the perfusion reservoir. All agents used (see below) are from Sigma and were added from their stock solutions directly to the perfusate in a cumulative way; all concentrations mentioned are those in the perfusate. After having passed the renal vasculature, the perfusate flowed into the tissue bath surrounding the kidney. Thus, agents reached the arterioles from both luminal and abliminal
site. Vessel diameters were measured at baseline and ten minutes after the start of each dose-step, unless otherwise stated.

**Experimental protocols**

**Membrane depolarization and angiotensin II**

In a first series, flow and diameters of ILA and AA were measured at baseline and after the addition of potassium chloride (25 mmol/L, KCl). Following a 30 minute wash out period, in which flow and diameters returned to their baseline value, the kidneys were exposed to an increasing concentration of angiotensin II (0.01 - 1 nmol/L). At the end, the wet weight of the hydronephrotic kidney was determined.

**Diclofenac and PGE₂**

In a second series, flow and diameters of ILA and AA were measured at baseline and after the addition of angiotensin II (0.1 nmol/L). This was followed by a 30 minute wash out period in which flow and diameters returned to their baseline value. Kidneys were then pretreated with diclofenac (1 µmol/L) for 20 minutes. Diclofenac has been reported to inhibit COX-1 with an IC₅₀ value of 76 nmol/L and COX-2 with an IC₅₀ value of 26 nmol/L. Hence, the dose used (1000 nmol/L) will have inhibited both isoforms completely. Subsequently, angiotensin II (0.1 nmol/L) was added followed by increasing concentrations of PGE₂ (0.01 nmol/L-1 µmol/L). Thereafter, wet weight of the hydronephrotic kidney was determined.

**Analysis of data**

All data are expressed as the mean±standard error of the mean (sem). The n-value refers to the number of kidneys examined. Multiple vessels were studied in a kidney, but only the mean values obtained for each vessel type per animal were used. ANOVA or a t-test with Bonferroni correction was performed on the raw data to analyze differences. Plasma glucose and Hba1c levels were analyzed using Mann-Whitney U test. All analyses were performed using Graphpad Prism version 4.02 for Windows, Graphpad Software, San Diego California USA. P<0.05 was considered statistically significant.
Results

Table 4.1. General characteristics of control and diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Control*</th>
<th>Diabetic</th>
<th>P#</th>
</tr>
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<tbody>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose mmol/L</td>
<td>7.12 ± 0.1</td>
<td>19.8 ± 0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hba1c %</td>
<td>3.91 ± 0.0</td>
<td>5.27 ± 0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Protein concentration g/L</td>
<td>1.46 ± 0.4</td>
<td>1.21 ± 0.2</td>
<td>0.57</td>
</tr>
<tr>
<td>Organ weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left hydronephrotic kidney gr</td>
<td>1.15 ± 0.1</td>
<td>1.08 ± 0.1</td>
<td>0.64</td>
</tr>
<tr>
<td>Right normal kidney    gr</td>
<td>2.18 ± 0.1</td>
<td>2.49 ± 0.1</td>
<td>0.008</td>
</tr>
<tr>
<td>Heart gr</td>
<td>1.39 ± 0.0</td>
<td>1.37 ± 0.1</td>
<td>0.78</td>
</tr>
<tr>
<td>Lung gr</td>
<td>1.73 ± 0.0</td>
<td>1.55 ± 0.1</td>
<td>0.07</td>
</tr>
</tbody>
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*, control n=13, diabetic n=12, #, p-value diabetic vs. control rats, + average over the last 3 weeks.

Figure 4.1. Serial measurements of body weight, chow and water intake in control (square, n=13) and 4 week diabetic rats (round, n=12). Diabetic rats were injected with streptozotocin (STZ) on day zero and partial insulin substitution was started at day 3; control animals received the same handling using buffer solution only. Values are means±sem, $, p<0.05 for total dose-response curves of the two groups, *, p<0.05, **, p<0.01, ***, p<0.001 vs. control at same time point. If sem is not visible, it was smaller than bullet size.

Systemic parameters

Directly after STZ-injection diabetic rats started to loose weight. However, after the initiation of insulin supplementation (3 days later, 4.1 ± 0.1 units/day/rat) they again gained weight at a rate comparable to controls, see figure 4.1. Rat chow and water intake increased rapidly after the induction of diabetes, but when partial insulin substitution was started both parameters

74
decreased till a lower albeit still elevated level within 9 days. Table 4.1 shows that the higher blood glucose levels in diabetic rats caused significantly increased Hba1c values. The urinary protein concentration was not different between control and diabetic rats, nor did, left hydronephrotic kidney or heart weight. However, the diabetic rats showed right kidney hypertrophy, while lung weight tended to be lower.

**Baseline microvessel diameter and flow**

![Graphs showing baseline microvessel diameters and flows](image)

Figure 4.2. No effect of diabetes mellitus on potassium chloride (KCl), i.e. cell membrane depolarization, induced changes in the diameter (left two panels) of interlobular arteries (ILA) and afferent arterioles (AA), or in flow (right panel). Control (square, n=9) and diabetic (round, n=4) rats; values are means±sem.

Baseline microvessel diameters were not significantly different between control and diabetic kidneys (table 4.2). The same holds for basal perfusate flow, which was in control 18.8±1.7 and in diabetic kidneys 17.6±1.4 ml/min, (p=0.57).

**Renal vasoconstrictor responses to KCl and Angiotensin II**

Figure 4.2 (two left panels) shows that the vasoconstrictor responses to KCl-induced cell membrane depolarization were not different between control and diabetic preglomerular microvessels. Likewise, the flow decrease in response to this stimulus was of similar magnitude in control and diabetic kidneys (figure 4.2, right panel).

In contrast, diabetes decreased the responsiveness to angiotensin II in the two preglomerular microvessels.

| Table 4.2. Baseline diameters (in µm) of distal interlobular arteries (ILA) and afferent (AA) arterioles in control and diabetic rat kidneys perfused at 80 mm Hg. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Control (n=13)  | Diabetic (n=12) | p               |
| ILA              | 25.95 ± 0.4     | 25.67 ± 0.7     | 0.73            |
| AA               | 18.94 ± 0.4     | 18.95 ± 0.5     | 0.99            |
examine (figure 4.3; left two panels). In diabetic kidneys the concentration-response curves had shifted to the right as compared to control, indicating that their ILAs and AAs were less sensitive to angiotensin II. The change in renal flow due to angiotensin II (figure 4.3, right panel) showed in diabetic kidneys a comparable shift.
Recovery in diabetic kidneys of the attenuated response to angiotensin II by inhibiting cyclo-oxygenases

Figure 4.4 shows that in diabetic kidneys, diclofenac restored the impaired responsiveness to angiotensin II in both preglomerular microvessels. This was paralleled by a similar restoration of the decreased flow (figure 4.4, right panel). In control rats diclofenac did not significantly affect angiotensin II responsiveness.

Sensitivity to the major COX-derived prostanoid PGE\(_2\) is not changed in diabetic kidneys

To evaluate whether in addition to an increased renal production of vasodilatory COX-derived prostanoids in diabetes also changes in microvascular reactivity to such substances might play a role, the sensitivity to PGE\(_2\) was studied. Increasing concentrations of PGE\(_2\) dilated the preglomerular arterioles, preconstricted with angiotensin II and pretreated with diclofenac to inhibit the endogenous prostanoid production. However, no difference between control and diabetic kidneys could be observed. Moreover, no differences in changes in perfusion flow due to an increasing PGE\(_2\) concentration could under these circumstances be established between control and diabetic kidneys (right panel figure 4.5).

![Figure 4.5](image_url). No difference in sensitivity to PGE\(_2\) between control (square, n=5) and diabetic (round, n=6) kidneys under preconstriction with AngII (0.1 nmol/L) and following pretreatment with diclofenac (1µmol/L). The latter was given in a dose blocking both COX-1 and COX-2 completely. Relative changes in diameter (two left panels) of interlobular arteries (ILA) and afferent arterioles (AA) and in perfusion flow (right panel). Responses expressed as percentage change from baseline. Values are means±sem.
Discussion

Using an experimental type 1 diabetic model we found no difference with controls in baseline diameter of preglomerular arterioles and in renal perfusate flow. Furthermore, the responsiveness to membrane depolarization evoked by increasing the KCl concentration was not disturbed, indicating that the ability of preglomerular vessels to constrict is not impaired. However, vasoconstriction as induced by the receptor mediated stimulus angiotensin II was attenuated. The inhibition of COX restored in diabetic kidneys the responsiveness to angiotensin II, while this had no effect in control kidneys. This indicates that an increased production of vasodilatory prostanoids can be involved in the attenuated response to angiotensin II due to diabetes mellitus. This is strengthened by the finding that no difference between control and diabetic kidneys existed in sensitivity to the major renal COX-derived prostanoid PGE$_2$.

The present study shows that in diabetic kidneys an attenuated responsiveness to angiotensin II can be corrected by COX-inhibition, indicating that vasodilatory prostaglandins are involved. In the kidney two downstream products of COX are known which have dilatory effects: PGE$_2$ and PGI$_2$ (25). Our results show a normal responsiveness to the major downstream COX-product in the kidney, PGE$_2$, when this was added exogenously. This indicates that diabetic renal microvessels are not more sensitive to PGE$_2$, making an increase in the production of PGE$_2$ and/or PGI$_2$ more likely. This could be due to an increase in the amount of COX-protein present in diabetic kidneys, which would lead to more production of downstream COX-products, dependent on the presence and amount of subsequent more specific synthases. Another study, which observed in diabetic hydronephrotic kidneys an attenuated response to pressure, found that aselective COX-inhibition could restore the altered responsiveness (13). Subsequently, they showed that exogenously added PGE$_2$ could inhibit pressure-induced vasoconstriction in a concentration-dependent manner (13). That study, therefore, also showed an involvement of COX-derived prostanoids in a disturbed vasomotor response of preglomerular microvessels in diabetes mellitus.

The reduced reaction to angiotensin II in our type 1 diabetic rats could be corrected with the aselective COX-inhibitor diclofenac. In type 1 diabetic patients the aselective COX-inhibitors indomethacin and aspirin were found to reduce an increased glomerular filtration rate (9, 14). However, two other studies found no effect of indomethacin (6, 18), while all studies reported hyperfiltration in these diabetic patients. The involvement of COX-derived products in the processes leading to diabetic nephropathy therefore may vary between individuals and/or groups.
In this study moderate hyperglycemia was induced in rats with a high dose of streptozotocin (STZ) followed by 4-weeks of partial insulin substitution. This yielded an attenuated responsiveness to angiotensin II in renal preglomerular microvessels. In the literature, however, the renal responsiveness to angiotensin II in rat diabetic models induced by STZ showed diverse results with studies demonstrating a normal (1, 2, 32, 37), decreased (16, 17, 31) or increased (4, 19, 37) reaction. Differences in experimental set up like the duration of the diabetes and the control of hyperglycemia might explain some of the differences but not all, indicating that there may be a multifactorial origin and/or variance in compensatory reactions (see below).

In another study we observed, using the same diabetic model and with animals from the same supplier, that the reaction to angiotensin II in the smallest pre- and postglomerular microvessels could be similar to control (29). Also creatinine clearance, as an estimate of the glomerular filtration rate, was not different between control and diabetic rats in that study. In the present study the characteristics of the diabetic model were identical (e.g. blood glucose levels, Hba1c, weight etc), while now the reaction to angiotensin II was significantly decreased in the diabetic group. The reason for the discrepancy with our previous observation is not clear, but might be related to variations in functioning of the COX-pathway. In the present study the diabetic rats had a reduced reactivity to angiotensin II which could be restored by COX inhibition. In the other study, however, we observed that the COX-2 isoform could compensate for a reduced reaction to angiotensin II in diabetes via an increased thromboxane A₂ production. The latter is a vasoconstrictive prostanoid. Therefore, an altered COX-balance could lead to different vulnerabilities, and, hence, variation in the origination of diabetic nephropathy.

A potential consequence of the loss of preglomerular reactivity to angiotensin II in type 1 diabetic patients could be hyperfiltration, which is seen in 20-40% of them. In the present study, we did not measure glomerular filtration rate. The majority of publications that reported glomerular filtration rate, observed hyperfiltration in the diabetic state (e.g. (1, 3, 7, 19)). Nevertheless, there are also some studies reporting a normal glomerular filtration rate (20, 24, 33, 37) as we did in the study where COX-2 derived thromboxane A₂ compensated for a reduced reaction to angiotensin II (see above). We therefore suggest that differences in activity of the COX-pathway are one of the factors determining which diabetic patients will develop glomerular hyperfiltration or not.

Our study shows that the reaction to membrane depolarization as evoked by increasing the extracellular KCl concentration was not changed. This indicates that the basic ability to constrict is not impaired in preglomerular arterioles of
diabetic kidneys. Membrane depolarization is a physiological component of many signaling pathways (26). On the other hand, Carmines et al. showed that in diabetic rats the reaction to KCl was less in afferent arterioles and that a diminished function of voltage gated calcium channels was involved (4, 5). The same group also found increases with diabetes in both the functional availability and basal activation of $K_{ATP}$ channels, which tend to promote afferent arteriolar vasodilatation (15). These three studies have been performed at two weeks of diabetes mellitus duration while our study was performed at 4 weeks. However, in addition to a difference in diabetes duration other factors may have been involved as well.

In conclusion, our data indicate the involvement of a vasodilatory COX-product in diabetic renal microvessels in their reduced reaction to angiotensin II. We suggest that in kidneys of diabetic patients who will ultimately develop diabetic nephropathy, changes in the COX-pathway occur leading to an increased production of a vasodilatory prostanoid.

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References


