Increased production of thromboxane A$_2$ via the COX-2 pathway contributes to normal renal microvascular responsiveness to angiotensin II in experimental diabetes

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Submitted
Abstract

Why 20-40% of the diabetic patients develop nephropathy is currently unknown. The present study was performed to investigate the involvement of COX-2 derived prostanoids in renal microvascular responsiveness to angiotensin II. To this end we investigated in a 4 week type 1 diabetic rat model, partially supplemented with insulin, diameter changes of distal interlobular arteries, afferent and efferent arterioles, employing isolated perfused hydronephrotic kidneys.

A normal creatinine clearance was found and, in accordance with this, periglomerular microvessels showed an unchanged responsiveness to angiotensin II. However, pretreatment with a selective COX-2 inhibitor attenuated the vasoconstrictor effects of angiotensin II in diabetic kidneys but not in control. Protein levels of thromboxane synthase were increased in diabetic kidneys. Blockade of the thromboxane A$_2$ receptor reduced in diabetic kidneys renal vasoreactivity to angiotensin II to a similar extent as COX-2 inhibition.

In conclusion, the present study demonstrates in diabetic kidneys an adaptation of the COX-2/thromboxane synthase pathway, which results in an unaltered glomerular filtration rate, as well as an unchanged ability of periglomerular arterioles to respond to angiotensin II. Our finding warrants caution regarding the prescription of Cox-2 inhibitors in diabetic patients, even when kidney function is still normal, to avoid disturbing this new balance.
Introduction

Patients with type 1 diabetes mellitus will develop diabetic nephropathy in about 20-40% of the cases (18, 19). Why some patients are nephropathy-prone, while others are not, is at present unknown. Involvement of angiotensin II is indicated by the clinical use of angiotensin converting enzyme inhibitors which lead to a delay in the loss of kidney function in patients with diabetic nephropathy (21, 41). In addition, an increased risk for the development of diabetic nephropathy has been noted in carriers of the C1166 allele of the angiotensin II type 1 receptor (10).

Angiotensin II is an important regulator of the diameters of pre- and postglomerular arterioles and, hence of glomerular hemodynamics (25). In addition, it can stimulate the synthesis and release of various prostanoids (24, 31). These products of the cyclo-oxygenase (COX) pathway also play an important role in renal and glomerular hemodynamics (11, 12, 27). The COX-1 isoform is expressed constitutively in most tissues, while in the kidney this is also the case for COX-2 (15, 37). Involvement of COX-2 in diabetic nephropathy is indicated by studies in rats (7, 20). In the often used streptozotocin (STZ)-induced diabetic rat model several studies have focused on angiotensin II, yielding results varying from an increase in microvascular reactivity (6), no effect (6, 30) to a decreased reactivity (13, 14). However, so far a possible relationship to the COX-2 pathway has not been investigated. Moreover, in none of the above-mentioned studies, glomerular filtration rate has been measured.

In the present study we investigated in the STZ-induced rat diabetic model a possible effect of COX-2 inhibition on angiotensin II-induced vasoconstriction in pre- and postglomerular microvessels. In addition, we determined in these animals general parameters of renal function such as creatinine clearance as an estimate of the glomerular filtration rate. Interestingly, we noted that COX-2 inhibition unmasked a decreased microvascular responsiveness to angiotensin II in the diabetic animals only, which was compensated for by an increased COX-2 derived production of the prostanoid thromboxane A$_2$. 

Thromboxane A$_2$ and Cox-2 in diabetic kidneys
**Materials and/or Methods**

Male Sprague-Dawley rats ($n=47$, Charles-River, Crl:CD®(SD)BR, Sulzfeld, Germany) were randomly assigned to a control or diabetic group, and within each group to the hydronephrotic model ($n=32$) or use without hydronephrosis. The latter was employed among others for characterization of renal function with metabolic cages. They were housed individually and handled according to the guidelines of the Institutional Animal Care and Use Committee. Weight and water intake were monitored daily, and food consumption weekly. Rats without hydronephrosis were placed for 24 hours in a metabolic cage on day 27 after diabetes induction (see below), or the concomitant time in controls; on day 31 their renal flow was measured. In hydronephrotic kidneys, experiments were performed between day 27-31. The rats received standard chow (AM2, Hope farms, Woerden, The Netherlands) and water *ad libitum*.

**Diabetes mellitus type 1 and metabolic cage experiments**

Diabetes was induced by a single i.v. injection of 60 mg/kg STZ; control animals received the 0.02 mol/L sodium citrate buffer (pH=4.5) only. In the hydronephrotic group this injection was given two weeks after ligating the left ureter (see below). To obtain moderate hyperglycemia, blood glucose was kept on average below 23.6 mmol/L (26) with daily insulin injections (Insulatard, Novo Nordisk, The Netherlands). Its dose depended on measured blood glucose levels and water intake, i.e. whether the latter declined to near normal levels. Control animals were daily subjected to the same accompanying handling. Blood glucose levels were determined 3 times a week with a OneTouch Ultra meter (LifeScan, The Netherlands) in samples from the tail tip. The percentage glycated hemoglobins (Hba1c) was determined after their separation from non-glycated hemoglobins by boronate affinity chromatography. Subsequently, both fractions were analyzed by HPLC (Primus CLC 385) using the DCCT and IFCC secondary reference method.

Urine of rats without hydronephrosis was collected with metabolic cages (diabetic $n=8$, control $n=7$). At the end a blood sample was taken from the tail vein (~200 µl). Plasma and urinary creatinine were determined using the Jaffé method (Roche diagnostics, Mannheim, Germany), and sodium (plasma and urine) concentration with an ion selective electrode (Roche diagnostics). Total urinary protein was obtained with the U/CSF protein assay (Roche diagnostics) and osmolality by freezing-point depression (Fiske associates Norwood, MA, USA). Subsequently, creatinine clearance and fractional sodium reabsorption were calculated (34).
Isolated perfused hydrenephrotic or non-hydrenephrotic kidney

To make one kidney hydrenephrotic, rats were anesthetized with a mixture of isoflurane (3%, Abbott), O$_2$ (0.7 L/min) en N$_2$O (1.2 L/min). For pain relief during and after surgery they received Temgesic subcutaneously (0.03 mg/kg Buprenorfine 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 renal microvessels (32).

For in vitro perfusion of hydrenephrotic or non-hydrenephrotic kidneys (28), the 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). The left kidney was exposed through a wide abdominal incision. Its renal artery was cannulated via the abdominal aorta and perfusion 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 (all Sigma). The medium was equilibrated with 95% air - 5% CO$_2$ at 37°C. Under continuous single-pass perfusion, the kidney was excised and moved to an organ bath with a thin glass viewing port on its bottom surface. Perfusion pressure was monitored in the renal artery and kept constant at 80 mm Hg. Renal flow was measured with a flowsensor (1 N, TS410, Transonic systems Inc, Maastricht, The Netherlands). The isolated kidneys were allowed to equilibrate for at least 60 minutes; all preparations possessed myogenic reactivity (data not shown). After the kidney had been excised a urine sample was taken to determine its protein concentration. Heart, lung and right kidney were excised as well to determine wet organ weight, which in the left kidney was done after the angiotensin II experiments (see below).

Visualization of microvessels in a hydrenephrotic kidney

The organ bath was placed on an inverted microscope (Axiovert 100; Zeiss, Weesp, The Netherlands). A small region of the transparent cortex of the hydrenephrotic kidney was immobilized and transilluminated with a light rod. Distal interlobular arteries (ILA), afferent (AA) and efferent arterioles (EA) were visualized with an objective lens (Zeiss, 40X, NA 0.60) using a CCD camera (7020/20; Philips, Eindhoven, The Netherlands). Images were recorded for off-line analysis with a VHS video recorder (RTV 825; Blaupunkt, Hildesheim, Germany), and digitized for diameter measurements using a computer equipped with an acquisition board (model IVG-128; Datacube, Peabody, MA, USA). In
vessel segments of ~5 µm length the distance between vessel walls was scanned at ~1 Hz intervals for a period of 30 s at each pixel and averaged with an automated custom designed program (22). ILAs were measured just before the bifurcation of AAs, AAs just after branching from ILAs and EAs within 50 µm of the point where they emerged from the glomeruli.

**Renal reactions to AngII**

In non-hydronephrotic kidneys flow was measured at baseline and during a concentration response curve to angiotensin II (Sigma) added cumulatively to the perfusion medium, 0.01 - 1 nmol/L. Flow was measured ten minutes after the addition of each new concentration.

In hydronephrotic kidneys the same was done, while in addition the diameters of ILAs, AAs and EAs were determined. Thereafter they returned to their initial baseline values during a wash-out period of 1 hour. Then, the cumulative angiotensin II concentration response protocol was repeated starting 20 minutes after addition to the perfusion medium of either a selective COX-2 inhibitor or a selective thromboxane A₂ receptor antagonist. The latter, SQ29548 (Biomol, Heerhugowaard, The Netherlands), was used at 1 µmol/L. Selective COX-2 inhibition was achieved with 10 µmol/L NS398 (Sigma, initially dissolved in DMSO). As reported by the supplier, this substance inhibits COX-2 with an IC₅₀ value of 3.8 µmol/L while COX-1 activity remains unaffected at concentrations up to 100 µmol/L.

**Western blot analysis of hydronephrotic kidneys**

In a separate set of experiments, 4 diabetic and 4 control hydronephrotic kidneys were isolated as previously described and snap-frozen in liquid nitrogen. Samples were homogenized and dissolved in lysis buffer. Subsequently, the proteins were separated by gel electrophoresis and blotted onto a nitrocellulose membrane (semi dry blotting from Biorad). Fifty, 100 and 150 µg of protein from each sample was loaded in a lane. Staining of COX-2, COX-1 or thromboxane synthase were in this order performed on the same blot with a specific primary antibody (all 1:750 dilution, polyclonal, host rabbit; Cayman Chemicals). Crossreactivity of the COX-1 and COX-2 antibodies could not be detected at the concentrations used (1). Proteins were visualized with a chemiluminescence kit (Amersham), and quantified using a CCD camera (Fuji Science Imaging Systems) in combination with AIDA Image Analyzer software (Isotopenmessgeräte; Staubenhardt, Germany). The samples with 150 µg of protein were used for statistical analysis after dividing each protein amount by the mean of the controls; the other two concentrations showed a similar result.
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. Since multiple vessels were studied in a kidney, the mean values obtained for each vessel type per animal were used. ANOVA or a students 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. The areas under the angiotensin II concentration response curves (AUC) were measured, and the difference calculated between the one obtained under COX-2 inhibition or thromboxane A2 receptor blockade versus the corresponding untreated curve (delta AUC) by deducting the first from the latter. Delta AUC from the two treatment groups were subsequently analyzed with a students t test. All these analyses were performed using Graphpad Prism version 4.02 for Windows (Graphpad Software, San Diego California USA). P<0.05 was considered statistically significant.

Figure 3.1. Serial measurements of body weight, chow consumption and water-intake in hydronephrotic control (square, n=15) and diabetic rats (round, n=17). In the latter, injection of streptozotocin to induce diabetes (at t=0) was followed by partial insulin substitution starting at day three (black box); control animals received the same handling using buffer solution only. Values are means±sem. $, p<0.05 for total curves of the two groups; *, p<0.05, **, p<0.01 and ***, p<0.001 for diabetic vs. control rats at the corresponding time point. If sem is not visible, it was smaller than bullet size.
Results

Table 3.1. General characteristics of control and diabetic hydronephrotic rats. Control n=15, diabetic n=17 except for the urine data where both control values are n=11, and diabetic n=15 and 13, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>P†</th>
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<tbody>
<tr>
<td>Blood</td>
<td></td>
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<tr>
<td>Glucose‡</td>
<td>7.03 ± 0.10</td>
<td>20.86 ± 0.69</td>
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<tr>
<td>Hba1c</td>
<td>3.91 ± 0.05</td>
<td>5.31 ± 0.13</td>
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<td>Urine</td>
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<tr>
<td>Protein concentration</td>
<td>3.00 ± 0.94</td>
<td>1.90 ± 0.55</td>
<td>0.29</td>
</tr>
<tr>
<td>Osmolality</td>
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<td>803.6 ± 59.8</td>
<td>0.29</td>
</tr>
<tr>
<td>Organ weight</td>
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<td></td>
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<tr>
<td>Hydronephrotic kidney</td>
<td>1.33 ± 0.08</td>
<td>1.43 ± 0.19</td>
<td>0.67</td>
</tr>
<tr>
<td>Right normal kidney</td>
<td>2.15 ± 0.07</td>
<td>2.35 ± 0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>Lung</td>
<td>1.64 ± 0.04</td>
<td>1.55 ± 0.04</td>
<td>0.14</td>
</tr>
<tr>
<td>Heart</td>
<td>1.51 ± 0.06</td>
<td>1.36 ± 0.04</td>
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† P-value diabetic vs. control. ‡ Average over the last 3 weeks.

Figure 3.1 shows that following STZ-injection the diabetic rats lost weight (left panel) but that partial insulin substitution (started 3 days later, 3.8±0.1 U/day) reversed this to a weight gain similar to that of controls, while chow consumption was higher (middle panel). The same was seen in non-hydronephrotic rats (data not shown, insulin dose 3.3±0.3 U/day). In diabetic rats hardly or no body fat was noted during autopsy, indicating this as an important determinant of the difference in body weight. Diabetic rats became at first markedly polydipsic (figure 3.1, right panel), but partial insulin substitution decreased this to a lower but still elevated level within 10 days; in non-hydronephrotic diabetic rats a similar pattern was seen (data not shown). Table 3.1 presents the increased blood glucose and Hba1c levels in the hydronephrotic diabetic group; a similar picture was seen in non-hydronephrotic rats. Table 3.1 also shows for hydronephrotic rats that the diabetics did not significantly differ in urinary protein concentration or osmolality (middle part).
Table 3.2. Baseline diameters (in µm) at 80 mm Hg renal perfusion pressure of distal interlobular arteries (ILA), afferent (AA) and efferent arterioles (EA) from control (C) and diabetic rats (DM) in the initial (untreated) situation, in the presence of the Cox-2 inhibitor NS398 or with the thromboxane A$_2$ (TxA$_2$) receptor antagonist SQ29548.

<table>
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<th>ILA</th>
<th>AA</th>
<th>EA</th>
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<tr>
<td>Untreated</td>
<td>C</td>
<td>27.7±0.9</td>
<td>20.3±0.7</td>
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<td></td>
<td>DM</td>
<td>27.7±0.7</td>
<td>19.7±0.5</td>
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<td></td>
<td>n=11</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>n=13</td>
</tr>
<tr>
<td>Cox-2 inhibited</td>
<td>C</td>
<td>28.4±1.1</td>
<td>21.8±1.3</td>
</tr>
<tr>
<td></td>
<td>DM</td>
<td>27.3±1.6</td>
<td>21.0±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=6</td>
<td>n=6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n=7</td>
</tr>
<tr>
<td>TxA$_2$ receptor antagonist</td>
<td>C</td>
<td>27.3±0.4</td>
<td>19.3±0.2</td>
</tr>
<tr>
<td></td>
<td>DM</td>
<td>28.7±1.1</td>
<td>20.3±1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=5</td>
<td>n=6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>n=6</td>
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</table>

Normal creatinine clearance and no proteinuria in this diabetic model

Creatinine clearance was the same in control and diabetic non-hydronephrotic rats (2.47±0.17 vs. 2.48±0.13 ml/min respectively, p=0.95). Also when corrected for total kidney weight (see below), no difference was seen (0.61±0.05 vs. 0.61±0.04 ml/min/gr kidney weight respectively, p=0.92). Urine production of the diabetic rats was significantly increased (23.8±2.08 vs. 17.0±1.41 ml/24 hours in controls, p=0.02), but total protein excretion did not differ (0.022±0.002 g/24 hours in both groups, p=0.99). Fractional sodium reabsorption, a marker for tubular integrity, was not changed (314.1±25.1 vs. 313.2±29.8 mmol/min in controls, p=0.98) and plasma sodium concentration was
normal (127±7 vs. 128±8 mmol/L in controls, p=0.96), indicating that diabetic rats were not dehydrated.

**Unaltered renal microvascular diameters at baseline and with AngII**

Figure 3.2 shows that diabetes did not affect vasoreactivity to angiotensin II in all 3 types of microvessels. This was reflected in a similar angiotensin II-induced flow-reduction in both control and diabetic kidneys (figure 3.2 right panel), as was also observed in non-hydronephrotic kidneys (data not shown). Initial perfusion flow did not differ significantly between diabetic and control hydronephrotic kidneys (20.1±2.7 vs. 16.4±1.2 ml/min/gr, p=0.23). Table 3.2 (top row) shows that the initial (untreated) baseline diameters for all three renal microvessels did not differ between diabetic and control kidneys.
Selective Cox-2 inhibition attenuates the vasoconstrictor effects of AngII in diabetic kidneys but not in controls

In diabetic kidneys, but not in control kidneys, COX-2 inhibition caused a reduced angiotensin II-induced vasoconstriction in all three types of microvessels (figure 3.3). The same was seen in the angiotensin II-induced flow decrease (right panels). Baseline diameters (table 3.2, second row) in control and diabetic kidneys were not affected by COX-2 inhibition in all 3 types of microvessels examined. This was paralleled by a nearly identical basal flow as compared to the initial (untreated) situation (see above) in COX-2 treated diabetic (22.8±5.2 ml/min/gr, $p=0.61$) and non-diabetic kidneys (19.3±4.0 ml/min/gr, $p=0.20$).

Diabetes mellitus did not significantly change renal Cox-2 and Cox-1 protein levels but thromboxane synthase content increased

COX-2 or COX-1 protein levels in the hydronephrotic kidneys were not significantly influenced by the diabetes (figure 3.4, $p=0.49$ and 0.12, respectively). Interestingly, thromboxane synthase levels, which is a downstream mediator from COX, were significantly increased by diabetes ($p=0.03$, figure 3.4).

Thromboxane A$_2$ receptor blockade reduces in diabetic kidneys renal vasoreactivity to AngII to a similar extent as Cox-2 inhibition

In diabetic kidneys, thromboxane A$_2$ receptor blockade remarkably decreased angiotensin II-induced constriction in all 3 types of microvessels, which was paralleled by a similar decrease in flow (figure 3.5, upper panel). Figure 3.6 (upper panel) shows that the difference in AUC as compared to the untreated situation, i.e. area in between two concomitant concentration response curves in for example the upper panels of figure 3.3 and 3.5, is in diabetics for COX-2 inhibition (solid bars) not different from that under thromboxane A$_2$ receptor blockade (open bars) for all three microvessels examined as well as for the flow induced changes.
In control kidneys, thromboxane A2 receptor blockade also attenuated the vasoconstriction and flow decrease due to angiotensin II (figure 3.5, lower panel). However, in contrast to the diabetic group, this attenuation due to thromboxane A2 receptor blockade was significantly different from the concentration response curves obtained with COX-2 inhibition, as is indicated by figure 3.6 (lower panel).

No effect of thromboxane A2 receptor blockade was seen on baseline diameters of ILA, AA and EA in both control and diabetic kidneys (table 3.2, third row). In addition, basal flow was not affected by the thromboxane A2 receptor antagonist in diabetic (19.8±4.3 ml/min/gr, \( p=0.95 \)) or control (16.2±1.3 ml/min/gr, \( p=0.93 \)) kidneys.

Figure 3.4. Western blot analysis of COX-2, COX-1 and thromboxane synthase protein levels in control (\( n=4 \)) and diabetic hydronephrotic kidneys (\( n=4 \)). Photos above the graph show representative examples. The same 6 lanes are displayed for the different proteins. The lanes containing 150 µg of protein extract were used for the graph in the lower panel, presenting the protein amount divided by the mean value of the controls (COX-2 \( p=0.49 \), COX-1 \( p=0.12 \) and thromboxane synthase \( p=0.03 \) vs. control).
Organ weights

Table 3.1 shows that the weight of the hydronephrotic (left) kidney did not differ between diabetes and control, being on average in both 57% of the normal kidney weight. Right kidney weight (table 3.1) was 1.43 (diabetes) and 1.45 (control) times that in non-hydronephrotic animals. In the latter total kidney weight did not differ significantly between diabetes (2.1±0.1 g) and control (1.9±0.1 g, \( p=0.41 \)). The weight of the heart was less in diabetic animals (table 3.1), while lung weight did not differ (table 3.1).

![Graph showing the effects of angiotensin II on kidney weight](image_url)

Figure 3.5. Selective blockade of the thromboxane A2 receptor in diabetic (upper panel) and control (lower panel) hydronephrotic kidneys attenuates angiotensin II-induced changes in the diameter of distal interlobular arteries (ILA), afferent (AA) and efferent arterioles (EA), or flow (right panel). Number of ILA, AA and EA being 26, 44 and 24 in untreated diabetic kidneys (open round solid line, \( n=13 \)) and 12, 18 and 12 in those treated with the thromboxane A2 antagonist was used (closed round dashed line, \( n=6 \)). Number of ILA, AA and EA being 20, 38 and 17 in untreated controls (open square solid line, \( n=11 \)) and 9, 19 and 9 in controls treated with the thromboxane A2 antagonist (closed square dashed line, \( n=5 \)). $, \ p<0.05$ for total concentration response curves of the two groups; *, \( p<0.05 \) for thromboxane A2 receptor antagonist vs. untreated at the corresponding point. Values are mean±sem and based on the means per animals for each vessel-type. If sem is not visible, it was smaller than bullet size.
Figure 3.6. Comparison of the differences in area under the curve (delta AUC) between the concentration response curve obtained in the presence of the COX-2 inhibitor (black bars), or the thromboxane A2 receptor antagonist (open bars), with their concomitant control curve (see figures 3.3 and 3.5) in interlobular arteries (ILA), afferent (AA) and efferent arterioles (EA), or flow (right panel), in diabetic (upper panel) and control kidneys (lower panel). *, p<0.05 vs. COX-2 inhibition.
Discussion

The present study shows that in type 1 diabetic rats with a normal creatinine clearance, no proteinuria and normal diameters of the pre- and postglomerular arterioles, selective COX-2 inhibition attenuated the initially normal renal vasoconstriction to angiotensin II. In control animals it did not do so. COX-2 or COX-1 protein levels were not significantly changed in the diabetic animals, but those of thromboxane synthase, an enzyme downstream from COX in the prostanoid cascade, were increased. Indeed, in diabetic kidneys selective thromboxane A$_2$ receptor blockade could mimic the effects of COX-2 inhibition. Hence, our findings indicate that normality of the glomerular hemodynamics present in these diabetic kidneys was achieved by an adaptation of the COX-2/thromboxane synthase pathway, which kept the microvascular angiotensin II-induced reactivity unaltered.

In our diabetic model, creatinine clearance was normal as measured in awake non-hydronephrotic rats. The same has been reported by several other studies (18, 23, 35, 40). Wilkes et al. reported hyperfiltration in diabetic rats studied at 1 week, however, from 2-12 months on glomerular filtration rate was elevated only in about half of the cases. They observed no difference in mean systemic blood pressure between non-hyperfiltering and hyperfiltering diabetic rats while the latter had a significantly increased renal plasma flow (40). This suggests that the cause may be located in the periglomerular arterioles. Indeed, in our non-hyperfiltering diabetic rats no differences with controls were observed in renal flow and microvascular diameters in the absence or presence of angiotensin II. Nevertheless, the majority of publications that report on glomerular filtration rate, observed hyperfiltration in the diabetic state (e.g. 4, 5, 9, 17). Parameters like anesthetics, level of hyperglycemia, partial insulin substitution, diabetes duration or rat strain are not able to account for the difference why some studies report hyperfiltration and others do not, since their range varies widely in the literature suggesting a multifactorial etiology.

The present study shows that in a diabetic model with as yet normal glomerular hemodynamics, selective inhibition of COX-2 attenuated the vasoconstrictor effects of angiotensin II. This observation warrants caution regarding the prescription of selective COX-2 inhibitors to diabetic patients, even when their kidney function is still normal to avoid disturbing this new balance.
Both COX-2 and COX-1 protein levels were not significantly changed by PLA₂. Endoperoxides, Prostacyclin synthase, PGD synthase, PGE synthase, Thromboxane synthase, Prostacyclin (PGI₂), PGD₂, PGE₂, Thromboxane (TxA₂), Vessel tonus, dilatation, constriction, AngII, Receptor PLC, G₁₁, Angiotensin II (AngII), PLC phospholipase C; PIP₂, phosphatidylinositol 4,5-biphosphate; IP₃, inositol 1,4,5-triphosphate; DAG, 1,2-diacylglycerol and PKC protein kinase C; PLA₂, phospholipase A₂; AA arachidonic acid; PG prostaglandin (for I₂, D₂, and E₂).

Figure 3.7. Schematic overview of the cyclo-oxygenase (COX) pathways in vascular smooth muscle cells and how angiotensin II (AngII) can activate this balance (solid arrows). PLC phospholipase C; PIP₂, phosphatidylinositol 4,5-biphosphate; IP₃, inositol 1,4,5-triphosphate; DAG, 1,2-diacylglycerol and PKC protein kinase C; PLA₂, phospholipase A₂; AA arachidonic acid; PG prostaglandin (for I₂, D₂, and E₂).
Both COX-2 and COX-1 protein levels were not significantly changed by the diabetes in the hydronephrotic kidneys used in our study. By contrast, levels of thromboxane synthase, an enzyme downstream from COX in the prostanoid cascade (see figure 3.7), were increased. This indicates that the effect of selective COX-2 inhibition on microvascular reactivity to angiotensin II in diabetics was brought about by abolition of an extra COX-2 derived supply of endoperoxides to thromboxane synthase. Our findings in control animals show that thromboxane A$_2$ is involved in angiotensin II-induced renal microvascular constriction (see figure 3.5), as has also been indicated by others (29, 36, 38). In addition, we showed that the endoperoxides needed for the normal synthesis of thromboxane A$_2$ are not derived from COX-2 (figure 3.3, lower panel). To all likelihood they are supplied to the thromboxane synthase by COX-1. By contrast, in the diabetic animals also COX-2 must have contributed endoperoxides to the thromboxane synthase (figure 3.3, upper panel), thereby allowing for an increased production of thromboxane A$_2$ needed to keep the angiotensin II reactivity normal. This is corroborated by our finding that thromboxane A$_2$ receptor blockade could mimic the effect of COX-2 inhibition (figure 3.5, upper panel).

An upregulation during diabetes of the contribution of thromboxane A$_2$ in microvascular constriction has also been observed in type 2 diabetic mice; in their skeletal muscle arterioles the myogenic response was mediated via thromboxane A$_2$ originating from COX-2, while it did not in control arterioles (2). In addition, in human aortic endothelial cells a similar adaptation has been shown, where high glucose caused COX-2 upregulation and an increase in thromboxane A$_2$ production (8). Also in rabbit aorta thromboxane synthesis was increased after 6 hours exposure to high glucose levels (33). In glomeruli an increased production of the thromboxane A$_2$ derivative TxB$_2$ has been found 25 to 28 days after the induction of diabetes (9). Indeed, urinary thromboxane excretion is higher in diabetic patients than in controls (16). These data support our finding of an enhanced role for thromboxane A$_2$ in microvascular and/or kidney function during the early stages of diabetes.

An increased production of thromboxane A$_2$ in diabetic animals via the COX-2 pathway as an adaptation to keep the microvascular angiotensin II-induced reactivity normal, points to a decreased sensitivity to angiotensin II itself in diabetes. This is in line with reports on reduced glomerular angiotensin II receptor densities in diabetic animals (3, 39). Of note, the plasma levels of angiotensin II were not altered in diabetic rats (39). Hence, it seems that a disturbance in the microvascular reaction to angiotensin II in type 1 diabetes mellitus is not compensated for by an increase in angiotensin II, but by an
increase in the thromboxane A₂ contribution to the vasoconstrictive capacity of angiotensin II.

In conclusion, the present study demonstrates that renal pre- and postglomerular arterioles respond to angiotensin II to a similar extent in control and diabetic kidneys with normal glomerular filtration rate. However, the way this was achieved differed, with an increased role in diabetic kidneys for COX-2 derived constrictor prostanoids; an important one being thromboxane A₂. Our data provide insight into an adaptive mechanism recruited during type 1 diabetes mellitus to prevent or abolish a threatening hyperfiltration. Careful considerations should be taken in providing COX-2 inhibitors to diabetic patients, also when a normal kidney function is observed, to avoid disturbing this new balance.

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