Functional, biochemical, and molecular investigations of renal kallikrein-kinin system in diabetic rats


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The kallikrein-kinin system (KKS) is a complex multi-enzymatic system that has been implicated in the control of renal circulation, glomerular hemodynamics, and tubular function (28, 36). Abnormalities of the KKS associated with major diseases such as hypertension, heart failure, and diabetes mellitus (DM) have been reported (29, 42). With respect to DM, it has been postulated that the KKS contributes to the development of diabetic nephropathy (11, 15, 17, 19, 23, 30, 33, 41, 43, 49) by disturbing renal hemodynamics and tubular function in concert with other vasoactive systems, e.g., the renin-angiotensin system (1, 3, 4, 7, 35), nitric oxide (NO) (9), or prostaglandins (8, 11).

Most of the previous studies were performed in type I diabetic patients or insulin-treated streptozotocin (STZ)-induced diabetic rats, in which an increased renal kallikrein activity is correlated with a rise in glomerular hyperfiltration (11, 15, 17–19, 23, 30). Insulin may modulate renal kallikrein activity and renal function in a dose-dependent manner (17). This is supported by findings observed in non-insulin-treated STZ-diabetic rats, which show a reduced synthesis of renal kallikrein (15, 17, 19, 30, 43) accompanied by a decrease in glomerular filtration rate (GFR) and renal plasma flow (RPF) (19, 41, 43). Thus the renal KKS may be suppressed in this model of DM. However, components of the KKS may not change in a strictly parallel fashion (3, 41). To understand the role of this system in the different stages of diabetic nephropathy it is necessary to characterize the renal KKS also in the absence of insulin. Therefore, in the present study, we systematically analyzed relevant components of the renal KKS in non-insulin-treated, severely hyperglycemic STZ-diabetic rats in order to understand the role of the renal KKS in the absence of insulin. Therefore, in the present study, we systematically analyzed relevant components of the renal KKS in non-insulin-treated, severely hyperglycemic STZ-diabetic rats. In particular, we determined renal kallikrein activity and urinary BK excretion to examine whether a reduced renal kallikrein synthesis may also lead to a reduced renal kinin formation. Furthermore, we analyzed two main renal kinin-degrading enzymes, angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP) 24.11 and measured the renal expression of kininogens and the B2 receptor. Finally, we investigated blood BK levels and prekallikrein activity to address the contribution of the plasma KKS to changes in the renal KKS under the conditions of diabetic nephropathy.

METHODS

Animals and Study Design

Experiments were performed on male Wistar rats weighing 300–330 g (Dr. Karl Thomae, Biberach/Riss, Germany). All animals were allowed free access to distilled water and were maintained on a 12-h light-dark cycle. Control animals were fed a standard chow ad libitum. To prevent any influence of a high protein intake on the renal KKS and on kidney function (19), the diabetic rats were pair fed in the present study: the food consumption of a separate group of age-matched nondiabetic control rats was calculated daily and taken as the
standard amount of food for matched STZ-diabetic rats. DM was induced by a single intraperitoneal injection of STZ (70 mg/kg) prepared in 0.1 M sodium citrate buffer (pH 4.5; Sigma, Munich, Germany), and hyperglycemia was confirmed 48 h later by a reflectance meter (Acutrend, Boehringer, Mannheim, Germany). Only rats with blood glucose levels >300 mg/dl 3 days after STZ injection were used (n = 15). Rats treated with a single intraperitoneal injection of vehicle (n = 15) were used as controls.

At the end of the study, hemodynamic parameters and renal function were studied in nine conscious animals per group. These rats were then placed in metabolic cages, and 24-h urine was collected. Aliquots were stored at −20°C to measure total protein excretion by the pyrogallol red method (Analyticon, Burbach, Germany) as well as renal kallikrein-like activity. Finally, the animals were anesthetized with ether, and blood samples from the retrobulbar cavity were collected to determine the number of leukocytes, blood HbA1c content, and hematocrit as well as plasma levels of sodium, creatinine, glucose, total protein, BK, and plasma prekallikrein activity. Urinary protein and BK excretion were measured in six additional non-pair-fed STZ-diabetic rats. To determine kidney RNA expression and tissue activity of renal kallikrein, six anesthetized rats per group were exanguinated, and kidneys were excised. Renal cortices were macroscopically separated from renal medullae, rapidly frozen in liquid nitrogen, and stored at −80°C. Molecular biological analyses were performed in tissues of six to four rats per group.

Hemodynamic Parameters and Renal Function

After the 11th week, femoral arterial and venous catheters were implanted for determination of GFR by a single-inulin injection method (800 mg/kg) (24) and for measurement of mean arterial pressure (MAP) with a Statham P23DC pressure transducer connected to a Gould Brush 2400 recorder (40). Experiments were performed at least 24 h after femoral cannulation in conscious rats.

Tissue and Urinary Kallikrein and Prekallikrein Assays

Using an esterolytic assay with the synthetic peptide substrate S-2266 (Haemochrom, Düsseldorf, Germany) the activity of urinary kallikrein (n = 9/group) was measured in 24-h urine samples and in renal cortex and medulla (n = 9/group) as described recently (2, 17). Briefly, 10 µl of urine or homogenized tissues in PBS (0.14 M NaCl in 0.01 M Na2HPO4-NaH2PO4, pH 7.4) were incubated with 50 µl of S-2266 in a final volume of 100 µl of Tris buffer (pH 8.2). After 30 min at 37°C, the reaction was stopped by adding 50 µl of acetic acid (50%), and the samples were read against their own blank in a spectrophotometer at a wavelength of 405 nm. Plasma prekallikrein activity was determined using the chromogenic tripeptide substrate S-2302 (Haemochrom, Düsseldorf, Germany) after activation with a prekallikrein activator, an acid-phospholipid type containing factor XII and high-molecular-weight (HMW) kininogen (Haemochrom) as described recently (6). Briefly, 10 µl of citrated plasma were incubated at 37°C with 10 µl of prekallikrein activator and 30 µl of Tris buffer (pH 7.8). After 3 min, 50 µl of substrate S-2302 were added. Thirty minutes later, the reaction was stopped by adding 50 µl of acetic acid (20%), and the samples were read against their own blank in a spectrophotometer at a wavelength of 405 nm.

BK Immunassay

Urinary and blood BK levels (n = 9) were measured by RIA (12). Briefly, 24-h urine was collected in the metabolic cages in tubes containing ethanol (1:5) to prevent extrarenal kinin formation or degradation. The metabolic cages were treated with a silicon spray to prevent contact activation of the renal KKS. Blood samples from the retrobulbar cavity (2 ml) were collected in animals anesthetized with ether using a thin glass capillary filled with an inhibitor cocktail (10,000 kallikrein inhibitory units trasylol, 800 mg soybean trypsin inhibitor, 4 mg polybrene, 10 mg p-o-phenanthrene, 20 mg/ml EDTA) to prevent contact activation of the plasma KKS during this procedure. Blood was collected directly from the capillary into a tube containing ethanol (8 ml) to arrest kinin formation and degradation. After centrifugation, the ethanol extracts were evaporated to dryness under nitrogen and dissolved in 750 µl of Tris buffer (0.1 M, pH 7.4) containing 0.02% neomycin and 0.1% BSA. BK concentration was measured radioimmunologically using a highly specific antibody as described previously (12). Briefly, 50 µl of the sample were incubated with 500 µl of antisera (dilution 1:50,000) and 50 µl of 125I-labeled [tyr8]BK tracer (5,000 cpm) for 24 h. Antibody-bound tracer was separated from free tracer on 150 µl of charcoal (5 g Norit A in 100 ml Tris buffer and 50 ml BSA (1 g) in saline). After centrifugation, the supernatant was discarded and the free charcoal-bound tracer was measured with a gamma counter. The cross-reactivity of the BK antisera used was 24.3% with [des-Arg9]BK, 4.3% with kallidin, and 0.06% with T kinin. BK recovery was ~91%.

ACE Assay

ACE activity was assayed as described by Unger et al. (45). Briefly, after homogenization of the renal cortex (n = 6/group) in 0.3% Triton X-100, tissue ACE activity was detected by a fluorometric method using carbobenzoxyphenyl-alanyl-histidyl-leucine as a substrate. The reaction was started by adding 50 µl of a 10 mM substrate solution to the samples and incubation at 37°C. At various time intervals, the reaction was stopped by transferring 100-µl aliquots from the incubation into 1 ml of 0.1 N NaOH. All subsequent steps in the assay were performed in the dark; 25 µl of 1% orthophthalaldehyde solution in dimethyl sulfoxide were added to the samples. After 30 min, the reaction was terminated with 1 ml of 0.8 N HCl; precipitates were spun down by a 3,000 g centrifugation for 3 min, and fluorescence (λ 360 nm/λ 500 nm) was measured within 60 min. Zero blank values were subtracted from the corresponding test values. Protein content was analyzed and expressed as picomoles of His-Leu per milligram of protein per minute.

Molecular Biological Investigations

NEP 24.11 cDNA and kininogen cDNA. NEP 24.11 cDNA was synthesized from 4 µg of total RNA with the use of specific primer 5′-TGTGATTTCATGGCATCGCATC-3′ (HS70, bases 1,719–1,740) (26), MMLV-RT (GIBCO, Eggenstein, Germany) was used for RT as well as the reaction mixture recommended by the enzyme manufacturer in a volume of 20 µl. PCR was performed with the use of 4 µl of the resulting cDNA, with the upstream primer 5′-CTATCCTGATGACATCTTTC-3′ (HS72, bases 1,422–1,442) and the downstream primer used for RT. The expected size of the NEP 24.11 PCR product is 322 bp. This PCR fragment was subcloned using the TA Cloning system (Invitrogen, San Diego, CA) and sequenced using the Sequenase Version 2.0 Kit (United States Biochemical).
Rat kininogen cDNA was a gift from Dr. J. Wagner (Heidelberg, Germany) and contained a 746-bp fragment of the HMW kininogen (nucleotides 393–1,039), which is 100% homologous to low-molecular-weight (LMW) kininogen and >90% homologous to T kininogen mRNA (22).

Northern blot analysis. Northern blot analysis of renal cortices was done after homogenization and total RNA extraction using Trizol reagent (GIBCO) according to the manufacturer’s directions. For each blot, 20 µg of total RNA per lane were loaded and electrophoresed on a 0.8% formaldehyde-containing agarose gel, transferred to Hybond-N membranes (Amersham Life Science, Amersham, UK), and immobilized by baking for 2 h at 80°C. Northern blots were carried out with specific 32P-labeled 2B7 cDNA probes to detect mRNA containing agarose gel, transferred to Hybond-N membranes were loaded and electrophoresed on a 0.8% formaldehyde–sodium chloride–sodium citrate (SSC), 0.1% SDS], followed by a 30-min wash at 65°C in a high-stringency buffer (0.1 SSC, 0.1% SDS) and an additional 15-min wash in a low-stringency buffer (2× SSC, 0.1% SDS). The hybridized fragments were separated by electrophoresis on a denaturing gel and analyzed using the FUJIX BAS2000 phosphorimager system. Quantitative analysis was performed using the Ambion RPA II kit (ITC Biotechnology).

Radiolabeled cDNA probes were radiolabeled with [32P]UTP, and specific mRNAs were normalized to those of CHOB-B and β-actin, respectively, to correct for differences in RNA loading and/or transfer.

Ribonuclease protection assay. To analyze renal B2-receptor expression (n = 4/group), a RNase protection assay (RPA) was performed using the Ambion RPA II kit (ITC Biotechnology). Antisense RNA probes were generated by T7 polymerase transcription using linearized plasmids that contained a 179-bp fragment of the B2-receptor cDNA or a 105-bp fragment of rat cDNA probe used as an internal control. The probes were radiolabeled with [32P]UTP, and ~5 × 10^6 cpm of each probe were hybridized together with 20 µg of total RNA per sample. After RNase A/T1 digestion 178 bp and 150 bp, respectively, were protected from the B2-receptor cDNA and β-actin sequences. The hybridized fragments were separated by electrophoresis on a denaturing gel and analyzed using the FUJIX BAS2000 phosphorimager system. Quantitative analysis was performed by measuring the intensity of the B2-receptor bands normalized by the intensity of the β-actin band.

Statistical Analysis of Data

All data are expressed as means ± SE. Data were analyzed using Student’s t-test. The comparisons among control rats, pair-fed STZ-diabetic rats, and non-pair-fed STZ-diabetic rats were performed using a two-way ANOVA in conjunction with Bonferroni confidence intervals. P values < 0.05 were accepted as significant.

RESULTS

Functional and Biochemical Characteristics of Diabetic Rats

Pair-fed STZ-treated rats exhibited severe hyperglycemia (~700 mg/dl) accompanied by mild ketonuria throughout the 12-wk study period. They also showed polydipsia, polyuria, hypoproteinaemia, and increased plasma HbA1c levels (Table 1). No differences in plasma sodium, hematocrit, and leukocytes were observed between diabetic rats and controls, indicating that the diabetic animals were euvoletic and that symptoms of infection or inflammation were absent (Table 1). No change in MAP between the two groups was observed (97.2 ± 2.1 vs. 96.3 ± 1.7 mmHg). Twelve weeks after STZ injection, the rats had increased plasma creatinine levels and urinary protein excretion accompanied by an increase in kidney size, with an elevated kidney-to-body weight ratio and by a decrease in GFR compared with controls (Table 1). Non-pair-fed diabetic rats showed a significant increase in proteinuria (32.3 ± 1.8 mg/24 h) and food intake (39.9 ± 2.8 g/24 h) compared with pair-fed diabetic rats (Table 1). The results for STZ-diabetic rats presented refer to pair-fed diabetic rats unless stated otherwise.

Renal Kallikrein–Kinin System

Biochemical investigations. In control rats, the tissue activity of kallikrein was 31.5% lower in the renal medulla than in the renal cortex (Table 2). In diabetic rats, renal kallikrein activity was reduced in the renal cortex and in the renal medulla compared with controls (Table 2). This was associated with an up to threefold reduction in renal cortical activity of ACE (Table 2). Urinary kallikrein activity was also reduced, whereas urinary BK excretion was higher than that of controls (Fig. 1). BK excretion in non-pair-fed STZ-diabetic rats

Table 2. Effect of streptozotocin on components of plasma and renal kallikrein–kinin system after 12 wk

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STZ (pair fed)</th>
</tr>
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<tbody>
<tr>
<td>Glomerular filtration rate, ml/min</td>
<td>3.1 ± 0.7</td>
<td>1.9 ± 0.2*</td>
</tr>
<tr>
<td>Urine volume, ml/24 h</td>
<td>18.3 ± 1.7</td>
<td>95.2 ± 4.10*</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.20 ± 0.04</td>
<td>0.38 ± 0.08*</td>
</tr>
<tr>
<td>Kidney, g/weight, g</td>
<td>3.4 ± 0.10</td>
<td>6.3 ± 0.20*</td>
</tr>
<tr>
<td>Urine protein, mg/24 h</td>
<td>7.9 ± 1.6</td>
<td>20.6 ± 6.30*</td>
</tr>
<tr>
<td>Renal function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>180.2 ± 30.5</td>
<td>772.1 ± 72.5*</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>141.2 ± 5.8</td>
<td>141.2 ± 5.8</td>
</tr>
<tr>
<td>Urine protein, mg/24 h</td>
<td>7.9 ± 1.6</td>
<td>20.6 ± 6.30*</td>
</tr>
<tr>
<td>Glomerular filtration rate, ml/min</td>
<td>3.1 ± 0.7</td>
<td>1.9 ± 0.2*</td>
</tr>
</tbody>
</table>
| Values are means ± SE; n = 9 rats. STZ, streptozotocin. *P < 0.05.
(82.2 ± 7.2 ng/24 h) was increased by a factor of 7 compared with controls and increased 2.5-fold compared with pair-fed diabetic rats (Fig. 1). BK excretion did not significantly correlate with the respective urinary protein excretion in the control group or in the STZ-diabetic group.

Molecular biological investigations.

NEP 24.11. Compared with controls, renal cortical mRNA expression of NEP 24.11 was reduced 1.8-fold in pair-fed diabetic rats (Fig. 2).

KININOGENS. Northern blot analysis of renal cortical RNA revealed two different RNA bands of 2.3 kb and a large band from 1.8 to 1.6 kb corresponding to HMW kininogen and LMW kininogens (51) in controls and in STZ-diabetic rats (Fig. 3). Quantification of the autoradiographic signals by densitometry revealed that the mRNA levels of HMW and LMW kininogen fractions in the renal cortex were ~40% higher in diabetic rats than in controls.

B<sub>2</sub> RECEPTOR. Densitometric quantification of renal cortical B<sub>2</sub>-receptor expression revealed an up to 1.8-fold increase in diabetic rats compared with controls (Fig. 4).

DISCUSSION

Non-insulin-treated, severely hyperglycemic STZ-diabetic rats develop a decrease in GFR and RPF over time, which could be correlated with a decrease in renal kallikrein synthesis and activation (15, 30, 41, 43). These findings led several authors to propose that the renal KKS is suppressed in this animal model and may contribute to complications of diabetic nephropathy. This hypothesis was proven in the present study by systematically analyzing relevant components of the renal KKS. Our salient observation is the finding of increased BK excretion in severely STZ-diabetic rats despite very complex alterations of the renal KKS. The renal cortical kallikrein substrate supply was increased, whereas the activities of tissue and urinary kallikrein were reduced. The kinin-degrading systems, i.e., cortical ACE activity and renal NEP 24.11 expression were reduced, and cortical B<sub>2</sub> receptor and kininogen expression was increased. Interpretation of these data is further rendered difficult by the fact that the plasma KKS was activated in these proteinuric rats, which raises the possibility that plasma kallikrein, kininogens, and kinins may have been delivered to the kidneys by glomerular filtration.

In agreement with previous findings (15, 17, 19, 30, 41, 43), we also observed an increase in proteinuria and a reduction in GFR and renal tissue and urinary kallikrein activity in severely hyperglycemic STZ-diabetic rats. We also demonstrated a markedly reduced kallikrein activity in the renal cortex and medulla, indicating that no shift of kallikrein production occurred between both renal macroenvironments in STZ-diabetic rats. Because it is generally assumed that changes in urinary kallikrein reflect changes in the intrarenal formation and urinary excretion of kinins (31), we also expected, based on these findings, a reduction in urinary BK excretion. However, we found an up to threefold increase in urinary BK excretion in our model. We were concerned that this result could be biased by altered food intake and protein balance (14, 16, 19, 50). Indeed, uncontrolled hyperphagic rats with a high protein intake exhibited a very high BK excretion. However, increased urinary BK was still observed in pair-fed diabetic rats. These observations indicate that the generation of urinary BK is partly influenced by high protein intake and less dependent on renal kallikrein activity. The latter notion is also in agreement with previous observations by other authors who found no correlation between urinary kallikrein and kinin excretion in different animal models, even when urine was collected directly from the ureter and formation or destruction of kinins in the bladder was excluded (21, 37).
In view of the above findings and considerations, the question arises as to whether urinary BK originates at all from the kidney during this stage of diabetic nephropathy. Theoretically, at least three different mechanisms could be responsible for increased urinary BK levels: 1) a prerenal mechanism with glomerular filtration of plasma KKS components, 2) a renal mechanism with impaired intrarenal kinin degradation and/or increased kininogen supply, and/or 3) postrenal kinin formation.

**Prerenal Kinin Formation**

We found the plasma KKS to be activated in STZ-diabetic rats as indicated by increased BK levels and prekallikrein activity. It has been suggested that an activation of the plasma KKS (including kininogen, kallikrein, and ACE) in diabetic individuals is related to an activation of the coagulation system, an increase in vascular permeability, thrombosis, and hemorrhage in peripheral vessels. After vascular damage, exposure to nonendothelial vessel surfaces can be a contact phase stimulus for the system to generate kinins (27, 34, 44).

However, changes in blood kinin levels have only a small effect on urinary BK levels under basal conditions (31) and plasma kallikrein or kininogens do not undergo glomerular filtration to a significant extent (25, 48). On the other hand, in chronic renal failure all plasma KKS components can cross the glomerular basement membrane and can be excreted into the urine (47). Therefore, it is possible that filtered components of the plasma KKS contributed to the observed increase in BK excretion in proteinuric diabetic rats.

**Renal Kinin Formation**

Under basal conditions, filtered and intrarenally formed kinins are destroyed immediately by kininases (5, 39, 46). Thus the final intrarenal and urinary concentrations of kinins will be a result not only of their enzymatic formation and filtration but also of intrarenal degradation processes. Therefore, we analyzed renal kinin degradation to determine whether alterations in this system could be responsible for changes in BK excretion in STZ-diabetic rats. We found reduced kininase activity, i.e., ACE activity and NEP 24.11 expression.

Because diabetes-induced tubulointerstitial changes like interstitial fibrosis, tubular cell atrophy, or interstitial inflammatory infiltrates are thought to be responsible for a reduced tubular production of proteins (53), it is unlikely that other tubular kininases can compensate for the observed impairment of the renal kinin-degrading system. Therefore, we suggest that a reduced degradation of filtered or intrarenally formed kinins contributed to the increased BK excretion in STZ-diabetic rats.

If kinins can escape tubular degradation and reach the distal nephron, it is possible that renal and glomerular hemodynamics are influenced by filtered as well as intrarenally generated kinins passing the macula densa. Intervention studies with ACE inhibitor or kinin receptor antagonists must prove this hypothesis.
Both the availability of kininogen as kallikrein substrate and the presence of kinin receptors are also important limiting factors for renal KKS function. Our results show an enhanced expression of renal cortical B2 receptors as well as of kininogens in STZ-diabetic rats. Although the mechanism remains unknown, it is possible that these findings reflect an attempt of the organism to compensate for reduced renal kallikrein activity by an increase in kallikrein substrate and kinin receptor supply. Thus, despite an up to 50% reduction in renal kallikrein activity, a rise in the renal production of kininogens could still engender an increase in BK formation. An additional argument for the existence of a direct activation of the renal KKS resides in the fact that proteinuria and BK excretion did not correlate. It is noteworthy, in this regard, that hypofiltration in severely diabetic rats originates from glomerular hypoperfusion as the result of an imbalance between increased vasoconstrictor and reduced vasodilator effects on renal arterioles and glomerular mesangial cells (13, 20, 32). The renal KKS can modulate and counterbalance glomerular vasoconstrictors (10, 38). Therefore, it is possible that an increase in tubular kinin levels as well as an enhanced number of B2 receptors belong to a mechanism to counterbalance vasoconstrictor-induced glomerular hemodynamic changes or other impaired tubular functions during this state of diabetic nephropathy.

Postrenal Kinin Formation

Kinins are formed mainly in the distal nephron with the highest concentration located in its final segment or even in the renal papilla and pelvis (36). Because of urinary kininase activity, in rats kinins are mostly degraded when stored in the bladder (52). Because increased urinary kininogen levels were found in diabetic individuals (44), we cannot exclude the possibility that, under this condition, urinary kinin-formation also occurs in the bladder and is at least partly responsible for an increase in urinary BK excretion. However, our data suggest that these kininogens are of prerenal and/or renal origin and reflect an activation of the renal KKS. Together with the demonstrated reduction in urinary kallikrein activity and the reported increase in urinary kininase activity of STZ-diabetic rats (27), it appears unlikely that postrenal kinin formation constitutes a significant pathway.

In conclusion, our results show that the renal BK excretion in nontreated, severely hyperglycemic STZ-diabetes rats is increased independently of renal kal-
likrein activity. This finding may partly be the result of diabetes-induced proteinuria leading to a filtration of components of the activated plasma KKS associated with a reduction of the renal kinin-degrading system. On the other hand, our observations are also consistent with a direct activation of the renal KKS. It must be proven in further studies whether the observed alterations in the KKS are part of an attempt of the organism to maintain GFR and RPF and to counterbalance increased renal vasoconstrictor systems in this stage of diabetic nephropathy.

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