Mechanisms through which bradykinin promotes glomerular injury in diabetes

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Mechanisms through which bradykinin promotes glomerular injury in diabetes. Am J Physiol Renal Physiol 288: F483–F492, 2005; doi: 10.1152/ajprenal.00165.2004.—In diabetes, mesangial cell proliferation and extracellular matrix expansion are critical components in the development of glomerulosclerosis. We reported that diabetes alters the activity of the kallikrein-kinin system and that these alterations contribute to the development of diabetic nephropathy. The present study examined the influence of streptozotocin-induced diabetes on the renal expression of bradykinin (BK) B² receptors (B²KR), connective tissue growth factor (CTGF), transforming growth factor-β (TGF-β), and TGF-β type II receptor (TGF-βRII) and assessed the signaling mechanisms through which B²KR activation may promote glomerular injury. Eight weeks after the induction of diabetes, renal mRNA levels of B²KR, CTGF, and TGF-β as well as protein levels of CTGF and TGF-βRII were measured in control (C), diabetic (D), and insulin-treated diabetic (D+I) rats. Renal B²KR and TGF-β mRNA levels expressed relative to ß-actin mRNA levels and CTGF and TGF-βRII protein levels were significantly increased in D and D+I rats compared with C rats (P < 0.03, n = 5). To assess the contribution of B²KR activation on modulating the expression of CTGF, TGF-βRII, and collagen I, mesangial cells (MC) were treated with BK (10⁻⁸ M) for 24 h and CTGF and TGF-βRII protein levels were measured by Western blots and collagen I mRNA levels were measured by RT-PCR. A two- to threefold increase in CTGF and TGF-βRII protein levels was observed in response to BK stimulation (P < 0.001, n = 6). In addition, a marked increase in collagen I mRNA levels was observed in response to BK stimulation. Treatment of MC with BK (10⁻⁸ M) for 5 min significantly increased the tyrosine phosphorylation of p60src kinase and of p42/p44 MAPK (P < 0.05, n = 4). Inhibition of src kinase by PP1 (10 μM) inhibited the increase in p42/p44 MAPK activation in response to BK. Finally, to determine whether BK stimulates CTGF, TGF-βRII, and collagen I expression via activation of MAPK pathways, MC were pretreated with an inhibitor of p42/p44 MAPK (PD-98059) for 45 min, followed by BK (10⁻⁸ M) stimulation for 24 h. Selective inhibition of p42/p44 MAPK significantly inhibited the BK-induced increase in CTGF, TGF-βRII, and collagen I levels. These findings are the first to demonstrate that BK regulates the expression of CTGF, TGF-βRII, and collagen I in MC and provide a mechanistic pathway through which B²KR activation may contribute to the development of diabetic nephropathy.

glomerulosclerosis; diabetic nephropathy; connective tissue growth factor

DIABETIC NEPHROPATHY IS A major cause of morbidity and mortality in diabetes. It is the single most common cause of end-stage renal failure (24, 36). A very characteristic and initial event of the development of diabetic nephropathy is glomerulopathy, which is featured by increased thickness of the glomerular basement membrane, and a widening of the mesangium with accumulation of extracellular matrix (ECM). Furthermore, the degree of mesangial expansion is strongly related to the clinical manifestations of diabetic nephropathy, such as albuminuria and decreased glomerular filtration rate (GFR) (7, 29).

The risk factors and pathophysiology for glomerulosclerosis in diabetes are continuing to be defined from clinical and animal models, cellular and subcellular investigations. Although inherent susceptibility seems to influence the rate at which complications develop, the abnormal milieu of the diabetic state (hyperglycemia) is the primary driving force for cellular damage (40). The development of glomerulosclerosis is clearly dependent on hyperglycemia as intensive control of glycemia in type I diabetic patients was associated with a reduction of glomerular lesions (34). High-glucose concentrations induce first proliferation of mesangial cells, followed by the development of hypertrophy, which eventually progress into glomerulosclerosis (3). The initiating and sustaining signals that link hyperglycemia to glomerular sclerosis are not fully realized. Hyperglycemia and its metabolites can directly or indirectly stimulate the synthesis and release of factors from resident renal cells, which in turn can stimulate mesangial cell growth, as well as ECM production in an autocrine or paracrine manner (6, 25). In this regard, the cytokine transforming growth factor (TGF)-β has been shown to play a pivotal role in mesangial cell expansion and matrix deposition (33). Recent evidence indicates that the profibrotic signals initiated by TGF-β are mediated via activation of connective tissue growth factor (CTGF) (8).

CTGF was originally identified as a product of human umbilical vein endothelial cells that was both chemotactic and mitogenic for fibroblasts (2). It is now known that CTGF belongs to a new gene family, CCN [named after prototype members of this family CTGF, Cyr61, and nov (1)]. The molecular mass of CTGF-like factors varies between 35 and 40 kDa, and the structure of these molecules consists of four modules: an NH₂-terminal IGFBP-like domain, a Von Willebrand factor type C repeat domain, a thrombospondin type 1 repeat domain, and a COOH-terminal dimerization domain (1). The biological actions of CTGF are pleiotropic and seem to be cell specific, but the cellular mechanisms of its actions are still undefined. An emerging role of CTGF is that of a profibrotic factor. Renal expression of CTGF is upregulated by the diabetic state and by other progressive renal diseases (17). In addition, CTGF has been shown to increase collagen I expression in human mesangial cells (10).

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Accumulating evidence supports a relationship between activity of the kallikrein-kinin system (KKS) and renal impairment. We previously showed that type 1 diabetic patients, at risk for developing nephropathy (those with elevated glomerular hemodynamics), show increased renal kallikrein and kinin production (14). In addition, diabetic rats with moderate hyperglycemia show increased renal and urinary excretion of active kallikrein and kinin, in conjunction with reduced renal vascular resistance (RVR) and increased GFR and renal plasma flow (RPF). Acute treatment of hyperfiltering diabetic rats with aprotinin, a kallikrein inhibitor, or with a B$_2$-kinin receptor antagonist increases the RVR and reduces GFR and RPF (15, 21).

Although most of the physiological actions of the KKS are attributed to the generation of bradykinin (BK) and activation of B$_2$-kinin receptors, virtually nothing is known about the multiple intracellular signals initiated on activation of BK B$_2$ receptors (B$_2$-KR) leading to expression of prosclerotic factors that ultimately result in glomerular injury. Therefore, the present study examined the influence of diabetes on the expression of glomerular B$_2$-kinin receptors and delineated the transduction pathway through which B$_2$-KR activation promotes the production of CTGF, TGF-$\beta$ type II receptor (TGF-$\beta$RII), and collagen I in mesangial cells.

**METHODS**

**Induction of Diabetes**

Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 200–220 g were used in all studies. Rats were housed two to three per cage in a light- and temperature-controlled room and had free access to food and water. Diabetes was induced by a single intravenous injection of streptozotocin (STZ), 65 mg/kg body wt through the tail vein. After 24 h, diabetes was confirmed in STZ-treated rats by tail vein plasma glucose levels. Glucose levels and body weights were measured at predetermined intervals to characterize the diabetic state. Age-matched control (C) rats were also studied.

The diabetic rats were divided into two groups. The first group (D) received no insulin treatment and displayed severe hyperglycemia. Plasma glucose levels were 240–400 mg/dl throughout the 8-wk study period. The second group of diabetic rats (D+I) was implanted subcutaneously in the back with insulin capsules that maintained a constant release of insulin (1 U) for 2 mo (Limbiln, Linshin, Canada). Insulin therapy began 24 h after induction of diabetes, and plasma glucose levels were maintained in the moderate hyperglycemic range (250 mg/dl) over the 8-wk study period.

**Mesangial Cell Culture**

Rat glomerular mesangial cells were prepared by a modification of the method of Lovett et al. (26). Glomerular cells collected as described above were incubated in PBS plus 0.1% gentamycin solution and 1% antibiotic antimycotic, pH 7.4, containing collagenase (5 mg/ml), at 37°C for half an hour to remove epithelial cells, leaving the glomerular cores containing mesangial and endothelial cells, vortexed every 10 min during the incubation. The cores were diluted in 1.5 ml RPMI 1640 medium plus HEPES per kidney, containing 0.1% gentamycin solution, 1% antibiotic antimycotic, 0.5% insulin-transferring solution, and 20% FBS, conditions that favor growth of mesangial cells. Cells were incubated at 37°C in a humidified atmosphere of 95% air-5% CO$_2$. Cell viability was assessed by standard dye exclusion techniques, using 0.1% Trypan blue. Mesangial cells were identified by the following criteria. Mesangial cells stained positive for intracellular cytoskeletal fibrils of actin and smooth muscle cell-specific myosin (indicative of contractile cells), desmin, and vimentin and negative for cytokeratin and factor VIII antigens. Morphologically, the mesangial cells had an elongated and stellate or spindle-shaped morphology. Mesangial cells isolated by this procedure were homogenous and were used in all studies between passages 3-8.

**RNA Extraction**

Kidneys from control and diabetic rats were removed under anesthesia and cortices were isolated to extract RNA using a combined method of TriZOL and RNeasy Midi Kit for total RNA extraction from animal tissue (Qiagen). Briefly, the cortices were homogenized using an appropriate volume of TriZOL (1 ml TriZOL/100 mg tissue). Chloroform (0.2 ml/ml TriZOL used) was then added to separate the aqueous phase from protein phase. Total RNA was dissolved in the aqueous phase. The RNA concentration was determined in a spectrophotometer (Ultraspac III, Pharmacia) by absorbance at 260 nm.

Total RNA from mesangial cells was extracted with RNeasy Midi Kit (Qiagen) according to the handbook’s protocol. The purified RNA was eluted in 30 µl of RNase-free water, and the RNA concentration was determined in a spectrophotometer (Ultraspac III, Pharmacia) by absorbance at 260 nm.

**RT-PCR**

RNA (2 µg) was converted to cDNA using MLV Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer’s protocol at 37°C for an hour. In PCR reactions, the following rat primers were used: (TGF-$\beta$RI) 5'-AAC CAC CCG GGT TGT GTC GTT GGT T-3', 3'-GTG ACC AAG GCC TTA CAG ACT G-5'; (collagen I) 5'-AAC GAT GGT GGC AAA GGT GAT-3', 3'-CAA CCA GGA CGA CCG TTC TTA-5'; (CTGF) 5'-CTA AGA CCT GTG GAA TGG GC-3', 3'-CCC CTG TTA CTG TAG AAA CTG-5' (Integrated DNA Technologies). The PCR was performed in 25 µl total volume containing 1X PCR buffer, 200 µM dNTPs, 2 ng/µl primers, 10–80 ng cDNA, and 1 U Taq (Taq DNA polymerase Kit, Qiagen). Reaction condition of TGF-$\beta$RI was as follows: 95°C for 30 min, 57°C for 45 min, 72°C for 60 min, total 29 cycles. Reaction condition of collagen I was 94°C for 60 min, 60°C for 60 min, 72°C for 60 min, total 30 cycles. Reaction condition of CTGF was 95°C for 45 min, 60°C for 45 min, 72°C for 45 min, total 25 cycles. The PCR products were visualized on a 1% agarose gel, photographs were taken, and the densitometric analysis was performed using the National Institutes of Health (NIH) image program.

**Real-Time PCR**

Total RNA (2 µg) obtained as described above was converted to cDNA using MLV Reverse Transcriptase (Promega) according to the manufacturer’s protocol at 37°C for 1 h. To determine the validity of primers and appropriate Tm for real-time PCR, the primers were first amplified in a PCR reaction to ensure that only one band is amplified. The following primers were designed so that all of the PCR products are within 75–150 bp (Integrated DNA Technologies): collagen I, 5'-CAC ACA TTC TGT GTC TCT TCC CAT-3'; 5'-GAT CAA GCA GCA TAC CTC GGG TTT CCA-3'; CTGF 5'-TTT CTG CCC TTG TCA CTG CT-3'; 5'-GGT GGA TAC TCG GTG GGT TA-3'; β-actin, 5'-ACT GCC GCA TCC TCT TTC TCT-3'; 5'-CCG CTC GTT GCC AAT AGT GA-3'.

For each target gene, a standard curve was established. This was achieved by performing a series of threefold dilutions of the gene of interest. Negative control was made using the same volume of RNase-free water instead of sample. The master mix was prepared as follows: 2× SYBR Green Supermix (cat. no. 170–8880, Bio-Rad) 12.5 µl, forward and reverse primer 0.25 µl, respectively, and ddH$_2$O 12 µl. For each well, 22 µl of master mix were loaded first, followed by 3 µl of sample, and mixed well to get total reaction volume of 25 µl. For plate setup, SYBR-490 was chosen as fluorophore. The plate was covered with a sheet of optical sealing film. PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 58°C for 1 s.
min (for β-actin and collagen I), then 95°C for 1 min, 55°C for 1 min, and 100 cycles of 55°C for 10 s. All of the reactions were done in duplicate. The correlation coefficient is between 0.99 and 1; PCR efficiency is between 80 and 120%. The mRNA levels were expressed relative to β-actin mRNA. Real-time PCR using iCycle iQ optical system software (version 3.0a) was used in our studies.

Protein Extraction and Western Blot Analysis

Kidney tissue. Cortices from C, D, and D+I rats were minced and homogenized with a polytron for 1 min in 1.5 ml of lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF, and protease inhibitor cocktail, pH 8.0. The homogenate was ultracentrifuged for 15 min, and the resultant supernatant was used for measurements of CTGF and TGF-βRII and actin protein levels by Western blots.

Cell extracts. Mesangial cells were washed twice, scraped in PBS containing 2 mM sodium vanadate, and centrifuged at 3,000 g for 5 min. Pellets were resuspended in 100 μl of lysis buffer (20 mmol/l Tris, 130 mmol/l NaCl, 10% glycerol, 10 mmol/l CHAPS, 1 mmol/l PMSF, 2 mmol/l Na vanadate, 100 μM/ml aprotinin, 0.156 mg/ml benzamidin, pH 8.0), sonicated for 5 s, incubated on ice for 30 min, and centrifuged at maximum speed for 5 min. The supernatant was used as the protein source and its concentration was determined by the method of Lowry et al. (27).

Western Blotting of CTGF and TGF-βRII

Quiescent mesangial cells were stimulated with BK (10^-8 M) for 24 h in the presence and absence of either a MAPK inhibitor (PD-98059, 40 μM) and/or the src kinase inhibitor PP1 (10 mM). Soluble protein (20–25 μg) obtained as described above was separated by SDS-PAGE (12%) under reducing conditions and transferred to PVDF membranes using semidy transfer system (20 V, 45 min) and immunoblotted with anti-TGF-βRII polyclonal antibody (1:1,000 dilution) and with anti-CTGF polyclonal antibody (1:500 dilution) overnight at 4°C followed by incubating the membranes in a secondary antibody conjugated to horseradish peroxidase (HRP). The immunoreactive bands were visualized using the chemiluminescence reagent Renaissance (New England Nuclear Life Science Products, Boston, MA), according to the procedure described by the supplier. Membranes were exposed to Kodak LS film and bands were measured by densitometry and quantified by NIH image program.

MAPK and p60^src Kinase Assay

Quiescent mesangial cells stimulated with BK (10^-8 M) for 5 min were suspended in 250 μl of lysis buffer (20 mM Tris, 130 mM NaCl, 10% glycerol, 10 mM CHAPS, 1 mM PMSF, 2 mM sodium vanadate, 100 mM/ml aprotinin, 0.15 mg/ml benzamidine, pH 8.0), sonicated for 10 s, and centrifuged at 13,000 g for 10 min. Twenty-five to thirty micrograms of cytosolic fraction were analyzed by SDS-PAGE and the separated proteins were transferred to PVDF membranes and immunoblotted with anti-phospho-MAPK polyclonal antibody (1:3,000 dilution, New England BioLabs, Beverly, MA) and/or anti-phospho-p60^src kinase antibody (1:250 dilution). Immunoreactive bands were visualized using the chemiluminescence reagent Renaissance (New England Nuclear Life Science Products), according to the procedure described by the supplier. Membranes were exposed to Kodak LS film and bands were measured by densitometry and quantified by NIH image program.

Statistical Analysis

All data are expressed as means ± SE and were analyzed by ANOVA or Student’s t-test for unpaired analysis. Values were considered significant if P < 0.05.

RESULTS

Characteristics of the Diabetic State

Plasma glucose levels were markedly elevated the day after STZ injection (402 mg/dl, P < 0.0001 vs. C). In the D group, plasma glucose levels remained elevated throughout the study, rising to 460 mg/dl after 8 wk (Fig. 1A). In contrast, plasma glucose levels in the D+I rats were lowered by the insulin treatment and were maintained between 200 and 300 mg/dl throughout the study (Fig. 1A). Initial body weights were not significantly different between diabetic rats and their age-matched controls. However, D rats had a significantly reduced body weight after 8 wk compared with C and D+I rats (289 ± 25 vs. 454 ± 14 g, D vs. C, P < 0.05, n = 6; Fig. 1B). Body weight gain in the D+I rats was normal and the final body weight was not significantly different from C rats (420 ± 11 vs. 454 ± 14 g, D+I vs. C, P > 0.05).

Upregulation of B2-Kinin Receptors by the Diabetic State

The expression of B2-kinin receptor mRNA levels in the renal cortex of D and D+I rats was measured by RT-PCR and compared with levels in C rats. The results shown in Fig. 2 demonstrate that a significant increase in the expression of B2KR was observed in D and D+I rats compared with C rats. Diabetes resulted in a 1.71-fold increase in B2KR mRNA levels and insulin-treated diabetic rats resulted in a twofold increase in B2KR mRNA levels, compared with the levels in C rats, P < 0.01. The B2KR mRNA levels were not significantly different between D and D+I rats. β-Actin mRNA levels measured in the same reaction were not different between C, D, and D+I rats.

Fig. 1. Plasma glucose levels (A) and body weights (B) in streptozotocin (STZ)-induced diabetic rats not treated with insulin and that displayed moderate hyperglycemia (D), diabetic rats implanted subcutaneously with insulin capsules that maintained a constant release of insulin (1 U) and that displayed severe hyperglycemia (D+I), and control rats (C). Final plasma glucose level was 95 ± 16, 460 ± 66, and 282 ± 41 mg/dl in C, D, and D+I, respectively. Body weight gain in D rats was significantly lower than C and/or D+I rats. *P < 0.05 vs. C and D+I. †P < 0.01 vs. D.
BK MODULATES CTGF AND TGF-βRII EXPRESSION

Induction of CTGF and TGF-βRII by BK in Mesangial Cells

Studies were initiated to explore whether BK will modulate the expression of CTGF and TGF-βRII in mesangial cells. Mesangial cells were stimulated with BK (10^{-8} M) for 24 h. The protein levels of CTGF and TGF-βRII and actin were measured by Western blots using specific anti-CTGF, anti-TGF-βRII, and anti-actin antibodies. The results shown in Fig. 5, A and B, represent the intensities of CTGF and TGF-βRII protein levels in control and BK-stimulated mesangial cells.

The results demonstrate that BK resulted in a significant increase in the production of CTGF levels and TGF-βRII levels compared with unstimulated control cells. These findings provide the first demonstration that BK can induce the expression of CTGF and TGF-βRII in mesangial cells.

Activation of MAPK (p42^{mapk} and p44^{mapk}) by BK

The MAPK signal transduction pathway represents an important mechanism by which growth factors regulate cell proliferation (32). To assess whether BK activates MAPK, mesangial cells were treated with BK (10^{-8} M) for 5 min. Cell lysate was analyzed by SDS-PAGE and the separated proteins were transferred to PVDF membranes and immunoblotted with anti-phospho-MAPK antibody (1:4,000 dilution, New England Nuclear BioLabs).

The results shown in Fig. 6 demonstrate that BK increased the tyrosine phosphorylation of p42^{mapk} and p44^{mapk} compared with unstimulated cells (P < 0.01, n = 4 experiments). To identify the cytoplasmic kinases involved in BK-induced MAPK phosphorylation, we examined the effects of specific cell-permeable p60^{src} kinase inhibitor PP1 (10 μM, Biomol Research Laboratories) and/or the cell-permeable MAPK kinase inhibitor PD-98059 (20 μM, New England Nuclear BioLabs). The increase in MAPK phosphorylation induced by BK was significantly reduced by the MAPK kinase inhibitor and by the p60^{src} kinase inhibitor (P < 0.01, Fig. 6).

Activation of p60^{src} Kinase by BK

Our findings demonstrate that activation of MAPK in response to BK is inhibited by the src kinase inhibitor PP1. Therefore, to determine whether BK would result in the activation of p60^{src} kinase, mesangial cells were treated with BK (10^{-8} M) for various times (0, 5, 30, 60 min), and the phosphorylation of p60^{src} was measured by Western blots using anti-phospho src kinase antibodies (1:300 dilution). The results shown in Fig. 7 demonstrate that the tyrosine phosphorylation of p60^{src} was significantly increased by BK with a peak response at 5 min poststimulation.

These findings provide the first evidence that BK stimulates p60^{src} activation and that p60^{src} is upstream of the MAPK pathway.

Role of MAPK in CTGF and TGF-βRII Production in Response to BK

To examine if MAPK will modulate the expression of CTGF and TGF-βRII, mesangial cells were pretreated for 45 min with the MAPK inhibitor (PD-98095), followed by BK (10^{-8} M) stimulation for 24 h. The protein levels of CTGF and TGF-βRII were measured by Western blots. The results shown in...
The results demonstrate that the BK once again produced a significant increase in the protein levels of CTGF and TGF-βRII compared with unstimulated cells (*P = 0.02, BK vs. control, \(n = 10\)). However, this increase in CTGF and TGF-βRII protein levels induced by BK was significantly inhibited by the MAPK inhibitor. The MAPK inhibitor had no significant effect on the basal levels of CTGF and TGF-βRII. These findings provide the first evidence that BK results in enhanced expression of CTGF and TGF-βRII in mesangial cells and that this effect is modulated by the MAPK pathway.

**Role of MAPK and in Collagen I Expression in Response to BK**

To examine if MAPK will modulate the expression of collagen I, mesangial cells were pretreated for 45 min with the MAPK inhibitor (PD-98095), followed by BK stimulation for 24 h. The mRNA levels of collagen I were measured by RT-PCR and collagen I protein levels were measured by Western blots. Bar graph represents the intensities of CTGF mRNA levels/β-actin mRNA levels and of TGF-βRII protein levels. Data are expressed as means ± SE. *P < 0.05 vs. C. †P < 0.04 vs. C.
RT-PCR. The results shown in Fig. 9 represent the intensities of collagen I mRNA levels as well as β-actin mRNA levels. The results demonstrate that the expression of collagen I was undetectable in control untreated mesangial cells. However, collagen I mRNA levels were increased dramatically in mesangial cells treated with BK compared with unstimulated control cells. This increase in collagen I mRNA levels induced by BK was completely abolished in the presence of the MAPK inhibitor (PD-98059).

These findings implicate that the MAPK pathway plays a pivotal role in modulating the expression of collagen I in response to BK in mesangial cells.

BK-Induced Upregulation of CTGF and Collagen I mRNA Levels Is Mediated Via Activation of B2-Kinin Receptors

To determine whether BK stimulates collagen I and CTGF levels via activation of its B2-kinin receptors, mesangial cells were pretreated for 30 min with the B2-kinin receptor antagonist Icatibant (10^-6 M) followed with stimulation with BK (10^-8 M) for 6 h. Collagen I and CTGF mRNA levels were measured by real-time PCR and expressed relative to β-actin mRNA levels. The results shown in Fig. 10 indicate that the addition of Icatibant to mesangial cells significantly reduced the BK-induced upregulation of collagen I and CTGF mRNA levels, demonstrating that BK mediates its effects via activation of B2-kinin receptors.

DISCUSSION

The contribution of the renal KKS to the development of diabetic nephropathy has been the subject of intensive investigation by our research group as well as others (2, 8, 10, 14, 15, 17, 21). We previously showed that the diabetic state is associated with increased expression of glomerular B1- and B2-kinin receptors, abnormal renal prokallikrein synthesis, and activation as well as altered renal and urinary active kallikrein (4, 19). These changes in renal KKS activity were associated with the abnormal renal hemodynamics that are a characteristic manifestation of diabetic nephropathy (14, 15, 21). In the
present study, we demonstrate that prolonged duration of diabetes and insulin therapy are associated with increased renal expression of B2KR and of prosclerotic growth factors CTGF, TGF-β, and its receptor TGF-βRII. Furthermore, our data also demonstrate that activation of B2KR by BK stimulates the expression of CTGF, TGF-βRII, and collagen I in mesangial cells. This increase in CTGF, TGF-βRII, and collagen I production in response to BK involves activation of src kinase and the MAPK pathway. These findings provide the first evidence that BK stimulates several key signaling pathways that participate in matrix dysregulation in mesangial cells.

The pathogenesis of diabetic nephropathy is multifactorial and involves direct effects of high extracellular glucose concentrations on glomerular, vascular, and tubular function. Metabolic imbalances associated with high tissue glucose levels in the diabetic state may directly influence many signal transduction pathways that contribute to the pathogenesis of diabetic glomerular injury. One of the most appreciated effects of high glucose is the bioactivation of TGF-β and the stimulation of ECM protein synthesis (6, 25). Recent evidence indicates that TGF-β may mediate its profibrogenic effects via activation of CTGF. The expression of CTGF is induced by TGF-β in mesangial cells, indicating that CTGF is a downstream target for TGF-β activation (12). Mesangial cells have been shown to induce collagen I and fibronectin expression in response to CTGF stimulation, thus implicating a role for CTGF in matrix deposition and mesangial expansion (10, 39). Furthermore, the glomerular expression of CTGF is induced by experimental diabetes and by high glucose, suggesting that hyperglycemia regulates the expression of CTGF (30, 31). Our data also demonstrate that the renal expression of CTGF, TGF-β, and TGF-βRII is upregulated in severe hyperglycemic diabetic rats, indicating that hyperglycemia may be the trigger for modulating the expression of CTGF, TGF-β, and TGF-βRII. Interestingly, we observed that the renal expression of CTGF, TGF-β, and TGF-βRII in moderately hyperglycemic insulin-treated diabetic rats did not differ significantly from severe hyperglycemic rats, indicating that other factors besides hyperglycemia may contribute to the expression of these genes.

Our finding of increased expression of renal B2KR supports our previous results showing that glomerular B2KR gene expression is activated within 3 wk after induction of diabetes. However, the present study demonstrates that B2KR expression is maintained at high levels for more prolonged duration of diabetes, suggesting that sustained activation of kinin receptor signaling is necessary to mediate glomerular injury. Although our in vivo data only point to an association between increased expression of B2KR and increased expression of
CTGF and TGF-βRII, we did however demonstrate that direct activation of B₂-kinin receptors by BK results in a marked induction of CTGF, collagen I, and TGF-βRII in mesangial cells. In addition, inhibition of B₂KR by Icatibant significantly reduced the increase in collagen I and CTGF mRNA levels in response to BK challenge. These findings provide the first evidence that BK can modulate the expression of CTGF, collagen I, and TGF-βRII in mesangial cells. Given the functional significance of CTGF and TGF-βRII upregulation and their consequences on matrix expression, our data point to a potential mechanistic pathway through which B₂KR activation may contribute to mesangial cell expansion in diabetes.

Although the molecular and cellular signals that promote mesangial expression of CTGF and collagen I in response to diabetes or hyperglycemia are not fully defined, recent findings implicated a pivotal role for the MAPK pathway in this process (13, 16). MAPKs represent a family of serine-threonine kinases that are rapidly activated in response to growth factor stimulation. In mammalian cells, these include ERK1 and ERK2 or p44mapk and p42mapk, JNK, and p38mapk (32). These kinases integrate multiple signal inputs and activated MAPKs are capable of phosphorylating a variety of diverse targets including effector kinases and transcription factors involved in the regulation of genes associated with cellular proliferation and hypertrophy (32). Recent evidence indicates that ligands signaling through G protein-coupled receptors can induce cellular responses that are mediated by activating key effector molecules known to be critical for receptor tyrosine kinase signaling such as src kinase, Ras, Raf, and MAPK (28, 37). This observation, together with the evidence demonstrating that activation of the MAPK cascade is important for receptor tyrosine-mediated growth regulation, suggests that MAPK could be a convergence point for growth signals originating from different G protein-coupled receptors and tyrosine kinase receptors. In this regard, our findings demonstrate that activation of B₂KR in response to BK stimulation results in rapid activation of p60src kinase and of p42/p44mapk.

To further delineate the role of cytoplasmic tyrosine kinases in BK-induced p42/p44mapk activation, the effects of cell-permeable-specific inhibitors of src kinase and MAPK kinase were examined. Pretreatment of mesangial cells with the src kinase inhibitor and/or the MAPK kinase inhibitor completely eliminated the increase in p42/p44mapk phosphorylation induced by BK. This finding demonstrates that p42/p44mapk is a downstream target for src kinase and lends further support to our previous results in vascular smooth muscle cells, showing that BK activates p42/p44mapk via p60src kinase, PKK-e, and MAPK kinase (37). The initiating and sustaining signals that link B₂KR activation to CTGF, TGF-βRII, and collagen I expression in mesangial cells are still not fully defined. In the present study, we provide evidence that the activation of p42/p44mapk by BK is critical for the production of CTGF, TGF-βRII, and collagen I. Pretreatment of mesangial cells with the MAPK kinase inhibitor PD-98059 prevented the BK-induced increase in CTGF and TGF-βRII protein levels and the increase in collagen I mRNA levels. One possible mechanism through which p42/p44mapk may modulate BK-induced gene expression is via transcriptional activation of the AP-1 complex. We previously showed that BK can promote nuclear translocation of p42/p44mapk and can induce the expression of protooncogenes c-fos and c-jun and the formation of AP-1 complex transcription factor (20). The increase in c-fos mRNA levels and the formation of AP-1 complex in response to BK were mediated via activation of p42/p44mapk (9, 11). The promoter regions of collagen I gene and the TGF-β gene have been shown to be regulated by transcription factors such as AP-1 (5, 23). There-

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**Fig. 9.** Role of MAPK in BK-induced collagen I expression. Quiescent mesangial cells were preincubated with a cell-permeable inhibitor of MAPK kinase PD-98059 (20 μM) for 45 min, followed by BK (10⁻⁸ M) stimulation for 24 h. RNA was extracted from the cells and collagen I mRNA levels were measured by real-time PCR. β-Actin mRNA levels were measured at the same time in the same samples. Blot is representative of 3 separate experiments.

**Fig. 10.** BK-induced collagen I and CTGF mRNA levels are mediated via B₂-kinin receptors. Quiescent mesangial cells were preincubated with Icatibant (10⁻⁶ M) for 30 min, followed by BK (10⁻⁸ M) stimulation for 6 h. Collagen I and CTGF mRNA levels were measured by real-time PCR. β-Actin mRNA levels were measured at the same time in the same samples. Blot is representative of 4 separate experiments. *P < 0.01 vs. BK.
fore, one can speculate that activation of B2KR by BK in mesangial cells results in activation and nuclear translocation of p42/p44

leuk, leading to the formation of the transcription factor AP-1, which leads to transcriptional activation of TGF-β gene and collagen I gene. Alternatively, once expression of TGF-β gene is induced by BK it can directly stimulate the expression of CTGF and collagen I. Thus interventional strategies aimed at the level of B2KR inhibition may be a useful approach to prevent this cascade of intracellular events that eventually result in mesangial cell expansion as a result of matrix deposition, a hallmark feature of diabetic nephropathy.

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