Targeted deletion of B$_2$-kinin receptors protects against the development of diabetic nephropathy

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DIABETIC NEPHROPATHY is a major cause of morbidity and mortality in diabetes. It is the single most common cause of end-stage renal failure (4, 22). A very characteristic and initial event of the development of diabetic nephropathy is glomerulosclerosis, which features increased thickness of the glomerular basement membrane and a widening of the mesangium with accumulation of extracellular matrix. Furthermore, the degree of mesangial expansion is strongly related to the clinical manifestations of diabetic nephropathy, such as albuminuria and decreased glomerular filtration rate (8, 24). Since nephron hyperfiltration and hyperperfusion occurs early in some of the conditions that predispose to development of glomerulosclerosis, it is postulated that hemodynamic factors are responsible for the initiation and progression of glomerular injury (5). Evidence for a role for hemodynamic factors in the pathophysiology of diabetic nephropathy comes from studies of animal models. Hostetter et al. (13) showed that streptozotocin-diabetic rats treated with insulin to maintain hyperglycemia between 200 and 300 mg/dl display vasodilatation of both the afferent and efferent arterioles, with the afferent more vasodilated than the efferent. This disproportionate vasodilatation of glomerular arterioles results in increased glomerular filtration rate (GFR), renal plasma flow (RPF), and glomerular capillary pressure (P$_{GC}$). In these hyperfiltering diabetic rats, angiotensin-converting enzyme inhibitors, by further dilating the efferent arteriole, or low-protein diet, by reversing afferent vasodilatation, lower P$_{GC}$ and prevent the development of glomerulosclerosis (36, 37). In addition, the development of glomerulosclerosis in diabetes is considered a consequence of hyperglycemia, and intensive control of glycemia in type 1 diabetic patients has been associated with a reduction in the development and progression of nephropathy (33).

Although the specific biochemical and cellular mechanisms that promote renal injury in diabetes are still undefined, accumulating evidence supports a relationship between activity of the kallikrein-kinin system (KKS) and renal impairment. Our group (11) previously reported that diabetic rats with moderate hyperglycemia show increased renal and urinary excretion of active kallikrein and kinin, in conjunction with reduced renal vascular resistance and increased GFR and RPF. Type 1 diabetic patients with hyperfiltration also have increased renal excretion of active kallikrein compared with diabetic subjects with normal GFR or nondiabetic control subjects (12). Acute treatment of hyperfiltering diabetic rats with aprotinin, a kallikrein inhibitor, or with a B$_2$-kinin receptor antagonist increases the renal vascular resistance and reduces GFR and RPF (15). Furthermore, feeding a low-protein diet to hyperfiltering-diabetic rats reduces renal kallikrein levels, GFR, and RPF to normal values (16). Recent findings by our group (17) in the DCCT/EDIC cohort of type 1 diabetic patients demonstrated that increased plasma prekallikrein activity is associated with increased albumin excretion rate (AER).

Whereas most of the physiological actions of the KKS are attributed to the generation of bradykinin (BK) and activation of B$_2$-kinin receptors (B2KR), the intracellular signals initiated upon activation of B2KR leading to expression of pro sclerotic factors that ultimately result in glomerular injury are just beginning to be defined. Activation of B2KR by BK resulted in marked induction of connective tissue growth factor (CTGF), collagen I, and transforming growth factor-β type II receptor (TGF-βRII) in mesangial cells (31). Inhibition of B2KR by...
Icatibant significantly reduced the increase in collagen I and CTGF mRNA levels in response to BK challenge (31). Furthermore, the glomerular expression of B2KR is increased by diabetes. However, the contribution of these receptors to the development diabetic nephropathy is still undefined. Therefore, the present study was designed to investigate the functional consequences of targeted deletion of B2KR on the initiation and progression of diabetic nephropathy.

**METHODS**

**Study design.** To address the contribution of B2KR to the development of diabetic nephropathy, we studied B2KR knockout mice (B2KR−/−) and their wild-type littermates (B2KR+/+). Male B2KR−/− mice (strain B6 129S-BdKrb2; Jackson Laboratories, Bar Harbor, ME) and B2KR+/+ mice (strain B6 129 SF2/J; Jackson Laboratories) weighing 20–30 g were used in our studies. Mice were housed three per cage in a light- and temperature-controlled room and had free access to food and water. Diabetes was induced by daily intraperitoneal injection of streptozotocin (STZ; 50 mg/kg body wt) for 3–5 days. Diabetes was confirmed in STZ-treated mice by tail vein plasma glucose levels. We used a total of 48 mice for this study, divided into 4 groups, with 12 mice in each group: B2KR knockout group (B2KR−/− D); B2KR knockout controls (B2KR−/− C); wild-type diabetic controls (B2KR+/+ C); group 4, B2KR knockout diabetic mice (B2KR−/− D). Glucose levels and body weights were measured at predetermined intervals to characterize the diabetic state and to ensure adequate metabolic control (15). Every week, mice were placed in metabolic cages (Nalgene) for 24 h to acclimate, and then 24-h urine collections were obtained from all mice to measure AER and other biomarkers of renal injury. Half of the mice were killed at 3 mo, and the remaining mice were killed at 6 mo after the induction of diabetes. Kidneys were immediately excised. One kidney was used for histological studies, and the other kidney was used for RNA extraction to assess expression of specific genes. The number of mice studied for each group at each time point were as follows: from weeks 1–11, 12 animals were studied for each group; thereafter, 6 animals were studied per group.

**Histological analysis of renal pathology in control and diabetic mice.** The left kidney was removed and sectioned into two pieces in a longitudinal-coronal direction through the renal hilum. One section was placed into a plastic cassette, labeled, and fixed overnight in 4% paraformaldehyde solution at 4°C. The tissue was then embedded in paraffin and sectioned into 4-μm-thick sections onto glass slides. After the paraffin was removed, the tissue sections were rehydrated, and hematoxylin-eosin, Periodic acid Schiff (PAS), and Masson’s trichrome stains were performed. The slides were observed under the light microscope attached to a digital camera system for capturing the images of the glomeruli and renal tubules to be used for analysis. Twenty glomeruli were chosen from each kidney that showed the most prominent mesangial areas with reddish pink tincture on PAS stain. After microscopic images were taken with a digital camera at ×400 magnification, the proportion of mesangial areas (MA) to the glomerular tuft areas (GTA) was calculated. On the digital image of each glomerulus, MA and GTA in pixel units were measured at the computer using Adobe Photoshop 7.0 software. The average ratio of 20 glomeruli in each kidney was calculated.

The 20 most prominently dilated distal convoluted tubules were selected at random in any given kidney. Digital photo images were taken at ×400 magnification of PAS-stained slides. The biggest 20 glomeruli in each kidney was calculated. Digital photo images were taken at ×400 magnification of PAS-stained slides. The biggest 20 dilated distal convoluted tubules in each kidney was calculated. The mean of the average ratio of MA to GTA in each group and the mean cross-sectional diameter of distal convoluted tubules in each group were compared between the two groups using Student’s t-test.

**RNA extraction from renal tissue.** Kidneys from control and diabetic mice were removed under anesthesia, and cortices were separated to extract RNA. For RNA extraction and purification, a method combining Trizol and RNeasy midi kit for total RNA isolation from animal tissue (Qiagen) was used. Briefly, the cortices were homogenized using an appropriate volume of Trizol (1 ml of Trizol per 100 mg of tissue), followed by addition of chloroform (0.2 ml per 1 ml of Trizol used) to separate the aqueous phase from the protein phase. Total RNA was dissolved in the aqueous phase. RNA purification followed the protocol of the RNeasy kit handbook. The RNA concentration was determined in a spectrophotometer (Ultraspec III; Pharmacia) with absorbance at 260 nm (A260). The ratio of A260 to A280 was calculated to check the RNA purity.

**Real-time PCR.** Total RNA (2 μg) extracted from the renal cortex of control and diabetic mice was converted to cDNA using MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer’s protocol at 37°C for 1 h. To determine the validity of primers and appropriate melting temperature for real-time PCR, the primers were first amplified in a PCR reaction to ensure that only one band was amplified. The following primers were designed so that all of the PCR products were within 75–150 bp (Integrated DNA Technologies): B2, 5′-act ttg aac gat ctc ctg-3′ and 5′-ata ctc aca cca tgg gca gta gtc-3′; B1, 5′-cac ttg gca agg atg tgt gag tgg-3′ and 5′-ggg gcc gag cat tgt atg gta gta gaa-3′; renin, 5′-tca aag gtt tcc tca gcc agg act-3′ and 5′-tca aac tgt ggc agg atc-3′ and 5′-tca aac tgg ggc agg atg aag ggg-3′; AT1, 5′-cgg caa tca cct act tgt taa gaa-3′ and 5′-cgg agg gga gga gtc ttc atc-3′; AT2, 5′-ag aatt acc cgt gac gac cca gct cgg-3′ and 5′-cat cca cgg cag atc caa gca cct ctg-3′; CTGF, 5′-tgt gtt gga ttc ctc ctt-3′ and 5′-gct gtt ggg tgt ggt tgt-3′; and β-actin, 5′-act gcc tcc tct tcc tc-3′ and 5′-cgg ctt ggc aat gtt 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: 12.5 μl of 2× SYBR green Supermix (catalog no. 170-8880; Bio-Rad), 0.25 μl of forward and reverse primer, respectively, and 12 μl of double-distilled H2O. For each well, 22 μl of master mix were loaded first, followed by 3 μl of sample, mixed well to get a 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 min for β-actin or, for CTGF, 62°C for 1 min, followed by 95°C for 1 min, 55°C for 1 min, and 100 PCR cycles of 55°C for 10 s. All of the reactions were done in duplicate. The correlation coefficient was between 0.99 and 1; PCR efficiency was between 80 and 120%. The mRNA levels were expressed relative to β-actin mRNA and analyzed using Students t-test for unpaired analyses and/or ANOVA. Real-time PCR using the iCycler iQ optical system software (version 3.0a) was used in our studies.

**Urinary AER.** The urinary AER was measured with a murine microalbuminuria ELISA kit (Exocell, Philadelphia, PA) according to the manufacturer’s suggestions.

**Statistical analyses.** Results are means ± SE, unless stated otherwise. All data were analyzed using SAS (version 8; SAS Institute, Cary, NC). We used t-tests to analyze continuous outcomes versus each covariate separately and χ² tests to analyze discrete outcomes versus each covariate. We used ANOVA to compare mean values.
across three or more groups and, similarly, ANCOVA to compare mean values across three or more groups when adjusting for other covariates. Bonferroni correction was used to adjust for inflated type I error when making multiple comparisons. Statistical significance was determined using a two-sided test, and significance was assumed for \( P \) values <0.05.

Generalized linear models and generalized estimating equations were used to compare urine volumes and AER within mice and across groups over time. Groups were defined as wild type and knockout, both including diabetic and control mice within each group. AER was modeled with a time and group main effect and a time by group interaction. Similarly, urine volume was modeled with a time and group main effect and a time by group interaction. Transformations of covariates, such as the quadratic transformation of time, were added to the models to check for the respective significance and were excluded when nonsignificant. Since each mouse was measured up to 15 times over 23 wk, the correlation within each mouse was also taken into account in the modeling. In particular, we tested different correlation models and found that compound symmetry was the best fit, i.e., measurements taken at adjacent time points have the same correlation as those taken more distant in time. A \( \chi^2 \) test was used to compare control with diabetic mice within the knockout group and the wild type group, respectively. Similarly, a \( \chi^2 \) test was used to compare wild type with knockout mice in the control group and the diabetic group, respectively. At each time point of the study when measurements were taken, e.g., week 2, week 3, and so on, we computed model-based estimates of the difference in AER and urine volume between controls and diabetics. These differences were computed for knockout mice and wild-type mice separately, and the estimated values were plotted over time.

**RESULTS**

**Characteristics of the diabetic state.** Initial body weights were not significantly different between diabetic and nondiabetic mice. However, B2KR\(^{+/+}\) D and B2KR\(^{-/-}\) D mice had significantly reduced body weight after 11 wk compared with B2KR\(^{+/+}\) C and B2KR\(^{-/-}\) C mice, and this reduction in body weight was maintained for the duration of the study (Fig. 1A). Plasma glucose levels were markedly elevated 1 wk after STZ injection in both B2KR\(^{+/+}\) D and B2KR\(^{-/-}\) D mice compared with their nondiabetic controls and remained elevated throughout the study period (Fig. 1B).

**Urinary AER.** The urinary AER results are presented in Fig. 2. In our initial analyses we compared the overall AER between groups. Overall AER in B2KR\(^{+/+}\) D and B2KR\(^{-/-}\) D mice was significantly greater than in B2KR\(^{+/+}\) C and B2KR\(^{-/-}\) C mice (\( P < 0.0001 \)). Overall AER in B2KR\(^{+/+}\) D mice was found to be significantly greater than that in B2KR\(^{-/-}\) D mice (\( P < 0.0001 \)). No significant differences in AER were observed between B2KR\(^{+/+}\) C and B2KR\(^{-/-}\) C mice (\( P = 0.4087 \)).

Our next set of analyses involved the use of generalized linear models and generalized estimating equations to compare AER within mice and across groups over time. AER was modeled with a time and group main effect and a time by group interaction effect. Since AER in each mouse was measured up to 15 times over 26 wk, the correlation within each mouse was taken into account in the modeling. At each time point when measurements were taken, e.g., week 2, week 4, we computed model-based estimates of the difference in AER between B2KR\(^{+/+}\) D and B2KR\(^{+/+}\) C, B2KR\(^{-/-}\) D and B2KR\(^{-/-}\) C, B2KR\(^{+/+}\) D and B2KR\(^{-/-}\) D, and, finally, B2KR\(^{+/+}\) C and B2KR\(^{-/-}\) C groups. These differences were computed, and the estimated values were plotted over time in Fig. 2, Band C, which provides a more specific assessment of the AER over time.

Analyses showed that B2KR\(^{+/+}\) D and B2KR\(^{-/-}\) D mice had significantly higher AER than B2KR\(^{+/+}\) C and B2KR\(^{-/-}\) C mice at every time point in the study (\( P < 0.0001 \); Fig. 2B). No significant differences in AER were observed between B2KR\(^{+/+}\) C and B2KR\(^{-/-}\) C mice at any time point in the study (Fig. 2C). However, the AER in B2KR\(^{+/+}\) D mice was significantly greater than that in B2KR\(^{-/-}\) D mice at every time point in the study, except for the last point at week 23 (Fig. 2C). These findings are the first to demonstrate the involvement of B2KR in the development of albuminuria in diabetes.

Analyses also were performed to compare the overall urine volume output between groups. The urine volume in B2KR\(^{+/+}\) D and B2KR\(^{-/-}\) D mice was significantly greater than that in B2KR\(^{+/+}\) C and B2KR\(^{-/-}\) C mice (\( P < 0.0001 \)). The overall urine volume in B2KR\(^{+/+}\) D mice was found to be significantly greater than that in B2KR\(^{-/-}\) D mice (\( P < 0.0018 \); Fig. 3A).
from B2KR we performed histological analysis of renal tissue obtained to protect against diabetes-induced glomerular and tubular injury, to determine whether deletion of B2KR will protect diabetic mice.

0.6047). At 6 mo, the relative MA in B2KR+/+ D mice was significantly less that in B2KR−/− D mice (P < 0.01; Fig. 4). Although the relative MA of B2KR−/− D mice at 6 mo was higher than at 3 mo, the ratio of MA in B2KR−/− D mice was significantly different from that in B2KR+/+ D mice (P < 0.01; Fig. 4) and was not significantly different from that in B2KR+/+ C and B2KR−/− C mice (P = 0.264). These findings constitute the first dem-

Additional analyses also were performed based on estimates of the difference in urine output between B2KR+/+ D and B2KR−/− D mice (Fig. 3B). The results demonstrate that the urine output in B2KR+/+ D mice was significantly greater than that in B2KR−/− D mice at every time point in the study up to week 14 (P < 0.0001). Beyond week 14, no significant difference in urine volume was observed between the two groups. No significant differences in urine output were observed between B2KR+/+ C and B2KR−/− C mice (P = 0.6047).

Histological analysis of renal pathology in control and diabetic mice. To determine whether deletion of B2KR will protect against diabetes-induced glomerular and tubular injury, we performed histological analysis of renal tissue obtained from B2KR+/+ and B2KR−/− mice at 3 and 6 mo after the induction of diabetes. Renal glomerular mesangial areas were well distinguished in all kidney sections by deep pink tincture on PAS stains from surrounding basement membranes of capillary tufts, which had a thin and robust threadlike nature. B2KR+/+ D mice showed widened mesangial areas with more prominent reddish pink tincture in the glomeruli, suggesting a diffuse type of glomerulosclerosis.

To detect whether morphological changes exist in the mesangium between the experimental groups, we determined the proportion of MA relative to GTA in pixel units. The results demonstrate that the mean GTA was not significantly different among the experimental groups at 3 and 6 mo, whereas the ratio of MA in B2KR+/+ D mice significantly increased by 57% compared with that of B2KR+/+ C mice (P < 0.01, 3 mo; Fig. 4).

In contrast, the relative MA in B2KR−/− D mice was not significantly different from that in B2KR+/+ C mice but was significantly lower than that in B2KR+/+ D mice (P < 0.01, 3 mo; Fig. 4). At 6 mo, the relative MA in B2KR+/+ D mice displayed an even greater increase in the ratio of MA (150%) compared with B2KR+/+ C mice (P < 0.01; Fig. 4). Although the relative MA of B2KR−/− D mice at 6 mo was higher than at 3 mo, the ratio of MA in B2KR−/− D mice was significantly different from that in B2KR+/+ D mice (P < 0.01; Fig. 4) and was not significantly different from that in B2KR+/+ C and B2KR−/− C mice (P = 0.264). These findings constitute the first dem-

Fig. 2. Urine albumin excretion rate (AER) in diabetic (B2KR+/+ D and B2KR−/− D) and control (B2KR+/+ C and B2KR−/− C) mice. Urine AER was measured by ELISA 1 wk after induction of diabetes and subsequently every week for 26 wk. A: AER (µg/24 h) for all 4 groups. B and C: estimates of AER between the groups as indicated. Overall, the AER in B2KR+/+ D mice was significantly higher than in B2KR−/− D mice (P < 0.0001). AER was not significantly different between B2KR+/+ C and B2KR−/− C mice.

significant difference in MA was observed between B2KR among the experimental groups of mice also were examined on demonstration that deletion of B2KR protects against diabetes-induced mesangial injury.

Morphological differences in the distal convoluted tubules among the experimental groups of mice also were examined using light microscopy at 3 and 6 mo after induction of diabetes. At 3 and 6 mo, B2KR+/+ D and B2KR−/− D mice showed dark red intranuclear inclusions and tiny red globular and sometimes granular cytoplasmic inclusions in the renal tubular cells on PAS stains. Most of these findings were along the distal convoluted tubules, but occasional cytoplasmic inclusions were found in the proximal convoluted tubules as well. These findings were localized at the same sites where distal convoluted tubules were markedly dilated, which seemed most prominent in the B2KR+/+ D group. The lining tubular epithelial cells in the dilated portions looked atrophic with smaller sizes and shorter heights. There was no significant difference in proximal convoluted tubules among the groups with no dilation or atrophic change. The results shown in Fig. 5 demonstrate that at 3 mo, B2KR+/+ D mice displayed a significantly greater tubular dilation compared with either B2KR+/+ C or B2KR−/− D mice (P < 0.001). In contrast, no significant difference in tubular dilation was observed in B2KR−/− D mice compared with either B2KR+/− C or B2KR+/− C mice (P = 0.381; Fig. 5). When kidney sections were analyzed from mice taken at 6 mo, the tubular diameter in B2KR+/+ D mice was again found to be significantly greater than that in B2KR+/+ C mice (P < 0.001). In contrast, no significant differences in tubular diameter were observed between B2KR−/− D and B2KR−/− C mice (P = 0.111). We did, however, observe that the tubular diameter in B2KR−/− C mice was significantly greater than in B2KR+/+ C mice (P < 0.001; Fig. 5).

Kinin receptor expression in control and diabetic mice. The expression of B2KR and B1-kinin receptor (B1KR) mRNA levels in the renal cortex of B2KR+/+ D, B2KR−/− D, B2KR+/+ C, and B2KR−/− C mice at 3 and 6 mo of diabetes was measured using real-time PCR and expressed relative to β-actin mRNA levels measured in the same reaction. The results demonstrated that the mRNA levels of B2KR were significantly elevated in B2KR+/+ D mice at 3 (Fig. 6A) and 6 mo (Fig. 6B) compared with B2KR+/+ C mice (P < 0.01, n = 5 mice per group). As anticipated, no B2KR mRNA levels were detected in B2KR−/− C or B2KR−/− D mice (Fig. 6).

B1KR mRNA levels were not different between B2KR+/+ D and B2KR+/+ C mice at 3 or 6 mo of diabetes (Fig. 6, Cand D). However, targeted disruption of B2KR markedly induced the expression of B1KR. The mRNA levels of B1KR in B2KR−/− C mice were significantly greater than the levels in B2KR+/+ C mice (*P < 0.001; Fig. 6C). Furthermore, the mRNA levels of B1KR in B2KR−/− D mice were significantly greater than the levels in B2KR−/− C mice at 3 mo, and this effect was accentuated at 6 mo of diabetes (P < 0.0001). These novel findings demonstrate for the first time that deletion of the B2KR results in renal induction of B1 receptors that is markedly accentuated in the presence of diabetes.

Influence of deletion of B2KR and diabetes on expression of renin-angiotensin system components. To define mechanisms by which deletion of B2KR conferred protection against the development of diabetic nephropathy, we explored the possible role of components of renin-angiotensin system (RAS) in this process. To explore whether deletion of B2KR will influence the expression of renin, we measured the renal expression of renin mRNA levels in B2KR+/+ D, B2KR−/− D, B2KR+/+ C, and B2KR−/− C mice at 6 mo of diabetes by using real-time PCR. The results shown in Fig. 7 demonstrate that diabetes upregulated the mRNA levels of renin in wild-type diabetic (B2KR+/+ D) mice compared with wild-type control (B2KR+/+ C) mice (P < 0.009). In contrast, the increase in renin mRNA levels induced by diabetes in wild-type mice was prevented in B2KR knockout mice. The renin mRNA levels in B2KR−/− D mice were not significantly different from those in

Fig. 4. Renal morphology in control and diabetic mice. Histological analyses of glomerular mesangial area were performed in control and diabetic mice at 3 and 6 mo after induction of diabetes. The ratio of mesangial area (MA) to glomerular tuft area (GTA) was calculated for each group and is represented on the bar graph. The results demonstrate that the ratio of MA in B2KR+/+ D mice was significantly higher than in B2KR−/− D mice at 3 and 6 mo. No significant difference in MA was observed between B2KR+/− C and B2KR−/− C mice. *P < 0.01; **P < 0.01 vs. B2KR+/+ C. †P < 0.01; ††P < 0.01 vs. B2KR−/− D.
either B2KR \(^{+/+}\) C or B2KR \(^{−/−}\) C mice but were significantly less than the levels in B2KR \(^{+/+}\) D mice \((P < 0.02; \text{Fig. 7})\).

Renal angiotensin-converting enzyme (ACE) mRNA levels are shown in Fig. 7B. The results demonstrate that the renal expression of ACE was significantly suppressed in both groups
To further explore the relationship between B2KR and AT₁ and AT₂ receptors, the mRNA levels of AT₁ and AT₂ receptors were measured in mesangial cells stimulated with BK (10⁻⁸ M) for 24 h by using real-time PCR and were expressed relative to β-actin mRNA levels. Treatment of mesangial cells with BK significantly reduced the mRNA levels of AT₂ receptors by more than 50% compared with unstimulated cells (P < 0.01, n = 5; Fig. 9). In contrast, BK did not significantly alter the mRNA levels of AT₁ receptors (Fig. 9). These findings provide the first demonstration that BK suppresses the expression of AT₂ receptors in mesangial cells.

CTGF expression in control and diabetic mice. To determine potential mechanisms by which deletion of B2KR conferred renal protection, we determined the expression of renal CTGF in B2KR⁺/⁺ D, B2KR⁻/⁻ D, B2KR⁺/⁺ C, and B2KR⁻/⁻ C mice at 6 mo of diabetes by using real-time PCR. CTGF mRNA levels in wild-type diabetic (B2KR⁺/⁺ D) mice were significantly greater than levels in wild-type control (B2KR⁺/⁺ C) mice (P < 0.04; Fig. 10). Targeted deletion of B2KR did not significantly alter the expression of CTGF in B2KR⁻/⁻ C mice compared with B2KR⁺/⁺ C mice. However, the increase in CTGF mRNA levels induced by diabetes in B2KR⁺/⁺ D mice was prevented in B2KR⁻/⁻ D mice (P < 0.05; Fig. 10). These findings suggest that a functional B2KR is required for CTGF to be induced by diabetes.

Angiotensin receptor expression in control and diabetic mice. To explore whether B2KR modulate the expression of renal angiotensin receptors, we determined the mRNA levels of AT₁ and AT₂ receptors in B2KR⁺/⁺ D, B2KR⁻/⁻ D, B2KR⁺/⁺ C, and B2KR⁻/⁻ C mice at 6 mo of diabetes by using real-time PCR. AT₁ and AT₂ mRNA levels were expressed relative to β-actin mRNA levels measured in the same samples at the same time. The results demonstrate that the expression of AT₁ receptors was not significantly altered by diabetes or by the deletion of B2KR. AT₁ mRNA levels were not significantly different among B2KR⁺/⁺ D, B2KR⁻/⁻ D, B2KR⁺/⁺ C, and B2KR⁻/⁻ C mice (Fig. 8).

On the other hand, deletion of B2KR resulted in a significant increase in the mRNA levels of AT₂ receptors in B2KR⁻/⁻ C mice compared with B2KR⁺/⁺ C mice (P < 0.01; Fig. 8). AT₂ receptor mRNA levels were also significantly greater in B2KR⁻/⁻ D mice than in B2KR⁺/⁺ D mice (P < 0.01; Fig. 8). In contrast, no effect of diabetes was observed on the mRNA levels of AT₂ receptors in wild-type diabetic (B2KR⁺/⁺ D) mice compared with wild-type control (B2KR⁺/⁺ C) mice. These novel findings are the first to demonstrate that deletion of B2KR results in the upregulation of renal AT₂ receptors.
intrarenal levels of kinins under the settings of diabetes or studies have demonstrated that ACE inhibitors increase the diabetic nephropathy are mediated via kinins. However, no that some of the beneficial effects of ACE inhibitors on same time inhibit the catabolism of BK. It is thus presumed inhibitors inhibit the generation of angiotensin II and at the tive signals leading to renal failure are still undefined. ACE stage renal disease. The factors responsible for these maladap- effects, diabetic patients progress with time to develop end-ers (23, 33, 34). Despite these interventions and beneficial ACE inhibitors and, more recently, angiotensin receptor block- glycermic control, blood pressure control, and treatment with of renal disease in diabetic patients. These include intensive glycemic control, blood pressure control, and treatment with ACE inhibitors and, more recently, angiotensin receptor blockers (23, 33, 34). Despite these interventions and beneficial effects, diabetic patients progress with time to develop end-stage renal disease. The factors responsible for these maladap- tive signals leading to renal failure are still undefined. ACE inhibitors inhibit the generation of angiotensin II and at the same time inhibit the catabolism of BK. It is thus presumed that some of the beneficial effects of ACE inhibitors on diabetic nephropathy are mediated via kinins. However, no studies have demonstrated that ACE inhibitors increase the intrarenal levels of kinins under the settings of diabetes or

DISCUSSION

The findings of the present study provide the first evidence that targeted deletion of B2KR protects against the development of diabetic nephropathy. Diabetic B2KR−/− mice display reduced AER, as well as reduced glomerular and tubular injury, compared with diabetic B2KR+/+ mice. The renoprotection conferred by deletion of B2KR was associated with increased renal expression of B1KR and angiotensin II AT2 receptors and decreased expression of the prosclerotic CTGF. In addition, our findings demonstrate that BK downregulates the expression of AT2 receptors in mesangial cells. These findings demonstrate that activation of renal B2 receptors promotes, and deletion of B2 receptors protects, against the development of diabetic nephropathy. This process involves interplay among B1, AT1, and AT2 receptors, resulting in activation/inhibition of key signaling pathways that ultimately alters mesangial cell structure and function.

Few interventions have been shown to slow the progression of renal disease in diabetic patients. These include intensive glycemic control, blood pressure control, and treatment with ACE inhibitors and, more recently, angiotensin receptor blockers (23, 33, 34). Despite these interventions and beneficial effects, diabetic patients progress with time to develop end-stage renal disease. The factors responsible for these maladaptive signals leading to renal failure are still undefined. ACE inhibitors inhibit the generation of angiotensin II and at the same time inhibit the catabolism of BK. It is thus presumed that some of the beneficial effects of ACE inhibitors on diabetic nephropathy are mediated via kinins. However, no studies have demonstrated that ACE inhibitors increase the intrarenal levels of kinins under the settings of diabetes or

mediate the renoprotection conferred by ACE inhibitor therapy. In addition, angiotensin receptor blocker therapy, which does not potentiate BK levels, has been shown to improve albuminuria in diabetic patients similar to ACE inhibitor therapy, thus ruling out a potential beneficial role for BK (6). It is noteworthy to point out that recent studies by Ignjatovic et al. (14) have demonstrated that ACE inhibitors can directly activate B1KR to stimulate nitric oxide independently of kinin potentiation.

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 (10, 11, 13–15). We have previously shown that the diabetic state is associated with increased expression of glomerular B1KR and B2KR and abnormal renal prokallikrein synthesis and activation, as well as altered renal and urinary active kallikrein (7, 19). These changes in renal KKS activity were associated with the abnormal renal hemodynamics that are a characteristic manifestation of diabetic nephropathy (11, 12). Furthermore, our studies demonstrated that prolonged duration of diabetes and insulin therapy are associated with increased renal expression of B2KR and prosclerotic growth factors CTGF and TGF-β, as well as its receptor, TGF-βRII (31). 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 Scr kinase and the MAPK pathway (31). In the present study we have demonstrated that increased renal expression of CTGF by diabetes is suppressed in B2KR−/− mice, indicating that functional B2KR are required for CTGF to be induced by diabetes.

Morphological changes induced by diabetes in the mesangium and tubular dysfunction have been implicated as key mechanisms responsible for albuminuria. These include enlargement of glomerular mesangial area, accumulation of extracellular matrix, thickness of glomerular basement membrane, and tubulointerstitial injury (10, 35). In this regard our studies demonstrate that the increase in MA relative to GTA in B2KR−/− D mice was significantly suppressed in B2KR−/− D mice. In addition, the morphological changes related to tubular dilation that were observed in B2KR−/− D mice were also significantly reduced in B2KR−/− D mice. These findings indicate that targeted deletion of B2KR retards the progression of

Fig. 9. Effect of bradykinin (BK) on angiotensin receptor expression in mesangial cells. Mesangial cells were stimulated with BK (10−8 M) for 24 h. AT1 (A) and AT2 (B) mRNA levels were measured using real-time PCR. β-actin mRNA levels were measured at the same time in the same samples. *P < 0.01 vs. control (n = 5).

Fig. 10. Renal connective tissue growth factor (CTGF) expression in control and diabetic mice. Renal CTGF mRNA levels in control and diabetic mice were measured at 6 mo after induction of diabetes. Renal CTGF and β-actin mRNA levels were measured using real-time PCR. *P < 0.05 vs. B2KR−/− C. †P < 0.05 vs. B2KR−/− D.
morphological changes induced by diabetes in glomerular and tubular cells.

The findings of the present study also point to a relationship between B2KR and AER. The increase in AER in B2KR−/− D mice as a result of diabetes was significantly decreased in B2KR−/− D mice, indicating that targeted deletion of B2KR protects against the development of albuminuria. Another study assessed the effects of B2KR and progression of diabetic nephropathy in the Ins2Akita model of diabetes. The renal expression of B1KR in the Ins2Akita mouse was increased 12-fold, whereas the renal expression of B2KR was increased 2-fold compared with wild-type mice (21). Interestingly, the double mutant In2Akita/B2KR−/− mice were associated with increased albuminuria as well as increased renal expression of B1 receptors (25-fold) compared with Ins2Akita mice (21). It is important to point out that this study did not provide any evidence as to whether the increase in albuminuria observed in the double knockout is the result of targeted deletion of B2 receptors or simply due to the increase in renal expression of B1 receptors. Another limitation of this study is that the data are based solely on one measurement of AER taken at 6 mo, and no information is provided regarding the temporal changes in AER over the study period.

In an attempt to understand the basis for renoprotection conferred by deletion of B2KR in diabetes, we assessed the contribution and interactions between the KKS and the RAS in this process. Although the primary roles of the KKS and the RAS under physiological conditions are quite divergent, they often function in concert with each other under pathological conditions such as renal and cardiovascular disease (18, 20, 25). The two systems can interact and influence the activity of each other at many levels. In this regard, ACE, which converts angiotensin I to angiotensin II, mediates the catabolism of BK and nitric oxide, and AT2 receptor blockade reduced interstitial fluid, and the induction of B2 receptors in response to angiotensin II is blocked (25). The two systems can interact and influence the activity of each other at many levels. In this regard, ACE, which converts angiotensin I to angiotensin II, also mediates the catabolism of BK (25). Renin modulates the production of BK in renal interstitial fluid, and the induction of B2 receptors in response to angiotensin II are mediated via activation of AT1 receptors (27, 32). Stimulation of renal AT2 receptors leads to generation of BK and nitric oxide, and AT2 receptor blockade reduces renin levels of BK and nitric oxide in response to angiotensin II (28–30). AT1 receptors mediate the vast majority of the actions of angiotensin II on cell growth and vasoconstriction, whereas AT2 receptors seem to functionally antagonize the actions mediated by AT1 receptors (2). Finally, functional heterodimerization between B2KR and AT1 receptors and B2KR and AT2 receptors was recently demonstrated (1, 3). In this regard, our studies have demonstrated that the increase in renal renin expression as a result of the diabetic state was eliminated in the presence of targeted deletion of B2KR. Furthermore, deletion of B2KR was associated with increased renal expression of AT2 receptors. This finding is quite intriguing, since it may provide a mechanistic pathway through which deletion of B2KR may confer renoprotection.

Finally, our studies also have revealed that deletion of B2KR resulted in a marked increase in the renal expression of B1KR, and this effect was further induced by diabetes. Other studies also have shown that deletion of B2KR results in increased expression of B1KR (9, 26). Whether the increase in B1KR contributes to the renoprotection in diabetic nephropathy is yet to be determined.

In summary, our study provides the first evidence that targeted deletion of B2KR protects against the development of diabetic nephropathy. Our study also demonstrates that deletion of B2KR resulted in the upregulation of renal B1KR and AT2 receptors, and the expression of these receptors was further accentuated by diabetes. The functional significance of the induction of these receptors in impeding/promoting nephropathy in B2KR−/− D mice is not yet known. Interventional studies on going in our laboratory aimed at teasing out the contribution of B1KR and/or AT2 receptors in mediating the renoprotection against the development of diabetic nephropathy could provide insights into novel therapy targeting the actions of kinins, which may further impede the progression of diabetic renal disease.

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