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Antihypertensive and renoprotective effect of the kinin pathway activated by potassium in a model of salt sensitivity following overload proteinuria

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Ardiles L, Cardenas A, Burgos ME, Drogue A, Ehrenfeld P, Carpio D, Mezzano S, Figueroa CD. Antihypertensive and renoprotective effect of the kinin pathway activated by potassium in a model of salt sensitivity following overload proteinuria. Am J Physiol Renal Physiol 304: F1399–F1410, 2013. First published April 3, 2013; doi:10.1152/ajprenal.00604.2012.—The aim of this study was to test the hypothesis that the activation of the kinin pathway by high-potassium diet can improve renal function. Participation of the kinin system in several models of renal damage has been explored to date (54). This evidence and the fact that the kinin system becomes activated in several models of renal damage suggest that the kinin system may be a potential target for the treatment of renal failure. In this study, we tested the hypothesis that the activation of the kinin system by high-potassium diet can improve renal function.

RENAL FIBROSIS IS THE FINAL common stage of chronic nephropathies and is characterized by an increased synthesis of extracellular matrix (ECM) proteins and a reduction in their catabolism directed by, among others, transforming growth factor-β (TGF-β) via Smads signaling (44). This cytokine can be secreted by renal cells or by infiltrating leukocytes, and three isoforms of TGF-β exist in mammals: TGF-β1, TGF-β2, and TGF-β3. The most studied isoform is TGF-β1, which acts as a mitogen for mesenchymal cells and indirectly stimulates synthesis of ECM proteins by favoring the epithelial-mesenchymal transition (EMT; Ref. 38). This effect is a well-known action of TGF-β that causes the transformation of epithelial tubular cells into cells that display a fibroblast-like phenotype. This process comprises four key stages: 1) loss of epithelial cell adhesion molecules, such as E-cadherin, 2) de novo synthesis of α-smooth muscle actin (α-SMA) and reorganization of actin filaments, 3) rupture of the tubular basement membrane by metalloproteases, and 4) cell migration. Furthermore, cells that acquire a mesenchymal phenotype also acquire the ability to produce and secrete collagen and vimentin, among other ECM proteins (33). Using a model of chronic renal damage, Tu et al. (49) demonstrated that the nonapeptide bradykinin significantly attenuated the EMT induced by TGF-β1 as observed from changes in cellular expression of E-cadherin, α-SMA, and Smad4, an important member of the TGF-β/Smad signaling pathway that is upregulated following TGF-β1 stimulation. Similarly, salt sensitivity is a common acquired defect in models of tubulointerstitial damage that, after discontinuation of the initial injury, is manifested by an elevation in blood pressure after animals are exposed to a high-sodium diet (2), highlighting the relevance of inflammation and upregulation of the renin–angiotensin–aldosterone system (32, 40). Overload proteinuria is a nonimmune animal model of tubulointerstitial damage, widely used to study acquired salt-sensitive hypertension and the role of proteinuria in the pathogenesis of renal fibrosis (2, 16, 17, 62). We have previously shown that massive proteinuria is associated with damage to kallikrein-producing tubules (connecting tubules) that in turn reduces tissue expression of the enzyme and lowers its urinary activity concomitant with an elevation in blood pressure. In addition, a significant antihypertensive effect was achieved when renal kallikrein synthesis was stimulated by a high-potassium diet (5).

Potential participation of the kallikrein-kinin system (KKS) in progressive renal damage has been demonstrated previously in experiments where bradykinin therapy improved cardiac remodeling and perivascular fibrosis in models of renovascular hypertension, an effect that may be blunted by cotreatment with a B₂ receptor (B₂R) antagonist (48). A similar result has been reported using models of unilateral ureteral obstruction where the genetic ablation of B₂R or its pharmacological blockade increased interstitial fibrosis by reducing ECM degradation (44). However, the existence of a link among the kinin system, a high-potassium diet, and changes in TGF-β has not been explored to date (54). This evidence and the fact that acquired salt sensitivity can follow overload proteinuria (2).
prompted us to investigate the effects of albumin overload on the kinin system and TGF-β in the kidney using both animal and in vitro models. The following experiments provide new evidence that stimulation of the kinin system may not only reduce salt sensitivity but may also have an extra renoprotective effect of reducing renal fibrosis by downregulating renal TGF-β.

**MATERIAL AND METHODS**

**Animals**

The Animals Ethics Committee of the Universidad Austral de Chile approved all experimental procedures performed in this study. Sprague-Dawley normotensive female healthy rats (aged 6–12 wk and weighing 180 g at the beginning of the experiments) were obtained from the Department of Anatomy, Histology and Pathology of the Universidad Austral de Chile. Animals were maintained at a constant room temperature with a 12-h light-dark cycle and had unrestricted access to food and water.

**Experimental Design and Groups**

Overload proteinuria and salt sensitivity. The main experimental protocol lasted a total of 8 wk. The first period (induction of tubulo-interstitial damage) comprised an overload proteinuria with a normosodic diet for 2 wk followed by a washout recovery period lasting another 2 wk (also with a normal sodium diet). Overload proteinuria without previous nephrectomy (to avoid the effects of renal mass reduction) was performed using one intraperitoneal injection per day for 14 days of 2 g BSA (Fraction V, A-4503; Sigma) administered as 6 ml of a 33.3% BSA solution dissolved in 0.9% sterile saline, as previously described (2, 5, 9, 15, 16).

The diet used during the protein overload and washout periods was a normal rodent chow containing 0.4% NaCl (normosodic diet). Following these periods, animals were given a high-sodium (4% NaCl) chow to uncover salt sensitivity during the final 4 wk of the experiment.

**HS group.** The disease group (n = 8), representing the model, received the protein overload followed by 2 wk of washout and 4 wk of hypersodic diet (Fig. 1).

**K+—HS group.** This group (n = 6) received 2% potassium chloride in tap water from 4 wk before the protein overload and until the end of the experiment, reducing to 1% during the salt sensitivity phase to avoid chloride excess. The basic experimental model (protein overload, washout, and high-salt period) was as for the HS group (Fig. 1).

**K+—HS-L group.** These animals (n = 5) were submitted to the same protocol as the above potassium group (K+—HS), but the kinin B2R antagonist icatibant (JE049; Sanofi-Aventis) was administered during the high-salt phase (4 final wk) at a dose of 500 μg·kg⁻¹·day⁻¹ using subcutaneous osmotic minipumps (ALZET; Fig. 1).

**Kinin B2R Blockade in Normal Rats**

To evaluate the effect of B2R blockade in normal animals without proteinuria, a group of six adult female rats fed a normosodic diet were submitted to subcutaneous administration of icatibant (500 μg·kg⁻¹·day⁻¹) during 4 wk and were compared with five normal rats without the treatment.

**Hipersodic Diet in Normal Rats**

To evaluate the renal histological effects of a high-sodium diet in normal animals, a group of five adult female rats fed a 4% NaCl diet during 4 wk were compared with five normal rats under normosodic diet (0.4% NaCl).

**Blood Pressure, Urine and Sera Collection, and Tissue Preparation**

Tail-cuff systolic blood pressure (SBP) was measured in prewarmed, conscious animals before the experiments, after the induction and washout, and at the end of the salt-sensitivity period using an ultrasonic Doppler flow detector 811-B (PARKS Electronic). Five readings were obtained at each time point, and a mean value was recorded for each animal. Twenty-four-hour urine collections were performed using individual metabolic cages during which food was withheld to avoid fecal contamination, and water or potassium in tap water was offered ad libitum. Animals were killed at the end of the salt-sensitivity period by aortic exsanguination under general anesthesia. Serum was obtained for biochemical analysis, and kidneys were removed, washed with saline, and horizontal slices were fixed in 4% formalin-PBS and embedded in paraffin wax.

**Urine and Serum Chemistry**

Proteinuria was measured using a turbidimetric method (UCSF Protein; Roche Diagnostics, Mannheim, Germany), electrolytes using an ion-selective autoanalyzer, and creatinine with a modification of the Jaffé reaction. Proteinuria and urinary electrolytes are expressed as milligrams or milliequivalents per milligrams of urinary creatinine to allow for differences in urinary volumes.

**Urinary Kallikrein Activity Assay**

Enzymatic activity was determined using the amidase method using the synthetic substrate d-Val-Leu-Arg-p-nitroanilide (Sigma) in the presence of a mixture of inhibitors (EDTA and soybean trypsin inhibitor; Sigma-Aldrich) to block all other kallikrein-like activities. Control experiments have shown that these inhibitors have no effect on the enzymatic activity of purified rat salivary and urinary kallikreins. Moreover, enzymatic activity obtained under these conditions was completely abolished by antirat urinary kallikrein antiserum but not by nonimmune serum, indicating that the activity indeed corresponds to kallikrein activity (3, 5, 50). Values, expressed as milliunits per milliliters, were calculated relative to milligrams of urinary creatinine to allow for differences in volume.

**Histological Evaluation**

**Tubulointerstitial lesions.** Hematoxylin-eosin-stained sections were analyzed using an optical microscope to evaluate the severity of tubular dilation, atrophy, and cellular infiltration. The percentage of the section affected by these endpoints was then calculated. Damage was graded (0 to 4+) based on a modification of the scale of Zoja et al. (63) where 0 = changes in <10% of the histological section; 1+ denoted changes in up to 25%; 2+ denoted changes in up to 50%; 3+ denoted changes in up to 75%; and 4+ denoted changes in >75% of the section. The entire cortical region of the section was examined, and the average score
obtained in each biopsy was used as “tubulointerstitial score,” as previously published (5).

**Tubulointerstitial fibrosis.** Masson’s trichrome staining was used to assess the degree of renal fibrosis, quantified as the percentage of the tubulointerstitial area, using a KS 300 imaging system 3.0 (Zeiss, München-Hallbergmoos, Germany).

**Glomerulosclerosis.** Hematoxylin-eosin-stained sections were examined for globally or segmentally sclerotic glomeruli, which were defined by partial collapse of the capillary loops, typically accompanied by Bowman’s capsule adhesion or synechiae, hyalinosis and/or increasing mesangial matrix material. A semiquantitative (0 to 3) score was used, where 0 denoted no glomerular lesions, 1+ denoted <25% of glomeruli involved, 2+ corresponded to any global or segmental scar in 25 to 50% of the glomeruli, and 3+ denoted >50% of glomeruli showing scarring.

**Immunohistochemistry**

Tissue sections (5-μm thick) were dewaxed, hydrated, washed in 50 mM Tris-HCl, pH 7.8, and then incubated with a rabbit anti-rat urinary kallikrein antibody (5) diluted 1:40,000 or a rabbit anti-TGF-β1 antibody 1:300, sc-146; Santa Cruz Biotechnology) diluted using the same buffer containing 1% immunoglobulin-free BSA. Cross-reactivity of the anti-rat urinary kallikrein antibody, assessed using a dot-blot immunoassay, was strong for rKLK1, moderate for rKLK2 and rKLK7, and minimal for rKLK9 (50). For SMA, a mouse monoclonal antibody (1:100) was used (clone IA4; Dako, Carpinteria CA). Bound immunoglobulins were detected using the LSAB + HRP System (Dako) or the Vectastain pk 7200 system (Vector). Negative controls included the omission of the first antibody and its replacement by nonimmune rabbit serum at the same dilution.

**In Situ Hybridization for Renal TGF-β**

Dewaxed and rehydrated sections were treated with 2× SSC (standard saline citrate 1×: 0.15 M NaCl in 0.015 M trisodium citrate pH 7) at 60°C for 10 min and washed with diethylpyrocarbonate-treated water. The sections were then treated with 5 mM levamisole for 30 min at room temperature and digested with proteinase K (5 μg/ml in 0.05 M Tris, pH 7.6) for 40 min at 37°C. After inhibition of endogenous biotin using a biotin blocking system (Dako) and washing with diethylpyrocarbonate-treated water, sections were immersed in 0.4% p-formaldehyde for 20 min at 4°C and then incubated with a prehybridization solution (Dako mRNA in situ hybridization solution) for 60 min at 37°C. The hybridization reaction was performed overnight at 37°C in 100 μl of the biotin-labeled antisense TGF-β probe 5'-ATGGTAGCCCTTGGGCTCGTCGAT CCACTT-3' (20 ng/μl) in a humidified chamber for 18 h (24). The slides were then washed with 4× SSC, 2× SSC, and 0.2× SSC with 30% formamide at 37°C for 10 min and then with Tris-buffered saline containing 2% Triton X-100 at room temperature for 15 min. Sections were incubated in alkaline phosphatase-labeled avidin (Dako) for 30 min at room temperature and then washed with Tris-buffered saline containing 1% BSA, and enzyme activity was developed using the NBT-BCIP substrate for 30 min at 37°C. Finally, sections were dehydrated and mounted with Canada balsam. The specificity of the reaction was confirmed by demonstrating the disappearance of hybridization signal after the sections were treated with RNase (100 μg/ml; Sigma), by using a negative control (plasmid DNA; Dako), and by omission of probe.

**Image Analysis of Immunohistochemistry and In Situ Hybridization**

The percentage and intensity of the labeled section area on immunohistochemistry or in situ hybridization sections were evaluated using the KS 300 Imaging System 3.0. The degree of staining was calculated from suitable binary threshold images as the ratio of the total field area, integrating the intensity of the staining in the specific areas. Thus potential differences in the amount of total tissue examined are controlled. For each sample, a mean value was obtained following analysis of 20 different fields (at ×20), reading glomerular and tubulointerstitial areas independently by segmentation. The procedure was performed independently twice, and interassay variability was not statistically significant. The staining score was expressed as density per millimeters squared. This method of quantification has been validated in previous publications (4, 5).

**Cell Culture**

HK-2 cells (human kidney proximal tubular cell line, catalog no. CRL-2190; ATCC Manassas, VA) were grown in RPMI containing 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, 100 mg/ml streptomycin, insulin-transferrin-sodium selenite, and hydrocortisone in 5% CO2 at 37°C. At 60–70% confluency, the cells were grown arrested using a serum-free medium for 24 h and then pretreated with 10 nM bradykinin for 24 h before incubating with 10 mg/ml BSA for 48–72 h. In other experiments, icatibant was added 30 min before bradykinin treatments.

**Morphology of Cultured Cells**

Image acquisition was performed using an OmniVID LW scientific camera, coupled to an OmniVID LW inverted phase contrast microscope. In the acquired images, the cells were segmented by manually outlining the cell boundaries. Morphological assessment was performed using the SCIAN-Soft image analysis software (SCIAN-Lab, University of Chile, www.scian.cl) written in the IDL language (Exelisiv, Boulder, CO). Initially, cell segmentation was performed using filters for region detection and refinement. Secondly, the ratio perimeter²/area (P²/A) was computed for each segmented region (cell). For controls, an average value of P²/A was computed for nontreated cells. Segmentation and computation of P²/A were then applied to the treated-cell images, and cells with a P²/A value greater than the control average were considered fusiform. Finally, the percentage of fusiform cells for each experimental condition was calculated.

**Western Blotting**

Proteins were separated using polyacrylamide gel electrophoresis under denaturing conditions and then transferred onto PVDF membranes (Millipore), as previously described (10). Proteins were detected using a specific primary mouse monoclonal anti-αSMA antibody (Dako). A peroxidase-labeled polyclonal goat anti-mouse IgG secondary antibody was then used, and specific reactions were detected using a chemiluminescence detection kit (Pierce). The efficacy of protein loading and transfer to membranes was assessed using GAPDH. Quantification of immunoreactive bands was performed by densitometric analysis using ImageJ 1.45 (National Institutes of Health).

**Measurement of TGF-β1 by ELISA**

The concentration of TGF-β1 in cell culture supernatants was assessed using a commercial ELISA kit (R&D Systems) following the manufacturer’s recommendations. Briefly, wells of microtiter plates (Polysorp F96; Nunc, Glostrup, Denmark) were coated with capture antibody (anti-TGF-β1) overnight at 4°C. The next day, plates were washed three times with PBS-0.05% Tween 20 and blocked with 5% BSA in PBS for 1 h at room temperature. After three washes, 100 μl of standard or supernatants were added, and after 2 h of incubation at room temperature and five washes, 100 μl of detection antibody and enzyme reagent mixture were added to each well and the plate was incubated for 1 h at room temperature. Unbound detection antibody and enzyme mixture were removed with seven consecutive washes with PBS-0.05% Tween 20. A total of 100 μl of substrate solution was added and incubated for 30 min in the dark at room temperature.

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The reaction was stopped by addition 50 μl of stop solution, and absorbance was measured at 450 nm using a microplate scanning spectrophotometer (Infinite M200; Tecan).

Statistical Analysis

A commercial statistical package (GraphPad InStat, version 3.01 for Windows 95/NT, San Diego, CA) was used. The nonparametric Kruskal-Wallis ANOVA test, followed by Mann-Whitney tests with Welch’s correction was used to examine differences between groups. The nonparametric Spearman test was used to measure correlations between variables. Values are expressed as the means ± SE, and P values < 0.05 were considered statistically significant.

RESULTS

Urinary Protein, Electrolytes, Blood Pressure, and Renal Function

Massive proteinuria was observed in all groups during the BSA overload. All groups showed a tendency for this proteinuria to disappear after the washout period. Interestingly, protein excretion after the induction was reduced in both groups receiving potassium compared with the HS-untreated group. Low levels of protein excretion persisted up to the end of the experiment, when a slight but nonsignificant reduction was observed in the K⁺-HS potassium-treated group compared with the HS group that did not receive potassium (Table 1). High kaliuresis confirmed the high intake of potassium in both groups receiving potassium in drinking water. Sodium excretion was not changed by the high-potassium diet, but it was significantly higher in all groups receiving potassium compared with the HS group that did not received potassium (Table 1). Sodium excretion was again significantly higher in groups receiving potassium than in the untreated group (Table 1). Blood pressure was not changed by the high-potassium diet, but it was significantly lower at the end of the experiment compared with the HS group that did not received potassium (Table 1). Renal function, evaluated by serum creatinine and creatinine clearance at the end of the experiment, was not significantly different when comparing between the three experimental groups (Table 2).

Urinary Kallikrein Activity

Basal (preinduction of proteinuria) levels of urinary kallikrein in groups pretreated with potassium were significantly higher than those recorded in the group that did not receive this treatment, confirming the effect of potassium in increasing urinary kallikrein activity. Kallikrein activity was significantly reduced after the overload challenge in all groups, but it was significantly higher in both groups receiving potassium at the end of the washout period. The unexpected higher urinary kallikrein activity in the K⁺-HS-I group, specially at the end of the washout phase, certainly does not represent the effect of icatibant since at that phase the animals were not receiving the B₂R antagonist. At the end of the experiment, kallikrein activity was again significantly higher in groups receiving potassium than in the untreated group (Table 1).

Tubulointerstitial Damage

At the end of the experiment, histopathological evaluation revealed tubulointerstitial damage comprising foci of mononuclear cells in the renal interstitium, the presence of tubular casts, and tubular atrophy in all groups. No differences in tubulointerstitial damage scores were observed between groups. With respect to the glomerular damage, a lower but nonsignificant glomerulosclerosis index was found in the K⁺-HS group when compared with HS rats (Table 2).

The examination under Masson’s trichromic staining (Fig. 2) verified important differences between groups because the potassium-treated K⁺-HS animals showed a significant reduction in the

<table>
<thead>
<tr>
<th>Table 1. Proteinuria, blood pressure, urinary electrolytes, and urinary kallikrein activity at different stages of the experiment</th>
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<tr>
<td><strong>Proteinuria, mg protein/mg creatinine</strong></td>
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<td><strong>Basal</strong></td>
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<td>Basal</td>
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<tr>
<td>End of protein overload phase</td>
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<td>End of washout phase</td>
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<td>End of salt-sensitivity phase</td>
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<td><strong>Systolic blood pressure, mmHg</strong></td>
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<td><strong>Basal</strong></td>
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<td>End of washout phase</td>
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<td>End of salt-sensitivity phase</td>
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<td><strong>Urinary potassium, μmol/mg creatinine</strong></td>
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<td><strong>Basal</strong></td>
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<tr>
<td>End of salt-sensitivity phase</td>
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<tr>
<td><strong>Urinary kallikrein activity, mU/mg creatinine</strong></td>
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Values are means ± SE. HS, high-sodium group; K⁺-HS, high-sodium high-potassium diet; K⁺-HS-I, high-sodium high-potassium diet plus icatibant. *P < 0.05 vs. HS; †P < 0.05 vs. HS-K⁺.
percentage of fibrotic area compared with the untreated HS rats. The importance of kinins and the B2R was confirmed by the significant increase in fibrosis observed when this receptor was blocked by icatibant (Fig. 2; Table 2).

**Kallikrein Immunohistochemistry**

As expected, immunoreactive renal tissue kallikrein was found exclusively in connecting tubules of the distal nephron in all groups. The HS group that did not received potassium showed a significant reduction in expression of tissue kallikrein when compared with kidneys of healthy, normal, nonprotein-uric rats [HS group: 1,358 ± 161 vs. healthy normal rats (n = 5): 4,124 ± 256 density/mm²; P < 0.05], and a clear although nonsignificant trend to reverse this decreased was observed in the group that also received icatibant (K⁺-HS-I = 360 ± 40 density/mm²). Glomerular staining was also significantly reduced by potassium stimulation (HS = 170 ± 37 vs. K⁺-HS = 35 ± 3 density/mm²) and reversed in the presence of icatibant (K⁺-HS-I = 63 ± 9 density/mm²; Fig. 3).

**Renal Expression of TGF-β**

**TGF-β protein.** Immunoreactivity was observed primarily in tubulointerstitial inflammatory foci of the deep cortex and glomeruli. Renal tubular cells also expressed the TGF-β protein but with less intensity. Tubulointerstitial TGF-β was significantly reduced by the high-potassium diet (HS rats = 743 ± 115 vs. K⁺-HS = 257 ± 50 density/mm²; P < 0.05), and a clear although nonsignificant trend to reverse this decreased was observed in the group that also received icatibant (K⁺-HS-I = 360 ± 40 density/mm²). Glomerular staining was also significantly reduced by potassium stimulation (HS = 170 ± 37 vs. K⁺-HS = 35 ± 3 density/mm²) and reversed in the presence of icatibant (K⁺-HS-I = 63 ± 9 density/mm²; Fig. 3).

**TGF-β mRNA.** Expression of TGF-β mRNA was primarily in renal tubules and was less intense in glomeruli. Tubular expression was significantly reduced in potassium-treated rats
compared with untreated HS group, but it was increased following addition of the B2R antagonist. Glomerular expression of TGF-β mRNA was detected in untreated rats (HS rats = 131 ± 21 density/mm²) but was absent in the K⁺-HS group, with a trend to reemerge in animals cotreated with icatibant (K⁺-HS-I = 8.0 ± 6.9 density/mm²; Fig. 4).

Effects of B2R Antagonism in Normal, Nonproteinuric Rats Fed a Normosodic Diet

These animals did not show elevated blood pressure or renal dysfunction, but significant fibrosis associated with upregulated renal TGF-β protein and mRNA was observed (Table 3).

Histological Effects of an Hypersodic Diet in Normal Rats.

The kidneys of these animals, evaluated by a Masson staining, evidenced significant fibrosis (1.1 ± 0.21%) when compared with controls rats fed a normosodic diet (0.28 ± 0.02%; P < 0.05), and a trend (nonstatistically significant) to express more interstitial α-SMA (hypersodic diet: 42 ± 6.6 vs. normosodic diet: 32 ± 3.3 density/mm²; Fig. 5).

Albumin Overload in HK-2 Proximal Tubule Cells

Exposure of HK-2 cells to BSA induced morphological alterations characterized by a change from round to a fusiform shape that was similar to fibroblasts (Fig. 6). After acquiring this new phenotype, cells overexpressed α-SMA and TGF-β. These changes were inhibited when cells were pretreated with bradykinin before albumin exposure. The beneficial effect produced by bradykinin was reduced by preincubating the cells with icatibant, indicating that the effects were specifically mediated by activating the B2R (Fig. 6).

Correlations

Significant positive correlations were found between area of fibrosis and with glomerular (r = 0.53) and tubulointerstitial (r = 0.55) expression of TGF-β protein, and with tubulointerstitial density.

Fig. 3. Renal tubulointerstitial expression of immunoreactive transforming growth factor-β (TGF-β) protein in HS, K⁺-HS, and K⁺-HS-I groups. Control denotes immunohistochemistry (IHC) performed in the absence of anti-TGF-β antibody. Original magnifications: ×200 (control: ×400). Data are of the quantified expression levels (dens, density). *P < 0.05, compared with HS.
stitial expression of TGF-β mRNA ($r = 0.57$). A significant correlation was also found between fibrosis and systolic blood pressure at the end of the experiment ($r = 0.71$). Negative correlations were observed between the expression of immunoreactive tissue kallikrein and with the area of fibrosis ($r = -0.77$) and systolic blood pressure ($r = -0.63$; Fig. 7).

**DISCUSSION**

We present experimental data demonstrating the benefits of the kinin pathway activated by a high-potassium diet in a rat model of salt-sensitivity postoverload proteinuria, which not only lowered blood pressure but also reduced renal TGF-β expression and the associated renal fibrosis. Similarly, our in vitro experiments showed that HK-2 proximal tubule cells submitted to protein overload suffer epithelial mesenchymal transition that may be prevented by bradykinin via B₂R.

The use of a high-potassium diet as an approach to induce upregulation of the kinin pathway is well supported by previous experimental evidence in the same rat strain (36). This maneuver increases kallikrein synthesis and release, evidenced

Table 3. *Effect of 4 wk of subcutaneous administration of icatibant to normal female rats fed a normosodic diet*

<table>
<thead>
<tr>
<th></th>
<th>Without Icatibant</th>
<th>With Icatibant (500 μg·kg⁻¹·day⁻¹)</th>
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<tbody>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>128 ± 2.7</td>
<td>128 ± 0.5</td>
</tr>
<tr>
<td>Creatinine clearance, μL/min</td>
<td>596 ± 149</td>
<td>741 ± 107</td>
</tr>
<tr>
<td>Proteinuria, mg/mg creatinine</td>
<td>3.1 ± 0.3</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>Urinary kallikrein activity, mU/mg creatinine</td>
<td>35.6 ± 0.3</td>
<td>40.1 ± 8.2</td>
</tr>
<tr>
<td>Fibrosis, %tubulointerstitial area</td>
<td>0.25 ± 0.03</td>
<td>1.77 ± 0.3*</td>
</tr>
<tr>
<td>TGF-β protein expression, dens/mm²</td>
<td>Undetectable</td>
<td>176 ± 27*</td>
</tr>
<tr>
<td>TGF-β mRNA expression, dens/mm²</td>
<td>Undetectable</td>
<td>270 ± 39*</td>
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Values are means ± SE. *$P < 0.05$, compared with without icatibant.
by mRNA upregulation, hypertrophy, and hyperplasia of connecting tubule cells and their organelles namely Golgi complex, rough endoplasmic reticulum, and a large number of kallikrein secretory vesicles (28, 51). It is interesting to highlight that connecting tubule cells also participate in potassium secretion (31) and that high-luminal potassium concentrations increase kallikrein secretion by depolarizing the tubular cell membrane (47). The stimulatory effect of potassium is not restricted to kallikrein secretion since it also increases the B2R mRNA.

Our results add more evidence for a role of the kinin cascade in salt sensitivity, which occurs by inducing hypertension in animals with a chronic downregulation of the system when exposed to a high-sodium diet (1, 30, 31, 34). The significant antihypertensive effect obtained by the stimulation of kinin system with a high-potassium diet and inhibited by the B2R antagonist may be coincidental with the proposed role of the system as a safety valve for excess sodium intake (31). Although the expected differences in urinary sodium excretion were not demonstrated in our experiments, it may be explained by the methodological approach used (we did not feed the animals during urine collection and at that stage of the experiment they were already in sodium balance). A vasodilator effect induced by a combination of a sustained high level of kinins and upregulation of the B2R may explain the reduction in blood pressure.

Tubulointerstitial fibrosis is widely accepted as a good marker of progression to renal failure (14, 61), and the experimental model of intraperitoneal BSA overload has been useful to study the role of proteinuria on its pathogenesis (17). An initial important observation was the significant reduction in proteinuria in both potassium-pretreated groups at the end of induction of tubulointerstitial damage, suggesting that stimulation of the kinin system could induce renoprotection. Remarkably, tubulointerstitial damage with tubular atrophy, inflammation, and fibrosis persisted to the end of the experiment, 6 wk after the overload proteinuric challenge has ceased; this fact was complemented with the tricromic Masson’s staining, which showed a significant reduction in fibrosis in potassium-pretreated animals. It is important to emphasize that the favorable effect of kinin system stimulation on tubulointerstitial fibrosis is reinforced by its loss obtained by blocking the B2R with icatibant.

How could the kallikrein secreted by connecting tubules mediate histological changes in the glomerulus and other parts of the nephron? The anatomical proximity of the connecting tubule to the glomeruli and more proximal segments of the nephron in the renal cortex has been shown earlier, highlighting that the connecting tubule passed outward to the renal capsule from whence it returned to the glomerular vascular pole and then ran alongside the afferent arteriole for a variable distance before reaching the collecting tubule (22). Such a morphological relationship has been confirmed in the rat (6) and human (52) and may explain the measurement of kinins in the renal interstitial compartment (45, 46), venous effluent, and lymph (21). Moreover, kinins act on mesangial cells B2R by modulating collagen synthesis through changes in TGF-β (7).

Initial experimental data on the potential renoprotective effect of the kinin system were found following the administration of purified rat kallikrein to Dahl salt-sensitive rats to reduce proteinuria, an effect abolished after blockade of B2R (25). Stronger evidence has been shown using a model of subtotal nephrectomy, where expression of the human kallikrein gene induced a reduction in blood pressure and albuminuria and protected the kidney from sclerosis and tubulointerstitial damage (55); these renoprotective effects were mediated by reducing oxidative stress and via the expression of TGF-β (12, 13, 49, 60, 61). Our experimental model was designed inducing an upregulation of the kinin system previous to the proteinuric challenge, where proteinuria induces the activation of tubular cells and a fibrogenic state in the whole kidney. In parallel, the experiments using HK-2 cells show that upregulation of TGF-β and the consequent EMT induced by
albumin overload are reduced when the cells are pretreated with bradykinin. We postulate that the renal tissue surrounded by an enriched kinin environment may be less sensitive to a proteinuric injury.

A close interaction among kinins, eicosanoids, and the two potent vasodilator agents nitric oxide (NO) and endothelial-derived hyperpolarizing factor (EDHF) has been proposed to mediate the benefits produced by kinins in the kidney (37, 42). Indeed, an enhanced renal function has been demonstrated in a B2R transgenic mice (53), a finding associated to increased urinary NO levels and renal cGMP and cAMP. Both NO and cGMP are vascular relaxing factors generated by kinins able to reduce vascular tone and enhance renal hemodynamics. Examining the mechanisms by which kallikrein attenuates gentamicin-induced kidney damage Bledsoe et al. (8) showed that a reduced renal inflammation and apoptosis were accompanied by an increase in NO together with a decrease in oxidative stress, JNK activation, and intercellular adhesion molecule-1 and TGF-β1 protein levels. Tissue kallikrein may also protect against salt-induced renal function impairment and fibrosis in hypertensive Dahl salt-sensitive rats through a similar mechanism. In these interesting experiments, increased NO levels were in conjunction with reduced NADH/NADPH oxidase and superoxide anion formation, confirming that kallikrein antifi-

![Fig. 6. Effect of BSA on HK-2 proximal tubule cells. HK-2 cells were directly exposed to 10 mg/ml BSA for 72 h or after pretreating with 10 nM of the kinin B2 receptor agonist bradykinin (BK) for 24 h or 1,000 nM of icatibant (I) for 30 min and then to bradykinin for 24 h. At the end of experiment the percentage of fusiform cells, the amount of active TGF-β and the levels of α-SMA were determined. *P < 0.05, compared with untreated; **P < 0.05, compared with BSA; &P < 0.05, compared with BK-BSA.](image)

![Fig. 7. Spearman rank correlations showing a positive association between the extension of fibrosis and the tubulointerstitial (TI) protein expression of TGF-β, from IHC, or mRNA, from in situ hybridization (ISH). A negative correlation of fibrosis with IHC expression of kallikrein was found.](image)
brotic effects may be mainly mediated by the NO/cGMP pathway (60).

It is difficult to speculate if the potassium/kinin interactions reduce blood pressure by attenuating tubular injury or whether the reduction in blood pressure reduces injury and TGF-β expression. In our opinion, the vasodilator/hypotensive effect produced after upregulation of the kinin pathway may induce renoprotection by reducing blood pressure, but this event cannot be separated from the favorable effects generated by an enhanced release of NO and downregulation of profibrotic mechanisms. We cannot exclude that part of our results may be explained by the induced changes in blood pressure because hypertension is one of the main factors favoring progression of renal disease (35). In this respect, there are previous publications proposing that kinins may have beneficial properties dependent on the B2R (11) and that this effect may account for the renoprotective outcome observed in spontaneously hypertensive rats, independently of changes in blood pressure (20). We agree with other authors (53) in that a reduction in blood pressure and attenuation of renal injury, as well as renal dysfunction, are equally important for the treatment of hypertensive renal diseases and stimulation of the kinin system may accomplish both approaches.

Our findings on TGF-β may be relevant to explain the antifibrotic effect of the kinin pathway in this model since TGF-β is a fibrogenic cytokine inducer of tubular and podocyte EMT, key events in renal fibrosis that may be mediated by Smad proteins (10, 33, 56). The expression changes evaluated from protein or mRNA signals and the correlations found with the extension of fibrotic area suggest that TGF-β might be modulated by the B2R. Although the increase in immunoreactive TGF-β in tubular cells did not reach statistical significance, it could be explained by a high delivery of this protein into the tubular lumen or interstitial tissue space, an idea supported by our findings on HK-2 cells where high levels of active TGF-β were found in the supernatants.

Our experiments using icatibant in normal rats under normosodic diet also support our hypothesis because these animals developed fibrosis in parallel with an upregulation of TGF-β but without an increase in blood pressure. This observation seems to be in disagreement with the absence of fibrosis or inflammation described in mice lacking the B2R (29, 44), but these type of experiments are almost entirely limited to mice (31) whereas we have used Sprague-Dawley rats. It is tempting to speculate that, during their development, knockout animals may establish compensatory mechanisms that result in absence of tissue damage whereas wild-type animals submitted to receptor blockade during their adult life may suffer an unexpected disruption that is difficult to compensate. We do not have experimental data to evaluate whether the effects produced by albumin plus high salt may be exaggerated under B2R blockade because in our model the addition of icatibant was designed to block the effects of a kinin system previously activated by potassium. Nevertheless, we have observed that animals submitted to overload proteinuria in combination with B2R blockade (in absence of potassium) show a significant increase of interstitial α-SMA when compared with proteinuric animals without icatibant (unpublished data).

Our data obtained in cultured HK-2 proximal tubule cells are also consistent with our animal models. We showed, in an isolated system, free of hemodynamic influences, that albumin induces changes compatible with EMT, which is the most accepted pathophysiological mechanism to explain renal fibrosis in progressive renal disease. This important fact has been recently demonstrated in normal rat tubular cells NRK52E (27), and here we added that EMT may be prevented by bradykinin, an effect that is reversed by icatibant.

The issue of salt itself as inducer of fibrosis or EMT was evidenced by a significant but mild increase in fibrosis and a trend to express more α-SMA by normal rats fed hypsosodic diet only, suggesting that, in this model, fibrosis may be mainly attributed to the effect of proteinuria. Salt may induce myocardial and renal fibrosis in normotensive and hypertensive rats in coincidence with oxidative stress (41) and upregulation of TGF-β1 mRNA (59), and an increase in renal expression and urinary excretion of TGF-β has also been described in the renal cortex of Sprague-Dawley rats submitted to a high-sodium diet without a significant increase in blood pressure (57, 58).

We cannot provide data on the potential effect of our complex dietary approach involving salt administered simultaneously with potassium and the potential changes induced on aldosterone. Although an increase in aldosterone had been described as a physiologic response to a high-potassium diet and aldosterone itself could stimulate kallikrein secretion (26), a recent study shows that a high-potassium diet increases renal kallikrein secretion in both control and aldosterone synthase-deficient mice, indicating that aldosterone is not required for the stimulatory effect of potassium intake on kallikrein secretion (18, 19). Since an increase in aldosterone might not be consistent with the proposed antihypertensive/antifibrotic effect observed in our experiments, it is important to note that a high-potassium diet has attenuated the hypertensive injury in subtotal nephrectomy by downregulating TGF-β, upregulating renal Smad7, and lowering blood pressure (54) and a similar effect has been observed in spontaneously hypertensive rats independently of blood pressure reduction (20). Even more relevant is that potassium supplementation in DOCA-salt rats has attenuated the development of hypertension (23, 43), suggesting that the renoprotective effect of high-kinin levels might overcome the negative effect of a potentially elevated aldosterone at least at the doses of potassium and the level of stimulation achieved in our experimental design.

Finally, our results support pharmacological strategies focused on the stimulation and/or preservation of renal kallikrein, its effector peptides, and/or the B2R, deserving to be considered when designing antihypertensive therapies due to its added advantage of a renoprotective effect.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS


REFERENCES


