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Up-regulation of the kinin B_2 receptor pathway modulates the TGF-β/Smad signaling cascade to reduce renal fibrosis induced by albumin

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ABSTRACT

The presence of high protein levels in the glomerular filtrate plays an important role in renal fibrosis, a disorder that justifies the use of animal models of experimental proteinuria. Such models have proved useful as tools in the study of the pathogenesis of chronic, progressive renal disease. Since bradykinin and the kinin B_2 receptor (B_2R) belong to a renoprotective system with mechanisms still unclarified, we investigated its anti-fibrotic role in the *in vivo* rat model of overload proteinuria. Upon up-regulating the kinin system by a high potassium diet we observed reduction of tubulointerstitial fibrosis, decreased renal expression of α-smooth muscle actin (α-SMA) and vimentin, reduced Smad3 phosphorylation and increase of Smad7. These cellular and molecular effects were reversed by HOE-140, a specific B_2R antagonist.

In vitro experiments, performed on a cell line of proximal tubular epithelial cells, showed that high concentrations of albumin induced expression of mesenchymal biomarkers, in concomitance with increases in TGF-β1 mRNA and its functionally active peptide, TGF-β1. Stimulation of the tubule cells by bradykinin inhibited the albumin-induced changes, namely α-SMA and vimentin were reduced, and cytokeratin recovered together with increase in Smad7 levels and decrease in type II TGF-β1 receptor, TGF-β1 mRNA and its active fragment. The protective changes produced by bradykinin *in vitro* were blocked by HOE-140.

The development of stable bradykinin analogues and/or up-regulation of the B_2R signaling pathway may prove value in the management of chronic renal fibrosis in progressive proteinuric renal diseases.

1. Introduction

Proteinuria is a common feature of renal glomerular diseases, which mirrors the severity of the underlying pathological changes, and contributes to the development of interstitial fibrosis [1]. Angiotensin II, a powerful vasoconstrictor peptide, is involved in both the inflammatory and the fibrotic processes in the kidney [2], and contributes to significant amount of proteinuria [3]. Experimental data support the renoprotective effects of angiotensin-converting enzyme (ACE) inhibitors, which reduce proteinuria, delay fibrosis and preserve renal function [4]. ACE inhibitors not only reduce the formation of active angiotensin II, but also inhibit degradation of bradykinin (BK), thereby increasing the circulating and interstitial tissue levels of this potent vasodilator [5].

BK is a vasoactive nanopeptide enzymatically formed by tissue kallikrein, which exerts its biological actions by activating the kinin B_2 receptor (B_2R), a member of the superfamily of G protein-coupled receptors. A renoprotective role of BK, mediated by B_2R, has been proposed since in animal models its activation reduces renal fibrosis whereas its pharmacological blockade or deletion exacerbates tubulointerstitial damage [6–10]. Human studies have shown that B_2R polymorphism predisposes to increased urinary albumin excretion [11], and specific B_2R polymorphism is more frequently observed in patients with end-stage renal disease [12]. In previous animal studies, we have shown that a high potassium diet induces up-regulation of the kinin cascade, and thereby reduces proteinuria in rats overloaded with albumin [13]. Furthermore, we recently

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described the renoprotective properties of this system in a model of salt-sensitive hypertension, induced by overload proteinuria, in which we observed a reduction in blood pressure and renal fibrosis, and down-regulation of TGF-β [14].

Tubular interstitial fibrosis involves an excessive deposition of extracellular matrix in the tubular interstitium by activated fibroblasts. A characteristic feature of these cells is the expression of α-smooth muscle actin (α-SMA), and therefore they are designated as myofibroblasts, but their origin is still matter of debate [15]. Although the mechanisms involved in the anti-fibrotic action of BK remain unknown, evidence suggests regulation by G-protein-coupled signaling pathway involving TGF-β1, and also by epithelial-mesenchymal transition (EMT) since BK attenuates the loss of E-cadherin expression and up-regulation of α-SMA, concomitantly with modulation of the Smad4 (Co-Smad) expression induced by TGF-β1 in HK-2 cells (human kidney proximal tubular cells) [16]. Urinary proteins, including albumin, have been shown to induce EMT of renal tubular cells *in vitro* [17–20], an event that may contribute, at least in part to the pathogenesis of chronic kidney disease [21]. The albumin overload proteinuria model resembles the damage of the tubular cells by exposure to elevated protein concentrations, as occurs in proteinuria-related renal diseases, and has proven to be valuable to investigate the relationship between proteinuria and renal damage [1]

The current study was designed to evaluate *in vivo* and *in vitro* the renoprotective role of BK and B_2R in albumin-induced renal damagę engineered through changes to the TGF-β Smad signaling pathway and further prevent EMT progression, with a consequent reduction in renal fibrosis.

2. Material and methods

2.1. Animals

The Animal 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 weeks, 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, and maintained at constant room temperature with a 12 h light/dark cycle and unrestricted access to food and water.

Differences in the intensity of proteinuria have been described in the model of albumin overload according to the rat strain and gender used [44]. Taking advantages of a local availability we preferred, like others, Sprague-Dawley female animals [13,14]. In fact, there are illustrative reports using female Lewis [22,36,45] or Sprague-Dawley rats [39].

2.2. Experimental design and animal groups

Overload proteinuria without previous nephrectomy (to avoid the effects of renal mass reduction) was commenced with one intraperitoneal injection per day for 14 days of 2 g of bovine serum albumin (BSA, Fraction V, A-4503, Sigma) given as 6 ml of a 33.3% BSA solution dissolved in 0.9% sterile saline [1,13,14,22], previously filtered (Milipore-Sterivex HV 0.45 um). The diet used during the overload and wash/out phases was a normosodic rodent chow (0.4% NaCl). Overload was followed by a wash/out recovery period lasting a further 2 weeks to explore the chronic profibrotic effect induced by BSA exposure *in vivo*, out of the reach of the acute effect of a proteinuric stage, and to provide sufficient time to develop histological fibrosis. Thereafter, animals were euthanized.

The experimental groups that were designed are listed in Fig. 1a, and comprised the following: (i) **CTRL** ($n = 5$): Control group; healthy female rats receiving normal rat chow and free access to water; (ii) **BSA** ($n = 5$): Disease group, representing the animal model, received protein overload followed by 2 weeks of wash/out; (iii) **KCl + BSA** ($n = 8$): As previously described, this group was pretreated with 2% KCl in tap water to up-regulate the kinin cascade and generate over-expression of both renal kallikrein and B_2R at the mRNA and protein levels [13,14,23] from 4 weeks prior to protein overload and until the end of the experiment. Three animals of this group were euthanized at the end of the 4th week of high potassium diet to determine changes in B_2R and tissue kallikrein by Western blotting and immunohistochemistry; (iv) **HOE + KCl + BSA** ($n = 5$): These animals were submitted to the same protocol as the above potassium group (KCl + BSA), but the kinin B_2R antagonist HOE-140 (Sanofi-Aventis, Germany) was administered during the albumin overload at a dose of 500 μg/Kg/day using subcutaneous osmotic minipumps (ALZET).

At the end of each experiment, 24 h of urine collection was performed to determine proteinuria (U/CSF Protein; Roche Diagnostics, Mannheim, Germany), urinary potassium (ion-selective autoanalyzer) and creatininuria (with a modification of the Jaffé reaction). Animals were euthanized by aortic exsanguination under general anesthesia; kidneys were removed, washed with saline, and prepared for immunohistochemistry (horizontal slices fixed in 4% formalin-PBS and embedded in paraffin wax) or homogenized for Western blot analysis.

2.3. B_2 inhibition in normal rats

To evaluate the effect of B_2R blockade in the kidneys of normal animals without proteinuria, a group of six adult female rats fed a normosodic diet were submitted to subcutaneous administration of HOE-140 (500 μg/kg per day) during 4 weeks and compared with five normal rats without treatment. Fibrosis was evaluated histologically by Masson's trichromic staining and TGF-β mRNA was determined by in situ hybridization.

2.4. Histological evaluation

Hematoxylin-eosin tissue sections were analyzed under optical microscopy 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. [24]; the entire cortical region was examined, and the average score obtained in each tissue sample was used as the "tubulointerstitial score" as published [13,14]. The degree of renal fibrosis was assessed histologically with the aid of Masson's trichromic staining and quantified as the percentage of the tubulointerstitial area.

2.5. Cell culture

HK-2 cells (human kidney proximal tubular cell line, ATCC Catalog CRL-2190, Manassas, VA, USA) were grown in RPMI 1640 containing 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, 100 mg/ml streptomycin, insulin-transferrin-sodium selenite (ITS) and hydrocortisone in 5% CO_2 at 37 °C. At 60–70% of confluence, the cells were growth-arrested in serum-free medium for 24 h before 10 nM BK (Sigma–Aldrich, St Louis, MO, USA) was added for 24 h, and then 10 mg/ml bovine serum albumin (BSA; Sigma) was added for 48–72 h. This albumin concentration was determined from dose response experiments (5 to 30 mg/ml) to induce EMT and providing the best balance between cytotoxicity and cell viability; it is coincident with that published by Ibrini et al. [17] in their studies on EMT using NRK52E cells and by others investigating albumin transport *in vitro* [25,26].

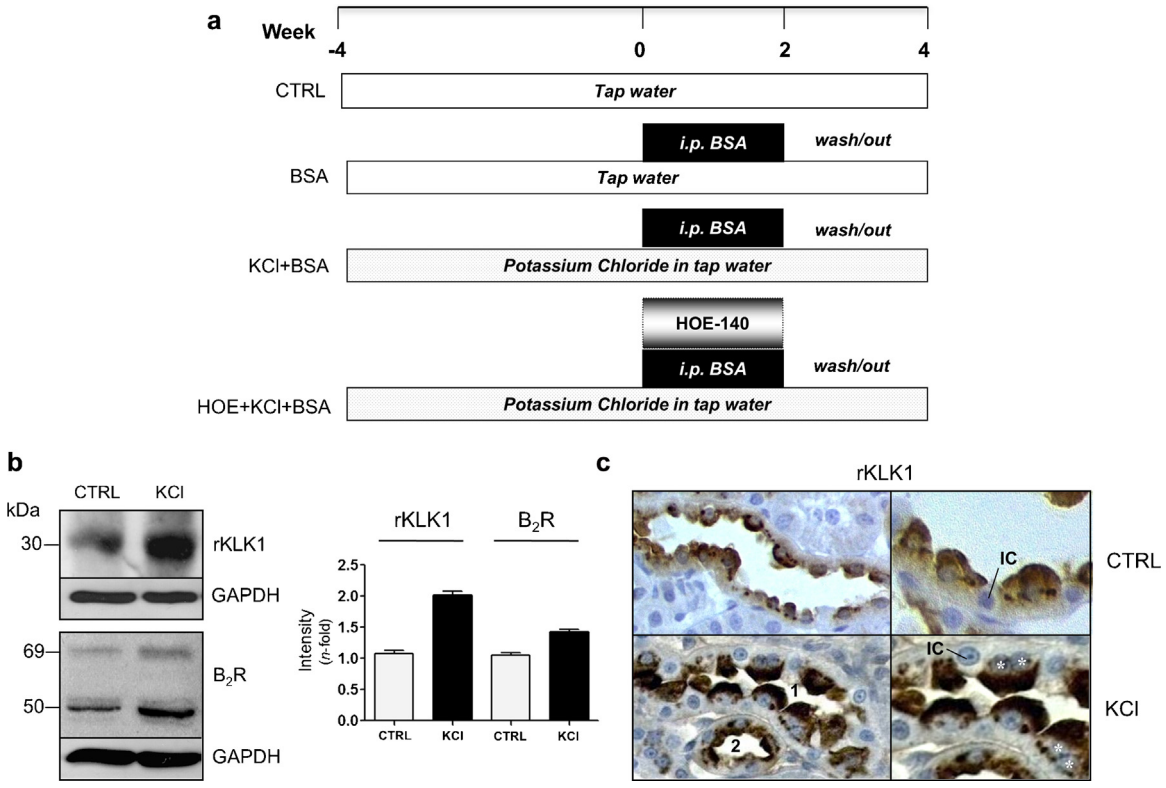


Fig. 1. (a) Schematic representation of the various experimental groups included in the study. CTRL, control group of healthy animals; BSA (bovine serum albumin), animals that received protein overload; KCl + BSA, animals that received potassium chloride in the their drinking water for 4 weeks prior to protein overload; HOE + KCl + BSA, group submitted to the same protocol as the KCl + BSA group except that the kinin B_2R antagonist, HOE-140, was administered during the protein overload phase. The last 3 groups were evaluated after a washout period of 2 weeks. i.p., intraperitoneally. b, c) High potassium diet up-regulates renal kallikrein (rKLK1) and the B_2R in Sprague-Dawley rats. Kidneys were homogenized, the proteins separated by polyacrylamide gel electrophoresis and then immunoblotted for kallikrein (rKLK1) or B_2R (b) or were fixed and processed to visualize renal kallikrein by immunohistochemistry (c). Kallikrein immunoreactivity is concentrated in the apical pole of connecting tubule cells of both controls (CTRL) and potassium-treated animals (KCl). Hypertrophy of kallikrein-producing cells is clearly observed in two connecting tubules (1, longitudinal section; 2, cross-section) of potassium-treated rats when compared with control; cell hypertrophy includes presence of binucleated immunoreactive cells (asterisks). Intercalated cells (IC), the other cell type present in connecting tubules are not hypertrophied. Images are representative of 4 animals per condition. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

B_2R activity was blocked by preincubating the cells for 30 min with 100 nM of the B_2R antagonist, HOE-140 (Sanofi-Aventis, Germany). When necessary, TGF-β was used at 10 ng/ml for 48 h.

2.6. Cell morphology

Image capture was obtained with an OmniVID LW camera coupled to an OmniVID LW inverted phase contrast microscope. Cell morphology assessment involved the use of SCIAN-Soft image analysis software (SCIAN-Lab, University of Chile, www.scian.cl) written in IDL language (Exelisvis, Boulder, Colorado, USA). Initially, cell segmentation was performed using filters for region detection and refinement. Secondly, the ratio perimeter^2/area (P^2/A) was computed for each segmented region (cell). For controls, an average value of P^2/A was computed for non-treated cells. Segmentation and computation of P^2/A was then applied to the treated-cell images. The cells with a P^2/A value greater than those of the control average were considered fusiform, and the percentage of fusiform cells recorded for each experimental condition was calculated.

2.7. Western blotting

Cells were homogenized using RIPA buffer (25 mM Tris-HCl pH 7.6; 150 mM NaCl; 1% IGEPAL; 1% sodium deoxycolate; 0.1% SDS) supplemented with a protease/phosphatase inhibitor cocktail (Pierce). Proteins were separated using polyacrylamide gel electrophoresis under denaturing conditions and then transferred onto PVDF membranes (Millipore, USA) as previously described [27]. For specific protein detection, primary antibodies were used as follows: (i) mouse monoclonal against vimentin, (BD Pharmingen, San Diego, CA, USA); (ii) rabbit monoclonal against pSmad3 (Abcam, Cambridge, UK); (iii) rabbit monoclonal against TGF-β1 (Cell Signaling Technology, Danvers, MA, USA); (iv) rabbit polyclonal against the type II receptor of TGF-β (Santa Cruz Biotechnology); (v) rabbit polyclonal against Smad7 (Abcam); (vi) mouse monoclonal against GAPDH (Millipore); (vii)rabbit polyclonal against rat urinary kallikrein [13] and (viii) rabbit polyclonal against B_2R (Abcam). Next, membranes were incubated with peroxidase-labeled polyclonal goat anti-mouse or anti rabbit IgG secondary antibody, and a chemiluminescence detection kit was utilized (Pierce). GAPDH was used as control of protein loading. Quantification of immunoreactive bands was performed by densitometric analysis using ImageJ 1.45 (National Institutes of Health, USA). In experiments involving animals, proteins were extracted from a piece of the whole kidney (1 mm-thick), which was homogenized in T-PER lysis buffer (Pierce) supplemented with a protease/phosphatase inhibitors cocktail (Pierce), and the samples were processed as described for cell studies.

2.8. Cell and tissue labeling

Cells were grown on coverslips, fixed in 4% *p*-formaldehyde, permeabilized with 0.1% Triton X-100 and then incubated with primary antibodies followed by incubation with a secondary anti-

body conjugated to Alexa-fluor 488. Nuclei were stained with DAPI (Invitrogen), and samples mounted with Mowiol and examined using confocal microscopy (Leica). Antibodies employed were a mouse monoclonal against vimentin (BD Pharmigen, San Diego, CA, USA) and a mouse monoclonal against pan-cytokeratin (Sigma). For animal studies, tissue sections (5 μm-thick) were dewaxed, hydrated, washed three times with 50 mM Tris–HCl pH 7.8 and then incubated with a mouse monoclonal anti-α-SMA antibody (Dako, Carpinteria CA, USA) or an anti-urinary kallikrein antibody [13], diluted in the same buffer containing 1% immunoglobulin-free BSA. Bound immunoglobulins were detected using the LSAB + HRP System (Dako) and peroxidase activity developed with 3,3′-diaminobenzidine. Negative controls included omission of the primary antibody and its replacement by non-immune rabbit serum at the same dilution. Renal TGF-β was evaluated by *in situ* hybridization as previously described [14].

2.9. Image analysis

The percentage and intensity of the labeled tissue section area visualized by immunohistochemistry, *in situ* hybridization or Masson's trichromic staining were evaluated with the KS300 Imaging System 3.0 (Zeiss, München-Hallbergmoos, Germany). The degree of staining was calculated by the ratio of suitable binary threshold images and the total field area, integrating the intensity of the staining in the specific areas. Determining the values using these methods enabled the avoidance of the potential differences in the amount of total tissue examined. For each sample, a mean value was obtained by the analysis of 20 different fields (at x20), reading the tubulointerstitial areas by segmentation, and excluding the vessels. The procedure was performed twice, independently, and the inter-observer variability was not significant. The staining score was expressed as density/mm^2, as previously published [13,14].

2.10. qPCR

Total RNA was isolated with TRIzol (Gibco, Invitrogen, Carlsbad, CA, USA), and gene expression was analyzed by real-time PCR performed on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The assay ID used for human TGF-β1 was Hs99999918_m1. For normalized data, 18s ribosomal RNA expression was used (assay IDs: 4.310.893E). Each specimen was evaluated twice independently, and the data expressed as the mean ± SE.

2.11. Measurement of TGF-β1 by ELISA

Concentration of TGF-β1 in cell culture supernatants was measured using a commercial ELISA kit (R&D Systems, USA) 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. After that, all steps were performed at room temperature. The next day, plates were washed three times with PBS-0.05% Tween 20 and blocked with 5% BSA in PBS for 1 h. After three washes, 100 μl of standard or supernatants were added and after 2 h of incubation and five washes, 100 μl of detection antibody and enzyme reagent mixture was added to each well, and the plate incubated for 1 h. Unbound detection antibody and enzyme mixture were removed with seven consecutive washes with PBS-0.05% Tween 20. Wells were incubated with 100 μl of substrate solution for 30 min in the dark. The reaction was stopped by adding 50 μl of stop solution, and the absorbance was measured at 450 nm using a microplate scanning spectrophotometer (Infinite M200, Tecan).

2.12. Statistical analysis

A commercial statistical package (GraphPad InStatR, version 3.01 for Windows 95/NT, San Diego CA, USA) was utilized. The nonparametric Kruskal–Wallis ANOVA test followed by the Mann–Whitney test was used to examine the differences between the groups. Values are expressed as the mean ± SE, and P values <0.05 were considered significant.

3. Results

3.1. Stimulation of the kinin cascade ameliorated the renal interstitial fibrosis in the overload proteinuria animal model

Up-regulation of the renal kinin cascade, induced by the high potassium diet and observed in the kidneys of rats prior to the protein overload was indicated by the overexpression of both B_2R and renal kallikrein (Fig. 1b). The anti-B2R antibody used recognizes two major bands, one 69 kDa band and another 50 kDa band, which are similar to those described in previous reports on HF-15 fibroblasts lysates [28]. In addition, the presence of hypertrophied kallikrein-containing cells (connecting tubule cells) on immunohistochemistry (Fig. 1c) when compared with the control group, confirmed previously published results [13,14,23,29,30]. Values for kaliuresis at the end of the experiment in the groups receiving potassium confirmed the high intake of KCl (KCl + BSA: 156 ± 21 and HOE + KCl + BSA: 364 ± 11 μmol/mg of creatinine vs control animals: 71 ± 17 μmol/mg creatinine and BSA group: 83 ± 2 μmol/mg creatinine).

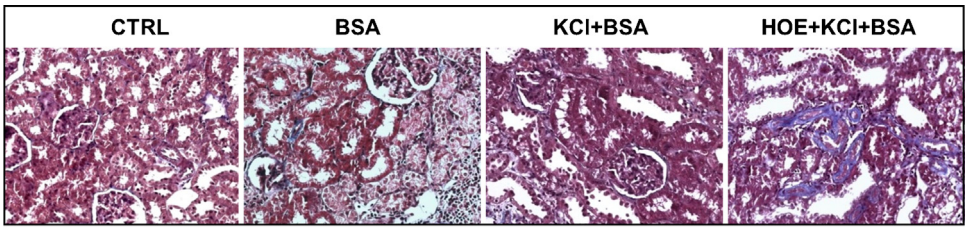
The profile of proteinuria after BSA overload in animals pretreated with or without potassium, and with or without the addition of B_2R antagonist, has been reported [13,14]. In the current design we evaluated urinary protein excretion only at the end of the experiment and, although the values were low (samples were collected after the washout), they remained higher in the BSA group than in control animals. Interestingly, potassium treatment significantly reduced proteinuria in albumin overloaded rats, but it was reversed when B_2R blockade was included (Fig. 2). Masson's trichrome staining of renal tissue sections showed histological changes of tubulointerstitial injury and collagen accumulation in the kidneys of rats exposed to albumin when compared to controls animals. In contrast, animals subjected to a potassium diet displayed significantly less tubulointerstitial fibrosis, a protective effect that was reduced when the B_2R antagonist HOE-140 was added (Fig. 2). The protection provided by potassium and its dependence on the B_2R was also observed in the tubulointerstitial score (Fig. 2).

3.2. The B_2blockade up-regulates TGF-β and induces renal fibrosis in normal non-proteinuric rats

A significant increase in fibrosis, accompanied by increase in the expression of TGF-β mRNA was observed after 4 wk of B_2R antagonist administration (Fig. 3).

3.3. Up-regulation of the kinin system prevented the over-expression of EMT markers induced by albumin overload in vivo

Immunohistochemistry showed that α-SMA (a myofibroblast marker) was weakly expressed in the renal tubule-interstitium of control rats, but its presence and intensity were prominent in BSA treated animals, especially in the fibroblast-like cells present in the inflammatory infiltrate (Fig. 4a and c). The α-SMA staining of cells in renal sections of animals on the potassium diet was strongly reduced, whereas B_2R antagonism reversed this effect (Fig. 4b).



	CTRL	BSA	KCl+BSA	HOE+KCl+BSA
Proteinuria (mg/mg creatinine)	0.49 ± 0.05	2.34 ± 0.6 *	0.96 ± 0.2 * #	3.72 ± 1.2 * &
TI fibrosis	0.22 ± 0.01	0.94 ± 0.1 *	0.42 ± 0.01 * #	0.95 ± 0.11 * &
TI score	0.17 ± 0.1	1.1 ± 0.08 *	0.72 ± 0.05 * #	1.33 ± 0.18 * &

Fig. 2. Renal tissue appearance under various experimental conditions. Renal tissue sections were stained by trichromic Masson's staining and blue staining is showing stained collagen deposited in the peritubular space. A representative image of each group is shown. x250. Bottom: Quantification of proteinuria, tubulointerstitial (TI) fibrosis and TI damage score. $^{*}P<0.05$ vs CTRL; $^{\#}P<0.05$ vs BSA, $^{\&}P<0.05$ vs KCl+BSA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

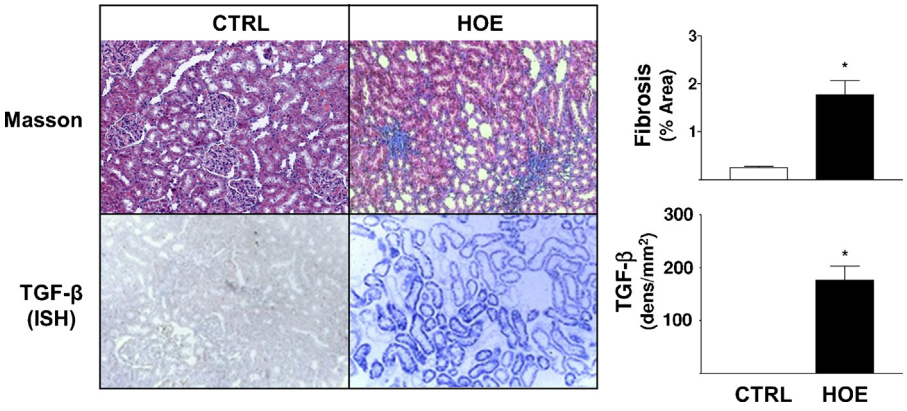


Fig. 3. Renal tissue effects after 4 weeks of subcutaneous administration of HOE-140 to normal female rats fed a normosodic diet. Pharmacologic B_2R antagonism induces significant fibrosis in the tubulointerstitial area as revealed by Masson trichromic staining showing collagen deposition in the peritubular space (blue) and TGF-β mRNA up-regulation. ISH, *in situ* hybridization. Images are representative of each condition. Original size x150. Stainings were quantified and results expressed as mean ± SE ($n=6$ per group) for each experimental group. $^{*}P<0.05$ vs CTRL. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Observation of selected tubular cells containing immunoreactive α-SMA was suggestive of EMT initiating *in vivo* (Fig. 4c). The values for vimentin (a mesenchymal marker) evaluated in renal extracts were considerably increased in BSA rats when compared to control animals, in which it was almost undetectable. Up-regulation of the kinin cascade prior to the protein overload significantly reduced vimentin levels and, importantly, it was abolished by treatment with the B_2R antagonist HOE-140 (Fig. 5a).

3.4. Up-regulation of the kinin cascade down-regulated the Smad signaling pathway in vivo

Albumin overload increased the expression of TGF-β1 and phosphorylated Smad3 levels compared to control animals (Fig. 5b and c). In contrast, potassium administered to BSA animals significantly attenuated the elevated levels of phosphorylated Smad3 and simultaneously up-regulated Smad7 (Fig. 5d), although it was not able to reduce precursor TGF-β1 levels. Changes induced by potassium on both components of the Smad pathway were reversed when the B_2R was antagonized pharmacologically (Fig. 5).

3.5. Albumin and BK modulated expression of the TGF-β/Smad signaling cascade in a dose-dependent manner in vitro

To investigate further the mechanisms involved in albumin-induced tubular damage, *in vitro* studies were done exposing HK-2 cells to BSA (5–30 mg/ml), the range most commonly used to evaluate the toxicity of proteinuria in PTEC. Proximal tubular fluid albumin concentration of rats with immunologically induced nephrotic syndrome has been reported in about 7 mg/dl in micropuncture studies [31] but urine protein concentrations measured in our rats at the first week of BSA injections (data not shown) were considerably higher. Since the glomerular ultrafiltrate suffers multiple processes ending in a different concentration of proteins in the collected urine, it is not easy to set-up *in vitro* studies based on clinical data, especially if we know that the *in vivo* tubular changes observed in nephrotic conditions are manifested only after a prolonged exposure of proximal tubules to filtered proteins.

We could see that HK-2 cells, when subjected to increasing concentrations of BSA, showed progressive and significantly increased phosphorylation of Smad3 and expression of TGF-β and α-SMA together with a down-regulation of Smad7 (Fig. 6). On the other hand, in these cells BK induced a less pronounced but significant

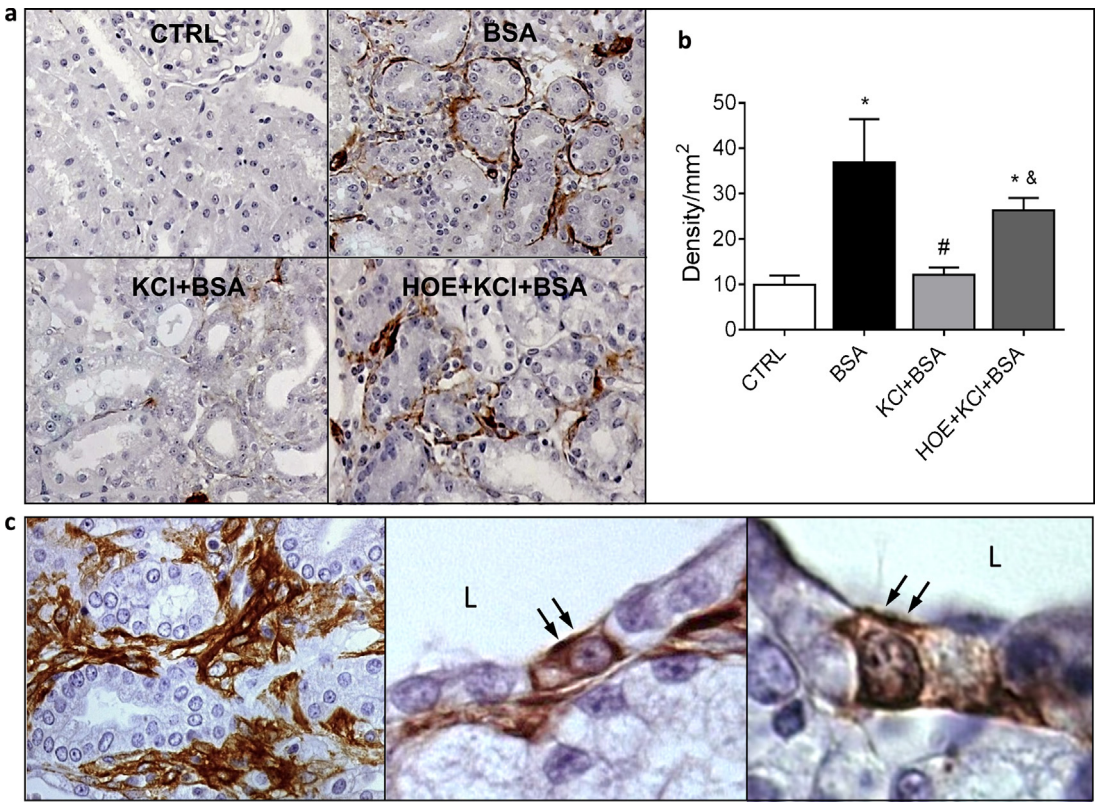


Fig. 4. Immunoreactivity to α-SMA in various experimental conditions. (a) Immunohistochemistry showing α-SMA in the peritubular space (brown staining). (b) Quantification of α-SMA staining in each experimental group expressed as the mean ± SE ($n = 5$ per group). $^{*}P < 0.05$ vs CTRL; $^{\#}P < 0.05$ vs BSA; $^{\&}P < 0.05$ vs KCl + BSA. (c) Representative images showing interstitial accumulation of α-SMA immunoreactive cells (left) and individual tubular epithelial cells expressing α-SMA (double arrows) in animals of the HOE + KCl + BSA group (center and right). L, tubular lumen. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reduction in the expression of the type II TGF-β receptor (TβRII), depending on the dose used (data not shown).

3.6. BK prevented phenotypic changes and the expression of mesenchymal markers induced by albumin in HK-2 cells

Albumin exposure induced significant morphological changes in which tubular cells transformed from a rounded shape to a fibroblast-like, fusiform shape (Fig. 7a) with the corresponding over-expression of vimentin and down-regulation of pan-cytokeratin when visualized by immunofluorescence (Fig. 8). Interestingly, BK treatment, prior to albumin, significantly prevented these changes that were reversed when the cells were preincubated with the B_2R antagonist, HOE-140, prior to incubation with BK (Figs. 7 and 8). Western blot analysis indicated significant over-expression of α-SMA and vimentin after exposure to albumin, but they were attenuated by pretreatment with BK. The effects produced by BK on the mesenchymal biomarkers were reversed by HOE-140, confirming the primary involvement of B_2R (Fig. 9).

3.7. BK prevented the phenotypic changes induced by TGF-β in HK-2 cells

Morphological changes caused by TGF-β were identical to those produced by albumin; renal tubular cells were transformed from a round shape to a fusiform fibroblast-like one. BK added to the incubation medium prior to TGF-β, significantly prevented these changes. The effect of BK was inhibited when the cells were initially incubated with HOE-140 (Fig. 7b).

3.8. BK attenuated the expression of TGF-β1 and modulated the Smad signaling pathway activated by albumin in HK-2 cells

Albumin exposure, in vitro, induced up-regulation of TGF-β1, visualized as an increase in its mRNA expression, precursor protein and active peptide levels, which were significantly reduced by pretreatment of the cells with BK and reverted by B_2R antagonism (Fig. 10). Albumin produced also a significant increase in TβRII protein levels, phosphorylation of Smad3 and a slight increase in Smad7 protein levels, a negative regulator of the TGF-β1 signaling. In contrast, pretreatment of cells with BK, prior to adding albumin, significantly attenuated the augmentation of TβR-II and up-regulated the levels of Smad7, molecular events that were antagonized by HOE140. Smad3 phosphorylation was not affected by BK (Fig. 10).

4. Discussion and conclusions

Our experiments support the protective role of BK in an animal model of proteinuria, functionally bioregulated by important pathogenic mechanisms such as the TGF-β/Smad pathway and EMT.

The overload proteinuria rat model is a non-immunologic model [22] where immune complex formation does not play a significant pathogenic role [32]. The kidneys show significant tubulointerstitial infiltrate consisting almost entirely of mononuclear cells [1,13,33] and express proinflammatory and profibrotic factors, including chemokines, cytokines, monocyte chemotactic proteins and growth factors which leads to tubulointerstitial inflammation and fibrosis [22,34–37].

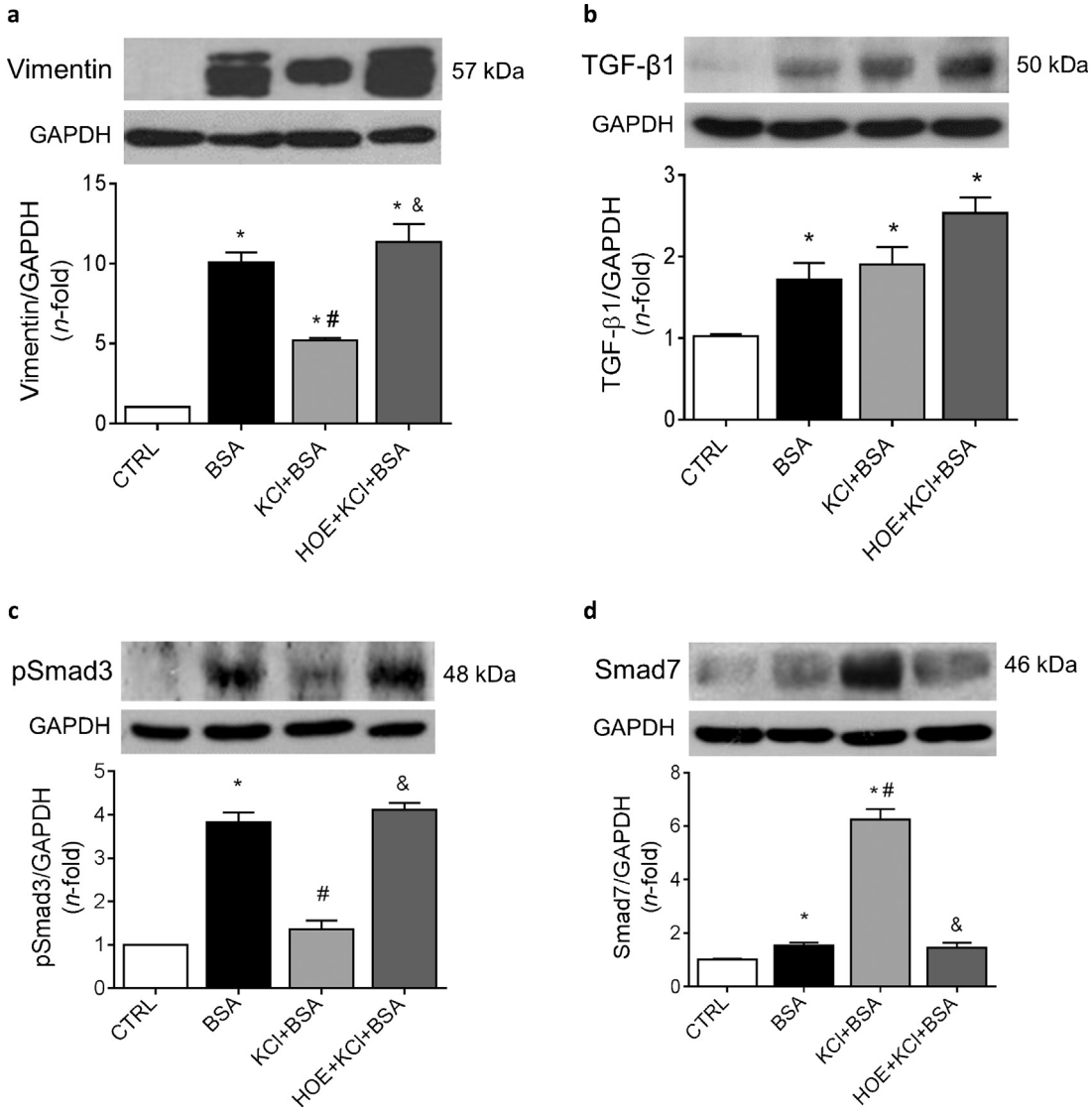


Fig. 5. Up-regulation of the kinin system in vivo modulates the phenotype of renal cells and the TGF-β/Smad signaling pathway. Renal tissue was homogenized, and the proteins were separated by polyacrylamide gel electrophoresis (a) Vimentin, (b) TGF-β1 (precursor protein), (c) phosphorylated Smad3 (pSmad3) and (d) Smad7 were assessed by Western blotting and data expressed as means ± SE ($n = 5$ per group). Values are expressed as n-fold over CTRL. $^{*}P < 0.05$ vs CTRL; $^{\#}P < 0.05$ vs BSA; $^{\&}P < 0.05$ vs KCl + BSA. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

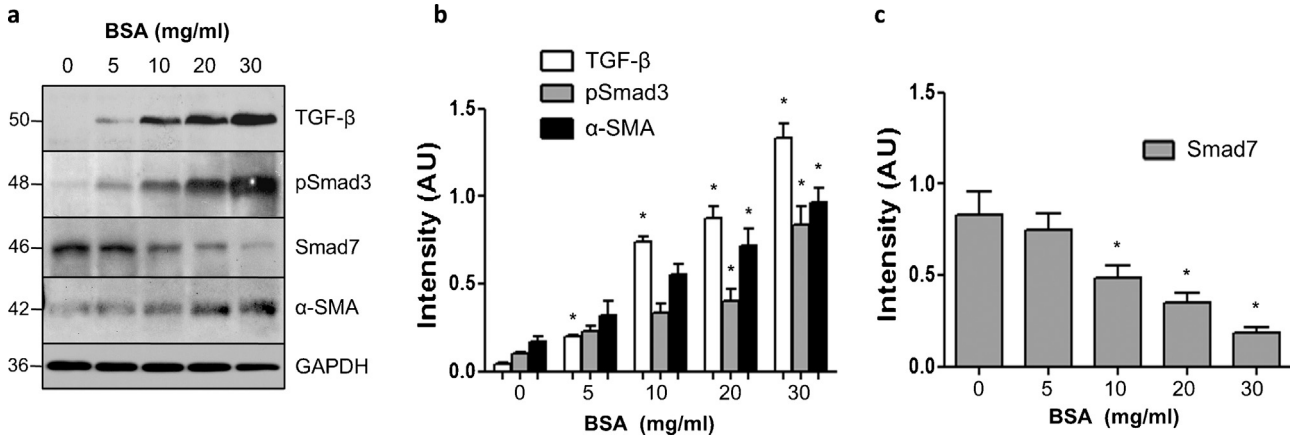


Fig. 6. Albumin up-regulates the TGF-β/Smad pathway in a dose-dependent manner. HK-2 cells were incubated with 5–30 mg/ml of albumin (BSA) for 72 h, homogenized and proteins separated by polyacrylamide gel electrophoresis and then assayed by Western blotting. BSA increases the expression of TGF-β, phosphorylated Smad3 (pSmad3), and α-SMA and reduces Smad7 levels in a dose-dependent manner. Bands corresponding to immunolabeled proteins are expressed as arbitrary units of intensity (AU). Values are shown as the mean ± SE of 3 independent experiments. $^{*}P < 0.05$ vs CTRL. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

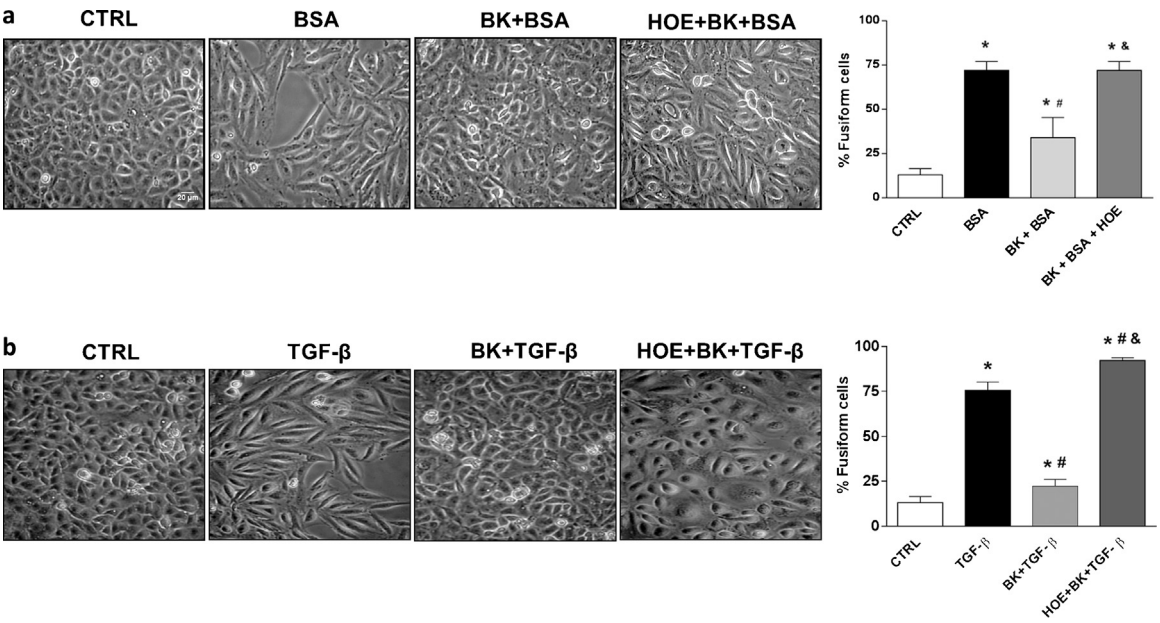


Fig. 7. Bradykinin prevents the phenotypic changes induced by BSA and TGF-β in HK-2 cells. Morphological appearance of HK-2 cells under phase contrast microscopy after (a) treatment with BSA, BK + BSA and HOE + BK + BSA and after (b) incubation with 10 ng/ml of TGF-β alone or following pretreatment with 10 nM bradykinin (BK + TGF-β) or with 100 nM HOE-140 to antagonize B_2R mediated effects (HOE + BK + TGF-β). Serum-deprived unstimulated cells were used as controls (CTRL) in all experiments. Quantification of morphological changes is represented in bar graphs in which values are shown as the mean ± SE of 3 or 4 independent experiments. $^{*}P < 0.05$ vs CTRL; $^{\#}P < 0.05$ vs BSA or TGF-β; $^{\&}P < 0.05$ vs BK + BSA or BK+TGF-β.gr7

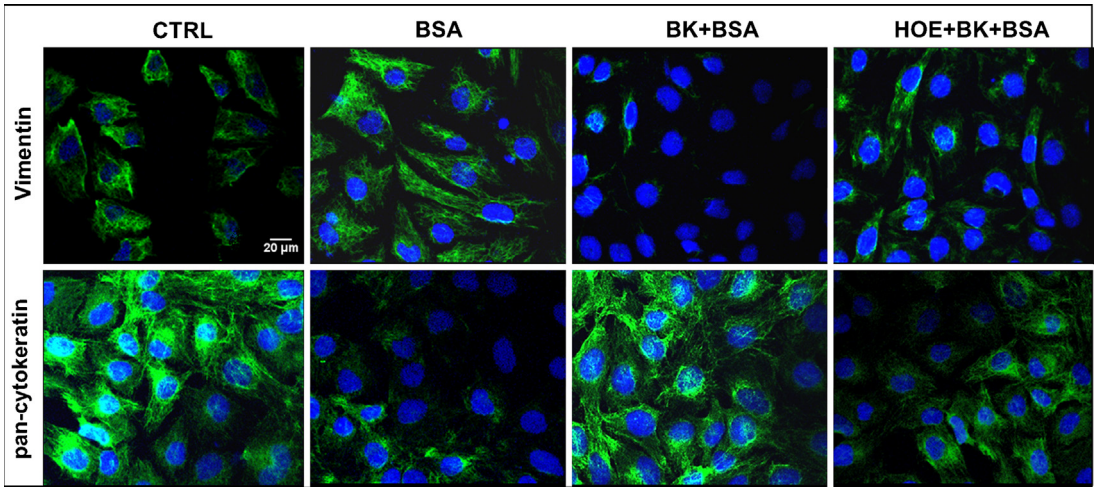


Fig. 8. Immunolabeling of HK-2 cells after treatment with BSA, BK+BSA and HOE+BK+BSA. Cells were incubated directly with BSA or pretreated with bradykinin (BK) or with the B_2R antagonist HOE140 prior to incubation with BSA. After fixation and permeabilization cells were immunostained with an antibody directed to vimentin (Upper row, green) or to all keratins (pancytokeratin, Bottom row, green). Nuclei were counterstained with DAPI (blue). Serum-deprived unstimulated cells were used as controls (CTRL). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The exposure of proximal tubule epithelial cells (PTEC) to high amounts of albumin and its processing has been proposed as crucial in the triggering of the renal fibrotic process. Reabsorption of albumin, via receptor-mediated endocytosis in PTEC, is initiated by its binding to apical clathrin-coated pits, followed by endocytosis and degradation in lysosomes; binding sites have been characterized, but the responsible receptors have not been fully identified. Two giant glycoproteins, cubilin and megalin, constitute important endocytic receptors exposed on the luminal membrane of PTEC [38]. Megalin might also possess intrinsic signaling properties and the ability to regulate cell signaling pathways and gene transcription after processing regulated intramembrane proteolysis. Other pathways for reabsorption might be mediated by the neonatal Fc receptor and CD36 [39,40]. Studies of direct toxicity by myeloma light chains on PTEC, point to proteinuria as a mechanism of kidney injury, confirming that protein overloading and increased endocytosis might be important. Furthermore, gene knockdown of megalin/cubilin protects HK-2 cells from the toxic effects of excessive light chains, including EMT, suggesting that they may be related to endocytosis [41].

BSA-associated damage may be also explained by the induction of mitochondrial reactive oxygen species/endoplasmic reticulum stress [42,43] which may serve as an endogenous danger-associated molecular pattern (DAMP) stimulus for the cytoplasmic Nlrp3 inflammasome [37,42,43] which stimulates caspase-1 activation to promote processing and secretion of proinflammatory cytokines [44].

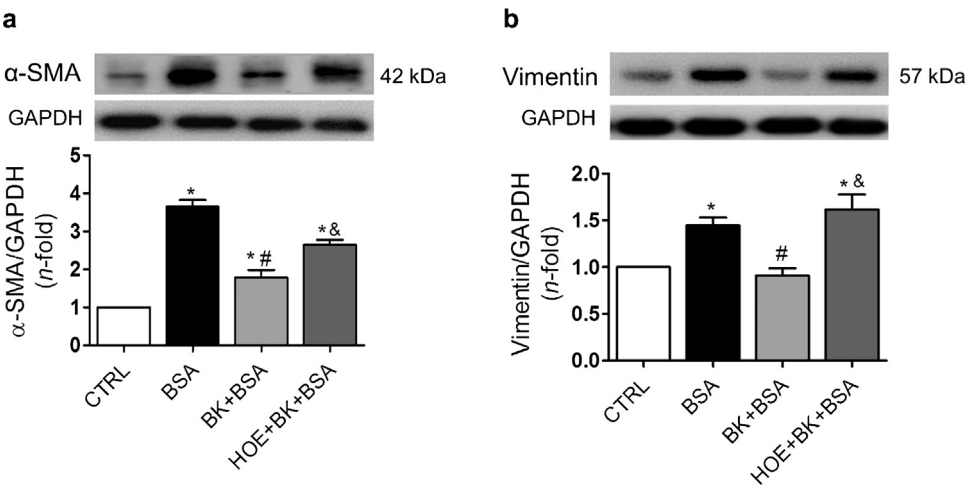


Fig. 9. Immunophenotype of HK-2 cells after exposure to BSA, BK+BSA and HOE+BK+BSA. After various treatments, cells were homogenized and proteins separated by polyacrylamide gel electrophoresis and then immunoprinted with antibodies directed to α-SMA (a) or vimentin (b). Serum-deprived unstimulated cells were used as controls (CTRL). GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Protein levels are expressed as the *n*-fold over the CTRL, and values are shown as the mean ± SE of 3 or 4 independent experiments. $^{*}P < 0.05$ vs CTRL; $^{\#}P < 0.05$ vs BSA; $^{\&}P < 0.05$ vs BK + BSA.

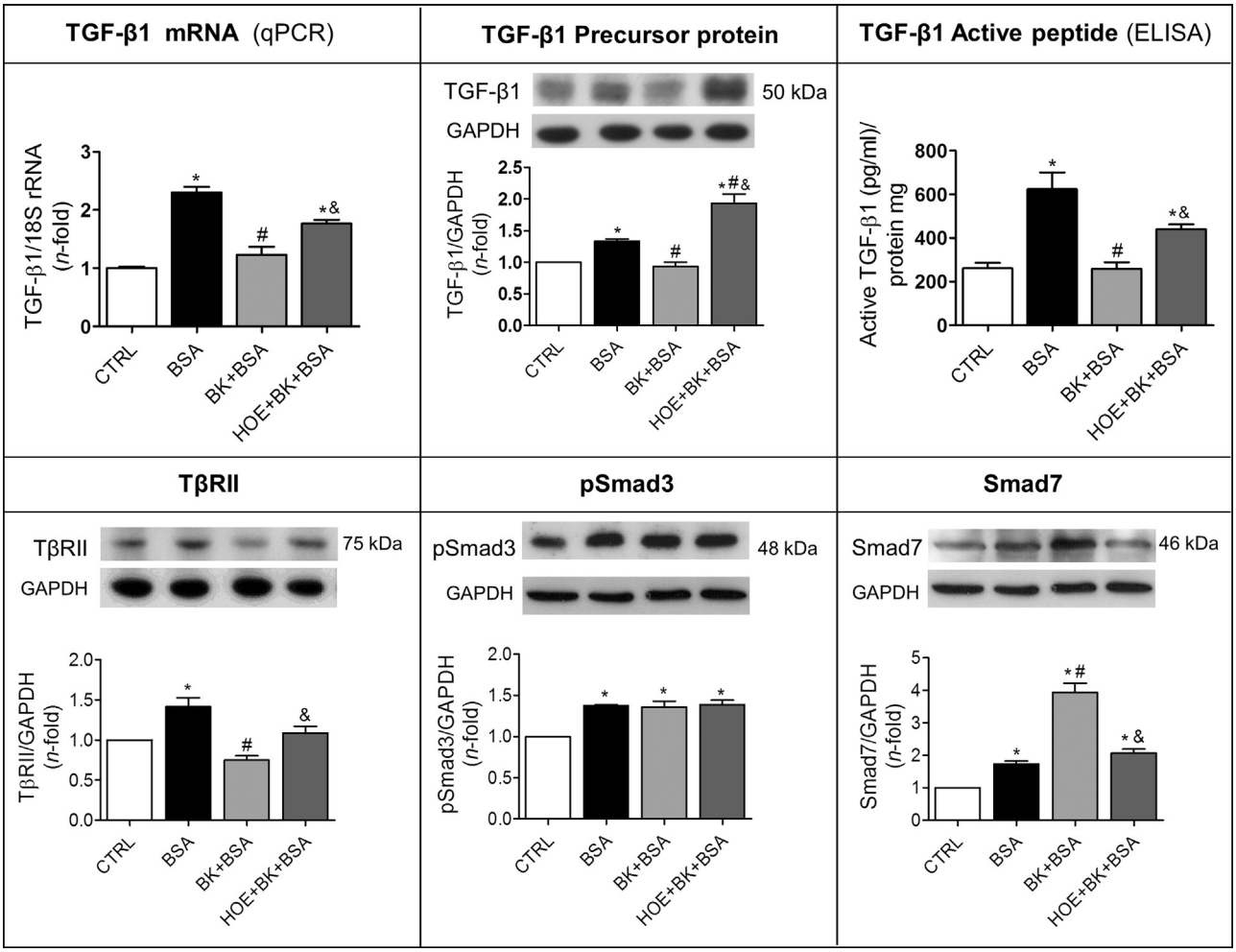


Fig. 10. Bradykinin modulates the changes induced by albumin on the TGF-β/Smad signaling pathway in HK-2 cells. Upper row: Cells were directly incubated with albumin or pre-treated with bradykinin (BK + BSA) or also with HOE-140 (HOE + BK + BSA). TGF-β1 active peptide levels are expressed as pg/ml normalized by the supernatant protein content. Values represent the mean ± SE of 3 or 5 experiments, and the mRNA and protein levels are expressed as the *n* -fold over the CTRL; 18S rRNA and GAPDH were used as house-keeping genes for qPCR and Western blotting, respectively. $^{*}P < 0.05$ vs CTRL; $^{\#}P < 0.05$ vs BSA; $^{\&}P < 0.05$ vs BK + BSA. Lower row: Proteins present in the HK-2 cell homogenates were separated by polyacrylamide gel electrophoresis and then assayed by Western blotting. Immunolabeled proteins are expressed as the *n* -fold over CTRL. Values are shown as the mean ± SE of 3 or 4 independent experiments. $^{*}P < 0.05$ vs CTRL, $^{\#}P < 0.05$ vs BSA, $^{\&}P < 0.05$ vs BK + BSA. TβRII, Type II TGF-β receptor; pSmad3, phosphorylated Smad3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Our experiments suggest that elevated concentrations of albumin could contribute to renal damage by regulating TGF-β1 signaling and EMT-related profibrotic events in PTEC. The key cellular player during kidney fibrosis is the activated fibrogenic fibroblast that may be derived either by proliferation of resident fibroblasts or from multiple parental lineages, including tubular epithelial, endothelial and bone marrow endosteal cells as well as pericytes [45,46]. EMT involves a phenotypic conversion of primary epithelial cells into mesenchymal cells, leading to a fibroblast-like phenotype characterized by down-regulation of E-cadherin and up-regulation of mesenchymal markers such as α-SMA, distinctive of activated myofibroblasts [20]. During this process, myofibroblasts synthesize collagens I and IV, laminin and fibronectin, which initiates deposition and accumulation of extracellular matrix in the interstitial compartment, generating tubulointerstitial fibrosis and chronic kidney disease progression [47,48]. Conversion of various cells into myofibroblasts may be driven by factors released from local resident cells responding to disease-induced stress such as hypoxia, hyperglycemia or proteinuria and from inflammatory and immune cells, which infiltrate the renal interstitium [49]. Previous work has shown that PTEC exposed to albumin acquire a mesenchymal phenotype and show activation of the TGF-β1 signaling pathway *in vitro* [17–19,50,51], as determined in our dose-dependent response experiment. The original hypothesis involving EMT as the main contributor to the increased population of activated fibroblasts has been recently challenged [52] by arguing that the process has not been confirmed in vivo. Nevertheless, much evidence raised against EMT comes from experiments performed using the unilateral ureteral obstruction (UUO) model of fibrosis in mice and recent data supports that disparities may arise from varying experimental conditions, different disease models, mouse strain and the type of genetic modification used [53]. The rat model we used showed a *de novo* expression of the mesenchymal marker SMA in some tubular cells, supporting the involvement of PTEC in EMT. This model, even though shares many of the pathophysiological changes observed in UUO-mediated fibrosis, differs not only in the species used but also in the main triggering factor (massive proteinuria), characteristic of the albumin overload rat model, and relevant to the pathogenesis of human progressive glomerular diseases [34].

During the last 20 years, many studies have reported the renoprotective properties of BK relying on the functionality of B_2R [7–9,13,14,16,54]. Although evidence focused on the mechanisms involved in this effect are scarce, it has been shown [16] that activation of B_2R in PTEC reduces myofibroblastic transformation and down-regulates α-SMA expression and Smad4 induced by TGF-β1, suggesting that B_2R antagonizes Smad signaling triggered by TGF-β1.

Animals fed on a high potassium diet over-express both kallikrein and B_2R proteins in the kidney, accompanied by high levels of the enzyme in the urine [13,14,23,29,30]. By up-regulating the kinin B_2R pathway we were able to prevent the phenotypic changes triggered by exposure to albumin *in vitro* demonstrating also the involvement of the TGF/Smad signaling pathway and obtaining a significant reduction in proteinuria and renal fibrosis in vivo. Appropriately, the B_2R antagonist, HOE-140, clearly reduced these beneficial effects both *in vivo* and in vitro. Wang and colleagues have reported that the administration of potassium to 5/6 nephrectomized rats causes a considerable increase in the expression of Smad7 and a decrease in collagens I, III and TGF-β1 levels in the remnant kidney [55]. Our view is that those changes are induced by up-regulation of B_2R. In the current study we have confirmed that albumin increases the mRNA, precursor protein, and active peptide of TGF-β1 in HK-2 cells, as reported by others [17–19,56–58], but in addition we have demonstrated that pretreatment with BK decreases the levels of expression and synthesis of TGF-β1. Moreover, these effects are abolished by B_2R blockade, suggesting that the anti-fibrotic effects are due to modulation of TGF-β by the B_2R. We could speculate that transcription, translation, secretion and/or activation of the latent molecule to an active form of TGF-β could be negatively regulated by the B_2R-dependent signaling pathways [59–63]. The antifibrotic role of B_2R and its modulation of TGF-β signaling are supported also by the administration of HOE-140 to normal (non-proteinuric) rats where B_2R blockade up-regulates TGF-β mRNA and induces tubulointerstitial renal fibrosis, an effect that may be independent of changes in blood pressure [14]. Our animal model showed that albumin overload induced an increase of the TGF-β1 precursor, as described previously [22], but it was not reversed by the stimulation of B_2R, a result not consistent with the data published by Wang et al. [55]. It is likely that potassium effects may depend on the experimental model, suggesting that variable pathogenic mechanisms operate when the albumin overload model is compared with the 5/6 nephrectomy one. Although it is reasonable to postulate that TGF-β1 precursor levels are not converted into the active TGF-β1 peptides (not measured in our *in vivo* study) we cannot exclude that the BK-induced down-regulation of TβRII may also decrease the activity of TGF-β1 by reducing its interaction sites. In fact, potassium administration did not reduce TGF-β1precursor but decreased Smad3 phosphorylation and up-regulated Smad7 expression, both functionally more important. Our *in vitro* experiments showed that BK did not affect the phosphorylated Smad3 levels as observed in vivo, possibly because the physiological environment included several regulatory factors that are absent in cell culture. *In vitro* results do not discard the synergic, circulatory benefits of kinins on endothelial cells improving nitric oxide production among other important influences [64]. Alternative TGF-β profibrotic pathways, including their possible interactions with Smad signaling (unexplored in our experimental design), may provide additional explanations to the differences found between our *in vivo* and *in vitro* experiments

The Smad signaling pathway, activated by TGF-β1, is negatively self-regulated by Smad7 [65–67]. The increase in Smad7 levels induced by B_2R activation, both *in vivo* and *in vitro,* supports the renoprotective role of BK mediated by antagonizing the pathophysiological role of the Smad pathway. This finding may also explain why, although we did not observe a reduction in Smad3 phosphorylation *in vitro,*BK was able to antagonize the increased expression of mesenchymal markers. Furthermore, Smad7 binds to promoter genes of the Smad pathway induced by TGF-β1 [68,69] through its MH2 domain, disrupting the formation of the Smad protein-DNA complex, preventing the final steps of this signaling pathway [68,69]. When Smad7 levels are elevated, as observed during B_2R stimulation, we expect a reduction in the biological effects of phosphorylated Smad3 because Smad7 also recruits ubiquitin ligases and phosphatases to the TβRI-activated protein that leads to proteosomal degradation or dephosphorylation, respectively [65,70].

Previous studies have demonstrated that the fibrotic process can be modulated by inhibiting the TGF-β signaling pathway. Thus, the specific inhibitor of TβRI (SD-208) attenuates TGF-β-induced Smad signaling, reducing human lung fibroblast transformation to myofibroblasts *in vitro* and lung collagen deposition *in vivo* [71]. Equivalent results have been reported in cardiac remodeling after myocardial infarct [72] and in renal fibrosis. The immunological neutralization of TGF-β may also reduce the progression of renal injury and α-SMA renal expression suggesting a potential influence in EMT [73]. We found also that treatment of HK-2 cells with TGF-β induces morphological changes that are identical to those produced by BSA. These changes can be reverted also by BK and are mediated by B_2R, in agreement with Tu et al. [16]. The role of BK in fibrogenesis is not restricted to renal cells, and BK-binding sites have been described in myocytes and cardiac fibroblasts, showing an anti-thropic role for kinins in attenuating cardiac and vascu-

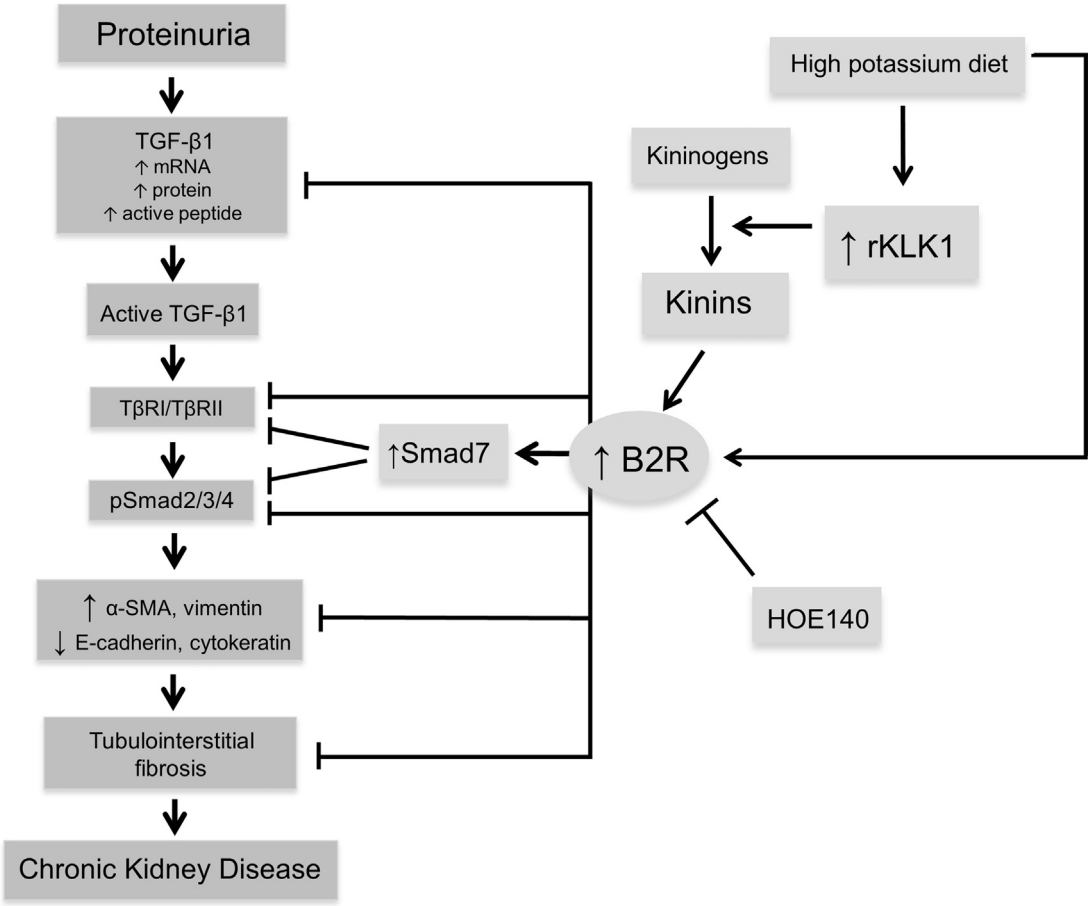


Fig. 11. Schematic representation of the protective role of kinins and B_2R in the kidney. Abundant protein in the glomerular ultrafiltrate results in proteinuria and contributes to the up-regulation of the TGF-β1 axis, which triggers epithelial-mesenchymal transition (EMT) and activates the SMAD signaling pathway in tubular cells, promoting development of renal tubulointerstitial fibrosis. A high potassium diet up-regulates the expression of both kallikrein (rKLK1) and B_2R, which counterbalances the effects produced by TGF-β1 and the SMAD signaling pathway, resulting in a reduction of tubulointerstitial fibrosis.

lar remodeling. BK might be a reciprocal regulator of collagen turnover by suppressing fibroblast/myofibroblast-induced growth in the heart and studies with ACE inhibitors and/or B_2R antagonists emphasize the physio/pathological benefits of B_2R signal transduction [74].

The role of kinin B_1 receptor (B_1R) was not addressed in our experiments but their potential deleterious effects deserve some commentaries since B_1R is up-regulated under pathological conditions and it has been proposed as a key antifibrotic target [75]. We did not find information on the participation of B_1R *in vivo* or *in vitro* during albumin loading but it has been reported that B_1R blockade/deletion has a curative antifibrotic effect [76], equivalent to that obtained with valsartan, in UUO [77] and in a model of glomerulonephritis [78].

We used a high potassium diet strategy to induce kinin system activation knowing the classical relationship that has been described between potassium, adrenal aldosterone secretion and its effects on tubular kallikrein-synthesizing cells [58]. That interaction, that could have influence on the analysis of our results, has been challenged by recent evidence showing that high-potassium diets increases renal kallikrein secretion in both control and aldosterone synthase deficient mice, indicating that aldosterone would be not required for the stimulatory effect of potassium intake on kallikrein secretion [59,60]. Furthermore, the renoprotective effect of this approach may overcome the potential profibrotic influence of aldosterone as it is suggested by the reduction of renal fibrosis induced by rich potassium diets in the model of subtotal nephrectomy [61] and spontaneously hypertensive rats [62].

Finally, we cannot provide information on the particular mechanism as to how albumin or B_2R regulate the molecular biology of TGF-β but, when subjected to albumin overload, B_2R modulates the Smad signaling pathway by regulating the availability of the active TGF-β1 peptide, TβRII and Smad7 levels. These effects are linked to decreased expression of mesenchymal biomarkers in PTEC, a change that may be relevant to patients with proteinuria. In conclusion, we propose that increased BK levels, through B_2R signal transduction, modulate TGF-β family pathway in order to resolve tubulointerstitial fibrosis. Drug design involving stable analogues of bradykinin may be of therapeutic value in the treatment of chronic renal fibrosis (Fig. 11).

Conflict of interest

None.

Acknowledgements

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