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ABSTRACT

Renal fibrosis is the common histological feature of advanced glomerular and tubulointerstitial disease leading to end-stage renal disease (ESRD). However, specific anti-fibrotic therapies to slow down the evolution to ESRD are still absent. Because persistent inflammation is a key event in the development of fibrosis, we hypothesized that the pro-inflammatory kinin B1 receptor (B1R) could be such a new target. Here we show that, in the unilateral ureteral obstruction model of renal fibrosis, the B1R is over-expressed and that delayed treatment with an orally-active non-peptide B1R antagonist, blocks macrophage infiltration leading to a reversal of the level of renal fibrosis. *In vivo* bone marrow transplantation studies as well as *in vitro* studies on renal cells show that part of this anti fibrotic mechanism of B1R blockade involves a direct effect on resident renal cells by inhibiting chemokine CCL2 and CCL7 expression. These findings suggest that blocking the B1R is a promising antifibrotic therapy.

Key words: kidney disease, bradykinin, unilateral ureteral obstruction, chemokine, reversion.

The incidence of chronic kidney disease (CKD) leading to end-stage renal disease (ESRD) has significantly increased and may reach epidemic proportions over the next decade (1). Regardless of the initial insult, the progression of most forms of renal disease results in tubulointerstitial fibrosis, which is the main histological hallmark of CKD (2). The presence of fibrosis in CKD is closely correlated to the future appearance of renal failure and has therefore been associated with poor long-term prognosis (3). Interstitial fibrosis is characterized by the progressive accumulation of extracellular matrix (ECM) proteins in the tubulointerstitial compartment. A multitude of events and factors (4) were identified to be involved in the development of renal fibrosis, potentially leading to new antifibrotic strategies and compounds (5-7). However in human, blockade of the renin–angiotensin aldosterone system remains the only effective therapy (1). These therapies only slow down the progression towards ESRD and alternative molecules or therapies are still necessary.

As a general rule the acute inflammatory response protects against infection and injury, however the chronicity of inflammation is often deleterious (8). Chronic inflammation is a key event in CKD that is mainly characterized by monocyte/macrophage accumulation in the renal interstitium and is well correlated with the progression of CKD (9, 10). Consequently, any strategy or agent able to limit or attenuate chronic renal inflammation should significantly slow down the rate of progression of CKD.

Lys-bradykinin and Lys-des-Arg^9-bradykinin are peptides intimately linked to inflammation (11). These kinin peptides interact with two different G-protein-coupled receptors: the bradykinin B2 receptor (B2R) and the B1R, respectively (11). Constitutively expressed in most tissues, the B2R has been shown to mediate most of the physiological actions of kinins. The role of the B2R has been studied in experimental CKD. We and others have observed that blockade of the B2R increased experimental renal fibrosis (12-15), but the ubiquitous expression of the B2R does not make it an ideal target.

In contrast, the kinin B1R, which is hardly detectable under physiological conditions, is overexpressed under inflammatory conditions in a variety of different tissues (11) and in turn stimulates inflammation upon activation. These data suggest that the B1R might be a more suitable target in chronic inflammatory renal disease.

A large number of *in vitro* and *in vivo* studies have shown that B1R induction is controlled by many pro–inflammatory cytokines and growth factors including IL1-ß, TNFα, Interferon gamma, as well as EGF (11). There is now clear evidence that induction of the B1R by many of these factors involves activation of transcription factor NF-κB and, conversely, B1R

stimulation activates NF-κB (11, 16). The activated B1R stimulates release of TNFα and IL-1 (17) and is also involved in leukocyte accumulation and activation (18, 19). The B1R is known to be expressed on macrophages (20) and on fibroblasts *in vitro* (20). Furthermore we have previously shown *in vivo* that experimental inflammation induces functional B1R expression in renal epithelial cells (16, 21). Its role in acute renal disease (ischemia-reperfusion) has been studied, but yielded contradictory results most probably due to compensation in the genetically engineered B1R, B2R single and B1R/B2R double knockout mice (22, 23). However, to the best of our knowledge, the role of the B1R in chronic renal inflammation and fibrosis has never been studied.

Taken together, specific induction of the B1R in renal inflamed tissues, its role in inflammation and the role of chronic inflammation in renal fibrosis led us to hypothesize that blocking the B1R could be an efficient approach to control the progression of CKD.

MATERIALS AND METHODS

Drug

B1R-antagonist SSR240612 was synthesized at Sanofi-Aventis R&D Montpellier-France (24). For *in vivo* experiments this compound was dissolved in water containing 2% dimethylsulfoxide (DMSO) to obtain a 1 g/L solution. The SSR240612 solution was diluted with distilled water and administered by gavage at a dose of 10 mg/kg/d. Final DMSO concentration was 0.01%.

Animals

Mice invalidated for the B1R (B1-/-) were obtained as described previously (25). Briefly, mice were generated by gene targeting on a mixed genetic background (129/SvJ x C57Bl/6J) and backcrossed ten times to C57Bl/6J (Harlan) as previously reported (26). We used C57Bl/6J (Harlan) as their wild type (B1+/+) control littermates. The mice were housed in a pathogen-free environment. All experiments reported were conducted in accordance with the NIH guide for the care and use of laboratory animals and were approved by a local animal care and use committee.

Unilateral Ureteral Obstruction (UUO)

B1+/+ and B1-/- male mice of 8 weeks of age were used for these experiments. The unilateral ureteral ligation was performed as previously described (12). Briefly, under oxygen-isoflurane anesthesia and through a longitudinal, left abdominal incision, the ureter was exposed and ligated with a 6/0 nylon thread at the uretero-pelvic junction. In sham operations, the ureter was exposed but not ligated and repositioned. Mice were maintained on a standard mouse chow and tap water. Treatments with the B1 receptor antagonist were initiated either 1 day before obstruction or 3 days after and continued throughout the time of obstruction. A control group received only the vehicle (0.01% DMSO solution). At the end of the different protocols, mice were sacrificed, and the kidneys were removed and divided in different parts according to the different protocols employed.

Cell culture

HEK293T (Human Embryonic Kidney) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) without pyruvate and with 4.5g/L glucose and 10% fetal calf serum at 37°C, 5% CO_2. Cells were seeded in 6-well plates and were transiently

transfected with either a pcDNA3 plasmid containing the GFP gene (Green Fluorescent Protein, pGFP) or a pcDNA3 plasmid containing the gene encoding for the human B1 receptor (phB1R) (#BDKB10TN00; Missouri S&T cDNA resource center) using JetPEI transfection reagent (Ozyme). Two days later, cells were pretreated or not with the peptidic antagonist des-Arg^{10}, Leu^9 kallidin (10^{-6} M) (NeoMPS) for 15 minutes and then treated with the human B1 receptor agonist des-Arg^{10}kallidin (10^{-6} M) (NeoMPS) for 4 hours.

Bone marrow transplantation

Bone marrow transplantation was performed as previously described (27). Briefly, 8 week-old B1+/+ male recipient mice were lethally irradiated with 10 Gy and put on acidified water (pH2) with antibiotics (neomycin 100 mg/L and polymyxin B sulphate 60000 U/L, Gibco) until 4 weeks after the transfer. Bone marrow cells were isolated from femurs and tibias of 12 week-old B1+/+ or B1-/- donor mice by flushing with PBS. Cells were then passed trough a 21G needle, centrifuged (250xg, 5 min, 4°C) and resuspended in RPMI 1640 (Gibco), 2% FCS, 5 U/mL heparin. Recipient mice received $5x10^6$ bone marrow cells in 100 μL by tail vein injection. UUO was performed 4 weeks after transplantation. At the time of sacrifice peritoneal macrophages were isolated from abdominal cavity by peritoneal lavage and blood was harvested. Haematologic analysis was performed by counting red blood cells, platelets, total leucocytes, neutrophils, monocytes and lymphocytes using a Micros-60CS/18 automated counter (ABX-diagnostics). The genomic DNA was extracted and the genotype was determined by PCR to verify the reconstitution of bone marrow after transplantation.

Histological analysis and immunohistochemistry

Four-micrometer paraffin-embedded sections were cut and used for routine staining (hematoxylin-eosin and periodic acid-Schiff (PAS) staining) and immunohistochemistry. Sections were first de-waxed in toluene and rehydrated through a series of graded ethanol washes before endogenous peroxidase blockage. Specific primary antibodies were incubated (1 hour at room temperature) for the detection of collagen type III (1/500) (Interchim), of F4/80 positive inflammatory cells (macrophages) (anti-mouse F4/80, 1/250) (RM2900; Caltag laboratories Inc., Burlingame, California, USA) and α-smooth muscle actin (α-SMA, Dako Epos method, U7033; Dako S.A., Trapppes, France). For visualization we used the Dako Envision system. Sections were finally counterstained with hematoxylin. Negative controls for the immunohistochemical procedures included substitution of the primary antibody with nonimmune sera.

Histomorphometric analyses were performed as previously described (12) using a commercially available image-analysis software which allows rebuilding of a kidney section from adjacent individual captures (Explora Nova Mosaïc software, La Rochelle, France).

Isolation of RNA

Total RNA was isolated from mouse tissue or cells using Qiagen RNeasy Mini kit, eluted in 20 µl RNase-free water and treated by DNase (TURBO DNA-free kit, Ambion) according to the manufacturer's protocol. 1.5 µl of this solution was used for quantitation by a NanoDrop instrument (ND-1000 spectrophometer).

Quantification of gene expression by real-time quantitative PCR

Real-time PCR was performed using the ABI PRISM 7900 HT. PCR amplification was performed in a total volume of 25 µl containing 25 ng of cDNA sample, 300 nM of forward and reverse primer and 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems). The reaction mixture was preheated at 95°C for 10 min, followed by 40 cycles at 95°c for 15 s and 60°C for 1 min. In our experimental models the most stable mouse housekeeping gene was the 18S ribosomal RNA (18S). The primers used in this study are listed in **Table 1**.

Statistical analysis

Data are expressed as mean plus or minus SD. ANOVA with post hoc Tukey alpha test was performed for comparison between the different groups. P values less than 0.05 were considered statistically significant.

RESULTS

Renal B1 receptor expression is induced in mice during unilateral ureteral obstruction (UUO).

We have used the *in vivo* model of UUO-induced renal fibrosis. This model has the advantage to mimic, in an accelerated manner, the main stages leading to interstitial fibrosis: macrophage infiltration, myofibroblast appearance and ECM-accumulation (28). Mice were subjected to UUO and kidneys were harvested at different time points after obstruction (figure 1). Macrophage infiltration was quantified by immunohistological staining for F4/80, and B1R mRNA expression was analyzed by real-time PCR. F4/80 positive cells infiltration started 24 h after UUO and their number clearly increased all along the progression of the pathology (figure 1A). 24 h of UUO induced a slight increase in B1R mRNA expression, which became significant at day 3 and 8 (figure 1B). This induction was restricted to the obstructed kidney since UUO did not induce B1R mRNA expression in the controlateral kidney nor in other tissue including aorta, brain, mesenteric tissue, or lung (figure 1C).

Genetic and pharmacological blockade of the B1 receptor reduce significantly the development of UUO-induced renal fibrosis.

We next assessed the effect of B1R blockade on the development of renal fibrosis. Interstitial collagen accumulation was studied by histomorphometric analysis in Sirius red-stained renal sections as an index of the fibrotic response to UUO of B1-/-, B1+/+ and mice pretreated with a B1R antagonist (B1Ra, 10mg/kg/d) (figure 2A-B). After 8 days of UUO, interstitial fibrosis was significantly reduced in B1-/- (figure 2A) and mice pretreated with the B1Ra (figure 2B) compared to the B1+/+ group or wild-type mice treated with the vehicle only. In the different control groups (sham) no significant difference in interstitial fibrosis was observed.

B1 receptor blockade decreases inflammatory cell infiltration and myofibroblast appearance.

In the tubulointerstitium of the control groups (sham) of both B1+/+ and B1-/- we found a very low level of F4/80 positive macrophages (figure 2C-D) and α-SMA positive myofibroblasts (figure 2E-F). As expected, UUO induced a significant increase in these renal fibrosis markers. Very interestingly, this increase was prevented by the genetic and pharmacological blockade of the B1R (figure 2C-F).

Delayed B1R antagonist treatment blunts the development of UUO-induced renal fibrosis.

To more closely mimic the clinical situation, we investigated whether B1R blockade would result in improvement of established renal fibrosis and inflammation. We thus studied the effect of delayed B1R blockade on UUO-induced fibrosis. Three days of UUO resulted in significant B1R expression (figure 1B) and kidney sections were also already strongly positive for macrophages, myofibroblasts and collagen accumulation (figure 3A, black line). We therefore decided to start B1Ra treatment 3 days after UUO (figure 3A, arrow). B1Ra halted F4/80 positive macrophage infiltration and this was associated with a significant decrease in interstitial myofibroblasts and fibrosis (figure 3A, blue line). This effect was confirmed at the mRNA level, since we found a significant decrease in collagen type I, III and IV mRNA expression (figure 3B). Treatment with B1Ra did not alter B2R mRNA expression (not shown).

Delayed B1R antagonist treatment blunts CTGF, CCL2 and CCL7 mRNA expression.

To better understand the mechanism by which B1R blockade is reducing renal fibrosis, we analyzed mRNA expression of the profibrotic cytokines TGFβ and CTGF, and two chemokines shown to be critical for monocyte/macrophage recruitment, CCL2 (MCP-1) and CCL7 (MCP-3) (29, 30). As shown in figure 4, the UUO-induced expression of CTGF, CCL2 and CCL7 was blunted by delayed oral B1Ra administration. However, the treatment did not significantly affect TGFβ.

Activation of the B1R directly induces CTGF, CCL2 and CCL7 mRNA expression *in vitro*.

To investigate whether the B1R is able to directly stimulate cytokine and chemokine expression, we used a B1R-transfected HEK293 cell line, which is a classical cellular model commonly used to evaluate the effects of the B1R (24, 31). We analyzed expression of CCL2, CCL7 and CTGF in these cells transfected with a GFP containing plasmid (pGFP), as a control, or a human B1 receptor containing plasmid (phB1R) (figure 5). Cells were treated either with the human B1R agonist, des-Arg[10] kallidin (1μM), or the peptidic B1 receptor antagonist, des-Arg[10], Leu[9] kallidin (1μM). Treatments with either B1R-agonist or -antagonist alone were without effect on chemokine expression in control GFP transfected cells. In B1R

overexpressing cells, the B1R agonist induced rapid expression (4h) of CCL2, CCL7 and CTGF, and this effect was blunted by B1Ra treatment. No effect was observed on TGFβ expression (not shown).

Specific deletion of the B1R on blood circulating cells does not affect the development of UUO-induced fibrosis.

As the B1R is expressed on infiltrating macrophages as well as on resident renal cells in the inflamed kidney we investigated whether specific B1R knockout on blood circulating cells (including inflammatory cells) modifies the development of UUO-induced fibrosis in B1+/+ mice. In order to verify that the mice were successfully reconstituted with bone marrow, peritoneal macrophages and blood of the recipient mice were harvested before the sacrifice, counted and genotyped. As shown in figure 6A, no difference was observed in the number of circulating hematopoietic cells in wild-type mice with B1+/+ or B1-/- marrow. Moreover, in wild-type mice reconstituted with B1-/- marrow, the B1R mRNA corresponding band was not detected in the blood as well as in peritoneal macrophages (figure 6B). These results demonstrated that the mice were successfully reconstituted and that blood-circulating cells were primarily from the donor. B1-/- bone marrow (B1-/-Bm) transplanted wild-type mice subjected to 8 days UUO did not display significant differences in chemokine mRNA expression (CCL2, CCL7), macrophage infiltration (F4/80 immunohistochemistry) and fibrosis development (Sirius Red) compared to obstructed B1+/+Bm transplanted mice (figure 6C). These results strongly support the hypothesis that the effect of B1R blockade on interstitial fibrosis is mainly mediated by resident renal cells, most likely by reducing chemokine expression and subsequently reduced inflammatory cell recruitment.

DISCUSSION

The main characteristics of most forms of chronic kidney disease (CKD) are persistent inflammation and tubulointerstitial fibrosis which are strong prognostic factors for progression towards ESRD (2, 3, 32). As recently reviewed (33), chronic inflammation is one of the novel risk factors contributing to the increased mortality seen in CKD patients. Therefore it is hypothesized that reduction of renal interstitial inflammation has the potential to reduce the progression of interstitial fibrosis and thus prevent ESRD (7, 34). To the best of our knowledge, even if a number of promising strategies are emerging (7, 34), we still do not have effective therapeutic strategies or molecules able to specifically target and reduce chronic renal inflammation without blocking other important pathophysiological functions.

We present here data that support the hypothesis that specific blockade of the kinin B1 receptor might be a promising strategy to reduce renal chronic inflammation and subsequently blunt the development of renal fibrosis. Several lines of evidence support this hypothesis. We first demonstrated that the B1R contributed to the progression of UUO-induced renal inflammation and fibrosis. We next showed that delayed treatment with an orally-active non-peptide B1R antagonist in the UUO model significantly decreased the progression of established renal fibrosis. We further showed, using the UUO model associated to bone marrow transplantation experiments and *in vitro* studies, that this effect was partly mediated via a mechanism involving the inhibition of CTGF and chemokine expression by resident renal cells. To our knowledge this is the first *in vivo* study reporting that B1R blockade could become an efficient antifibrotic strategy.

We first demonstrated that during UUO, B1R mRNA expression was significantly increased in the obstructed kidney. Very interestingly, this induction seemed to be restricted to the site of the pathology since there was no marked difference of B1R expression in other tissues. The effects of genetic ablation of the B1R (B1-/-) showed reduced macrophage recruitment associated with a reduction in interstitial fibrosis. This suggests that B1R blockade is modifying the inflammatory response. This was consistent with the attenuated inflammatory response observed in B1-/- mice in different inflammatory models including pleurisy, paw edema (25), as well as in a model of intestinal ischemia and reperfusion injury (35).

It is well known that compensatory mechanisms occur in genetically engineered animals. We have previously reported increased renal B1R expression in B2-/- mice (36).

Similarly, increased renal B2R mRNA expression in B1-/- mice has been reported (37). We have also shown that *in vivo* B2R activation reduces UUO-induced renal fibrosis (12). Therefore it is possible that the B2R is involved in the effects observed in B1-/- mice. However, B1R antagonist treatment, mimicking the effects observed in B1-/- mice did not induce renal B2R mRNA expression. Thus, the reduction in UUO-induced renal fibrosis observed B1-/- mice is most likely not due to the modification of kinin B2R expression.

How does B1R blockade reduces the inflammatory response in this model of renal fibrosis? As shown by a number of studies, interstitial inflammation mediated by macrophages constitutes an early and major event in response to UUO (38, 39). The precise molecular mechanism of the development of renal fibrosis is not yet fully elucidated, but key mediators including the major profibrotic cytokines TGF-ß and CTGF were clearly identified (4, 28, 40, 41). As expected, we observed an increased TGF-ß and CTGF mRNA expression in UUO-induced fibrosis. Interestingly, the blockade of the B1R was not associated with a decrease in TGFβ mRNA expression, but reduced its downstream mediator CTGF. This observation confirms *in vitro* experiments reporting that activation of the B1R stabilized CTGF mRNA, without modifying TGFβ expression (20). In addition, this is the first demonstration that B1R antagonism reduces CTGF mRNA expression *in vivo*. Furthermore a number of chemokines are involved in renal monocyte/macrophage infiltration (9). The role of CCL2 (MCP-1) (30) and even more recently the role of CCL7 (MCP-3) (29) were clearly established in this process. Our data showed reduced renal expression of these two chemokines in response to B1Ra treatment.

The next issue was to determine whether the B1R is directly involved in this attenuated chemokine response observed *in vivo*. To verify this hypothesis we used human embryonic kidney (HEK) cells transfected with the human B1R to study the effect of B1R stimulation on CTGF and chemokine expression. We observed that B1R stimulation is able to rapidly (4h) induce a significant increase in CTGF, CCL2 and CCL7 mRNA expression, which was abolished by a specific B1R antagonist. Although the mechanism by which the B1R stimulates CTGF mRNA overexpression has been previously shown to be mediated by mRNA stabilization (20), the effects of B1R activation on CCL2 and CCL7 mRNA expression most probably involves NF-κB as it was shown that the B1R activates this transcription factor (42). In addition, down regulation of CCL2 and CCL7 expression was observed by inhibition of NF-κB (43, 44).

These results thus suggest that direct stimulation of CTGF and chemokine expression by the

B1R could be involved in the pro-inflammatory and pro-fibrotic actions of the B1R, although the identity of the cell type involved in this effect remains to be determined. Almost any renal cell type can express functional chemokines *in vivo* (30) and the B1R is potentially expressed on infiltrating macrophages and also on resident renal cells in the inflamed kidney. We were not able to perform co-localization experiments, as antibodies against the mouse B1R are not commercially available. Therefore, we carried out bone marrow transplantation experiments to clarify whether decreased expression of CTGF and chemokines induced by B1Ra treatment could be mediated by infiltrating inflammatory or resident renal cells, or both. We performed UUO in wild-type mice reconstituted with either B1+/+ bone marrow or bone marrow lacking the B1R gene (B1-/-). Eight days after UUO, there was no difference between mice with B1+/+ or B1-/- bone marrow, showing that the absence of the B1R on monocytes/macrophages was without effect on inflammatory cell infiltration, chemokine expression and interstitial fibrosis. Thus, although the B1R is also expressed on monocytes/macrophages (11), these apparently did not participate in the anti-inflammatory and anti-fibrotic effects of B1R antagonism. These data strongly suggested that the beneficial effect of the B1R blockade was mainly mediated by resident renal cells.

As recently reviewed, a number of experimental data showed that the progression of renal fibrosis is a reversible process (45). Our data showed that, in UUO-induced fibrosis, delayed B1Ra administration leads to reversion of fibrosis. However, we must remain cautious in our interpretation because the UUO model is an accelerated model of interstitial fibrosis. We are currently validating these data on chronic kidney disease models where interstitial fibrosis appears more progressively.

Because of its inducible character, mainly localized at the site of inflammation, it has been suggested that blocking the B1R will be potentially without significant side effects. This has drawn attention to the B1R as a new therapeutic target for the treatment of pathologies related to chronic inflammation such as airway inflammation, diabetic neuropathy, arthritis and chronic and neuropathic pain (46). An additional advantage over other potential antifibrotic agents is that the B1R antagonist is already orally available (24).

Because failure of many organs (lung (47), liver (48), heart (49)) is often associated to the development of fibrosis, our study should be considered as a "proof of concept" of the therapeutic potential associated with B1R blockade.

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TECHNICAL HELP

We thank S Schaak for advice and help.

FIGURE LEGENDS

Table 1

Primer sequences used for real-time PCR.

Figure 1

B1R mRNA expression is induced locally in the kidney by UUO. Kinetics of macrophage infiltration was quantified by immunohistological staining for F4/80 (*A*) and B1R mRNA expression was analyzed by real-time PCR expression (*B*) during UUO. B1R mRNA expression was quantified by real-time PCR in aorta, brain, mesenteric tissue, lung and controlateral kidney of mice submitted to 8 days UUO (grey bars) compared to sham operated mice (white bars) (*C*). *$p<0.05$ versus time 0. n= 10/group.

Figure 2

Genetic and pharmacological blockade of the B1R reduce UUO-induced renal inflammation and fibrosis. The effect of B1R knockout (B1-/-) (*A, C, E*) or B1R antagonist (B1Ra) treatment (*B, D, F*) after 8 days UUO was assessed by comparison to wild-type mice (B1+/+) or vehicle treated mice respectively. Collagen accumulation was quantified by sirius red staining (*A, B*), macrophage infiltration by anti-F4/80 immunochemistry (*C, D*) and myofibroblast appearance by anti-α-smooth muscle actin (α-SMA) immunochemistry (*E, F*). Pictures display representative areas of kidneys from vehicle-treated or B1Ra-treated, sham or obstructed mice. *$p<0.05$ versus sham. #$p<0.05$ versus UUO 8 days. n= 10/group.

Figure 3

UUO-induced renal inflammation and fibrosis is reduced with delayed B1R antagonist treatment. (*A-B*) Mice were subjected to UUO for 8 days (black line) while oral B1R antagonist (B1Ra) treatment started (arrow, blue line) 3 days after the obstruction. (*A*) Kidney sections were analyzed by immunohistological staining for macrophages (F4/80), myofibroblasts (α-SMA) and collagen (sirius red) accumulation. (*B*) Type I, III, and IV collagen mRNA expression was analyzed by real-time PCR. *$p < 0.05$ versus sham operated mice. #$p < 0.05$ versus UUO 8 days vehicle-treated mice. n= 10/group.

Figure 4

Delayed administration of B1R antagonist blunts UUO-induced chemokine and cytokine overexpression. We analyzed mRNA expression of the profibrotic cytokines (TGFβ, CTGF) and chemokines (CCL2, CCL7) by real-time PCR during UUO in vehicle-treated mice (open bars) or in B1R antagonist (B1Ra) treated mice (filled bars). *$p < 0.05$ versus sham operated mice. #$p < 0.05$ versus UUO 8 days vehicle-treated mice. n= 10/group.

Figure 5

B1R stimulation induces CCL2, CCL7 and CTGF overexpression in HEK293 cells. CCL2, CCL7 and CTGF mRNA expression analysis by real-time PCR in HEK293T cells transfected with GFP (pGFP) or human B1 receptor (phB1R). Cells were pretreated with B1R antagonist (B1Ra) (des-Arg^{10}, Leu^9 kallidin 10^{-6} M) for 15 min and then treated with B1 receptor agonist (B1R ago) (des-Arg^{10} kallidin 10^{-6} M) for 4 hours. *$p < 0.05$ versus unstimulated cells. #$p < 0.05$ versus B1R agonist alone. n=3.

Figure 6

Absence of B1R in bone marrow-derived cells does not affect the development of fibrosis during UUO in wild-type mice. The successful reconstitution of wild-type mice with either wild-type (B1+/+) or B1 knockout (B1-/-) bone marrow was confirmed by counting the number of circulating hematopoietic cells (*A*) and by genotyping blood cells *(B, upper panel)* and peritoneal macrophages using PCR (*B, lower panel*). The effect of 8 days UUO in wild-type mice with either B1+/+ or B1-/- bone marrow (Bm) transplantation was assessed by measurement of CCL2 and CCL7 mRNA levels and by immuno-histological staining for F4/80 and collagen (sirius red). *$p < 0,05$ versus sham operated mice (white bar). n=7/group.

REFERENCES

1. Meguid El Nahas, A., and Bello, A. K. (2005) Chronic kidney disease: the global challenge. *Lancet* **365**, 331-340
2. Klahr, S., Schreiner, G., and Ichikawa, I. (1988) The progression of renal disease. *N Engl J Med* **318**, 1657-1666
3. Nath, K. A. (1992) Tubulointerstitial changes as a major determinant in the progression of renal damage. *Am J Kidney Dis* **20**, 1-17
4. Iwano, M., and Neilson, E. G. (2004) Mechanisms of tubulointerstitial fibrosis. *Curr Opin Nephrol Hypertens* **13**, 279-284
5. Boor, P., Sebekova, K., Ostendorf, T., and Floege, J. (2007) Treatment targets in renal fibrosis. *Nephrol Dial Transplant* **22**, 3391-3407
6. Nguyen, T. Q., and Goldschmeding, R. (2008) Bone Morphogenetic Protein-7 and Connective Tissue Growth Factor: Novel Targets for Treatment of Renal Fibrosis? *Pharm Res*
7. Strutz, F. (2001) Potential methods to prevent interstitial fibrosis in renal disease. *Expert Opin Investig Drugs* **10**, 1989-2001
8. Nathan, C. (2002) Points of control in inflammation. *Nature* **420**, 846-852
9. Kluth, D. C., Erwig, L. P., and Rees, A. J. (2004) Multiple facets of macrophages in renal injury. *Kidney Int* **66**, 542-557
10. Eddy, A. A. (2005) Progression in chronic kidney disease. *Adv Chronic Kidney Dis* **12**, 353-365
11. Leeb-Lundberg, L. M., Marceau, F., Muller-Esterl, W., Pettibone, D. J., and Zuraw, B. L. (2005) International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* **57**, 27-77
12. Schanstra, J. P., Neau, E., Drogoz, P., Arevalo Gomez, M. A., Lopez Novoa, J. M., Calise, D., Pecher, C., Bader, M., Girolami, J. P., and Bascands, J. L. (2002) In vivo bradykinin B2 receptor activation reduces renal fibrosis. *J Clin Invest* **110**, 371-379
13. Pawluczyk, I. Z., Patel, S. R., and Harris, K. P. (2004) The role of bradykinin in the antifibrotic actions of perindoprilat on human mesangial cells. *Kidney Int* **65**, 1240-1251
14. Okada, H., Watanabe, Y., Kikuta, T., Kobayashi, T., Kanno, Y., Sugaya, T., and Suzuki, H. (2004) Bradykinin decreases plasminogen activator inhibitor-1 expression and facilitates matrix degradation in the renal tubulointerstitium under angiotensin-converting enzyme blockade. *J Am Soc Nephrol* **15**, 2404-2413
15. Seccia, T. M., Belloni, A. S., Guidolin, D., Sticchi, D., Nussdorfer, G. G., Pessina, A. C., and Rossi, G. P. (2006) The renal antifibrotic effects of angiotensin-converting enzyme inhibition involve bradykinin B2 receptor activation in angiotensin II-dependent hypertension. *J Hypertens* **24**, 1419-1427
16. Marin-Castano, M. E., Schanstra, J. P., Praddaude, F., Pesquero, J. B., Ader, J. L., Girolami, J. P., and Bascands, J. L. (1998) Differential induction of functional B1-bradykinin receptors along the rat nephron in endotoxin induced inflammation. *Kidney Int* **54**, 1888-1898
17. Tiffany, C. W., and Burch, R. M. (1989) Bradykinin stimulates tumor necrosis factor and interleukin-1 release from macrophages. *FEBS Lett* **247**, 189-192
18. Ahluwalia, A., and Perretti, M. (1996) Involvement of bradykinin B1 receptors in the polymorphonuclear leukocyte accumulation induced by IL-1 beta in vivo in the mouse. *J Immunol* **156**, 269-274
19. Bockmann, S., and Paegelow, I. (2000) Kinins and kinin receptors: importance for the activation of leukocytes. *J Leukoc Biol* **68**, 587-592
20. Ricupero, D. A., Romero, J. R., Rishikof, D. C., and Goldstein, R. H. (2000) Des-

Arg(10)-kallidin engagement of the B1 receptor stimulates type I collagen synthesis via stabilization of connective tissue growth factor mRNA. *J Biol Chem* **275**, 12475-12480

21. Schanstra, J. P., Marin-Castano, M. E., Praddaude, F., Tack, I., Ader, J. L., Girolami, J. P., Bascands, J. L., and Jeunier, B. (2000) Bradykinin B(1) receptor-mediated changes in renal hemodynamics during endotoxin-induced inflammation. *J Am Soc Nephrol* **11**, 1208-1215

22. Kakoki, M., McGarrah, R. W., Kim, H. S., and Smithies, O. (2007) Bradykinin B1 and B2 receptors both have protective roles in renal ischemia/reperfusion injury. *Proc Natl Acad Sci U S A* **104**, 7576-7581

23. Wang, P. H., Cenedeze, M. A., Pesquero, J. B., Pacheco-Silva, A., and Camara, N. O. (2006) Influence of bradykinin B1 and B2 receptors in the immune response triggered by renal ischemia-reperfusion injury. *Int Immunopharmacol* **6**, 1960-1965

24. Gougat, J., Ferrari, B., Sarran, L., Planchenault, C., Poncelet, M., Maruani, J., Alonso, R., Cudennec, A., Croci, T., Guagnini, F., Urban-Szabo, K., Martinolle, J. P., Soubrie, P., Finance, O., and Le Fur, G. (2004) SSR240612 [(2R)-2-[((3R)-3-(1,3-benzodioxol-5-yl)-3-[[(6-methoxy-2-naphthyl)sulfonyl]amino]propanoyl)amino]-3-(4-[[2R,6S)-2,6-dimethylpiperidinyl]methyl]pheny 1)-N-isopropyl-N-methylpropanamide hydrochloride], a new nonpeptide antagonist of the bradykinin B1 receptor: biochemical and pharmacological characterization. *J Pharmacol Exp Ther* **309**, 661-669

25. Pesquero, J. B., Araujo, R. C., Heppenstall, P. A., Stucky, C. L., Silva, J. A., Jr., Walther, T., Oliveira, S. M., Pesquero, J. L., Paiva, A. C., Calixto, J. B., Lewin, G. R., and Bader, M. (2000) Hypoalgesia and altered inflammatory responses in mice lacking kinin B1 receptors. *Proc Natl Acad Sci U S A* **97**, 8140-8145

26. Mori, M. A., Araujo, R. C., Reis, F. C., Sgai, D. G., Fonseca, R. G., Barros, C. C., Merino, V. F., Passadore, M., Barbosa, A. M., Ferrari, B., Carayon, P., Castro, C. H., Shimuta, S. I., Luz, J., Bascands, J. L., Schanstra, J. P., Even, P. C., Oliveira, S. M., Bader, M., and Pesquero, J. B. (2008) Kinin B1 receptor deficiency leads to leptin hypersensitivity and resistance to obesity. *Diabetes* **57**, 1491-1500

27. de Winther, M. P., and Heeringa, P. (2003) Bone marrow transplantations to study gene function in hematopoietic cells. *Methods Mol Biol* **209**, 281-292

28. Bascands, J. L., and Schanstra, J. P. (2005) Obstructive nephropathy: insights from genetically engineered animals. *Kidney Int* **68**, 925-937

29. Tsou, C. L., Peters, W., Si, Y., Slaymaker, S., Aslanian, A. M., Weisberg, S. P., Mack, M., and Charo, I. F. (2007) Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest* **117**, 902-909

30. Segerer, S., Nelson, P. J., and Schlondorff, D. (2000) Chemokines, chemokine receptors, and renal disease: from basic science to pathophysiologic and therapeutic studies. *J Am Soc Nephrol* **11**, 152-176

31. Ni, A., Yin, H., Agata, J., Yang, Z., Chao, L., and Chao, J. (2003) Overexpression of kinin B1 receptors induces hypertensive response to des-Arg9-bradykinin and susceptibility to inflammation. *J Biol Chem* **278**, 219-225

32. Becker, G. J., and Hewitson, T. D. (2000) The role of tubulointerstitial injury in chronic renal failure. *Curr Opin Nephrol Hypertens* **9**, 133-138

33. Kovesdy, C. P., and Kalantar-Zadeh, K. (2008) Novel targets and new potential: developments in the treatment of inflammation in chronic kidney disease. *Expert Opin Investig Drugs* **17**, 451-467

34. Khwaja, A., El Kossi, M., Floege, J., and El Nahas, M. (2007) The management of

CKD: a look into the future. *Kidney Int* **72**, 1316-1323

35. Souza, D. G., Lomez, E. S., Pinho, V., Pesquero, J. B., Bader, M., Pesquero, J. L., and Teixeira, M. M. (2004) Role of bradykinin B2 and B1 receptors in the local, remote, and systemic inflammatory responses that follow intestinal ischemia and reperfusion injury. *J Immunol* **172**, 2542-2548

36. Marin-Castano, M. E., Schanstra, J. P., Neau, E., Praddaude, F., Pecher, C., Ader, J. L., Girolami, J. P., and Bascands, J. L. (2002) Induction of functional bradykinin b(1)-receptors in normotensive rats and mice under chronic Angiotensin-converting enzyme inhibitor treatment. *Circulation* **105**, 627-632

37. Seguin, T., Buleon, M., Destrube, M., Ranera, M. T., Couture, R., Girolami, J. P., and Tack, I. (2008) Hemodynamic and renal involvement of B1 and B2 kinin receptors during the acute phase of endotoxin shock in mice. *Int Immunopharmacol* **8**, 217-221

38. Chevalier, R. L. (2006) Obstructive nephropathy: towards biomarker discovery and gene therapy. *Nat Clin Pract Nephrol* **2**, 157-168

39. Sung, S. A., Jo, S. K., Cho, W. Y., Won, N. H., and Kim, H. K. (2007) Reduction of renal fibrosis as a result of liposome encapsulated clodronate induced macrophage depletion after unilateral ureteral obstruction in rats. *Nephron Exp Nephrol* **105**, e1-9

40. Border, W. A., and Noble, N. A. (1997) TGF-beta in kidney fibrosis: a target for gene therapy. *Kidney Int* **51**, 1388-1396

41. Okada, H., Kikuta, T., Kobayashi, T., Inoue, T., Kanno, Y., Takigawa, M., Sugaya, T., Kopp, J. B., and Suzuki, H. (2005) Connective tissue growth factor expressed in tubular epithelium plays a pivotal role in renal fibrogenesis. *J Am Soc Nephrol* **16**, 133-143

42. Schanstra, J. P., Bataille, E., Marin Castano, M. E., Barascud, Y., Hirtz, C., Pesquero, J. B., Pecher, C., Gauthier, F., Girolami, J. P., and Bascands, J. L. (1998) The B1-agonist [des-Arg10]-kallidin activates transcription factor NF-kappaB and induces homologous upregulation of the bradykinin B1-receptor in cultured human lung fibroblasts. *J Clin Invest* **101**, 2080-2091

43. Cuaz-Perolin, C., Billiet, L., Bauge, E., Copin, C., Scott-Algara, D., Genze, F., Buchele, B., Syrovets, T., Simmet, T., and Rouis, M. (2008) Antiinflammatory and antiatherogenic effects of the NF-kappaB inhibitor acetyl-11-keto-beta-boswellic acid in LPS-challenged ApoE-/- mice. *Arterioscler Thromb Vasc Biol* **28**, 272-277

44. Wuyts, W. A., Vanaudenaerde, B. M., Dupont, L. J., Demedts, M. G., and Verleden, G. M. (2003) Involvement of p38 MAPK, JNK, p42/p44 ERK and NF-kappaB in IL-1beta-induced chemokine release in human airway smooth muscle cells. *Respir Med* **97**, 811-817

45. Chatziantoniou, C., and Dussaule, J. C. (2008) Is kidney injury a reversible process? *Curr Opin Nephrol Hypertens* **17**, 76-81

46. Campos, M. M., Leal, P. C., Yunes, R. A., and Calixto, J. B. (2006) Non-peptide antagonists for kinin B1 receptors: new insights into their therapeutic potential for the management of inflammation and pain. *Trends Pharmacol Sci* **27**, 646-651

47. Meltzer, E. B., and Noble, P. W. (2008) Idiopathic pulmonary fibrosis. *Orphanet J Rare Dis* **3**, 8

48. Wallace, K., Burt, A. D., and Wright, M. C. (2008) Liver fibrosis. *Biochem J* **411**, 1-18

49. Diez, J., Gonzalez, A., Lopez, B., and Querejeta, R. (2005) Mechanisms of disease: pathologic structural remodeling is more than adaptive hypertrophy in hypertensive heart disease. *Nat Clin Pract Cardiovasc Med* **2**, 209-216