Inhibition of the TGFβ signalling pathway by cGMP and cGMP-dependent kinase I in renal fibrosis

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Keywords
- cGMP-dependent protein kinase I; cyclic guanosine monophosphate; renal fibrosis; soluble guanylate cyclase stimulation

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(Received 8 November 2016, revised 16 January 2017, accepted 23 January 2017)
doi:10.1002/2211-5463.12202

Agents that enhance production of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) ameliorate the progression of renal fibrosis. However, the molecular mechanism of this process is not fully understood. We hypothesize that the antifibrotic effects of cGMP and cGMP-dependent kinase I (cGKI) are mediated via regulation of the TGFβ signalling pathway, both via ERK and the Smad-dependent route. Kidney fibrosis was induced by unilateral ureter obstruction (UUO) in wild-type and cGKI-deficient (cGKI-KO) mice. The cGMP/cGKI signalling pathway was activated by application of the soluble guanylate cyclase (sGC) stimulator BAY 41-8543 (BAY), beginning 1 day after UUO. After 7 days, the antifibrotic effects of BAY were analysed by measuring mRNA and protein expression of characteristic fibrotic biomarkers. The effects of cGMP/TGFβ on cultured fibroblasts were also analysed in vitro. BAY application influenced the activity of the extracellular matrix (ECM)-degrading matrix metalloproteases (MMP2 and MMP9) and their inhibitor tissue inhibitors of metalloproteinase-1, the secretion of cytokines (e.g. IL-6) and the expression pattern of ECM proteins (e.g. collagen, fibronectin) and profibrotic mediators (e.g. connective tissue growth factors and plasminogen-activator inhibitor-1). Activation of the cGMP/cGKI signalling pathway showed protective effects against fibrosis which were mediated by inhibition of P-Erk1/2 and translocation of P-smad3. The elucidation of these signalling mechanisms might support the development of new therapeutic options regarding cGMP/cGKI-mediated antifibrotic actions.

Fibrosis is characterized by excessive expression of extracellular matrix (ECM). Fibrogenic factors promote the fibrotic process such as transforming growth factors (TGFβ), plasminogen-activator inhibitor-1 (PAI-1) or connective tissue growth factors (CTGF) [1]. TGFβ is involved in the differentiation of fibroblasts to myofibroblasts, which are characterized by the expression of α-smooth muscle actin (αSMA). Myofibroblasts synthesize ECM proteins including collagen and fibronectin, and they secrete cytokines, for example, IL-6.

Abbreviations
cGKI, cGMP-dependent protein kinase I; cGKI-KO, cGKI-knockout; cGMP, cyclic guanosine monophosphate; Co-IP, coimmunoprecipitation; Col1α1, collagen1a1; CTGF, connective tissue growth factor; ECM, extracellular matrix; ERK1/2, extracellular-signal regulated kinase; GTP, guanosine triphosphate; MMPs, matrix metalloproteinases; NO, nitric oxide; PAI-1, plasminogen-activator inhibitor-1; sGC, soluble guanylyl cyclase; TGFβ, transforming growth factor β; TIMP, tissue inhibitors of metalloproteinases; UUO, unilateral ureter obstruction; wt, wild-type; αSMA, α-smooth muscle actin.
In addition, synthesis and degradation of ECM proteins are determined by metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Their expression pattern is regulated by MAPK/Erk kinase, which promotes the progression of fibrosis [2,3].

We evaluated the effects of the soluble guanylate cyclase (sGC) stimulator BAY 41-8543 (BAY) on the fibrotic kidney. Under physiological conditions, sGC can be activated by nitric oxide (NO). Activated sGC synthesizes the second messenger cyclic guanosine monophosphate (cGMP) which then stimulates cGMP-dependent protein kinases (cGK) [4]. We have previously reported that cGMP suppresses renal fibrosis in particular via cGKIα, an isoform of cGK. cGKIα is expressed in fibroblasts and myofibroblasts, which are excessively produced after unilateral ureter obstruction (UUO) [5]. Protective effects of sGC stimulation on renal fibrosis in rats have already been shown [6,7]. Thereby, BAY reduced apoptosis and macrophage infiltration after relief of UUO [7], and Sharkovska et al. [6] reported that sGC stimulation improved creatinine clearance in hypertensive renin-transgenic rats. However, the molecular mechanism by which cGMP via cGKI affects the development of kidney fibrosis has not yet been fully elucidated. Therefore, we analysed the impact of BAY on fibrosis in a mouse model of UUO using cGKI-knockout (cGKI-KO)-mice. The present study investigates the functional role of sGC stimulation in the fibrotic process, the signalling pathway as well as the underlying mechanisms involved.

**Results**

**Effect of BAY and function of cGKI on the mRNA expression of different fibrotic biomarkers**

As marker for fibrosis induction, we examined the mRNA levels of αSMA, fibronectin, collagenα1 (Col1a1), CTGF, TIMP-1, PAI-1, MMP2 and MMP9 (Fig. 1). One week after UUO surgery, the mRNA levels were elevated in comparison to the contralateral control kidney. Especially, αSMA (Fig. 1A), Col1a1 (Fig. 1C), TIMP-1 (Fig. 1E), PAI-1 (Fig. 1F) and MMP2 (Fig. 1G) were strongly increased by UUO. In contrast, the mRNA expression of fibronectin (Fig. 1B) and CTGF (Fig. 1D) were only moderately upregulated, and MMP9 (Fig. 1H) was nearly unchanged.

To examine the role of NO/cGMP signalling in renal fibrosis, we injected the sGC stimulator BAY. A significant raise of cGMP in kidney tissues of BAY-treated mice in comparison to untreated mice indicating a BAY-induced stimulation of sGC was measured (Fig. S1). BAY treatment decreased the mRNA expression of αSMA, fibronectin, Colla1, CTGF, TIMP-1, PAI-1, MMP2 and MMP9 in wt-mice (Fig. 1). In contrast, cGKI-KO mice showed no significant decrease in the mRNA levels (Fig. 1).

![Fig. 1](image-url) Effect of BAY in wt- and cGKI-KO-kidneys on the mRNA expression levels of (A) αSMA, (B) fibronectin, (C) Colla1, (D) CTGF, (E) TIMP-1, (F) PAI-1, (G) MMP2 and (H) MMP9. In wt-mice, BAY caused a significant decrease in the mRNA expression of (A) αSMA, (B) fibronectin, (C) Colla1, (D) CTGF, (E) TIMP-1, (G) MMP2 and (H) MMP9 with the exception of (F) PAI-1. In cGKI-KO-mice sGC stimulation showed no significant decrease in the mRNA levels (A–H). The results are shown as the x-fold change in mRNA expression in the fibrotic kidney relating to the opposite healthy kidneys whose mRNA expression was set to one. In each mouse strain the untreated mice were compared with BAY-treated mice. Significant differences between two groups are indicated with asterisks (*P < 0.05, **P < 0.01). The columns show the number of animals which were used. The right columns illustrate the data of GKI-KO-mice and patterned columns the data of BAY-treated mice.
expression of all investigated biomarkers of fibrosis with the exception of PAI-1 (Fig. 1F). The expression of PAI-1 was reduced but the difference did not reach significance. To explore whether cGKI is involved in the impact on the fibrotic process, we analysed cGKI-KO-mice. As previously reported, untreated cGKI-KO-mice showed less mRNA expression than untreated wild-type (wt) mice [5]. However, the mRNA expression of cGKI-KO-mice was not influenced by BAY application (Fig. 1).

**Effect of BAY and role of cGKI on the protein expression of αSMA, fibronectin, Col1a1 and total collagen**

After UUO, the interstitial accumulation of protein expression of αSMA, fibronectin and Col1a1 was increased in wt- and cGKI-KO kidneys as demonstrated by immunofluorescence analysis (Fig. 2A–C). The quantitative analysis revealed that BAY significantly reduced the protein expression of αSMA, fibronectin and Col1a1 in wt-, but not in cGKI-KO-kidneys (Fig. 2A–C). The same pattern was present when we used the Sirius red/fast green staining for total collagen. In wt-, but not in cGKI-KO-kidneys, sGC stimulation by BAY significantly downregulated the level of total collagen (Fig. 2D).

**Effect of BAY and function of cGKI on the activity or protein expression of TGFβ target genes**

As expected, UUO increased the protein expression of the TGFβ target gene CTGF in comparison to the healthy kidney (Fig. 3A). The quantitative analysis, which compared only fibrotic kidneys, confirmed that the protein expression of CTGF was significantly diminished by BAY in fibrotic wt-kidneys. However, treatment of cGKI-KO-mice did not result in a reduction of CTGF (Fig. 3A). Figure 3B demonstrates that PAI-1-expression was not significantly influenced by BAY. The protein expression of TIMP-1 was not changed by UUO in comparison to the contralateral healthy kidney (Fig. 3C). Intriguingly, TIMP-1 was significantly higher in BAY treated than in untreated fibrotic wt-kidneys. In cGKI-KO-mice, we detected no increase in TIMP-1 expression following BAY administration (Fig. 3C). The latent and active forms of MMP2 and the latent forms of MMP9 were elevated, but the active forms of MMP9 were reduced by UUO (data not shown). In agreement with the increase in TIMP-1, which is an inhibitor of MMPs, the latent and active forms of MMP2 (Fig. 4A,B) and the latent forms of MMP9 (Fig. 4A,C) were significantly diminished by BAY. This was again only observed in wt-, but not in cGKI-KO-kidneys.

**Effect of cGMP/cGKI on the TGFβ signalling pathway**

At first we analysed the influence of cGMP/cGKI on the TGFβ/smad signalling pathway. Isolated fibroblasts of wt- (left side of Fig. 5A) and cGKI-KO-kidneys (right side of Fig. 5A) were pretreated with cGMP or vehicle followed by exposure to TGFβ or vehicle (Fig. 5A). We quantified the intranuclear and extranuclear fluorescence intensity of P-smad3 respectively. Figure 5B shows that TGFβ treatment significantly enhanced nuclear fluorescence intensity of P-smad3 but pretreatment with cGMP significantly limits nuclear translocation of P-smad3 in fibroblasts of wt-kidneys in the presence of TGFβ. cGMP alone had no effects (data not shown). Intriguingly in fibroblasts of cGKI-KO-kidneys pretreatment with cGMP did not change the translocation of P-smad3 (Fig. 5C). In contrast to P-smad3, P-smad2 was not influenced by preincubation with cGMP (data not shown). Isolated fibroblasts expressed sGC but during culturing the expression of sGC was downregulated (data not shown). Therefore, we stimulated the cells only with cGMP and not with the sGC stimulator BAY. Furthermore, we quantified the total cellular fluorescence intensity of P-smad3 which was significantly increased by TGFβ treatment but interestingly not significantly changed by cGMP pretreatment (Fig. S2). In pulmonary artery smooth muscle cells activation of cGMP/PKG limited TGFβ-induced nuclear translocation of smad3 by sequestering smad3 with cytosolic β2-tubulin [8]. Therefore, we performed a coimmunoprecipitation (Co-IP) of stimulated
fibroblasts to check whether smad3 and β2-tubulin form a cGMP-dependent complex. Figure 5D shows that β2-tubulin antibody precipitated cGKIα, P-smad3 and smad3 in TGFβ- and cGMP/TGFβ-stimulated fibroblasts. However, in contrast to Gong et al. [8], there was no increase in the intensity of the bands after pretreatment with cGMP.

Second, the phosphorylation of Erk1 and Erk2 (P-Erk1/2) was assessed. UUO increased the phosphorylation and the protein expression of Erk1 and Erk2 (Fig. 6A). Immunoblots with antibodies against total Erk1 and Erk2 demonstrated that their expression is increased by UUO, but not changed by Bay administration (Fig. 6A). Accordingly, in Fig. 6B, C only fibrotic kidneys are compared and P-Erk1/2 is normalized to Erk1/2 and related to untreated fibrotic wt-kidneys. sGC stimulation caused a significant decrease in P-Erk1 and P-Erk2 in fibrotic kidneys of...
treated in contrast to untreated wt-mice. This BAY-induced decrease of Erk1/2 phosphorylation in fibrotic wt-kidneys was not due to changed protein expression of Erk1/2. In cGKI-KO-kidneys, the Erk phosphorylation was not reduced by BAY (Fig. 6B,C).

Increased IL-6 levels in cGKI-KO-mice

It has been shown that IL-6 promotes fibrosis [9]. UUO significantly increased the IL-6 concentration in serum of wt-mice. Administration of BAY tends to result in diminished IL-6 levels in serum of wt-mice compared with untreated wt-mice. Interestingly, the IL-6 concentration was significantly higher in untreated and treated cGKI-KO-mice and fluctuated much more than in wt-mice (Fig. 7).

Effect of BAY and role of cGKI on the renal function examining serum creatinine

The serum level of creatinine increased significantly after 7 days of UUO. Following BAY administration, serum creatinine was decreased, but there was no significant difference between BAY treated and untreated wt-mice. Conversely, in cGKI-KO-mice, BAY influenced in no way the serum creatinine (Fig. 8).

Discussion

In the present study, we have investigated the functional role of sGC stimulation in regulating renal fibrosis. BAY reduced the mRNA- and protein...
expression of different fibrosis marker. The antifibrotic impact of sGC stimulation was not observed in cGKI-KO-mice, suggesting that cGKI mediates the repair process of renal fibrosis.

Our study confirmed that the serum creatinine, which is a parameter for renal function, is increased after UUO [10]. However, it was not significantly reduced by BAY in wt-mice and unchanged in cGKI-KO-mice. Our results are in line with the nephroprotective effects of PDE5 inhibitors which also enhance the cGMP pool [11–13]. cGKI-KO-mice have higher IL-6 levels [14,15] which exert profibrotic effects [9,16]. Conforming with our present study, the IL-6 levels were increased by UUO and treated, as well as untreated cGKI-KO-mice showed a higher IL-6 concentration than wt-mice. However, cGKI-KO-kidneys revealed no more pronounced fibrosis compared to wt-kidneys suggesting that other signalling pathways as IL-6 are important for induction of renal fibrosis. The application of BAY reduced the IL-6 concentration, but the difference was not significant. Considering the effects of the MAPK signalling, the phosphorylation of Erk promotes fibrosis [17]. In cardiac fibrosis the inhibition of Erk phosphorylation by cGMP has already been discussed [18]. Our results confirmed the decrease in phosphorylation of Erk after BAY application. Consistent with our data, Beyer et al. [19] have also identified that the stimulation of sGC decreased TGFβ signalling through the inhibition of Erk1/2 phosphorylation. Additionally, we observed that cGMP influenced via cGKI the phosphorylation of Erk because in cGKI-KO-mice, the effects of BAY were lower.

It is generally accepted that TGFβ acts by stimulation of its downstream mediator smad2 and smad3. Latest studies report that diminished smad2- as well as smad3 phosphorylation results in enhanced renal fibrosis [20–22]. However, it is also recently discussed that phosphorylation of smad2 and smad3 by TGFβ exerts reverse effects in renal fibrosis. Smad2 maybe plays a protective role negatively regulating the smad3 signalling. TGFβ activates smad2 which diminishes TGFβ1/smad3 signalling, including phosphorylation, nuclear translocation and the binding of smad3 to the Col1 promoter, leading to augmented collagen synthesis [23,24]. In our study, phosphorylated smad2 was unaffected by cGMP in renal fibroblasts (data not shown). However, nuclear translocation of P-smad3 was diminished by cGMP in the presence of TGFβ in wt-, but not in cGKI-KO-fibroblasts. Interestingly cGMP inhibited only the translocation of P-smad3, but not the phosphorylation of smad3 (Fig. S2). In contrast to our study, Beyer et al. [19] showed that nuclear P-smad2- and P-smad3 levels and smad reporter activity were unaffected by sGC stimulation in human fibroblasts. As already mentioned in pulmonary artery smooth
muscle cells, activation of cGMP/PKG limited TGFβ-induced nuclear translocation of smad3 by sequestering smad3 with cytosolic β2-tubulin [8]. However, in contrast we did not detect an increase in P-smad3–β2-tubulin interaction after pretreatment with cGMP. In our study exists a cGKIα–P-smad3–β2-tubulin interaction in fibroblasts, but the intensity of this interaction is not influenced by cGMP. Consequently, the observed inhibition by cGMP of TGFβ-induced nuclear translocation of P-smad3 cannot be explained by sequestering P-smad3 with cytosolic β2-tubulin. CTGF is downstream of TGFβ signalling and upregulated in response to TGFβ stimulation [25]. However, the regulation of CTGF expression via cGMP is controversially discussed. Hewitson et al. and Beyer et al. showed that cGMP is not able to decrease the CTGF expression in fibroblasts, [1,19] which contrasts our study illustrating reduced CTGF expression with BAY.

Expression of PAI-1, which acts profibrotic, is slightly attenuated by BAY. TGFβ1 activates PAI-1 and PAI-1, in turn, stimulates TGFβ1 [26]. The expression of PAI-1 is regulated via TGFβ1-induced Erk phosphorylation [27] which is significantly reduced by BAY.

After UUO, the MMP2 mRNA is adjusted much higher than MMP9. Therefore, MMP2 appears to be more crucial in the development of renal fibrosis than MMP9. Of importance is the fact that BAY-induced increase in TIMP-1 expression was accompanied by diminished MMP2 activity. TIMPs do not reveal a high specificity for any particular MMP [28], but we suppose that the diminished activity of MMP2 by BAY maybe caused by the regulation of substantial increased TIMP-1. The role of MMPs in developing renal fibrosis is very complex and subsequently differently discussed. On the one hand, MMP exert antifibrotic effects degrading diverse components of the ECM. On the other hand, they are implicated in pathological processes such as fibrosis and thereby degrading basal membrane. Especially, MMP2 degrades collagen IV, which is an essential part of the basal membrane [29]. A TGFβ-induced increase of the MMP2 protein and mRNA is also reported [30]. In turn, enhanced MMP activity can stimulate the TGFβ-complex, which afterwards activates fibroblasts and provokes the synthesis of collagen [31]. Accordingly, the BAY-induced reduction of MMP activity may lead to reduced TGFβ activity, which correlates with the observed decreased expression of TGFβ target genes. Considering this, the decrease in MMP2 activity by cGMP/cGKI can ameliorate the progression of renal fibrosis.

BAY application in rats was previously shown to ameliorate renal injury after relief of ureteral obstruction [32]. In the clinics, renal damage depends on the duration until relief of ureteral obstruction [33]. It has to be evaluated clinically in the future whether application of sGC stimulators might be a therapeutical approach to diminish renal fibrosis upon ureteral obstruction and to enhance renal recovery after relief.

**Conclusion**

The results of the present study suggest a therapeutic potential of BAY application in renal fibrosis. The antifibrotic effect of BAY is mediated via cGMP/cGKI by inhibition of Erk and smad3 signalling pathways (Fig. 9).

**Materials and methods**

**Mice**

129/Sv-WT and 129/Sv-cGKI-KO-mice [34] were bred and maintained in the animal facilities of the University of Regensburg.

The investigation conforms to the guide for the Care and Use of Laboratory Animals published by the US National
Institute of Health. The experimental protocols were approved by the local authorities for animal research (Regierung der Oberpfalz, Bayern, Germany; #54-2532.1-02/10) and were conducted according to the German law for animal care.

**Unilateral ureter obstruction**

The renal fibrosis was induced by UUO in 6–12-week-old mice as described in [5]. The application with BAY 41-8543 (BAY; daily, intraperitoneal, 4 mg·kg bw⁻¹) started 24 h after surgery using water, glycerol, PEG400 as vehicle. After 7 days, the fibrotic process was analysed.

**Quantitative RT-PCR**

Real-time PCR of αSMA, fibronectin, Collα1, CTGF, PAI-1, TIMP-1, MMP2, and MMP9 was performed as previously described [5]. 18S rRNA served as housekeeper gene. The results are shown as the x-fold change in mRNA expression (2ΔΔCt) in the fibrotic kidney relating to the opposite healthy kidneys whose mRNA expression was set to one.

**Sirius red/fast green**

Collagen levels were determined by Sirius red/fast green method [5]. We calculated the increase (%) of collagen (collagen/nonprotein collagen) after 7 days UUO related to the healthy kidney.

**Immunofluorescence**

The fixation, staining and quantification of kidney tissues were performed as previously reported [5]. The quantification of αSMA, fibronectin, Collα1 and total collagen was related to the contralateral healthy kidney. The fluorescence intensity was quantified using the metamorphic offline software (Visitron Systems, Puchheim, Germany).

**Western blot analysis**

The protein expression of CTGF, PAI-1 (Santa Cruz Biotechnology, Heidelberg, Germany), Erk1/2, P-Erk1/2 (Cell Signaling, Danvers, MA, USA) and TIMP-1 (Sigma Aldrich, Taufkirchen, Germany) was assayed by western blotting [5]. Representative immunoblots show the influence of UUO in comparison to the contralateral healthy kidney and the effects of BAY in healthy and fibrotic wt-kidneys. The graphs statistically compare exclusive fibrotic kidneys. The values of all markers of the fibrotic wt- and cGKI-KO-kidneys were related to the mean values of fibrotic untreated wt-kidneys. This ratio was set to one and normalized to the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling) respectively to Erk1/2 for P-Erk1/2 values. For quantification, ImageJ densitometry was used (BioRad, München, Germany).

**Gelatin zymography assay**

The activity of MMP2 and MMP9 was detected using gelatin zymography, which can distinguish between latent and active forms of proteinases.

Briefly, the culture medium was electrophoresed in a SDS/PAGE gel containing 0.1% gelatin. The gel was loaded with 70 μg/35 μg total protein for MMP2/MMP9. The gel was washed (100 mm NaCl and 2.5% Triton X-100 in 50 mm Tris-HCl, pH 7.5) to remove SDS and incubated in a reaction buffer (200 mm NaCl, 0.02% NaN3, 0.5 μm ZnCl2, 1 mm CaCl2, 2% Triton X-100, in 50 mm Tris-HCl, pH 7.5) for enzymatic reaction at 37 °C overnight. Finally, the gel was stained with Coomassie blue, and destained in 10% acetic acid/30% methanol and quantified using ImageJ software (open source). MMPs in fibrotic tissue were expressed as relative values of markers in kidneys from untreated wt-mice.

**Cell culture**

The fibroblasts of wt- and cGKI-KO-mice were isolated and stained as described previously [5]. The cells were pretreated with 8Br-cGMP (Biolog, Bremen, Germany; 1 mm, 1 h, 37 °C) or vehicle followed by exposure to TGFβ (Biomol, Hamburg, Germany; 2 ng·mL⁻¹, 1 h, 37 °C) or vehicle. Nuclei were stained with DAPI (gift from Armin Kurtz, University Regensburg). P-smad3 and P-smad2 (Cell Signalling, respectively, were detected using an Alexa647-conjugated anti-rabbit secondary-antibody (1 : 200; Invitrogen, Karlsruhe, Germany) for 2 h at room temperature. Coverslips were washed, mounted with glycerol and analysed using an Axiosvert 200 microscope (Zeiss, Jena, Germany). To ensure a valid comparison, images were randomly selected from different fields. The intranuclear and extranuclear fluorescence-intensity of three to six equal areas was measured respectively. Then, the mean value of intranuclear and extranuclear fluorescence intensity of P-smad3 was determined respectively. For the quantification of the fluorescence intensity all values were related to values of untreated wt-fibroblasts (control) using the metamorphic offline software.

**(Co-)immunoprecipitation**

The stimulated (TGFβ/cGMP+TGFβ) cells were lysed in 2% Lubrol-PX buffer [20 mm Tris; 150 mm NaCl, 2% Lubrol (nonaethylenyglycol-monododecylether)] containing phosphatase inhibitors (Roche, Mannheim, Germany) and protease inhibitors. After homogenization and centrifugation (18 000 g, 10 min, 4 °C) the protein concentration of the supernatant was determined by a Lowry-based method.
The reactions were completed with Co-IP buffer (50 mM Tris-HCl, pH 7.5, 15 mM EGTA, 100 mM NaCl, 0.1% Triton X-100) containing also phosphatase inhibitors (Roche) and protease inhibitors.

About 1000–1500 μg of cell lysates was given onto the beads. Two microgram β2-tubulin-antibody (Sigma Aldrich) was added and incubated on ice 90 min. Meanwhile 40 μL of protein-A-G-Sepharose beads (Thermo Scientific, Dreieich, Germany) were pretreated for each immunoprecipitation. They were washed three times with Co-IP buffer, then blocked with 3% BSA in Co-IP buffer and washed three times at least once more. After that, the incubated cell lysates were centrifuged (18 000 g, 10 min, 4 °C), then the supernatant was added to the washed and blocked Sepharose beads and rotated overnight at 4 °C. Following this, three washing steps were performed (100 g, 4 °C, 1 min) and the precipitate was eluted with Laemmli buffer 2×. Proteins were separated by SDS/PAGE (12.5%) and blotted to polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany). The blots were incubated with anti-smad3, anti-cGKIα [35] and anti-β2-tubulin, at 4 °C overnight. Bands were visualized by use of an ECL select Western Blotting Detection Reagent (GE Healthcare, Amersham, UK). Co-immunoprecipitation of cell extracts without antibody and Co-IP buffer with antibody, respectively, were used as controls for the specificity of the Co-IP analysis (data not shown).

Determination of IL-6 and cGMP

For measurement of IL-6 levels in serum, blood was drawn in anaesthetized (2% isoflurane) mice from the retrobulbar plexus and centrifugated (8 min, 1000 g). Afterwards, the IL-6 levels in the serum were determined with mouse IL-6 Quantikine ELISA Kit, (R&D Systems, Wiesbaden-Nordenstadt, Germany). For determination of cGMP concentration in tissue, the kidneys were removed and assessed with cGMP-EIA kit (IBL, Cayman, UK).

Serum creatinine

Serum creatinine was determined by HPLC as previously reported with minor modifications [5]. In brief, 10 μL serum was mixed with 50 μL perchloric acid to precipitate proteins. The tube was vortexed and kept at 4 °C for 15 min. Following centrifugation (5 min, 10 000 g), an aliquot of 5 μL of the supernatant was injected into the HPLC apparatus (Prominance LC20 series equipped with a LC20A photometric detector set at 234 nm; Shimadzu, Duisburg, Germany). Separation was performed using a Zorbas 300-SCX 5 μm, 150 × 4.6 mm, analytical column (Agilent, Waldbronn, Germany) and a mobile phase consisting of 5 mM sodium acetate (pH = 5.1)/acetonipectrol [800 : 200 (v : v)]. Creatinine eluted after 6.3–6.5 min at a flow rate of 1.0 mL-min⁻¹ (column temperature 35 °C).

Statistical analysis

All data are expressed as mean ± SEM. For calculation of statistical differences between two means, the unpaired Student’s t-test (two-tailed, confidence interval 95%) was used. If the difference between two groups was statistically significant, then it is indicated by asterisks (*P < 0.05; **P < 0.01; ***P < 0.001). n indicates the number of animals.

Acknowledgements

We thank Astrid Seefeld, Gertraud Wilberg, Katharina Wohlfart and Anna M’Bangui for their excellent technical assistance. The expert aid of Frank Schweda and Matthias Mack (University of Regensburg) is highly acknowledged. The work was supported by the Bavarian State and the Deutsche Forschungsgemeinschaft, SFB 699.

Conflicts of interest

PS and J-PS are employees at Bayer Pharma AG. VW is an employee at Novartis Pharma GmbH, Nuremberg. The PhD thesis of VW is funded by Novartis Pharma.

Author contributions

ES, VW and JS planned experiments, analysed data and wrote the manuscript; ES, VW, AS and FK performed experiments. FH, PS and HPS contributed reagents or other essential material. All authors critically read the manuscript.

References

1 Hewitson TD, Martic M, Darby IA, Keltnack KJ, Bisucci T, Tait MG and Becker GJ (2004) Intracellular cyclic nucleotide analogues inhibit in vitro mitogenesis and activation of fibroblasts derived from obstructed rat kidneys. *Neprhon Exp Nephrol* 96, e59–e66.

2 Li L, Cheng FW, Wang F, Jia B, Luo X and Zhang SQ (2014) The activation of TLR7 regulates the expression of VEGF, TIMP1, MMP2, IL-6, and IL-15 in Hela cells. *Mol Cell Biochem* 389, 43–49.

3 Cheng X, Gao W, Dang Y, Liu X, Li Y, Peng X and Ye X (2013) Both ERK/MAPK and TGF-Beta/Smad signaling pathways play a role in the kidney fibrosis of diabetic mice accelerated by blood glucose fluctuation. *J Diabetes Res* 2013, 463740.

4 Hofmann F, Bernhard D, Lukowski R and Weinmeister P (2009) GMP regulated protein kinases (cGK). *Handb Exp Pharmacol* 191, 137–162.
Inhibition of TGFβ signalling pathway by cGMP/cGKI

E. Schinner et al.
27 Matsui S, Yamane T, Kobayashi-Hattori K and Oishi Y (2014) Calcitonin gene-related peptide regulates mitogen-activated protein kinase pathway to decrease transforming growth factor beta1-induced hepatic plasminogen activator inhibitor-1 mRNA expression in HepG2 cells. Biosci Biotechnol Biochem 78, 787–790.

28 Chelladurai P, Seeger W and Pullamsetti SS (2012) Matrix metalloproteinases and their inhibitors in pulmonary hypertension. Eur Respir J 40, 766–782.

29 Ronco P, Lelongt B, Piedagnel R and Chatziantoniou C (2007) Matrix metalloproteinases in kidney disease progression and repair: a case of flipping the coin. Semin Nephrol 27, 352–362.

30 Eldred JA, Hodgkinson LM, Dawes LJ, Reddan JR, Edwards DR and Wormstone IM (2012) MMP2 activity is critical for TGFbeta2-induced matrix contraction–implications for fibrosis. Invest Ophthalmol Vis Sci 53, 4085–4098.

31 Kassiri Z, Oudit GY, Kandalam V, Awad A, Wang X, Ziou X, Maeda N, Herzenberg AM and Scholey JW (2009) Loss of TIMP3 enhances interstitial nephritis and fibrosis. J Am Soc Nephrol 20, 1223–1235.

32 Wang-Rosenke Y, Mika A, Khadzhynov D, Loof T, Neumayer HH and Peters H (2011) Stimulation of soluble guanylate cyclase improves renal recovery after relief of unilateral ureteral obstruction. J Urol 186, 1142–1149.

33 Lucarelli G, Ditoonno P, Bettocchi C, Grandaliano G, Gesualdo L, Selvaggi FP and Battaglia M (2013) Delayed relief of ureteral obstruction is implicated in the long-term development of renal damage and arterial hypertension in patients with unilateral ureteral injury. J Urol 189, 960–965.

34 Weber S, Bernhard D, Lukowski R, Weinmeister P, Worner R, Wegener JW, Valtcheva N, Feil S, Schlossmann J, Hofmann F et al. (2007) Rescue of cGMP kinase I knockout mice by smooth muscle specific expression of either isozyme. Circ Res 101, 1096–1103.

35 Geiselhoringer A, Gaisa M, Hofmann F and Schlossmann J (2004) Distribution of IRAG and cGKI-isosforms in murine tissues. FEBS Lett 575, 19–22.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Analysis of renal cGMP levels after BAY application.

Fig. S2. Analysis of the whole fluorescence intensity of P-smad3.