Enhancing cGMP in experimental progressive renal fibrosis: soluble guanylate cyclase stimulation vs. phosphodiesterase inhibition

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1Department of Nephrology and Center of Cardiovascular Research, Charité Campus Mitte, Humboldt University, Berlin, Germany; 2Department of Pediatrics, Second University Hospital, Sun Yat-sen University, Guangzhou, People’s Republic of China; and 3Department of Cell Biology and Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

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Wang, Yingrui, Stephanie Krämer, Tanja Loof, Sebastian Martini, Susanne Kron, Hiroshi Kawachi, Fujo Shimizu, Hans-H. Neumayer, and Harm Peters. Enhancing cGMP in experimental progressive renal fibrosis: soluble guanylate cyclase stimulation vs. phosphodiesterase inhibition. Am J Physiol Renal Physiol 290: F167–F176, 2006. First published July 26, 2005; doi:10.1152/ajprenal.00197.2005.—cGMP serves as the main second messenger of nitric oxide (NO). Antifibrotic effects of enhancing renal cGMP levels have recently been documented in experimental acute anti-Thy-1 glomerulonephritis. The present study compares the effects of the cGMP production-increasing soluble guanylate cyclase (sGC) stimulator BAY 41-2272 with those of the cGMP degradation-limiting phosphodiesterase inhibitor pentoxifylline (PTX) in a progressive model of renal fibrosis. At 1 wk after induction of anti-Thy-1-induced chronic glomerulonephrosis (cGS), rats were randomly assigned to groups as follows: cGS, cGS + BAY 41-2272 (10 mg·kg body wt−1·day−1), or cGS + PTX (50 mg·kg body wt−1·day−1). BAY 41-2272 and PTX reduced systolic blood pressure significantly. At 16 wk, tubulointerstitial expressions of sGC mRNA and NO-induced cGMP synthesis were increased in untreated cGS animals, whereas their glomerular activity was depressed compared with normal controls. Tubulointerstitial and glomerular cGMP production in response to NO were significantly enhanced in animals treated with BAY 41-2272, but not in those treated with PTX. BAY 41-2272 administration resulted in marked reductions of glomerular and tubulointerstitial histological matrix accumulation, expression of TGF-β1 and fibronectin, macrophage infiltration, and cell proliferation as well as improved renal function. In contrast, only moderate and nonsignificant renoprotective changes were observed in the cGS + PTX group. In conclusion, increasing renal cGMP production through BAY 41-2272 significantly improved renal NO-cGMP signaling and limited progression in anti-Thy-1-induced chronic renal fibrosis, whereas inhibition of cGMP degradation by PTX was only moderately effective. The findings indicate that pharmacological enhancement of renal cGMP levels by sGC stimulation represents a novel and effective antifibrotic approach in progressive kidney disorders.

BAY 41-2272; pentoxifylline; TGF-β1; progression

THE MAIN SECOND MESSENGER MOLECULE of the L-arginine-nitric oxide (NO) pathway, cGMP, mediates NO’s critical biological actions in mammalian physiology, such as vasodilatation, inhibition of platelet aggregation, leukocyte recruitment, and cell proliferation (11). NO-cGMP signaling takes place at the intracellularly located enzyme soluble guanylate cyclase (sGC, GTP pyrophosphate lyase (cycling), EC 4.6.1.2), which serves as the principal physiological target for the freely diffusible molecule NO (6, 10). sGC is a heterodimeric enzyme that is expressed predominantly as an α1β1-heterodimer in various tissues, including the kidney (23). cGMP subsequently activates further downstream mechanisms such as cGMP-regulated protein kinases, ion channels, and phosphodiesterases (PDE) to finally create NO’s biological actions (10).

In models of acute anti-Thy-1-induced glomerulonephritis, we and others recently reported that glomerular NO-cGMP signaling is markedly altered in the course of the disease and that specific pharmacological stimulation of sGC activity by BAY 41-2272 limits the fibrotic response that follows antibody injection (4, 20). To extend these findings from stimulating cGMP production to inhibiting cGMP degradation for enhancement of renal cGMP levels, the present study was designed to compare the effects of the sGC stimulator BAY 41-2272 (which increases cGMP synthesis) with those of the PDE inhibitor pentoxifylline (PTX, which decreases cGMP degradation) in a progressive model of renal fibrosis, i.e., anti-Thy-1 antibody-induced chronic progressive glomerulonephrosis of the rat. In this model, a single injection of anti-Thy-1 antibody into uninephrectomized rats induces a short-term acute mesangio proliferative glomerulonephritis that is followed by a slow primarily non-immune-mediated progression of the disease over several months toward glomerulosclerosis, tubulointerstitial fibrosis, and advancing renal insufficiency (7, 14, 18). BAY 41-2272 is a novel orally available pyrazolopyridine derivate that stimulates sGC activity directly and increases sGC’s sensitivity to NO (22). PTX inhibits PDE-1 to -5 and is used in treatment of patients with peripheral vascular disease (8). Interventions were started 1 wk after disease induction. At 16 wk, actions on proteinuria, blood pressure, renal sGC expression and activity, tubulointerstitial and glomerular matrix protein expression, macrophage infiltration, and cell proliferation, as well as kidney function, were determined.

METHODS

Materials. All materials, chemicals, and cell culture media, if not stated differently, were purchased from Sigma Chemical-Aldrich (Taufkirchen, Germany).

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Animal care, model of anti-Thy-1-induced glomerulosclerosis, and study design. Animal care and treatment conformed with the guidelines of the American Physiological Society and were approved by local authorities. Male Wistar rats (150–180 g; Charles River, Sulzfeld, Germany) were caged in a constant-temperature room with a 12:12-h dark-light cycle and fed a normal-protein (22.5%) diet (Altromin, Lage, Germany). Food and water intakes were monitored throughout the experiment.

Anti-Thy-1-induced chronic glomerulosclerosis (cGS) was induced by intravenous injection of MAb 1-22-3 (5 mg/kg body wt in PBS, pH 7.4) 3 days after uninephrectomy, as previously described (18). MAb 1-22-3 binds to a Thy-1-like antigen on mesangial cells and causes a fast complement- and NO-dependent mesangial cell lysis within the next 24 h (14, 18). The progression in cGS is linked to the uninephrectomy, which is performed before anti-Thy-1 antibody injection, because the glomerular disease resolves over 1–4 wk in animals with two kidneys (14, 24).

On the basis of the actual 24-h proteinuria achieved 1 wk after anti-Thy-1 antibody injection, the diseased animals were randomly assigned to the following groups: 1) untreated uninephrectomized, anti-Thy-1-injected animals (cGS, n = 11), 2) BAY 41-2272-treated uninephrectomized, anti-Thy-1-injected animals (cGS + BAY 41-2272, n = 11), and 3) PTX-treated uninephrectomized, anti-Thy-1-injected animals (cGS + PTX, n = 8). In addition, groups of four nonnephrectomized, PBS-injected controls and four uninephrectomized, PBS-injected controls were included. Because the results for the nondiseased control animals were very similar and did not provide additional information, they are presented as a combined group (control).

Drug administration. Treatments were started 7 days after antibody injection to avoid interference with the induction of disease by anti-Thy-1 antibody. BAY 41-2272 (5-cyclopropyl-2-[1-(2-fluoro-1H-pyrazol-4-yl)pyrimidin-3-yl]-4-ylamine; generously provided by Dr. Johannes-Peter Stasch, Pharma Research Center, Bayer, Wuppertal, Germany), a new orally available stimulator of sGC (22), was given with food at 10 mg·kg body wt·day⁻¹, on the basis of our previous report in acute anti-Thy-1 glomerulonephritis (20). PTX (Alexis, Grünberg, Germany) is a trimethylated xanthine derivative (12). As a nonselective PDE inhibitor, it can increase intracellular cGMP or cAMP to dilate blood vessels and smooth muscles and ameliorate microcirculation. PTX was given with the drinking water targeting a dose of 50 mg·kg body wt·day⁻¹, on the basis of a previous report in rats with five-sixths subtotal nephrectomy (8).

Design of analysis. At 16 wk, i.e., after 15 wk of treatment, the actions of enhancing renal cGMP levels by BAY 41-2272 and PTX on proteinuria, systolic blood pressure, renal sGC activity, matrix protein expansion, macrophage infiltration, cell proliferation, and kidney function were determined. Glomerular and tubulointerstitial changes were analyzed separately. Glomeruli were isolated by a graded sieving technique. Because the renal cortex consists mainly of tubulointerstitial tissue (>95%), it was used to represent the tubulointerstitium. Analysis of fibrosis involved a computer-based histological calculation of the matrix actually accumulated as well as molecular analysis of expression of the key fibrosis marker and mediator TGF-β1, the matrix protein fibronectin, which indicates matrix protein synthesis, and the protease inhibitor tissue inhibitor of metalloproteases (TIMP)-1, which reflects matrix protein degradation. Tubulointerstitial and glomerular macrophage infiltration and cell proliferation were analyzed by immunohistochemistry using an ED1 or a proliferating cell nuclear antigen (PCNA) antibody, respectively. In addition, mRNA expression of P-selectin was determined. Very recently, P-selectin was identified as a cGMP-regulated adherence molecule important for macrophage recruitment (1). Blood creatinine and urea concentrations, calculated creatinine clearance, and blood hematocrit served as markers of renal function. sGC signaling was analyzed by the mRNA expression of α₁s and β₁s-GC and by the cGMP production of renal tissues ex vivo at basal level and in response to a defined NO stimulus.

Blood pressure and proteinuria. Systolic blood pressure was assessed at 8 and 16 wk in trained conscious animals by tail-cuff plethysmography as previously described (18). Animals were housed individually in metabolic cages for 24-h urine collection at 1, 8, and 16 wk after disease induction. Urinary protein was determined by a pyrogallol red method (18) and is expressed as milligrams of protein per 24 h.

Death of the animals. The animals were anesthetized with 0.1 mg of ketamine hydrochloride (10%; Ketanest, WDT, Garbsen, Germany) and 0.01 mg of xylazine (2%; Rompun, Bayer Vital, Leverkusen, Germany) per 100 g body wt. After laparotomy, blood was drawn from the abdominal aorta into EDTA-coated tubes, and kidneys were subsequently perfused with 40 ml of ice-cold PBS. Materials and tissues were processed as described below.

Blood cell counts. Spectrometric enzyme-based assays were used to measure plasma and urine creatinine and plasma urea. Glomerular filtration rate was calculated subsequently on the basis of the corresponding urine volume (7). Hematocrit was determined after 10 min of centrifugation and is expressed as percentage of blood cells per total blood volume.

Histology and immunohistochemistry. All microscopic examinations were performed in a blinded fashion as previously reported (7). For histological examination, cortical tissue was fixed in 10% neutral-buffered formalin. Sections (3 μm) of paraffin-embedded tissue were stained with periodic acid-Schiff to analyze tubulointerstitial and glomerular fibrosis by a computer-based morphometric analysis. Renal sections were examined on a light microscope (model DM LB2, Leica Microsystems, Wetzlar, Germany) connected to a video camera (model PL-AL62, Karl Zeiss Vision, Munich, Germany) and an image analysis system (Axiovision 2.05, Karl Zeiss Vision) using a 10×10 orthographic grid overlaid on digital images. The relative degree of tubulointerstitial fibrotic lesions, i.e., matrix deposition, cell infiltration, tubular atrophy, and dilation, was calculated in 15 randomly selected cortical areas per animal observed at ×200 magnification. It is expressed as percentage of the area affected in relation to the total area analyzed. Glomerular matrix expansion was evaluated by calculating the relative degree of the mesangial matrix-occupying area (in percentage) of 15 glomeruli from each rat.

Renal macrophage infiltration and cell proliferation were analyzed on paraffin-embedded tissues incubated with a primary mouse anti-ED1 antibody (Serotec, Oxford, UK) in conjunction with a standard alkaline phosphatase-anti-alkaline phosphatase technique (DakoCyto- mation, Hamburg, Germany) and a primary mouse anti-PCNA-antibody (DakoCytonation) and a secondary goat anti-mouse antibody coupled with the Envision staining system (DakoCytonation) as previously described (7, 18). Renal macrophage infiltration and cell proliferation evaluated by ED1- and PCNA-positive cells, respectively, were counted in ≥15 glomerular sections and ≥15 randomly selected cortical areas from each rat observed at ×200 magnification.

Glomerular and cortical protein expression of TGF-β1, fibronectin, and TIMP-1. Glomeruli from individual rats were isolated by a graded sieving technique (160-, 125-, and 71-μm mesh metal sieves) and described previously (18). For cultures of renal cortical tissue, a piece of cortical tissue was weighed and minced extensively with a razor blade (19). Glomeruli or cortical tissues were suspended in DMEM supplemented with 0.1 U/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin at a density of 2,000 glomeruli/ml and 10 mg/ml, respectively. After 48 h of incubation at 37°C in 5% CO₂, supernatants were harvested and stored at −20°C until further analysis. After acid activation, TGF-β1 content of culture supernatant was measured using a commercially available ELISA kit (R & D Systems, Wiesbaden, Germany) according to the manufacturer’s instructions.
TIMP-1 levels were analyzed using another commercially available ELISA kit (R & D Systems). Fibronectin was measured with a modified competitive ELISA according to published methods (18). Three samples from each rat were analyzed.

**Glomerular and cortical basal and NO-stimulated cGMP production.** Cortical tissues and glomeruli (150 μl) were placed into a 96-well microplate at 37°C and 5% CO₂, as previously described (20). After 1 h, 5 mM IBMX (20 μl; Alexis, Grünberg, Germany) was added to block cGMP degradation. After 10 min of incubation at 37°C and 5% CO₂, the cortical and glomerular tissues were exposed to 20 μl of 1 mM diethylamine-NONOate, a fast NO-releasing compound (Alexis) and incubated for another 10 min at 37°C and 5% CO₂. The reaction was stopped by cooling the microplate on ice and adding 20 μl of 5% dodecyltrimethylammonium bromide to facilitate cell lysis. Supernatant of lysed cells was stored at −80°C until analysis (20). cGMP levels in cortical and glomerular lysates were measured by ELISA (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer’s instructions as described previously (20).

The results are expressed as femtomoles of cGMP per well for glomerular and cortical lysates.

**Cortical mRNA expression of endothelial NO synthase, α₁- and β₁-sGC, P-selectin, TGF-β₁, fibronectin, and TIMP-1.** Cortical total RNA was extracted and processed by a “two-step” RT-PCR as described previously (7, 20). Real-time PCR was performed using the LightCycler system and SYBR Green I as double-strand DNA-binding dye (Roche Diagnostics, Mannheim, Germany). The following primer pairs were used: 5'-TCCAGTAACACAGACCTCG-3' (sense) and 5'-CAGGAGTAAGTGAGACCTGG-3' (antisense) for endothelial NO synthase (eNOS; 61°C annealing temperature), 5'-CCACATCAACAGGGCTAAT-3' (sense) and 5'-GAAGTGCAAGTCTGATTC-3' (antisense) for α₁-sGC (62°C annealing temperature), 5'-CGGATGCCACGGTATTGTCT-3' (sense) and 5'-CTCTGGCCTTGGACGCAATT-3' (antisense) for β₁-sGC (62°C annealing temperature), 5'-ACCATGCGTGTATCAGGCC-3' (sense) and 5'-CTCTGCAGCAACTAGTACTG-3' (antisense) for P-selectin (61°C annealing temperature), 5'-GGTGCGAGGCGACGCGCTGA-3' (sense) and 5'-GCGATGGTGCCCTGGCCT-3' (antisense) for TGF-β₁ (64°C annealing temperature), 5'-GTCAGAATGCGT-CATGTTCCA-3' (sense) and 5'-CAATGGAGAATCGGTATGGG-3' (antisense) for fibronectin (64°C annealing temperature), 5'-CCCAAAGAACATCGAGAAG-3' (sense) and 5'-CCCTGGTGCGATTCCCCAGC-3' (antisense) for TIMP-1 (66°C annealing temperature), and 5'-CCCATCTCCAGGAGAGAT-3' (sense) and 5'-CATGTTCCCA-3' (antisense) for GAPDH (59°C temperature).

Results were quantified relative to the point at which the specific fluorescence rises above the background fluorescence (crossing point) as previously reported (7, 20). Finally, the initial number of molecules was calculated and compared in relation to the expression of GAPDH mRNA as housekeeping gene.

**Statistical analysis.** Values are means ± SE. One-way ANOVA and a subsequent Mann-Whitney U-test were used for statistical comparison of the groups. P < 0.05 was considered significant.

**RESULTS**

**Body weight and food and water intake.** At 16 wk, at the end of the experiment, body weights were significantly lower in all three cGS groups [478 ± 16, 472 ± 17, and 471 ± 28 g] for cGS, cGS + BAY 41-2272, and cGS + PTX, respectively, P < 0.01 vs. controls, P = not significant (NS) between cGS groups] than in the control group (567 ± 16 g), indicating chronic renal disease. Food and water intakes were not significantly different between the groups throughout the experiment (data not shown).

**Blood pressure.** Systolic blood pressure at 8 and 16 wk was moderately, but significantly, increased in the anti-Thy-1-induced cGS model (136 ± 3 and 137 ± 2 mmHg, respectively, P < 0.01 vs. controls; Fig. 1A). Both treatments normalized systolic blood pressure. In the BAY 41-2272-treated animals, blood pressures of 112 ± 5 and 114 ± 4 mmHg were achieved at 8 and 16 wk, respectively, whereas 124 ± 4 and 128 ± 3 mmHg were recorded in the PTX-treated animals at 8 and 16 wk, respectively (all P < 0.01 vs. cGS and P < 0.05 for cGS + BAY 41-2272 vs. cGS + PTX).

**Proteinuria.** Pretreatment proteinuria was equally increased in all three nephritic groups (Fig. 1B), indicating effective randomization: 175 ± 12, 174 ± 10, and 174 ± 14 mg/day for cGS, cGS + BAY 41-2272, and cGS + PTX (P = NS). Urinary protein loss increased gradually in all diseased animal
groups during the experiment. At 16 wk, proteinuria was lower, but not significantly, in the BAY 41-2272-treated animals: 438 ± 51, 554 ± 71, and 483 ± 70 mg/day for cGS + BAY 41-2272, cGS, and cGS + PTX, respectively (P = NS).

Tubulointerstitial matrix expansion. In untreated anti-Thy-1-induced cGS, there was a marked increase in histological tubulointerstitial matrix score (57 ± 5%) and protein expression of TGF-β1 (521 ± 44 pg/ml), fibronectin (15,041 ± 1,129 ng/ml), and TIMP-1 (8,378 ± 1,572 ng/ml) compared with nonnephritic control animals (all P < 0.01 vs. control; Fig. 2). mRNA expressions of TGF-β1, fibronectin, and TIMP-1 were elevated 9.0-, 9.2-, and 9.6-fold, respectively (all P < 0.01 vs. control). Treatment with BAY 41-2272 significantly reduced histological tubulointerstitial matrix accumulation (−42%, P < 0.05 vs. cGS) and protein and mRNA expressions of TGF-β1 (−38 and −30%, respectively, P < 0.05 vs. cGS for both), fibronectin (−49 and −53%, respectively, P < 0.05 vs. cGS for both), and TIMP-1 (−52 and −59%, respectively, P < 0.05 vs. cGS for both), whereas PTX only slightly and insignificantly reduced tubulointerstitial disease severity in the chronic anti-Thy-1 animals (Fig. 2).

Tubulointerstitial expression and activity of the NO-cGMP signaling cascade. Tubulointerstitial mRNA expression of α1- and β1-sGC was increased 2.6- and 3.1-fold, respectively, in anti-Thy-1-induced cGS (P < 0.01 vs. control; Fig. 3, A and B). Expression of tubulointerstitial eNOS mRNA was also elevated in the diseased animals (1.5-fold vs. control, P < 0.05) but was not further altered by BAY 41-2272 or PTX treatment in any significant way (2.3- and 1.9-fold increase vs. control, P = NS vs. cGS). Consistent with the tubulointerstitial sGC mRNA expression, NO-stimulated cGMP production of tubulointerstitial tissue was 2.7-fold higher in the chronic anti-Thy-1 group than in controls (P < 0.01; Fig. 3D). This documents BAY 41-2272’s pharmacological action as an enhancer of sGC activity. Tubulointerstitial mRNA expression and activity of sGC were not significantly altered by PTX treatment (Fig. 3).

Fig. 2. Effects of BAY 41-2272 and PTX on tubulointerstitial matrix protein expression 16 wk after induction of cGS. A: tubulointerstitial matrix accumulation. B, C, and D: protein expression of TGF-β1, fibronectin, and tissue inhibitor of metalloproteinase-1 (TIMP-1), respectively. Treatments were started 7 days after injection of anti-Thy-1 antibody into uninephrectomized rats. Control group is composed of nonnephritic animals with or without uninephrectomy. Relative degree of matrix accumulation was calculated by computer-based morphometric analysis. Matrix protein production was determined in extensively minced individual cortical tissues cultured at a density of 10 mg/ml for 48 h. *P < 0.05; **P < 0.01; ***P < 0.001 vs. cGS.
Glomerular matrix expansion. At 16 wk after induction of chronic anti-Thy-1 renal fibrosis, glomerular matrix protein accumulation was characterized by an increase in histological matrix score (57% vs. control; Fig. 4). While BAY 41-2272 lowered histological matrix accumulation (28%, P < 0.05 vs. cGS), TGF-β1 (25%), and fibronectin (46%, P < 0.05 vs. cGS), treatment with PTX produced only modest, and not significant, effects on glomerular matrix protein expression and accumulation. Glomerular TIMP-1 protein expression was significantly lower in untreated diseased than in control animals (918 ± 213 vs. 315 ± 133 ng/ml, P < 0.05). This decrease was partially prevented by BAY 41-2272 or PTX treatment.

Glomerular activity of the NO-cGMP signaling cascade. Basal glomerular cGMP production did not differ between the groups (Fig. 5). In contrast, NO-stimulated glomerular cGMP production was lower in untreated chronic anti-Thy-1 animals than in nonnephritic controls (−50%, P < 0.05 vs. control). Treatment with BAY 41-2272 increased NO-stimulated cGMP production significantly (46 ± 6 and 24 ± 3 fmol/well for cGS + BAY 41-2272 and cGS, respectively, P < 0.01), but no change was observed in PTX-treated animals (Fig. 5).

Renal function. Animals with chronic anti-Thy-1 glomerulosclerosis showed increases in plasma creatinine and urea levels, whereas creatinine clearances and blood hematocrit levels were decreased (all P < 0.01 vs. control; Fig. 6). Corresponding to the histological and molecular results on renal matrix expansion, administration of BAY 41-2272 lowered plasma creatinine (−57%, P < 0.05 vs. cGS) and urea (−47%) levels and preserved creatinine clearances (+73%, P < 0.05 vs. cGS) and blood hematocrit levels (46.2% vs. 40.9%, P < 0.05 vs. cGS). In contrast, renal function was not significantly affected by PTX treatment.

Renal macrophage infiltration, P-selectin expression, and cell proliferation. To further define the mechanisms underlying the renoprotective effect of BAY 41-2272 in chronic anti-Thy-1 glomerulosclerosis, we analyzed renal macrophage infiltration, P-selectin expression, and cell proliferation. In the untreated anti-Thy-1 animals, we found a marked renal macrophage infiltration and cell proliferation. At the tubulointerstitial level, ED1-positive cells (indicating macrophages) had increased 8.0- and 4.3-fold at the glomerular level, whereas PCNA-positive tubulointerstitial cells (indicating cell proliferation) were elevated by 19.7-fold and PCNA-positive glomerular cells by 2.0-fold (both P < 0.001 vs. control; Fig. 7).
Treatment with BAY 41-2272 markedly reduced tubulointerstitial and glomerular infiltration with macrophages (−50 and −42%) and tubulointerstitial and glomerular proliferation of cells (−31 and −31%; all P < 0.05 vs. cGS). In the PTX-treated animals, tubulointerstitial and glomerular macrophage number and cell proliferation were comparable to that in the untreated chronic anti-Thy-1 group. Consistent with the renal macrophage number, cortical P-selectin mRNA expression increased 5.5-fold in the chronic anti-Thy animals without treatment (P < 0.01 vs. controls). Cortical P-selectin expres-

Fig. 4. Effects of BAY 41-2272 and PTX on glomerular matrix protein expression 16 wk after induction of cGS. A: glomerular matrix accumulation. B, C, and D: protein expression of TGF-β1, fibronectin, and TIMP-1, respectively. Treatments were started 7 days after injection of anti-Thy-1 antibody into uninephrectomized rats. Control group is composed of nonnephritic animals with or without uninephrectomy. Matrix expansion was scored on periodic acid-Schiff-stained slides. Glomeruli were harvested from individual animals and cultured at a density of 2,000/ml for 48 h. *P < 0.05; **P < 0.01 vs. cGS. *P < 0.05 vs. cGS + PTX. †P < 0.05 vs. control.

Fig. 5. Basal (A) and NO-stimulated (B) glomerular cGMP synthesis 16 wk after induction of cGS. Treatments were started 7 days after injection of anti-Thy-1 antibody into uninephrectomized rats. Control group is composed of nonnephritic animals with or without uninephrectomy. cGMP generation was measured by ELISA in glomerular tissue harvested from individual animals in the presence or absence of the NO donor diethylamine-NONOate. **P < 0.01 vs. cGS. ***P < 0.01 vs. control.
sion was significantly reduced by BAY 41-2272 (−50%, P < 0.05 vs. cGS), but not by PTX (−16%, P = NS vs. cGS).

Taken together, the results in chronic anti-Thy-1-induced renal fibrosis show that enhancing renal cGMP levels by BAY 41-2272 administration significantly improved altered tubulo-interstitial and glomerular sGC activity and limited the progressive course of this model toward glomerulosclerosis, tubulointerstitial fibrosis, and renal insufficiency. In contrast, administration of the PDE inhibitor PTX had no significant impact on renal matrix protein accumulation and function.

DISCUSSION

The cellular levels of cGMP are defined by the balance between the activities of synthesizing enzymes (GC) and catabolizing enzymes (PDE) (11). A marked downregulation of sGC activity has recently been reported in the early mesangial cell lysis phase of acute anti-Thy-1-induced glomerulonephritis (20). In its subsequent matrix expansion phase, sGC-mediated cGMP synthesis was strongly upregulated, and the pharmacological stimulation of renal sGC activity and cGMP production markedly lowered glomerular TGF-β overexpression and matrix expansion (4, 20). Expanding on these findings, the present study in the progressive model of anti-Thy-1-induced renal fibrosis shows that enhancing renal cGMP levels by administration of BAY 41-2272 significantly limited tubulointerstitial fibrosis and preserved renal function, whereas using the PDE inhibitor PTX to block cGMP degradation was only moderately beneficial.

When two different agents are compared, dosing is always an important issue. The amount of BAY 41-2272 administered was based on previous reports in acute anti-Thy-1 disease, and judging from the profound effects observed, the dose can be considered appropriate. The dose of PTX was selected on the basis of an earlier study showing beneficial effects in experimental five-sixths nephrectomy and can be considered relatively high (8). Furthermore, the marked reduction in blood pressure achieved by the agent proves its bioavailability and should be regarded as an indicator for sufficient dosing. In an observation relevant for the conclusions of this study, an important difference between the two drugs became evident in the cGMP production of renal tissue in response to a defined NO stimulus. Although glomerular and tubular NO-dependent cGMP synthesis could be enhanced strongly by BAY 41-2272, PTX produced only minor effects. The latter probably remained below the detection limit of this system. The cGMP metabolism has a very fast turnover (6, 10); therefore, the PDE inhibitor IBMX must be added ex vivo to bring glomerular and tubulointerstitial cGMP production to a measurable range. Hence, the findings in chronic anti-Thy-1-induced glomerulosclerosis indicate that the sGC stimulator BAY 41-2272 has a greater ability to increase renal cGMP production than the PDE inhibitor PTX. Another reason for the differences in results

Fig. 6. Effects of BAY 41-2272 and PTX on markers of renal function 16 wk after induction of cGS. Plasma creatinine (A) and urea levels (B) and creatinine clearance [expressed as glomerular filtration rate (GFR)/100 g body wt (BW), C] are shown as markers of excretory kidney function. Blood hematocrit (D) indicates renal endocrine/erythropoietin-producing capacity. Control group is composed of nonnephritic animals with or without uninephrectomy. *P < 0.05 vs. cGS.
may be that blockade of PDE 1- to -5 by PTX inhibits not only the degradation of cGMP, but also other nucleotides such as the intracellular effector cAMP, which may lead to actions of its own on the course of the disease (12).

The outcome of PTX administration in anti-Thy-1 progressive renal fibrosis is consistent with a previous investigation of acute anti-Thy-1 glomerulonephritis in which PTX was antifibrotic when started before disease induction but ineffective when the glomerulonephritis was established (3). However, our results differ from those of a study of five-sixths nephrectomy (8). Here, PTX was remarkably effective, suggesting that, in situations in which high blood pressure dominates the progression of renal disease, this agent may be of greater value.

The findings of the present study expand our understanding of the regulation and the role of the L-arginine-NO-cGMP axis in renal disease. Most studies have concentrated on the impairment of the L-arginine-NO pathway resulting from NO deficiency, which has been mainly understood as the absolute or relative insufficiency of eNOS to generate adequate amounts of NO (17, 20). An active role of sGC enzymes in this cascade has mostly been neglected. With regard to the known NO physiological signaling effects, the increases in blood pressure, renal cell infiltration, proliferation, and matrix production seen in chronic anti-Thy-1-induced renal fibrosis indicate a functional insufficiency in NO action. The significantly blunted cGMP synthesis in response to NO, as observed in chronic anti-Thy-1 glomeruli, documents an absolute insufficiency in NO signal transduction. In tubulointerstitial tissue fibrosis of chronic anti-Thy-1 renal fibrosis, the sGC activity appeared to be relatively insufficient in nature, because its specific stimulation by BAY 41-2272 remarkably reduced blood pressure, kidney cell infiltration, proliferation, and matrix production. The increase in tubulointerstitial expression and activity of sGC in chronic anti-Thy-1 disease may, in all probability, rather, represent a counterregulatory, but insufficient, mechanism for slowing disease progression. The changes in NO-cGMP signaling in chronic anti-Thy-1 glomerulosclerosis are comparable to those found in a recent study comparing the vasodilator hydralazine with sGC stimulation (25). Furthermore, several other studies recently reported a decrease in the expression or activity of vascular sGC in functionally NO-deficient models, such as spontaneously hypertensive rats, angiotensin II infusion, myocardial infarction, lead-induced hypertension, diabetic Goto-Kakizate rats, and aging (2, 5, 9, 13, 21, 26).

Parallel to NO signal transduction deficiency as a new and important pathomechanism in renal fibrosis, the sGC stimula-
tor BAY 41-2272 has emerged as a novel therapeutic approach to effectively overcome NO signaling deficiency. The reductions in blood pressure, renal matrix accumulation, leukocyte infiltration, and cell proliferation achieved by BAY 41-2272 are consistent with enhanced NO-cGMP signal transduction. Regarding the subsequent underlying mechanism of this renoprotective effect, several pathways have to be considered. Because BAY 41-2272 and PTX normalized systolic blood pressure in anti-Thy-1-induced renal fibrosis, it may not be likely that prevention of hypertensive renal tissue injury can be considered the main reason for the observed differences in renoprotection. However, it may be that the moderately, but less significantly, lowered blood pressure of the PTX-treated animals had some influence. Apart from this potential hemodynamic factor, the present study provides strong evidence that two other pathways directly regulated through cGMP were of importance, i.e., a marked reduction in renal macrophage infiltration, and actively proliferating cells. Macrophages can release cytokines and growth factors, including TGF-
which are vital stimuli for the production of extracellular matrix (18). P-selectin, an important and cGMP-dependent adhesion molecule for macrophage recruitment (1), was specifically downregulated by BAY 41-2272. Cell proliferation is a prerequisite of matrix protein production in many renal diseases (15). In support of this interpretation, two recent studies show a blood pressure-independent reduction in macrophage infiltration and mesangial cell proliferation in acute anti-Thy-1 glomerulonephritis through BAY 41-2272 (4, 20).

The model of anti-Thy-1-induced, chronic progressive renal fibrosis is an experimental paradigm for a “one hit, self-progressing kidney disease,” as occurs in patients who progress after a single episode of glomerulonephritis (7). Beyond this, the model shares a number of features with other chronic kidney disorders as part of the common final pathway leading to end-stage renal disease (18). They include persistent TGF-β overexpression, renal cell proliferation, blood cell infiltration, and extracellular matrix accumulation. Hence, the findings of this study may be relevant for the pathomechanism and the therapeutic approach to other experimental and human chronic renal diseases, such as diabetic and hypertensive nephropathy. Regarding the future therapeutic potential of sGC stimulators, it is noteworthy that this novel drug class shows no tachyphylaxis, is devoid of unwanted free radical production, and acts precisely at the subcellular location where the entirety of the subsequent downstream signaling cascade is already lined up (20).

In conclusion, the present study shows that NO-cGMP signal transduction is markedly altered in the progressive course of anti-Thy-1-induced chronic renal fibrosis. Pharmacological stimulation of sGC through BAY 41-2272 enhances renal cGMP levels significantly more than the PDE inhibitor PTX and, thereby, effectively limits the progressive course of this model. Further studies are indicated to show whether this new pathomechanism and therapeutic approach will have an important impact on the progression of other chronic kidney disorders.

**REFERENCES**


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