Expression and activity of soluble guanylate cyclase in injury and repair of anti-thy1 glomerulonephritis

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Background. Activation of soluble guanylate cyclase and generation of cyclic 3′,5′-guanosine monophosphate (cGMP) is the main signal transducing event of the L-arginine-nitric oxide pathway. The present study analyzes the expression and activity of the nitric oxide-cGMP signaling cascade in and the effect of the specific soluble guanylate cyclase stimulator Bay 41-2272 on the early injury and subsequent repair phase of acute anti-thy1 glomerulonephritis.

Methods. Anti-thy1 glomerulonephritis was induced by OX-7 antibody injection in rats. In protocol 1 (injury), Bay 41-2272 was given starting 6 days before antibody injection. One day after disease induction, parameters of mesangial cell injury (glomerular cell number and inducible nitric oxide synthesis) were analyzed. In protocol 2 (repair), Bay 41-2272 treatment was started one day after antibody injection. On day 7, parameters of glomerular repair [glomerular matrix score, expression of transforming growth factor (TGF)-β1, fibronectin, and plasminogen-activator-inhibitor (PAI)-1, infiltration with macrophages and fibrinogen deposition (indicating platelet localization)] were determined. In both protocols, tail bleeding time, systolic blood pressure, plasma cGMP levels, glomerular mRNA expression of endothelial nitric oxide synthase (eNOS), α1 and β1 soluble guanylate cyclase, and basal and nitric oxide-stimulated glomerular cGMP production were analyzed.

Results. Bay 41-2272 prolonged bleeding time, reduced blood pressure, and increased plasma cGMP levels in both protocols. In the injury experiment, disease induction increased inducible nitric oxide synthesis and reduced glomerular cell number, while expression and activity of soluble guanylate cyclase was almost completely diminished. Bay 41-2272 did not affect parameters of mesangial cell injury and glomerular soluble guanylate cyclase expression and activity. In the repair protocol, expression and activity of soluble guanylate cyclase was markedly increased by disease. Bay 41-2272 further enhanced soluble guanylate cyclase expression and activity. This went along with significant reductions in proteinuria, glomerular matrix accumulation, expression of TGF-β1, fibronectin, and PAI-1, macrophage infiltration and fibrinogen deposition as compared to the untreated anti-thy1 animals.

Conclusion. Glomerular nitric oxide signaling via cGMP is markedly impaired during injury of anti-thy1 glomerulonephritis, while it is highly up-regulated during subsequent repair. Further pharmacologic soluble guanylate cyclase stimulation limits glomerular TGF-β1 overexpression and matrix expansion, suggesting that the soluble guanylate cyclase enzyme represents an important antifibrotic pathway in glomerular disease.

Nitric oxide is endogenously generated from the amino acid L-arginine and is a key messenger and effect molecule in a wide variety of mammalian functions ranging from vasodilatation and platelet disaggregation to neuronal transmission and immune defense [1]. The intracellularly located enzyme soluble guanylate cyclase [guanosine triphosphate (GTP) pyrophosphatase-lyase (cycling) EC 4.6.1.2] is the principal physiologic target for the freely diffusible molecule nitric oxide and represents its main signaling pathway via generation of cyclic 3′,5′-guanosine monophosphate (cGMP) [2, 3]. Soluble guanylate cyclase is a heterodimeric enzyme that is expressed predominately as α1β1 heterodimer in various tissues, including the kidney [3, 4]. Within the soluble guanylate cyclase, nitric oxide binds to a haem prosthetic group that is linked to the His-105 residue of the β subunit. This binding is thought to lead to a transformational protein conformation and to a subsequent activation of the enzyme with a several hundredfold increase in cGMP generation [3]. cGMP is the second messenger molecule of the L-arginine-nitric oxide pathway and regulates further downstream effector mechanisms such as cGMP-dependent protein kinases, cGMP-gated ion channels and cGMP-regulated phosphodiesterases to finally produce nitric oxide’s main biologic effects [5].

Research over the last decade has shown that the L-arginine-nitric oxide pathway is critically involved in pathologic matrix production and accumulation of the
kidney [6–10]. Using L-arginine administration to activate the L-arginine-nitric oxide pathway, previous studies have documented both beneficial and detrimental outcomes. In the rat model of anti-thy1-antibody–induced glomerulonephritis, L-arginine supplementation has been reported to limit glomerular overexpression of the key fibrosis mediator transforming growth factor (TGF)-β and matrix accumulation during its repair phase [11]. In a subsequent study using the same model, we have recently shown that this beneficial effect of L-arginine is mainly mediated via endogenous generation of nitric oxide and can be mimicked by pharmacologic nitric oxide donation [12]. However, in these studies it remained unclear, whether the protective, antifibrotic effect of nitric oxide is transduced by cGMP, and whether nitric oxide signaling via cGMP is affected during the course of anti-thy1 glomerulonephritis.

The present study was designed to characterize the role of the nitric oxide-cGMP signaling cascade during the course of anti-thy1 antibody-induced glomerulonephritis. In two separate protocols, the expression and activity of the nitric oxide-cGMP signaling cascade was analyzed during the early injury (day 1 after antibody injection) and the subsequent repair phase (day 7 after antibody injection). The early injury phase in anti-thy1 glomerulonephritis is characterized by an anti-thy1 antibody- and nitric oxide-dependent lysis of the mesangial cells [13]. The subsequent repair phase shows a rapid and marked glomerular TGF-β overexpression and matrix protein expansion [10]. To further characterize the role of nitric oxide-cGMP signaling in glomerular injury and repair, the novel, orally applicable compound Bay 41-2272 was given to nephritic animals in order to specifically enhance the activity of soluble guanylate cyclase [14]. Bay 41-2272 is a pyrazolopyridine derivate that amplifies cGMP generation specifically and multifoldly in the presence of given, physiologic or low nitric oxide concentrations, through interaction with the soluble guanylate cyclase enzyme. In addition, Bay 41-2272 is also capable of an nitric oxide-independent stimulation of the soluble guanylate cyclase.

METHODS

Materials

Unless otherwise indicated, materials, chemicals or culture media were purchased from Sigma Chemical-Aldrich Co. (Taufkirchen, Germany).

Animals

Male Wistar rats (180 to 250 g) were obtained from Charles River (Sulzfeld, Germany) and fed a normal protein diet (22.5% protein) (Altromin, Lage, Germany) for at least 3 days before the start of the experiment to allow equilibration. Body weight was determined at the beginning and end of each experiment. Animals were housed in a constant temperature room with a 12-hour dark/12-hour light cycle. Food and water intakes were monitored. Animal care and treatment were in conformity with the guidelines of the American Physiological Society and approved by local authorities.

Induction of acute anti-thy1 glomerulonephritis

Acute anti-thy1 glomerulonephritis was induced by tail vein injection of the monoclonal antibody OX-7 [1 mg/kg body weight in phosphate-buffered saline (PBS)] as previously described [12]. In the kidney, OX-7 binds to a thy1-like antigen on the surface of mesangial cells and causes complement- and nitric oxide-dependent mesangial cell lysis [13]. Control animals were injected with equal volumes of PBS only. OX-7 antibody was produced from a hybridoma cell line as previously described [12]. The antibody were diluted in PBS (pH 7.4) and stored at −70 °C until use.

Drug administration

Chemically, Bay 41-2272 is 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine. It belongs to a new class of drugs that markedly amplify the cGMP output of soluble guanylate cyclase in the presence of given amounts of nitric oxide [14]. In addition, Bay 41-2272 also has the ability to directly stimulate soluble guanylate cyclase activity in the absence of nitric oxide. This novel class of drugs has been designated as soluble guanylate cyclase enhancers or soluble guanylate cyclase stimulators. Bay 41-2272 is structurally related to YC-1, the first member of the class of soluble guanylate cyclase enhancers [3]. However, in contrast to YC-1, Bay 41-4272 is orally applicable and does not inhibit the activity of the cGMP-degrading phosphodiesterase-5 [3, 14]. Photoaffinity studies have suggested that the cysteine 238 or 243 region of the ® subunit of the soluble guanylate cyclase is target of Bay 41-2272. Bay 41-2272 has recently been shown to reduce the platelet activity in vitro, to decrease blood pressure, and to prolong survival in a high-renin, low nitric oxide model of hypertension [i.e., L-nitro-arginine methyl ester (L-NAME)-treated transgenic renin rats [14]].

In our experiments, Bay 41-2272 was given with the food in a dose of 10 mg/kg body weight/day. This dose has previously been reported to reduce sufficiently the mean arterial blood pressure in spontaneously hypertensive rats [14]. Bay 41-2272 was generously provided by Dr. Johannes-Peter Stasch, Pharma Research Center, Bayer AG, Wuppertal, Germany. The drug-containing food was produced in our laboratory by using the flour of the standard rat chow (22.5% protein, A1311) (Altromin) as previously described [15]. Briefly, Bay 41-2272 was mixed into the dry food flour in appropriate amounts, water was
added to form pellets, and the air-dried pellets were subsequently given to the animals.

**Experimental design**

The expression and activity of the nitric oxide-cGMP pathway was separately analyzed in the early injury phase (protocol 1) and the subsequent matrix expansion phase of acute anti-thy1 glomerulonephritis. The expression analysis of the nitric oxide-cGMP cascade included mRNA expression of endothelial nitric oxide synthase (eNOS) and α1 and β1 soluble guanylate cyclase. The activity of glomerular soluble guanylate cyclase was determined at basal level and in response to a defined amount of nitric oxide in freshly-isolated glomeruli ex vivo. In the injury experiment, glomerular cell number and inducible nitric oxide production were analyzed as indicators of mesangial cell injury. In the repair protocol, parameters of glomerular matrix expansion (glomerular matrix score and expression of TGF-β1, fibronectin, and plasminogen activator inhibitor type 1 [PAI-1] protein) were measured. In both protocols, an additional group of nephritic animals was treated with the soluble guanylate cyclase enhancer Bay 41-2272. In protocol 1 (injury), Bay 41-2272 treatment started 6 days before and continued until 24 hours after injection of anti-thy1-antibody. In protocol 2 (repair), Bay 41-2272 treatment started 1 day after and continued until day 7 after antibody administration.

**Soluble guanylate cyclase in the injury phase of acute anti-thy1 glomerulonephritis (day 1 after antibody injection) (protocol 1)**

Six days before antibody injection, Wistar rats were assigned to the following groups: (1) PBS-injected controls (N = 6); (2) anti-thy1 antibody-injected rats, no treatment (glomerulonephritis) (N = 10); and (3) anti-thy1 antibody-injected rats plus Bay 41-2272 (glomerulonephritis + Bay 41-2272) (N = 10).

One day after antibody injection, the histological degree of mesangial cell lysis and the release of basal and lipopolysaccharide (LPS)-stimulated nitrite of cultured glomeruli were analyzed. At this point, mesangial cell lysis is complete and inducible glomerular nitric oxide production is markedly increased [16].

**Soluble guanylate cyclase in the repair phase of anti-thy1 glomerulonephritis (day 7 after antibody injection) (protocol 2)**

One day after antibody injection, when the mesangial cell lysis had occurred and the fibrotic response had started [16], Wistar rats were assigned to the following groups: (1) PBS-injected controls (N = 6); (2) anti-thy1 antibody-injected animals, no treatment (glomerulonephritis) (N = 10); and (3) anti-thy1 antibody-injected rats plus Bay 41-2272 (glomerulonephritis + Bay 41-2272) (N = 12).

Seven days after disease induction, expression of the key fibrosis mediator and marker TGF-β served as the principal therapeutic target. To document that TGF-β expression reflected actual matrix accumulation, a histologic glomerular matrix score was used. In addition, renal expression of the matrix protein fibronectin was measured as an indicator for matrix protein production. The protease inhibitor PAI-1 was used as sensitive marker of the matrix degrading system. In acute anti-thy1 glomerulonephritis, the fibrotic response peaks 7 days after antibody injection [12]. In order to further elucidate mechanisms underlying potentially a beneficial effect of Bay 41-2272 on glomerular matrix expansion, its effects on glomerular macrophage infiltration and intraglomerular thrombosis in vivo as well as on TGF-β production of normal and nephritic glomeruli in vitro were analyzed. Since cGMP-dependent inhibition of tissue leukocytes recruitment has recently been related to a down-regulation of the adhesion molecule p-selection [17], immunohistologic detection of glomerular macrophage infiltration was paralleled by measuring the mRNA expression of p-selectin. Intraglomerular thrombosis is commonly seen during the repair phase of acute anti-thy1 glomerulonephritis and indicates glomerular deposition of platelets as platelet-fibrinogen aggregates [15]. Immunohistologic fibrinogen staining was used as indirect marker for glomerular platelet deposition.

**Protocols 1 and 2**

Bleeding time, blood pressure, and plasma cGMP levels were analyzed in the subgroups of both protocols (N = 4 to 6 per group per protocol). Since the results for the two protocols were very similar and did not provide additional information, they are as a combination presented in the Results section.

**Bleeding time**

Rat tail bleeding time was analyzed as previously reported in anesthetized animals with a standardized incision (10 mm long, 1 mm deep) on the dorsal part of the tail [15].

**Urine collection and measurement of proteinuria**

In protocol 2, 24-hour urine was collected from each rat the day before sacrifice, using metabolic cages. Urinary protein concentration was measured by a pyrogallol red method using a microplate technique [12]. Proteinuria is expressed as mg protein/24 hours.
Sacrifice
At the end of each experiment, animals were anesthetized with ketanest (50 mg/kg body weight) (Pharmacia GmbH, Erlangen, Germany)/xylazin (10 mg/kg body weight) (Bayer Vital GmbH, Leverkusen, Germany). Following a midline abdominal incision, 5 to 10 mL blood was drawn from the abdominal aorta into ethylene-diaminetetraacetic acid (EDTA)-coated syringes. The blood-filled syringes were immediately cooled on ice. In parallel, the rat kidneys were subsequently perfused with 40 mL ice-cold PBS. For histologic examination, cortical tissue was fixed in 10% neutral buffered formalin. The cooled rat blood was subsequently centrifuged at 4°C, and 20 μL of 5 mmol/L 3-isobutyl-1-methylxanthine (IBMX) (Alexis GmbH, Grünberg, Germany) was added to 980 μL of plasma to block cGMP degeneration. Samples were stored at −20°C until measurement of cGMP concentration.

Glomerular isolation and culture
Glomeruli from individual rats were isolated by a graded sieving technique (160, 125, and 71 μm mesh metal sieves) as described previously [12]. Glomeruli were suspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.1 U/mL insulin, 100 U/mL penicillin, and 100 μg/mL streptomycin at a density of 2000 per mL.

Basal and nitric oxide-stimulated glomerular cGMP production
Volumes of 150 μL of the resuspended glomeruli were placed into the wells of a 96-well microplate and pre-warmed at 37°C/5% CO2. After 1 hour, 20 μL 5 mmol/L IBMX was added to each well to block cGMP degradation. After 10 minutes of incubation at 37°C/5% CO2, the glomeruli were exposed to 20 μL 1 mmol/L diethylamine NONOate (DEA/NO), a fast nitric oxide-releasing compound (Alexis GmbH, Grünberg, Germany) and incubated for another 10 minutes at 37°C/5% CO2. To stop the reaction, the microplate was quickly put on ice, and 20 μL 5% dodecytrimethylammonium bromide was added to stop the reaction and to facilitate cell lysis. Thus results express total (intra- and extracellular) cGMP levels. Lysed cells were stored at −80°C until analysis.

Measurement of cGMP
cGMP levels in plasma and glomerular lysates were measured by enzyme-linked immunosorbent assay (ELISA) (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer’s instructions. Briefly, samples, standards and peroxidase-labeled cGMP conjugate were transferred to a microplate precoated with a cGMP-specific antibody. After addition of substrate, optical density was read at 450 nm using an automated plate reader (MRX II) (Dynex Technologies, Frankfurt am Main, Germany). The results are expressed as fmol per mL for plasma and fmol cGMP per well for glomerular lysates.

Basal and LPS-stimulated glomerular nitric oxide production
In protocol 1 (injury), glomeruli were cultured at a density of 2000 per mL for 48 hours (basal nitric oxide glomerular production). Additional samples were cultured in the presence of 10 μg LPS from Escherichia coli (serotype 0127:B8) to stimulate inducible nitric oxide production (stimulated glomerular nitric oxide production). Nitrite served as indicator of nitric oxide production and was determined by the Griess reaction in glomerular culture supernatants [18]. Briefly, 50 μL of sample was mixed with 100 μL Griess reagent (0.05% N-[1-naphthyl] ethylene diamine dihydrochloride, 0.5% sulfanilamide in 45% glacial acetic acid) in 96-well plates. After 10 minutes of incubation in the dark, absorbance was read at 570 nm in an automated plate reader (MRX II) (Dynex Technologies). Standard samples were prepared with sodium nitrite.

Effect of Bay 41-2272 on glomerular TGF-β1 production in vitro
To demonstrate that stimulation of the soluble guanylate cyclase regulates TGF-β production directly and independently of blood pressure, glomeruli from six normal and eight nephritic rats (day 7 after OX-7 injection, protocol 2) were re-suspended in DMEM culture medium at a density of 2000 per mL. The soluble guanylate cyclase stimulator Bay 41-2272 was added in increasing concentrations: 0, 0.1, 1, 5, and 10 μmol/L. After 48 hours’ incubation at 37°C/5% CO2, supernatants were harvested and stored at −20°C until analysis of glomerular TGF-β1 production. Two samples from each animal and Bay 41-2272 concentration were analyzed.

Glomerular production of TGF-β1, fibronectin, and PAI-1
Glomeruli were cultured at a density of 2000 per mL at 37°C/5% CO2. After 48 hours of incubation, supernatants were harvested and stored at −20°C until analysis. In previous experiments, we have shown that the TGF-β1, fibronectin, and PAI-1 production by glomeruli ex vivo is constant over 48 hours and closely correlates to the actual glomerular matrix accumulation in vivo [11]. TGF-β1 content of culture supernatant was measured after acid activation using a commercially available ELISA kit (TGF-β1 Duoset™, R&D Systems, Wiesbaden, Germany) according to the manufacturer’s instructions. Fibronectin and PAI-1 levels were measured with
inhibitory ELISA as previously described [12]. Three samples from each rat were analyzed.

**Glomerular mRNA expression of endothelial nitric oxide synthase (eNOS), α1 and β1 soluble guanylate cyclase, inducible nitric oxide synthase (iNOS), p-selectin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)**

Glomerular total RNA was extracted with Trizol™ reagent (Gibco BRL, Berlin, Germany) according to the manufacturer’s instructions. The mRNA expression of iNOS, eNOS, soluble guanylate cyclase and GAPDH were determined by a “two-step” reverse transcription-polymerase chain reaction (RT-PCR). A cDNA copy was created with reverse transcriptase from RNA PCR Core kit (Roche, Applied Biosystems, Branchburg, NJ, USA). Real-time PCR was performed using the LightCycler System and SYBR Green I as dsDNA binding dye (Roche Diagnostics GmbH, Mannheim, Germany). The following primer pairs were used (annealing temperature in parenthesis): eNOS sense 5′-TCCAGT AACACAGACAGTG-3′, antisense 5′-CAGGAAATGAA TGGAGGAGCTTG-3′ (61°C); α1 soluble guanylate cyclase sense 5′-CCACATCAACACAGGCTAAT-3′, antisense 5′-GAAGTGAAGGTTCAGTCTC-3′ (62°C); β1 soluble guanylate cyclase sense 5′-CGATGCACGGTATTGCTC-3′, antisense 5′-CTCTGGCTTACGACATT-3′ (62°C); p-selectin sense 5′-ACCATGACGTTCCAGCC-3′, antisense 5′-CTCTTGTCACACATGA-3′ (62°C); GAPDH sense 5′-CCAT GACGTTATCCAGGC-3′, antisense 5′-CTTCCAGGAGGAT-3′ (62°C); iNOS sense 5′-GATGA CTTTCCACACAGCT-3′ (59°C); and GAPDH sense 5′-ACCATGACGGTGACG-3′, antisense 5′-ACA CTTTGGTGATGAGGC-3′ (60°C). For analysis, a relative quantification method was used as previously described [19, 20]. Briefly, amplification is described as $N = N_0 \times E^{\Delta C_p}$ where $N$ is the number of amplified molecules; $N_0$ is the initial number of molecules; $E$ is the amplification efficiency; and $\Delta C_p$ is the crossing point deviation expressing as $\Delta C_p = C_p_{\text{target}} - C_p_{\text{GAPDH}}$. In this quantification method, measured $C_p$ is defined as the point at which the fluorescence rises above the background fluorescence. Finally, $N_0$ of the target gene was calculated and relatively compared to the expression of GAPDH mRNA as housekeeping gene.

**Light and immunohistochemistry microscopy**

All microscopic examinations were performed in a blinded fashion. Three μm sections of paraffin-embedded tissue were stained with periodic acid-Schiff (PAS). In
Fig. 3. Indices of glomerular mesangial cell lysis in rats 1 day after induction of acute anti-thy1 glomerulonephritis (GN) (protocol 1, injury). Shown are glomerular nuclei count (A) indicating mesangial cell lysis, basal (B), and lipopolysaccharide (LPS)-stimulated nitric oxide (NO) production (C) as well as inducible nitric oxide synthase (iNOS) mRNA expression (D). The rats were treated with the guanylate cyclase stimulator Bay 41-2272 from day 6 before disease induction until sacrifice. Anti-thy1 glomerulonephritis was induced by injection of OX-7 antibody. Normal control animals (control) were injected with phosphate-buffered saline (PBS). **P < 0.01 and ***P < 0.001 vs. control.

protocol 1, the number of cell nuclei was counted in 15 glomeruli of 80 to 100 μm diameter from each animal for calculation of mesangial cell lysis [16].

In protocol 2, glomerular matrix expansion was evaluated on PAS-stained slides by rating the percentage of the mesangial matrix occupying areas in 20 glomeruli from each rat (0% to 100%) [12]. The number of infiltrating macrophages was analyzed in 15 glomeruli of each animal using a primary mouse-anti-ED1 antibody (Serotec, Hamburg, Germany) and a secondary goat antimouse antibody coupled with the Envision™ staining system (DakoCytomation, Hamburg, Germany) [15]. Fibrinogen deposition was determined with a primary rabbit antifibrinogen antibody and a secondary goat anti-rabbit antibody coupled with the Envision™ staining system (DakoCytomation). Glomerular fibrinogen deposition is expressed as the percentage of fibrinogen-positive area in 15 glomeruli from each rat (0% to 100%) [15].

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis between the groups was performed by one-way analysis of variance (ANOVA) and a subsequent Mann-Whitney U test. A P value < 0.05 was considered significant.

RESULTS

Body weight, bleeding time, blood pressure, and plasma cGMP levels

In protocols 1 and 2, the animals’ body weights were not significantly different at the beginning and end of the experiments. Since results for rat tail bleeding time, systolic blood pressure, and plasma cGMP levels were similar in both protocols, they were combined. Rat tail bleeding time and systemic blood pressure were comparable between normal and disease control rats (bleeding time 268 ± 22 seconds and 298 ± 36 seconds) (Fig. 1A) (blood pressure 123 ± 2 mm Hg and 118 ± 2 mm Hg) (Fig. 1B) (both P = NS). Treatment with Bay 41-2272 prolonged bleeding time (471 ± 41 seconds) (P < 0.01) and reduced systemic blood pressure (102 ± 3 mm Hg) (P < 0.01) in comparison to disease controls. Plasma cGMP levels were significantly higher in the untreated anti-thy1 animals than in the normal controls (4130 ± 650 fmol/mL vs. 2120 ± 260 fmol/mL) (P < 0.05) and were significantly further increased by Bay 41-2272 treatment (6610 ±
Fig. 4. Glomerular mRNA expression of endothelial nitric oxide synthase (eNOS) (A), α1 soluble guanylate cyclase (α1 sGC) (B), and β1 soluble guanylate cyclase (β1 sGC) (C) in rats 1 day after induction of acute anti-thy1 glomerulonephritis (GN) (protocol 1, injury). The rats were treated with the guanylate cyclase stimulator Bay 41-2272 from day 6 before disease induction until sacrifice. Anti-thy1 glomerulonephritis was induced by injection of OX-7 antibody. Normal control animals (control) were injected with phosphate-buffered saline (PBS). mRNA was analyzed by a real-time polymerase chain reaction (PCR) method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene. mRNA expression is shown as a percentage of the normal control group. **P < 0.01 vs. control.

Fig. 5. Basal (A) and nitric oxide-stimulated (B) glomerular cyclic guanosine monophosphate (cGMP) synthesis in rats 1 day after induction of acute anti-thy1 glomerulonephritis (GN) (protocol 1, injury). The rats were treated with the guanylate cyclase stimulator Bay 41-2272 from day 6 before disease induction until sacrifice. Anti-thy1 glomerulonephritis was induced by injection of OX-7 antibody. Normal control animals (control) were injected with phosphate-buffered saline (PBS). cGMP generation was measured by enzyme-linked immunosorbent assay (ELISA) in glomeruli harvested from individual animals in the presence or absence of the nitric oxide donor DEA/NO. **P < 0.01 vs. control.

Soluble guanylate cyclase in the injury phase of acute anti-thy1 glomerulonephritis (protocol 1)

Compared to the normal control animals, injection of anti-thy1 antibody resulted in a significantly reduced glomerular cell number (43.9 ± 4.6 vs. 56.3 ± 1.1) (P < 0.001) (Fig. 3A) and significant increases in basal (6.9 ±
soluble guanylate cyclase 10 ± 2%, basal cGMP production 11 ± 1 fmol/well, and nitric oxide-stimulated cGMP production 25 ± 3 fmol/well) (P = NS for all parameters vs. glomerulonephritis) (Figs. 4 and 5).

Together, the results from protocol 1 consistently show that the expression and activity of the nitric oxide-cGMP pathway is almost completely disrupted 1 day after induction of anti-thy1 glomerulonephritis. Administration of Bay 41-2272 was unable to increase glomerular cGMP production and subsequently had no influence on the degree of mesangial cell injury during this phase of anti-thy1 glomerulonephritis.

Soluble guanylate cyclase in the repair phase of acute anti-thy1 glomerulonephritis (protocol 2)

Seven days after injection of anti-thy1 antibody, disease was characterized by significant increases in proteinuria (165.1 ± 9.5 mg/24 hours) (Fig. 6), histologic matrix accumulation (matrix score 73.2 ± 2.2%), glomerular production of TGF-β1 (816 ± 66 pg/mL), fibrinectin (69,587 ± 2886 ng/mL), and PAI-1 (1368 ± 40 ng/mL) (P < 0.01 vs. control for all parameters) (Fig. 7).

As compared to the normal control animals, untreated nephritics showed a slight decrease in eNOS mRNA expression (72% ± 10%) (P = 0.08 vs. control) (Fig. 8), while mRNA levels of α1 and β1 soluble guanylate cyclase were markedly increased (562 ± 76% and 330 ± 31%) (both P < 0.001 vs. control). In line with the mRNA expression data, basal and nitric oxide-stimulated cGMP synthesis was significantly increased in the nephritic animals (basal cGMP production 381 ± 155 fmol/well, nitric oxide-stimulated cGMP production 38,011 ± 17,528 fmol/well) (both P < 0.01 vs. control) (Fig. 9).

In the repair protocol, treatment with Bay 41-2272 significantly decreased proteinuria (110.4 ± 18.3 mg/24 hours (P < 0.05 vs. control) (Fig. 6), histological matrix accumulation (matrix score 64.8 ± 1.6%) and glomerular production of TGF-β1 (603 ± 49 pg/mL), fibrinectin (45,668 ± 2513 ng/mL), and PAI-1 (1156 ± 53 ng/mL) (P < 0.05 vs. glomerulonephritis for all parameters) (Fig. 7). In comparison to the untreated disease controls, administration of Bay 41-2272 was associated with marked increases in the expression of the nitric oxide-cGMP signaling cascade: eNOS mRNA expression 125 ± 19% (P < 0.05 vs. glomerulonephritis) (Fig. 8A), α1 guanylate cyclase mRNA 1750 ± 544% (P < 0.05 vs. glomerulonephritis) (Fig. 8B), and β1 guanylate cyclase 1217 ± 463% (P < 0.05 vs. glomerulonephritis). While basal glomerular cGMP production was similar in treated and untreated nephritics (381 ± 155 fmol/well vs. 380 ± 98 fmol/well) (Fig. 9A), glomerular nitric oxide-stimulated cGMP production was significantly further increased in the Bay 41-2272-treated animals (181,969 ±

Fig. 6. Urinary protein excretion 7 days after induction of acute anti-thy1 glomerulonephritis (GN) (protocol 2, repair). Treatment with Bay 41-2272 was started 24 hours after disease induction. Urine was collected for 24 hours using metabolic cages. *P < 0.05 vs. GN.
114,909 fmol/well) \( (P < 0.05\) vs. glomerulonephritis) (Fig. 9B).

**Effect of Bay 41-2272 on glomerular TGF-β1 production in vitro**

As shown in Figure 10, the effect of Bay 41-2272 was analyzed in cultured glomeruli harvested from normal and day 7 anti-thy1 nephritic animals. In both groups, increasing concentrations of Bay 41-2272 significantly decreased glomerular 48 hours TGF-β1 protein production in a dose-dependent manner. In normal glomeruli, 0.1, 1, 5, and 10 μmol/L Bay 41-2272 reduced TGF-β1 synthesis by 16%, 24%, 28%, and 26%, and in nephritic glomeruli, by 10%, 15%, 25%, and 21%, respectively.

**Effect of Bay 41-2272 on glomerular macrophage number and fibrinogen deposition**

In comparison to the normal controls, nephritic animals at day 7 showed a moderate elevation in glomerular p-selectin expression (132 ± 20%) \( (P = \text{NS vs. control})\) (Fig. 11A) and a highly significant increase in glomerular ED1-positive cells (9.2 ± 1.2 vs. 0.8 ± 0.2 cells per glomerular section) \( (P < 0.01)\) (Fig. 11B) and fibrinogen deposition (9.8 ± 2.2% vs. 0.4 ± 0.1% staining-positive area per glomerulus). Treatment with Bay 41-2272 limited significantly glomerular macrophage infiltration (6.2 ± 0.5 cells per glomerular section) \( (P < 0.05\) vs. glomerulonephritis) (Fig. 11B) and fibrinogen deposition (3.8 ± 0.7% staining-positive area per glomerulus) \( (P < 0.05\) vs. glomerulonephritis). In addition, the moderate increase of glomerular p-selectin expression seen in the untreated nephritic animals was prevented by Bay 41-2272 treatment (85 ± 10.2%) \( (P = \text{NS vs. glomerulonephritis})\) (Fig. 11A).

Taken together, the results of protocol 2 show that nitric oxide-cGMP signal transduction is markedly up-regulated during the repair phase of anti-thy1 glomerulonephritis. Treatment with Bay 41-2272 resulted in a further significant increase of soluble guanylante cyclase expression and activity and limited the fibrotic response characterizing this phase of anti-thy1 glomerulonephritis.

**DISCUSSION**

As it is the case in other organs, the nitric oxide-cGMP signaling cascade is spatially distributed to a high degree in the renal tissue [4, 21]. Since nitric oxide generally acts through paracrine diffusion to its target cell rather than by intracellular signaling, the expression of NOS and soluble guanylate cyclase differ at the cellular level. Expression
of soluble guanylate cyclase mRNA and protein has been demonstrated in mesangial cells of both the intracellular and extracellular mesangium and in podocytes, but not in glomerular endothelial cells [4, 22]. Outside the glomerulus, soluble guanylate cyclase has been documented in cortical and interstitial fibroblasts as well as along the renal vasculature [4]. The two NOSs that provide the soluble guanylate cyclase with its main activator have been shown in close connection to soluble guanylate cyclase expressing cells [4, 21]. eNOS is exclusively expressed in endothelial cells and provides the main signal for soluble guanylate cyclase activation in the vasculature and the mesangium. Neuronal NOS (nNOS) is located in macula densa cells and is closely linked to the
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**Fig. 10. Effects of the soluble guanylate cyclase stimulator Bay 41-2272 on (A) and nephritic (B) glomerular normal transforming growth factor (TGF-β) production in vitro (protocol 2, repair).** Glomeruli were harvested from individual animals (normal nonnephritic and day 7-nephritic animals) and cultured at a density of 2000 per mL for 48 hours in the presence of the indicated concentrations of Bay 41-2272. Glomeruli without Bay 41-2272 served as control. *P < 0.05 vs. control.

The present study shows that expression and activity of soluble guanylate cyclase is both dramatically and contrarily altered during the course of anti-thy1 glomerulonephritis. Shortly after anti-thy1 antibody-induced mesangial cell injury had occurred, glomerular expression of α1 and β1 soluble guanylate cyclase was dramatically reduced and the activity of the glomerular soluble guanylate cyclase as determined as cGMP-production in exposure to nitric oxide was almost completely abolished. This contrasted to the subsequent glomerular matrix expansion phase, in which the glomerular expression of the soluble guanylate cyclase was strongly upregulated and the glomerular cGMP response to nitric oxide was manifoldly increased, respectively. This characteristic dual expression pattern of the glomerular soluble guanylate cyclase is likely to be explained by the destiny of the mesangial cell, which is the main glomerular host of the soluble guanylate cyclase [4] during the course of anti-thy1 diseases. In the injury phase, the loss of soluble guanylate cyclase expression and activity paralleled mesangial cell lysis, while during the following repair phase up-regulation of the soluble guanylate cyclase went along with mesangial cell expansion and proliferation.

The dramatic down- and up-regulation of soluble guanylate cyclase activity found in the course of anti-thy1 glomerulonephritis goes beyond our current understanding of the regulation of the L-arginine-nitric oxide-cGMP axis in glomerular disease. Previously, it had generally been assumed that the activity of this pathway is mainly depending on the capability of eNOS to generate nitric oxide [7, 9, 10]. In this sense, impaired renal nitric oxide production has been linked to insufficient supply with its main precursor L-arginine and the cofactor tetrahydrobiopterin as well as to increased deactivation of the nitric oxide produced, for instance, by free radicals [6, 10, 23]. From this point of view, the term nitric oxide deficiency has been mainly understood as deficiency in the synthesis of nitric oxide, and soluble guanylate cyclase has played a rather passive role. The soluble guanylate cyclase enzyme has been thought to be expressed constitutively and to transduce the nitric oxide signal without further modulation [3]. However, our findings in acute anti-thy1 glomerulonephritis reveal that soluble guanylate cyclase can be the object of a marked transcriptional regulation and this has a marked effect on the subsequent ability of nitric oxide to signal via cGMP. This conclusion became most evident in the injury protocol. Despite glomerular nitric oxide production being markedly increased, glomerular nitric oxide-cGMP signaling was disrupted because of a loss of the soluble guanylate cyclase enzyme. Although to lesser extent than in our study, a decrease in the expression or activity of vascular soluble guanylate cyclase has recently been reported in several disease models characterized by a functional nitric oxide deficiency. These models include spontaneously hypertensive rats, aging, myocardial infarction, angiotensin II infusion, lead-induced hypertension, and diabetic Goto-Kakizate rats [24–29]. In many of these studies, endothelial nitric oxide production was found to be normal or even increased as well. Thus, impaired expression or activity of the soluble guanylate cyclase enzyme can functionally mimic nitric oxide deficiency.

In addition to the focus on soluble guanylate cyclase, the specific pharmacologic soluble guanylate cyclase-stimulator Bay 41-2272 was used to further characterize the role of the nitric oxide-cGMP signaling pathway.
in anti-thy1 glomerulonephritis. In the injury protocol, Bay 41-2272 failed to amplify glomerular nitric oxide-induced cGMP generation, probably because of the loss of glomerular soluble guanylate cyclase expression. With this in mind, it is not surprising that Bay 41-2272 had no effect on the degree of anti-thy1 antibody-induced mesangial cell injury. In contrast, glomerular nitric oxide-induced cGMP production was found to be strongly elevated in the Bay 41-2272-treated animals during the subsequent repair phase. Although already up-regulated by the disease itself, the further amplification of glomerular cGMP generation by Bay 41-2272 significantly limited the fibrotic response of the repair phase, as shown by reductions in proteinuria, glomerular histologic matrix accumulation, and expression of TGF-β1, fibronectin, and PAI-1. Of interest in this context is the finding that soluble guanylate cyclase enhancer treatment not only significantly increased glomerular nitric oxide-induced cGMP generation, but also the mRNA expression of both subunits of the soluble guanylate cyclase. One could speculate that this may be a direct effect of Bay 41-2272 on soluble guanylate cyclase expression, however, it might as well be just a reflection of the improved glomerular wound repair in the treatment group.

Bay 41-2272’s antifibrotic effect in anti-thy1 glomerulonephritis expands on the findings of previous investigations in anti-thy1 glomerulonephritis using L-arginine supplementation or nitric oxide donation [11, 12]. Given the L-arginine-nitric oxide-cGMP axis, the very similar beneficial effects on glomerular TGF-β overexpression achieved by soluble guanylate cyclase enhancement strongly suggest that L-arginine supplementation and nitric oxide donation mediate its antifibrotic actions via cGMP signaling. Furthermore, the study suggests that pharmacologic enhancement of nitric oxide-cGMP signal transduction may be a reasonable strategy to overcome limitations of L-arginine supplementation or nitric oxide donation in treating fibrotic renal disease [10, 12, 30].

The antifibrotic effect of Bay 41-2272 in anti-thy1 glomerulonephritis can be related to blood pressure-dependent and -independent effects of increased cGMP production. The decrease in blood pressure by Bay 41-2272 may have played a role, although the fibrotic response in anti-thy1 glomerulonephritis is generally not altered by changes in the blood pressure level [10, 31, 32]. That pressure-independent, cGMP-dependent mechanisms were likely operating is suggested by several lines of evidence. First, the in vitro experiments in which Bay 41-2272 lowered the TGF-β production of cultured normal and nephritic glomeruli imply that cGMP interacts directly with the expression of this key fibrosis mediator. This finding is in line with a recent study showing that overexpression of the cGMP-dependent protein kinase G down-regulates thrombospondin 1 expression and TGF-β activity in mesangial cells exposed to high glucose [33]. Second, Bay 41-2272 treatment went along with significant decreases in glomerular macrophage infiltration and platelet deposition. Both can serve as relevant intraglomerular sources of increased TGF-β expression and both are known to be regulated by cGMP [14, 15, 17, 34]. Finally, cGMP has been found to inhibit renal cell proliferation in culture [35]. That this effect may be involved is suggested by a very recent study demonstrating that mesangial cell proliferation in anti-thy1 glomerulonephritis is decreased by stimulation of soluble guanylate cyclase [abstract; Hohenstein et al, J Am Soc Nephrol 14:63A, 2003].

The dual and contrary expression and activity pattern of soluble guanylate cyclase during anti-thy1 glomerulonephritis may be related the pathologic glomerular cell changes which are characteristic for this model. Based on the limiting actions of cGMP on cell proliferation and matrix production [33, 35, 36], one may speculate that the loss of soluble guanylate cyclase activity 1 day after induction of mesangial cell lysis could be related to the rapid and marked glomerular cell proliferation and matrix expansion that follows. In our model, glomerular
TGF-β and matrix protein synthesis peak around day 7 and thereafter decline over weeks until they are back to normal again. Based on the reestablishment and marked up-regulation of the soluble guanylate cyclase activity documented on day 7, one may speculate in turn that reestablished nitric oxide-cGMP signaling may have contributed to the subsequent resolution of the disease.

CONCLUSION

The present study shows that glomerular nitric oxide signaling via cGMP is markedly impaired during the injury phase of anti-thy1 glomerulonephritis, while it is highly up-regulated during subsequent repair. In the latter phase, further pharmacologic soluble guanylate cyclase stimulation limits glomerular TGF-β overexpression and matrix accumulation. The results suggest that soluble guanylate cyclase represents an important, at least partially blood pressure-independent, anti-fibrotic pathway in glomerular disease.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Deutsche Forschungsgemeinschaft (PE 558/2-3). Yingrui Wang is a recipient of a doctoral degree grant from the Deutscher Akademischer Austauschdienst (A/01/03941). Reprint requests to Harm Peters, M.D., Department of Nephrology, Charité, Campus Mitte, Humboldt University, Schumannstrasse 20/21, D-10099 Berlin, Germany. E-mail: Harm.Peters@charite.de

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