PDE-5 inhibition impedes TSP-1 expression, TGF-β activation and matrix accumulation in experimental glomerulonephritis

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Abstract

Background. Matrix expansion and mesangial proliferative glomerulonephritis are hallmarks of mesangial proliferative glomerulonephritis. Specific inhibition of PDE-5, an enzyme catalyzing the intracellular degradation of cyclic GMP, can be achieved by the inhibitor vardenafil. In this study, we investigated the effects of PDE-5 inhibition in the anti-Thy1 model in the rat in vivo.

Methods. After disease induction, rats received 10 mg/kg bw vardenafil twice a day via gavage. On Days 2 and 6, renal biopsies, as well as glomerular isolates, urine and blood samples were taken to compare vardenafil- and placebo-treated groups during the course of disease.

Results. Small amounts of PDE-5 were detected in healthy kidneys, but induced in a typical mesangial pattern during disease (by IHC and WB). Specific PDE-5 inhibition resulted in increased glomerular levels of cGMP. Treated animals demonstrated inhibition of MC proliferation and matrix accumulation while renal function and influx of inflammatory cells were not affected. Due to PDE-5 inhibition, the endogenous TGF-β-activating protein TSP-1 and the TGF-β-signalling protein p-smad-2/3 were decreased suggesting this as an antifibrotic mechanism of action of vardenafil in this model.

Conclusion. Considering the availability and safety profile of vardenafil, the beneficial antiproliferative and antifibrotic effect in experimental glomerulonephritis may potentially be applicable to the treatment of mesangial proliferative glomerulonephritis in man.

Keywords: anti-Thy1 model; matrix expansion; PDE-5 inhibition; TGF-β; TSP-1

Introduction

Mesangial proliferative glomerulonephritis (e.g. IgA-Nephropathy) is the most common form of glomerulonephritis in man within the Western world. Excessive mesangial cell (MC) proliferation and matrix expansion are hallmarks of this disease, but also occur in other types of renal disease such as lupus- and membrano-proliferative glomerulonephritis. To date, no specific antiproliferative treatment for mesangial proliferative glomerulonephritis has been established in man. The anti-Thy1 model is a well-established and frequently used rat model for studying inhibitors of MC proliferation and matrix expansion in vivo. After the intravenous injection of an antibody against the Thy1 antigen on the mesangium, complement-mediated mesangiolysis is followed by a marked proliferative activity of the mesangium starting on Day 2, peaking on Days 5/6 and ceasing afterwards. Diseased glomeruli transiently develop hypercellularity, variable amounts of proteinuria and a marked increase of extracellular matrix proteins, all of which are typical hallmarks of the corresponding human disease [1].

Nucleotide-degrading phosphodiesterases exist in >10 different isoforms [2]. Phosphodiesterase-5 is highly specific for cyclic GMP and a homodimer with two regulatory and one catalytic cGMP binding sites. The binding of cGMP to the catalytic domain leads to rapid degradation of cGMP to 5′-GMP [3]. In contrast, the binding of cGMP to the regulatory domain leads to a conformational change of the enzyme and subsequent phosphorylation and stimulation of enzyme activity [3–5].

PDE-5 has been described in various tissues and cell types such as lung, liver, kidney, brain and platelets [4,5]. PDE-5 is an important part of the NO/cGMP pathway in smooth muscle cells and the successful drug targeting of PDE-5 [6,7] contributed markedly to the further understanding of its functional role. So far, PDE-5 inhibition leading to increased intracellular cGMP has been approved clinically as a treatment for male erectile dysfunction and pulmonary hypertension [7].

In the present study, we sought to investigate the therapeutic potential of specific PDE-5 inhibition during experimental mesangial proliferative glomerulonephritis and showed that it prevents MC proliferation and expression of TSP-1, a major in vivo activator of the profibrotic molecule TGF-β.
Methods

Animal model and experimental design

All animal experiments were done according to American Physiological Society guidelines and duly approved by local government authorities. Experimental mesangial proliferative glomerulonephritis was induced in eight Sprague-Dawley rats per group (150–200 g; Charles River, Sulzfeld, Germany) by a single injection of 1 mg/kg bw of the mouse monoclonal anti-Thy1 antibody ER-4. All animals were fed standard rat chow (Altromin 1324, Spezialfutterwerke GmbH, Lage, Germany) and tap water ad libitum. In the placebo group, one rat was not successfully diseased and therefore excluded.

To ensure equivalent antibody binding in all rats and to avoid potential interference with the treatment, therapy was started not earlier than 1 h after disease induction. Animals received either a total of 20 mg/kg bw vardenafil (Bayer-Healthcare, Wuppertal, Germany) dissolved in water or an equal amount of the solvent via oral gavage per day. The first three applications were given within 18 h as loading doses followed by two daily doses of 10 mg/kg bw from Day 2 onwards. A 24-h urine collection was performed for assessment of proteinuria and creatinine clearance before Day 2 onwards. A 24-h urine collection was performed for the measurement of cGMP levels in the other kidney was fixed and processed for immunohistochemistry. To perform immunoperoxidase staining, tissue sections were incubated with the following primary antibodies described elsewhere [8,9].

Tissue processing and immunohistochemical staining

Renal biopsies were fixed in methyl Carnoy’s solution or 3% paraformaldehyde, embedded in paraffin, and cut into 5 µm sections for indirect immunoperoxidase staining as described elsewhere [8,9].

To perform immunoperoxidase staining, tissue sections were incubated with the following primary antibodies as indicated: a murine IgM monoclonal antibody (mAb) against the proliferating cell nuclear antigen (PCNA) [9] (19A2; Coulter Immunology, Hialeah, FL, USA); ED-1, a murine IgG1 mAb to a cytoplasmatic antigen present in monocytes, macrophages and dendritic cells (Serotec Ltd, Oxford, UK) [9]; OK-7, a murine IgG1 mAb specific for MC (Serotec) [9]; a polyclonal antibody to collagen IV (goat anti-human/bovine collagen IV; Southern Biotechnology Associates, Inc., Birmingham, AL, USA) [9] or an mAb against fibronectin (Gibco, Invitrogen, Karlsruhe, Germany) [10]; JG-12, an mAb against the AGE receptor for staining of glomerular capillaries (kindly provided by D. Kerjaschki, University of Vienna, Vienna, Austria) [11]; PL-1, a murine mAb against rat platelets (kindly provided by W.W. Baker, Groningen, The Netherlands) [12]; a polyclonal antibody against PDE-5A (Alexis Biochemicals, Gruenberg, Germany); Clone A6,1, a murine IgG1 mAb against Thrombospordin-1 (TSP-1) (Dunn, Asbach, Germany); a rabbit polyclonal antibody against phosphorylated smad-2/3 (New England Biolabs, Frankfurt, Germany); a polyclonal anti-TGF-β1 antibody, a chicken polyclonal anti-human active TGF-β1 (R&D systems, Germany) and a rabbit polyclonal Ab against TGF-β2 (both SantaCruz Biotech., Heidelberg, Germany). Negative controls for immunostaining included either deletion or substitution of the primary antibody with equivalent concentrations of an irrelevant murine mAb or preimmune rabbit IgG. All tissue sections were incubated with primary antibodies overnight at 4°C. Afterwards specific biotinylated secondary antibodies (all by Zymed, San Francisco, CA, USA) were applied followed by peroxidase conjugated Avidin D (Vector Lab., Burlingame, CA, USA) and colour development with diaminobenzidine with nickel chloride for nuclear staining and otherwise without nickel. Expression of collagen IV, fibronectin and TSP-1 were quantified using computer-assisted image analysis software (MetaVue, Visitron Systems, Munich, Germany) in a blinded fashion. Therefore, glomeruli were digitally photographed at a 400-fold magnification under standard lighting conditions (with respect to white balance and exposure). Using MetaVue Software, image stacks were built and thresholds were set under visual control by the investigator to assure exact measurements. The same threshold was used for one image stack and—if necessary—adjusted for the next stack. At least 50 glomerular cross-sections were analysed at a 400-fold magnification. Data are shown as percent positive area per glomerular cross-section. PCNA-positive nuclei, p-smad-2/3-positive cells, ED-1-positive cells and glomerular microaneurysms [13] as well as the number of all glomerular cells were counted separately in 50 consecutive glomeruli per section using a 400-fold magnification in a blinded fashion and shown as number per glomerular cross-section. Mesangiolysis and platelet influx were assessed using a 400-fold magnification in 50 consecutive glomeruli using a semiquantitative scoring system from 0 to 4 where 0 means 0%, 1 means 1–25%, 2 means 26–50%, 3 means 51–75% and 4 means 76–100% of the glomerular area lacks positive staining for the MC marker OX-7 or was positive for the platelet marker PL-1, respectively. Glomerular expressions of PDE-5A, TGF-β1 and TGF-β2 were evaluated using a semiquantitative scoring system from 0 to 4, where 0 means 0%, 1 means 1–25%, 2 means 26–50%, 3 means 51–75% and 4 means 76–100% positively stained area. All results are given as mean ± SD per glomerular cross-section. Active TGF-β was graded semiquantitatively and reflected changes in the area and intensity of mesangial staining: 0, very weak or absent staining; 1+, weak staining with <25% of the glomerular tuft showing focally increased staining; 2+, 25–49% of the glomerular tuft with focally increased staining; 3+, 50–75% of the glomerular tuft demonstrating increased staining and 4+, >75% of the glomerular tuft stained strongly.
**Immunohistochemical double staining**

To determine the number of proliferating MC, double immunostaining for PCNA, a marker of cell proliferation, and for OX-7 (MC-specific) was performed as previously described [9]. The number of proliferating MCs is given as mean ± SD per glomerular cross section.

**TUNEL assay**

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labelling assay as previously described [14]. Cells were regarded as TUNEL positive if their nuclei were stained black and displayed typical apoptotic morphology with chromatin condensation. The number of apoptotic cells was counted in 50 sequentially selected glomeruli and is given as the mean number ± SD per glomerular cross section.

**Glomerular preparations and measurement of cGMP**

Glomerular preparations were done as described elsewhere [9]. Glomerular extracts were homogenized on ice in homogenization buffer containing 5 × 10⁻⁴ mmol 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, Seelze, Germany) to block the activity of phosphodiesterases and snap frozen in liquid nitrogen. Measurement of cyclic GMP was performed in triplets using a commercial radioimmunoassay kit (IBL, Hamburg, Germany) as described before [15].

**Western blot analysis**

Protein samples containing 20 µg of protein from isolated glomeruli were separated by 12% SDS–PAGE and blotted onto PVDF membranes. Blots were blocked for 12 h at 4°C in a 1-block blocking solution (Tropix, Bedford, USA). After washing, blots were incubated with the primary antibodies against PDE-5A, TGF-β1, TGF-β2 or p-smad-2/3 described for immunohistochemistry for 1.5 h followed by a horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG (Amersham Biosciences, Buckinghamshire, UK). Immunoreactive bands were detected on the basis of chemiluminescence using an enhanced chemiluminescence kit (ECL). For TGF-β1 and 2, β-actin was detected simultaneously as loading control using an antibody from Abcam, Cambridge, UK. For p-smad-2/3, vinculin was detected as loading control using an antibody from Sigma-Aldrich, Taufkirchen, Germany. Quantitative analysis was performed using a computer-assisted system (AIDA Image Analyzer, raytest GmbH, Straubenhardt, Germany).

**Miscellaneous measurements**

Urinary protein was measured colorimetrically using a commercial test kit (Bio-Rad Lab., Hercules, CA, USA) based on the Bradford dye-binding procedure [16,17]. Serum and urinary creatinine were measured using an autoanalyser (Beckman Instruments, Brea, CA, USA).

**Statistical analysis**

All values are expressed as mean ± SD. Statistical significance (defined as $P < 0.05$) was evaluated by Student’s $t$-test.

**Results**

**Glomerular phosphodiesterase 5 is increased during experimental mesangial proliferative glomerulonephritis**

By immunostaining with a specific antibody against PDE-5A, healthy rat kidneys only demonstrated small amounts of PDE-5 (Figure 1A). During anti-Thy1 nephritis, glomerular PDE-5 increased during the phase of MC proliferation up to Day 6, where it appeared in a typical mesangial pattern (Day 2: Figure 1B, Day 6: 1C). By immunostaining, no differences occurred between placebo- and vardenafil-treated groups (Figure 1D). The glomerular area positive for PDE-5 was increased to 1.4 ± 0.27 in vardenafil and 1.3 ± 0.16 in placebo-treated rats versus 0.4 ± 0.11 in healthy controls ($P < 0.05$; Figure 1D) on Day 6. These results were verified by western blot analysis (Figure 1E), which demonstrated increased expression of PDE-5 on Day 6, but also on Day 2 compared to healthy controls.

**PDE-5 inhibition leads to intracellular accumulation of cGMP in vivo**

Administration of vardenafil inhibits specifically the intracellular degradation of cGMP by PDE-5 in vivo [18]. To confirm this effective pharmacological mechanism of PDE-5 inhibition in mesangial proliferative glomerulonephritis, glomerular isolates on Day 6 of disease were homogenized and cGMP levels were compared in vardenafil-treated and control animals. Glomerular cGMP levels were significantly increased in animals exposed to PDE-5 inhibition compared to controls indicating the efficacy of vardenafil treatment (Figure 1F).

**Inhibition of PDE-5 does not affect mesangiolysis**

To exclude alteration of the disease induction process by the treatment, application of vardenafil or placebo was started not earlier than 1 h after antibody induction of the anti-Thy1 model, when the maximal binding of the disease-inducing antibody had already occurred [19,20]. In addition, assessment of mesangiolysis on Day 2 via a semiquantitative scoring system verified equivalent disease induction in all rats independent of the treatment ($3.31 ± 0.16$ in placebo versus $3.16 ± 0.41$ in vardenafil-treated anti-Thy1 rats; Figure 1G). Since the formation of microaneurysms is thought to occur as a consequence of mesangiolysis and indirect as well as direct endothelial cell injury and the following repair reaction, the percentage of microaneurysm formation in kidneys of rats treated with the PDE-5 inhibitor or placebo was assessed, but was equivalent in both groups (Figure 1H).

**In vivo**
Fig. 1. PDE-5 is upregulated in glomeruli of the anti-Thy1 model, and PDE-5 inhibition leads to increased glomerular cGMP levels. Expression of PDE-5 was analysed after staining against PDE-5A in normal healthy rats (A), Day 2 (B) and Day 6 (C), and evaluated using computer-assisted image analysis (D). Glomerular isolates were used to verify changes in PDE-5 expression by western blot analysis (E) and for measurement of the glomerular cGMP content by RIA (F). Mesangiolysis (G) was assessed using a semiquantitative scoring system from 0 (no mesangiolysis) to 4 (complete mesangiolysis) after immunostaining for the MC marker OX-7. The number of microaneurysms per glomerular cross section was evaluated after JG-2 staining (H) (*P < 0.05).
Inhibition of PDE-5 reduces MC proliferation in experimental mesangial proliferative glomerulonephritis

Excess of mesangial proliferation is a hallmark of mesangial proliferative glomerulonephritis both in man and in the anti-Thy1 model. Therefore, we examined whether this response would be affected by PDE-5 inhibition in vivo. Identification of proliferating cells with an mAb against PCNA showed a decreased rate of total glomerular proliferation in rats with PDE-5 inhibition on Day 6 (peak of glomerular proliferation) compared to placebo-treated rats (13.3 ± 3.7 versus 20 ± 4.3; P < 0.05) (Figure 2A). Double staining for proliferating MCs (PCNA and OX-7-positive) demonstrated that vardenafil treatment significantly inhibited MC proliferation compared to placebo-treated rats (7.3 ± 0.93 versus 15.04 ± 0.83; P < 0.01) (Figure 2B). Figure 2C shows examples of proliferating MC as indicated by arrows of double-stained cells with black PCNA-positive nuclei (proliferation marker) surrounded by an OX-7-positive cytoplasm (MC). In addition, the glomerular cell number on Day 6 was significantly decreased in anti-Thy1 nephritic rats with PDE-5 inhibition compared to placebo-treated rats (34.3 ± 2 versus 39.8 ± 4.3; P < 0.05; Figure 2D). On Day 2, when non-MC (mainly glomerular endothelial cells) proliferation was predominant, no difference in glomerular or MC proliferation was detected between vardenafil- and placebo-treated rats (Figure 2A, B). Since the glomerular cell number was balanced by both cellular proliferation and cell death, apoptotic cells/glomerular cross sections were investigated using the TUNEL assay, but no significant differences were identified between the two groups on either Day 2 or 6 (Figure 2E).

Vardenafil therapy inhibits glomerular extracellular matrix expansion during anti-Thy1 nephritis

Increased mesangial proliferation during mesangial proliferative glomerulonephritis is usually accompanied by excessive matrix expansion leading to renal fibrosis. We therefore assessed glomerular matrix expansion in the anti-Thy1 model as indicated by collagen IV and fibronectin immunostaining in response to PDE-5 inhibition. Vardenafil treatment reduced glomerular collagen IV (percentage area positive for collagen IV was 31.6 ± 3.6 versus 45.4 ± 5.7; P < 0.05; Figure 3A) and glomerular fibronectin (percentage area positive for fibronectin was 3.1 ± 2.8 versus 12.9 ± 4; P < 0.001; Figure 3B) on Day 6 of anti-Thy1 nephritis compared to placebo-treated nephritic rats. Figure 3C shows representative images of the collagen IV immunostaining in vardenafil-treated animals compared to placebo-treated rats (Figure 3D). The differences in glomerular fibronectin...
Fig. 3. Vardenafil inhibits glomerular matrix expansion. Glomerular matrix accumulation was evaluated after immunostaining for collagen IV (A) and fibronectin (B) and quantified using computer-assisted image analysis as described in the Methods section. For both matrix molecules, PDE-5 inhibition led to a significant reduction on Day 6. Examples of collagen IV and fibronectin stainings are depicted in (C)/(D) and (E)/(F), respectively, demonstrating representative findings with reduced glomerular collagen IV (C) and fibronectin (E) in kidneys exposed to PDE-5 inhibition compared to placebo-treated kidneys (D for collagen IV, and F for fibronectin) (*P < 0.05).

 expression of PDE-5 inhibitor- and placebo-treated rats are depicted in Figures 3E and F, respectively. By both stainings, no differences were apparent on Day 2, a time when matrix expansion due to the peak mesangiolysis is not yet present.

**Vardenafil therapy reduces glomerular TSP-1 and TGF-β activation in anti-Thy1 nephritis**

Since previous studies identified TSP-1 as an important target of the NO-sGC-cGMP pathway [21], we also assessed changes of the glomerular expression of TSP-1 due to PDE-5 inhibition. On Day 6, glomerular TSP-1 was reduced in rats with PDE-5 inhibition compared to placebo controls (2.8 ± 3.4 versus 6.6 ± 3.1; *P < 0.05), whereas no differences occurred on Day 2 (Figure 4A). Since TSP-1 has been established as an endogenous activator of the latent TGF-β complex in vivo [22], we investigated whether the reduced TSP-1 expression is also translated into a reduction of TGF-β activation. We first assessed expression of the two TGF-β isoforms TGF-β1 and TGF-β2 by immunohistochemistry and western blot analysis. During the time course of disease, TGF-β1 was unchanged in PDE-5 inhibitor-treated kidneys compared to controls by immunohistochemistry (d2 score: 1.17 ± 0.8 versus 1.17 ± 0.6; n.s.; d6 score: 0.8 ± 0.5 versus 1.8 ± 1.2; n.s.) as well as western blot (6.6 ± 3.9 versus 4.8 ± 4.3 rel. TGF-β1 expression; n.s.; Figure 4B). In parallel, TGF-β2 was not significantly different in placebo- and PDE-5 inhibitor-treated kidneys by immunohistochemistry (d2 score: 0.4 ± 0.3 versus 0.4 ± 0.4; n.s.; d6 score: 0.4 ± 0.6 versus 0.5 ± 0.6; n.s.) as well as western blot analysis of glomerular isolates (3.4 ± 4.5
PDE-5 inhibition impedes TSP-1 expression

Fig. 4. Vardenafil inhibits the TGF-β-activating glycoprotein TSP-1 and subsequent TGF-β activation. Glomerular TSP-1 expression was evaluated after immunostaining and quantified using computer-assisted image analysis. (A) Quantification of glomerular TSP-1 staining demonstrated a significantly reduced glomerular TSP-1 on Day 6 in rats treated with the PDE-5 inhibitor vardenafil. Decreased glomerular TSP-1 by vardenafil was not associated with changes in levels of TGF-β1 (B) and TGF-β2 (C) on Day 6 as quantified by western blot from glomerular extracts. Assessment of active TGF-β using a specific antibody (D) as well as evaluation of the phosphorylated form of the TGF-β signalling molecule smad-2/3 demonstrated a clear reduction of p-smad-2/3 complexes on Day 6 by immunostaining (D) and western blot analysis (E) thereby indicating reduced TGF-β activation (*P < 0.05).

Table 1. Influx of inflammatory cells and parameters of renal function during anti-Thy1 nephritis and PDE-5 inhibition

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 2</th>
<th>Day 6</th>
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<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>PDE-5 inhibition</td>
</tr>
<tr>
<td>Platelet accumulation (score 0–4)</td>
<td>12.7 ± 10.8</td>
<td>5.0 ± 6.2</td>
</tr>
<tr>
<td>Macrophages/macrophages (n)</td>
<td>1.6 ± 0.7</td>
<td>1.6 ± 0.7</td>
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<tr>
<td>Proteinuria (mg/24 h)</td>
<td>7.3 ± 7.8</td>
<td>7.5 ± 8.5</td>
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<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.2 ± 0.03</td>
<td>0.2 ± 0.02</td>
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*aMean per 50 glomerular cross-sections. Platelet accumulation within glomeruli was assessed by a semiquantitative scoring system after staining for PL-1. The number of monocytes/macrophages was counted after staining for ED-1.

versus 6.1 ± 4.1 n.s.; Figure 4C). Analysis of the phosphorylated form of the smad-2/3 complex as an indicator of active TGF-β signalling clearly demonstrated (parallel to decreased glomerular TSP-1 expression) reduced TGF-β activation on Day 6 as indicated by immunohistochemistry (5.6 ± 2.5 versus 11 ± 2.1 p-smad-2/3-positive nuclei, Figure 4D) as well as by western blot analysis of glomerular isolates (2.4 ± 2.1 versus 5.4 ± 1.9 P < 0.05; Figure 4E). No differences regarding p-smad-2/3 occurred on Day 2 in PDE-5 inhibitor-treated versus placebo-treated rats (7.9 ± 3 versus 7.6 ± 4.3 p-smad-2/3 positive nuclei, n.s.).

Inhibition of PDE-5 affects neither influx of inflammatory cells nor renal function

Since intracellular accumulation of cGMP in platelets and other cells can potentially also modulate platelet aggregation [15,23] or inflammation, we investigated the glomerular accumulation of platelets (PL-1 positive) as well as monocytes/macrophages (ED-1 positive), which was equivalent in groups with and without PDE-5 inhibition (Table 1).

To evaluate whether changes in MC proliferation and matrix expansion were translated into functional changes
during experimental renal disease, we assessed proteinuria and serum creatinine values. Both parameters did not demonstrate significant differences due to PDE-5 inhibition (Table 1).

**Discussion**

Excessive matrix expansion and MC proliferation are common findings in IgA nephropathy and other human renal diseases and at the same time the hallmark of experimental anti-Thy1 nephritis in the rat. To date, no specific antifibrotic or antiproliferative treatment is available for human renal disease.

In this study, we have investigated specific inhibition of PDE-5 as a potential new treatment strategy. Hereby, we demonstrated that PDE-5 is expressed in glomeruli and increased during mesangial proliferative glomerulonephritis in the rat kidney in vivo [4,5]. As a consequence of PDE-5 inhibition by vardenafil, nephritic rat glomeruli were characterized by increased glomerular cGMP levels leading to subsequent inhibition of MC proliferation and matrix expansion, potentially via downregulation of the TGF-β activating glycoprotein TSP-1.

Cyclic GMP is the specific substrate of PDE-5 interacting with the regulatory domain of this enzyme [3]. PDE-5 inhibition leads to the intracellular accumulation of cGMP and increased levels of cGMP in glomerular extracts are the proof for glomerular expression and activity of PDE-5 besides our immunostaining and western blot results. Production of cGMP is catalyzed through soluble Guanylyl-cyclase (sGC) after activation by nitric oxide [24,25], which is thought to mediate NO-induced actions [26]. Besides PDE-5, six other PDEs contribute to the degradation of cGMP and increased levels of cGMP in glomerular extracts are the proof for glomerular expression and activity of PDE-5 besides our immunostaining and western blot results. Production of cGMP is catalyzed through soluble Guanylyl-cyclase (sGC) after activation by nitric oxide [24,25], which is thought to mediate NO-induced actions [26]. Besides PDE-5, six other PDEs contribute to the degradation of cGMP (PDEs 1, 2, 3, 6, 9, 10, 11) suggesting some redundancy in this system. PDEs 5, 6 and 9 are cGMP specific [2,6]. A twofold increase of glomerular cGMP levels by vardenafil on Day 6 followed by marked biological effects such as reduced proliferation and matrix expansion clearly demonstrates the importance of glomerular PDE-5 for cGMP degradation during experimental nephritis, even in the absence of consequences for kidney function.

Enhanced immunostaining for PDE-5 during the proliferative phase of this disease model and the antiproliferative effect on MCs (by OX-7/PCNA staining) suggest that localization and action are mainly restricted to the mesangium. Studies on PDE-5 expression in normal mouse and rat tissues [4,5] as well as in vitro studies in rat MCs that linked cGMP hydrolysis with the presence of PDE-5 [27,28] are consistent with our findings. The previously described absence of immunohistochemical localization in normal rat kidneys [5] might relate to insufficient sensitivity of the antibody used for this study. The link of enhanced PDE-5 expression with the proliferative response in MCs indicates that PDE-5 inhibitors can modulate cell proliferation in a relatively cell type specific manner. This might be especially important considering the importance of timely repair of the renal endothelium and the detrimental effects of inhibition of endothelial repair during experimental renal disease [29]. The fact that glomerular proliferation was not affected on Day 2, at a time when glomerular endothelial cell proliferation is predominant [13], is consistent with the interpretation of our findings. The fact that microaneurysm formation, which depends mainly on the degree of endothelial injury and subsequent repair, is also not affected by vardenafil therapy also indicates a specific effect on MCs. Since apoptosis as the major mechanism for resolution of hypercellularity in this model [30] is not affected by PDE-5 inhibition, the reduction of glomerular hypercellularity appears to be due to the sole inhibition of MC proliferation.

Reports on the antiproliferative effect of nonselective PDE inhibitors such as IBMX further strengthen our findings [31].

Inhibition of PDE-5 also markedly inhibited extracellular matrix accumulation, the second hallmark of renal disease progression, as indicated by collagen IV as well as fibronectin staining. Previous studies have frequently demonstrated a link between glomerular cell proliferation and matrix accumulation [32,33], although glomerular matrix expansion and MC proliferation can also be dissociated [34]. In a previously published study by Rodriguez-Iturbe et al. [35] beneficial long-term effects of PDE-5 inhibition have been demonstrated in a model of renal mass reduction. However, in contrast to the present study, long-term effects seemed to be mainly mediated by preservation of capillaries, reduced glomerulosclerosis and tubulointerstitial fibrosis as well as beneficial effects on the influx of inflammatory cells and apoptotic events [35].

PDE-5 inhibition via the intracellular increase of cGMP regulates various genes involved in cellular proliferation and matrix production [36]. The NO-cGMP pathway has also been shown to inhibit expression of genes mediating matrix synthesis directly or indirectly via the major profibrotic cytokine, transforming growth factor-beta (TGF-β). Studies in cultured MC demonstrated that the NO-cGMP system is able to downregulate the profibrotic mediator connective tissue growth factor (CTGF) [37] as well as the activator of the TGF-β procytokine complex (after glucose stimulation), TSP-1 [21,38]. In the present study, glomerular TSP-1 expression as well as the amount of ‘active TGF-β’ and TGF-β signalling (as indicated by the degree of phosphorylation of the TGF-β signalling molecule smad-2/3 by specific immunostaining and western blotting) but not the TGF-β1 or 2 procytokine complex was decreased in diseased glomeruli via PDE-5 inhibition. Hereby, this study further links the NO-cGMP-PDE pathway with the regulation of TSP-1 and the major profibrotic cytokine TGF-β in this nephritis model.

Since NO has been shown to be important in mediating MC injury in vitro and in this model [39,40], many effects could have been the consequence of interactions between PDE-5 inhibition and the disease induction process. To avoid this potential interference, treatment was initiated after maximal antibody binding had already occurred [1,41]. The equal degree of mesangiolysis, microaneurysm formation, cellular proliferation/matrix accumulation and influx of inflammatory cells on Day 2 of disease in both groups supports the conclusion that disease induction/MC injury was neither specifically nor unspecifically affected by vardenafil [40].
In conclusion, this study demonstrates that the therapeutic inhibition of PDE-5 via vardenafil leads to intracellular cGMP elevation within glomeruli during experimental mesangial proliferative glomerulonephritis. Subsequently, vardenafil inhibits MC proliferation and matrix expansion during experimental glomerulonephritis in vivo. Most likely the reduction of the TGF-β activating glycoprotein TSP-1 mediates this antifibrotic action. Since PDE-5 inhibitors are clinically established pharmacological drugs with an excellent safety profile, PDE-5 inhibition may be a promising treatment option for mesangial proliferative glomerulonephritis in man.

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Conflict of interest statement. None declared.

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