Cellular Distribution and Function of Soluble Guanylyl Cyclase in Rat Kidney and Liver

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Abstract. Soluble guanylyl cyclase (sGC) catalyzes the biosynthesis of cGMP in response to binding of l-arginine-derived nitric oxide (NO). Functionally, the NO-sGC-cGMP signaling pathway in kidney and liver has been associated with regional hemodynamics and the regulation of glomerular parameters. The distribution of the ubiquitous sGC isoform α1β1 sGC was studied with a novel, highly specific antibody against the β1 subunit. In parallel, the presence of mRNA encoding both subunits was investigated by using in situ hybridization and reverse transcription-PCR assays. The NO-induced, sGC-dependent accumulation of cGMP in cytosolic extracts of tissues and cells was measured in vitro. Renal glomerular arterioles, including the renin-producing granular cells, mesangium, and descending vasa recta, as well as cortical and medullary interstitial fibroblasts, expressed sGC. Stimulation of isolated mesangial cells, renal fibroblasts, and hepatic Ito cells with a NO donor resulted in markedly increased cytosolic cGMP levels. This assessment of sGC expression and activity in vascular and interstitial cells of kidney and liver may have implications for understanding the role of local cGMP signaling cascades.

Guanylyl cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] exists in two isoenzyme forms and catalyzes the biosynthesis of cGMP from GTP. The membrane-bound forms are monomers that are stimulated by different peptide hormones. Soluble guanylyl cyclase (sGC) is a heme-containing heterodimer consisting of one α subunit (73 to 88 kD) and one β subunit (70 kD) (for review, see reference 1). The α1β1 isoenzyme is thought to be the major form. In addition, two other subunits, α2 and β2, have been cloned, and an α2β1 isoform has been functionally characterized (2,3). sGC is the most well characterized receptor for nitric oxide (NO); binding of l-arginine-derived NO to the heme group of sGC results in marked stimulation of the enzyme, thus increasing the intracellular cGMP concentration (4). Increases in cGMP levels are responsible for cellular events that ultimately lead to decreases in intracellular calcium concentrations and smooth muscle relaxation (5) or to regulation of multiple genes through interactions with their respective promoters (6).

In the kidney, both vascular and tubular effects of NO have been observed (for review, see references 7, 8, and 9), and the cellular sources for constitutive NO synthase (NOS), which catalyzes the formation of NO, have been identified (10,11). Because of the wide diversity of cell types in this organ, a detailed knowledge of sGC distribution is required for an understanding of local, cGMP-mediated effects of NO. A number of earlier studies demonstrated the organ- and cell-specific presence of sGC mRNA, by PCR and Northern blot analyses (12–15), and of the immunoreactive protein, by immunohistochemical and Western blot analyses (16,17). To date, however, there is still disagreement with respect to the reported immunohistochemical distribution of sGC and functional data. This study was performed in kidneys, to test the hypothesis that more structures than previously established are involved in NO-sGC-cGMP signaling as a major regulatory pathway in end-organ perfusion and specific cell function. We used a newly generated, affinity-purified antibody against a carboxy-terminal domain of β1 sGC, which, because of its monospecificity in Western blotting analyses and its cellular localization spectrum, was clearly superior to previously used antisera. Immunohistochemical data obtained with this antibody were corroborated by Western blot analyses of extracts from tissues and isolated cell preparations, by in situ hybridization, by reverse transcription (RT)-PCR assays of tissues and isolated cell extracts (using probes for both α1 and β1 sGC), and by in vitro assessment of NO-dependent accumulation of cGMP in...
tissues and cultured cells. The liver was studied to compare cell type specificity of sGC localization and function with another end-organ with a well established role of the NO-sGC-cGMP pathway. Mechanisms involved in NO-dependent regulation of local hepatic microcirculation were previously identified (18–20). Common aspects of cGMP-dependent signaling in kidney and liver are discussed.

Materials and Methods

Animals and Tissue Preparation

Male Sprague-Dawley rats weighing between 200 and 450 g were obtained from the local animal facilities of the Departments of Anatomy, Charité, and Nephrology, University of Freiburg, and of the Department of Clinical Chemistry, University of Aachen. Male Wistar rats (Harlan-Winkelmann, Borchern, Germany) were used for the experiments on isolated renal fibroblasts. All animals had been maintained with standard chow and tap water. For morphologic and immunohistochemical evaluations, a total of six rats (approximate body weight, 200 g) were used. For perfusion-fixation, animals were anesthetized by intraperitoneal injection of Nembutal (40 mg/kg body wt; Sanofi, Hannover, Germany). Animals underwent cannulation of the abdominal aorta and perfusion with sucrose-phosphate-buffered saline (PBS) solution (330 mosmol, pH 7.3) for 15 to 20 s at a pressure of 220 mmHg, directly followed by perfusion with paraformaldehyde (3% in PBS, pH 7.3) for 90 s at 220 mmHg and then for 200 s at 100 mmHg. Fixative was removed from the animal by subsequent perfusion with sucrose-PBS solution for 60 s at 100 mmHg. Tissues were then removed and dissected for further preparations. For in situ hybridization and conventional immunohistochemical analyses, tissues were immersed in 800-mosmol sucrose-PBS solution (pH 7.3) for 12 h, shock-frozen in liquid nitrogen-cooled isopentane, and stored at −70°C. For pre-embedding immunohistochemical analyses, tissue blocks were postfixed for 12 h in paraformaldehyde (3% in PBS containing 0.5% glutaraldehyde) and stored in sucrose-PBS solution (330 mosmol) at 4°C until embedding in agarose and sectioning (30 μm) with a Vibratome (1000S; Leica, Wetzlar, Germany) tissue slicer.

Isolation of Glomeruli

Glomeruli were isolated as described previously (21). As for all other tissue isolation procedures described in this section, rats were anesthetized with sodium pentobarbital (50 mg/kg body wt). Kidneys were removed and prepared, under sterile conditions, in ice-cold RPMI 1640 medium (Seromed, Berlin, Germany). Cortices were sieved by using steel sieves with pore sizes of 150 μm and then 100 μm. The sieved structures were captured with a smaller sieve (pore size, 50 μm), transferred to 50-ml tubes, and centrifuged (4000 rpm, 4°C, 10 min). The pellet was then transferred into a small volume (2 to 3 ml) and divided into aliquots (100 μl). The aliquots were transferred to a microdissection chamber, 200 glomeruli/aliquot were microdissected, and aliquots were pooled and centrifuged (15,000 rpm, 1 min, 4°C). The resulting pellet was then prepared for mRNA extraction.

Isolation of Renal Mesangial Cells and Podocytes

Mesangial cells were isolated and cultured as described previously (22). In brief, glomeruli were obtained as described above, incubated with collagenase (1 g/L; Sigma, Deisenhofen, Germany) for 15 min, and suspended in RPMI 1640 medium supplemented with 170 g/L fetal calf serum, 2.5 mM l-glutamine, 0.1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/L streptomycin, 0.2 g/L nonessential amino acids (all from Seromed, Berlin, Germany), and 5 mg/L insulin-transferrin-sodium selenite supplement (Roche, Mannheim, Germany). Approximately 50 glomeruli/cm² were plated onto collagen-coated glass coverslips (Greiner, Nütingen, Germany) and incubated at 37°C in an incubator with a water-saturated atmosphere of 5% CO₂/95% air. Mesangial cells were morphologically characterized by phase-contrast microscopy. They stained positively for smooth muscle actin, desmin, and vimentin but not for cytokeratin and factor VIII, which demonstrates the absence of glomerular epithelial and endothelial cells. Cells responded to 10⁻⁴ M angiotensin II with increases in free cytosolic calcium concentrations.

Podocytes were isolated and cultured as described (23). Briefly, immortalized mouse podocytes carrying the thermosensitive variant of the SV40 T antigen inserted into the mouse genome were used. These podocytes proliferate at 33°C in the presence of interferon γ, whereas cells are transformed into the quiescent differentiated phenotype at 37°C in the absence of interferon γ. Podocytes then stain positively for the podocyte differentiation markers WT-1 and synaptopodin. Cells between passage 14 and passage 20 were seeded at 37°C onto collagen-coated plates and cultured for at least 7 d, until cells were differentiated, in standard RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/L streptomycin.

Isolation and Culture of Renal Medullary Fibroblasts

Detailed procedures for the isolation and culture of rat inner medullary fibroblasts were published previously (24). In brief, rats were euthanized by cervical dislocation. Kidneys were immediately removed, and the inner medulla was excised. Tissue was placed in 290-mosmol, ice-cold, Heps-Ringer’s buffer (118 mM NaCl, 16 mM H-Heps, 16 mM Na-Heps, 14 mM glucose, 3.2 mM KCl, 2.5 mM CaCl₂, 1.8 mM MgSO₄, 1.8 mM KH₂PO₄, pH 7.4), minced with a razor blade, and subsequently incubated for 75 min at 37°C in Heps-Ringer’s buffer containing 0.2% (wt/vol) collagenase (CLS II; Cooper, Frankfurt, Germany) and 0.2% (wt/vol) hyaluronidase (Roche Diagnostics, Mannheim, Germany). After completion of the incubation procedure, the majority of the collecting duct cells in suspension were removed by low-speed centrifugation. The supernatants from the first two low-speed centrifugations, containing the majority of interstitial cells, were further separated from collecting duct cells with the use of beads coated with Dolichos biflorus agglutinin, as described (24). The resulting cell suspension was then subjected to single-step density gradient centrifugation with Nycodenz (Nyegaard Co., Oslo, Norway). After centrifugation, interstitial cells were maximally enriched, with a density of 1.081 to 1.093 g/cm³. After removal of the Nycodenz, cells were plated in culture wells and maintained in Dulbecco’s modified Eagle’s medium/nutrient mixture Ham’s F-12 medium (1:1) supplemented with 2 mMglutamine, 1 mM sodium pyruvate, 1% (vol/vol) nonessential amino acids, 50 U/ml penicillin, 50 U/ml streptomycin, and 10% fetal calf serum (all from Life Technologies, Eggenstein, Germany). Passage 1 cultures were examined.

Isolation and Culture of Hepatic Ito Cells

The isolation and culture of liver Ito cells from male Sprague-Dawley rats (body weight, 500 to 600 g) were performed as described previously (25). In brief, nonparenchymal liver cells were isolated by using the pronase-collagenase method (26). Ito cells were purified by single-step density gradient centrifugation in Nycodenz (see above) and were identified on the basis of their typical light-microscopic appearance and vitamin A-specific autofluorescence. The mean purity
of freshly isolated cells was 90 ± 5%, cell viability was >95%, and the yield ranged from 30 to 50 × 10⁶ cells/liter. Ito cells were seeded at a density of 0.2 × 10⁶ cells/cm², in 2 ml of Dulbecco’s modified Eagle’s medium containing 4 mM L-glutamine, 10% fetal calf serum, 1000 U/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C. The medium was changed approximately 20 h after seeding, after which the purity of Ito cells was >97%. The second medium change was approximately 28 h after seeding, at which time fetal calf serum supplementation was reduced to 0.2%.

### Histochemical and Western Blotting Protocols

#### Primary Antibodies.
A polyclonal antibody against the carboxy-terminus of the β1 subunit (SRKNTGTEETEQDEN) of bovine lung sGC (27) was raised in rabbits and immunopurified using the antigenic peptide coupled to SulfoLink coupling gel (Pierce, Boston, MA). A rabbit polyclonal antibody against NOS1 purified from porcine cerebellum (28) was a gift from Bernd Mayer (Graz, Austria). A mouse polyclonal antibody against NOS1 purified from porcine cerebellum (28) was a gift from Bernd Mayer (Graz, Austria). A mouse monoclonal antibody against the podocyte-specific antigen podosynapsin was kindly provided by Peter Mundel (New York, NY). A mouse monoclonal antibody against human α-smooth muscle actin was acquired from Dako (Glostrup, Denmark). A mouse monoclonal antibody against human desmin was also acquired from Dako. A rabbit polyclonal antibody against ecto-5'-nucleotidase was a gift from Brigitte Kaissling (Zurich, Switzerland).

#### Western Blot Analyses.
Freshly isolated kidneys, lungs, skeletal muscle, and liver from rats were rapidly dissected and cut into small pieces. The cortex and medulla from kidneys were separated. Isolated glomeruli, mesangial cells, and interstitial cells were also assayed. These samples were homogenized on ice in homogenization buffer [175 mM NaCl, 1 mM ethylenediaminetetraacetate, 50 mM triethanolamine-HCl, pH 7.4, 2 mM dithiothreitol (DTT), 1 μM pepstatin, 0.2 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride], using a glass/glass homogenizer. The homogenate was then centrifuged at 4°C for 30 min at 200,000 × g. The supernatant (cytosol) was supplemented with 50% (vol/vol) glycerol and stored at −20°C. Cytosolic proteins (16 to 20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5%) and blotted onto nitrocellulose membranes. Blots were blocked for 30 min with 5% non-fat dry milk (NFDM) and resuspended in diethylpyrocarbonate-treated water. RNA from isolated glomeruli was extracted by using the guanidinium thiocyanate method (29). All samples were quantified by spectrophotometric measurement of specific antibody, as well as competition with the antigenic peptide.

### Ultrastructural Pre-Embedding Histochemical Analyses.
For fine structural immunolabeling and immunoperoxidase labeling, an established protocol was used (10); for incubation of 20-μm-thick slices generated with a Vibratome, anti-β1 sGC antibody was used at dilutions between 1:25 and 1:50. Sections were incubated overnight in microtiter plates, postfixed with 1% osmium tetroxide, rinsed in maleate buffer, stained en bloc with uranyl acetate, and flat-embedded in Epon 812. Semithin sections were produced and photographed by using a light microscope. Ultrathin sections were then cut and viewed by using an electron microscope. Control experiments were performed by replacing primary antibodies with skim milk-PBS controls.

### NADPH-Diaphorase Staining.
The catalytic activity of NOS was demonstrated by enzymatic reduction of nitro blue tetrazolium in the presence of NADPH (NADPH-diaphorase reaction) (28). Slides were washed in PBS and incubated for 15 to 20 min in 0.1 M phosphate buffer containing 0.3% Triton X-100, 0.01% nitro blue tetrazolium, and 0.1% NADPH. No reaction product was observed when NADPH was replaced by NADH.

#### In Situ Hybridization.
The mRNA expression of the α1 and β1 subunits of sGC was investigated by in situ hybridization using digoxigenin-labeled riboprobes made from the bovine cDNA coding for the respective subunits. According to the protocol provided by the manufacturer (Roche), sense and antisense riboprobes were generated by in vitro transcription of the 647-bp α1 or 2000-bp β1 sGC cDNA fragment, using T3 and T7 polymerases and digoxigenin-labeled UTP, followed by time-controlled alkaline hydrolysis. For in situ hybridization, 7-μm cryostat sections were treated according to an established protocol (10). Briefly, 10 ng sGC antisense mRNA/μl hybridization mixture was incubated for 18 h at 48°C. The slides were washed sequentially with decreasing concentrations of SSC at 40°C and then with buffer 1 (0.1 M Tris-Cl, 0.15 M NaCl, pH 7.5) at room temperature and were then incubated for 30 min with buffer 1 containing 1% blocking reagent and 0.5% bovine serum albumin. Sheep anti-digoxigenin-alkaline phosphatase conjugate (diluted 1:500 in blocking medium) was applied for 60 min at room temperature and then overnight at 4°C. The slides were washed twice with buffer 1 and rinsed in buffer 3 (0.1 M Tris-Cl, 0.1 M NaCl, 0.05 M MgCl₂, pH 9.5). A solution of 4-nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indolylphosphate, and levamisole dissolved in buffer 3 was then used for the color reaction. The reaction was stopped by two washes with buffer 4 (0.1 M Tris-Cl, 1 mM ethylenediaminetetraacetate, pH 8.0). As a control, sense probes were applied in parallel with antisense probes. Slides were rinsed with PBS and coverslipped with PBS-glycerol.

### RT-PCR.
Total RNA was isolated from kidney cortex, medulla, and liver by using a commercially available kit (InViTek, Berlin, Germany). RNA was extracted with phenol/chloroform, precipitated with isopropanol, and resuspended in diethylpyrocarbonate-treated water. RNA from isolated glomeruli was extracted by using the guanidinium thiocyanate method (29). All samples were quantified by spectrophotometric analyses at 260 nm. Five micrograms of total RNA from each sample were reverse-transcribed with 60 U of murine Moloney leukemia virus reverse transcriptase for 25 min at 37°C, in a total volume of 15 μl, according to the protocol provided by the manufacturer (Roche). The samples were then heated at 70°C for 5 min, to inactivate the enzyme. These cDNA were used to compare the amounts of α1 sGC mRNA or β1 sGC mRNA versus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in different tissues. PCR were performed with specific primers for α1 sGC (5'-CCACATCAAACCCG-
GCTAA-T-3' and 5'-GAAGTGCAAGDTTCCAGTCTC-3'), for β1 sGC (5'-CGGATGCCAGGTATTGCTCT-3' and 5'-CTCTTGTTCTGAAGGCACATT-3'), and for GAPDH (5'-TATTCGGTTGGAATCTGAC-3' and 5'-TGCTCAGGTTTCTTAC-3' or 5'-ACCACTGCTTGCATC-3' and 5'TCCAACCCCGTTGCTGTA-3'), cDNA fragments of the expected sizes of 331 bp (5'-CAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'), and for GADPH (5'-TATCCGTTGTGGATCT-3').

Quantitative RT-PCR

Quantitative RT-PCR is based on the assumptions that the cDNA template and a competitive internal template compete equally for the primers and that amplification is colinear. The PCR fragment obtained with the β1 sGC primers (see above) contains two sites for the restriction enzyme NlaIII. Digestion of the β1 sGC PCR product with NlaIII yielded three fragments, which were separated on agarose gels and then purified. The outer two fragments were ligated and served as a competitive template. A 0.3-μg sample of total RNA was reverse-transcribed in the presence of decreasing amounts of the internal standard (20 ng, 2 ng, 200 pg, 20 pg, and 2 pg), in 25 μl. Each assay included 0.06 U/ml Taq polymerase (InVitTek), 50 mM MgCl₂, 25 mM dNTP, 0.1 mM DTT, and 10 mM concentrations of the aforementioned β1 sGC primers. The cDNA were amplified in 30 cycles (20 s at 94°C, 20 s at 59°C/63°C, and 30 s at 72°C) with 0.06 U/ml Taq polymerase and 50 mM MgCl₂. The PCR products were separated on 3% agarose gels, stained with ethidium bromide, and observed with ultraviolet illumination.

Determination of sGC Activity in Cytosolic Fractions by RIA

Cytosolic proteins (10 μg each) from the indicated tissues were incubated in the presence of 300 μM GTP, 3 mM MgCl₂, 3 mM DTT, 0.5 mg/ml bovine serum albumin, 0.25 g/L creatine phosphokinase, 5 mM creatine phosphate, 1 mM 3-isobutyl-1-methylxanthine (RBI, Köln, Germany), and 50 mM triethanolamine hydrochloride (pH 7.4), in a total volume of 0.1 ml. Stimulation of sGC was performed by addition of 300 μM S-nitrosoglutathione (Alexis, Grünberg, Germany). The incubation was stopped by the addition of ice-cold ethanol (final concentration, 70%). Formed cGMP was measured by RIA, as described (30).

Measurements of Intracellular cGMP Levels in Isolated Cells

Cells were cultured in six-well plates, maintained at 37°C, and rinsed with physiologic Ringer's solution. After preincubation with 0.5 M 3-isobutyl-1-methylxanthine for 5 min, cells were exposed to S-nitroso-N-acetylpenicillamine (SNAP) (100 μM and, in the case of podocytes, 1000 μM; Biomol, Hamburg, Germany) for 30 or 60 min. In control experiments, 1H-1,2,4-oxadiazole[4,3-a]quinoxalin-1-one (10 μM; Alexis) was added simultaneously with SNAP for 30 min. For termination of the assay, the supernatants were rapidly removed and cells were rinsed with ice-cold 70% ethanol. After ethanol extraction, cGMP concentrations were measured with an enzyme-linked immunosorbent assay (Amerham Buchler, Braunschweig, Germany). To confirm the specificity of the cGMP pathway, we tested atrial natriuretic peptide (ANP) (1 μM; Sigma), which stimulates the membrane-bound guanylyl cyclase, for 30 min.

Results

Localization of sGC in Kidney

Histochemical staining revealed significant amounts of β1 sGC in the renal vasculature and in interstitial cells. Intra- and juxtaglomerular structures demonstrated marked selective staining with a polyclonal antibody against β1 sGC. In double-staining analyses with either anti-desmin, as a marker of the intra- and extravascular mesangium (Figure 1a and b), or anti-synaptotagmin, as a podocyte marker (Figure 1c, d and e), sGC immunoreactivity was clearly recognizable in the mesangial axes of the extravascular mesangium. sGC was also detected in the extravascular mesangium, including the contact areas with the macula densa, which was identified on the basis of NADPH-diaphorase and NOS1 immunostaining (Figure 2a, a and d). Ultrastructural immunoperoxidase labeling demonstrated uniform cytosolic distribution of sGC label exclusively in the mesangial cells (Figures 1e and 2e). Prominent signal was also detected in the mesangial areas, where extensions of the mesangial cells are connected to the glomerular capillaries (Figure 3a). The preglobular portion, containing the granular renin-producing cells, was also strongly labeled; in the granular cells, cytosolic labeling was obvious in the vicinity of the renin-containing granules (which were unreactive) (Figure 3c). Staining of the effenter arteriolar wall was immunostained up to the intraglomerular site where it branches into the glomerular capillaries (Figure 3a). The preglobular portion, containing the granular renin-producing cells, was also strongly labeled; in the granular cells, cytosolic labeling was obvious in the vicinity of the renin-containing granules (which were unreactive) (Figure 3c). Staining of the effenter arteriole wall was generally strong (Figure 3d) but was particularly intense in juxtedudillary nephrons, from which the descending vasa recta originate. In situ hybridization also demonstrated prominent labeling in the glomerular arteriolar walls, using probes for α1 and β1 subunits (Figure 3b). Intraglomerular structures did not reliably exhibit an in situ hybridization signal, which may be attributable to insufficient sensitivity of the method used. A weak signal was observed in the extravascular mesangium, sometimes in continuity with reactive portions of the glomerular arterioles. In the cortial interstitium, fibroblasts were labeled throughout the cortical labyrinth, the medullary rays, and the perivascular areas. The immunoreactive cells were identified by double-labeling with an antibody directed against ccto-5'-nucleotidase, an enzyme that is typically located along the cell membranes of cortical fibroblasts (Figure 4a and b). sGC-immunoreactive fibroblasts were further identified throughout the outer medulla and along the vascular bundles extending to the inner medulla. Ultrastructural immunoperoxidase staining revealed intense, evenly distributed, cytosolic β1 sGC staining in these cells, sparing all major organelles (Figure 4c). In situ hybridization also produced strong α1 and β1 sGC mRNA signals in peritubular and perivascular locations, with a distribution pattern analogous to that typical of interstitial fibroblasts (Figure 4d).

In the renal medulla, the descending vasa recta demonstrated continuous strong β1 sGC immunoreactivity within the con-
Figure 1. Identification of renal intraglomerular β1 soluble guanylyl cyclase (sGC) immunoreactivity. (a and c) Anti-β1 sGC (Cy3-labeled) (a) and anti-desmin (Cy2-labeled) (c) immunostaining. Prominent glomerular structures stained with anti-β1 sGC are the mesangial axes of the glomerular tuft and the afferent arteriolar wall (arrowhead); these structures are double-stained with anti-desmin. (c and d) Anti-β1 sGC (c) and antisyntaptopodin (Cy2-labeled) (d) immunostaining. Syntaptopodin is a selective podocyte marker. It should be noted that the two staining patterns are complementary. (e) Ultrastructural anti-β1 sGC immunoperoxidase labeling of a glomerular capillary loop, focusing on a mesangial angle. Selective β1 sGC signal is present in a mesangial cell process at the site where it anchors the glomerular basement membrane. Magnifications: ×250 in a to d; ×11,000 in e.

Figure 2. Renal β1 sGC immunoreactivity in the intra- and extraglomerular mesangium. (a to d) Two different views of the mesangium, using double-staining with anti-β1 sGC antibody (Cy3-labeled) (a and c), anti-nitric oxide (NO) synthase 1 (NOS1) antibody (Cy2-labeled) (b), and NADPH-diaphorase staining (d). The strong β1 sGC signal of the extraglomerular mesangium is located next to the β1 sGC-unreactive, NOS1-positive macula densa. The intraglomerular mesangium is also β1 sGC-positive; the continuity between extra- and intraglomerular immunostaining is evident (c), next to the diaphorase-positive macula densa (d). (e) Ultrastructural anti-β1 sGC immunoperoxidase labeling of the extraglomerular mesangium, demonstrating strong cytosolic labeling. An adjacent portion of the endothelium (arrowheads) is unreactive. Magnifications: ×250 in a to d; ×12,000 in e.
Figure 3. Cortical renal vasculature expressing β1 sGC. (a) Ultrastructural anti-β1 sGC immunoperoxidase labeling shows prominent staining of the glomerular afferent arteriolar wall, including the renin-containing granular cells (arrowhead). On the right, extraglomerular mesangium is also stained. (b) In situ hybridization shows prominent expression of β1 sGC mRNA in the wall of the glomerular arterioles. Intraglomerular expression is not detectable. (c) Renin-containing granular cells are shown at high resolution; labeling is restricted to the cytosol. Adjacent endothelium (arrowheads) and thick ascending limb epithelium (arrows) are unstained. (d) Muscular media cells of an afferent arteriole demonstrate strong β1 sGC signal. The endothelium is unstained. Magnifications: ×2000 in a; ×350 in b; ×4100 in c; ×3300 in d.

Figure 4. Renal β1 sGC expression in the cortical interstitium. (a and b) Double-immunostaining with anti-β1 sGC (Cy3-labeled) (a) and anti-ecto-5'-nucleotidase (Cy2-labeled) (b), showing that peritubular cells are double-stained, whereas the proximal tubule brush border is only anti-ecto-5'-nucleotidase-positive (arrowhead in b). (c) Ultrastructural anti-β1 sGC immunoperoxidase labeling, showing dense reactivity in a typical interstitial fibroblast. (d) In situ hybridization for sGC mRNA expression (α1 subunit), showing that sites of interstitial peritubular fibroblasts are prominently labeled. (e) In situ hybridization, demonstrating that, as in d, fibroblasts are labeled with a probe specific for sGC (β1 subunit). Magnifications: ×650 in a and b; ×3200 in c; ×220 in d and e.
tractile media along their initial portions; in the terminal portions, the remaining pericytes, which were typically arranged around the circumference of these vessels, were positively stained (Figure 5). Strong labeling in pericytes was particularly evident in the vascular bundles of the outer and inner stripes of the outer medulla. In the inner medulla, the number of immunoreactive cells progressively decreased toward the papillary tip. Immunoreactive perivascular fibroblasts in the vascular bundles were identified on the basis of their characteristic branched morphologic features, which are distinct from those of vascular pericytes (Figure 5a). Ascending vasa recta were not regularly accompanied by $\beta_1$ sGC-immunoreactive cells.

The locations of signals produced by in situ hybridization with probes for the $\alpha$ and $\beta$ subunits of sGC corresponded to the immunoreactivity distribution. We detected mRNA signal primarily in perivascular pericytes and fibroblasts of the vascular bundles (outer medulla), whereas interstitial fibroblasts in the inter-bundle regions were rarely labeled. In the inner medulla, only a few interstitial cells were positively stained.

Localization of sGC in Liver

In the liver, hepatic stellate (Ito) cells expressed significant levels of $\beta_1$ sGC immunoreactivity and $\alpha_1/\beta_1$ sGC mRNA, as revealed by in situ hybridization (Figure 6, a and b). In the periphery of a hepatic lobule, nearly all Ito cells were intensively immunostained for sGC; the intensity decreased toward the central vein, however, and no signal was detectable in the innermost region around the central vein. High-resolution immunoperoxidase labeling permitted identification of these cells on the basis of their typical perisinusoidal location in the space of Disse and their regular content of large lipid vacuoles, which were surrounded by an intense signal for the cyclase (Figure 6c). The walls of portal venules and larger veins were also observed to be sGC-immunopositive.

The specificity of the immunohistochemical labeling was verified by preabsorption of the $\beta_1$ sGC-specific antibody with the peptide used for immunization. No signal was observed in kidney or liver after incubation with this mixture. For in situ hybridization analyses, sense and antisense probes transcribed from $\alpha_1$ and $\beta_1$ cDNA, respectively, were routinely used on sections. No signals were obtained with the sense probes.

Measurements of NO-Stimulated cGMP Formation in Extracts from Tissues and Isolated Cells

Determination of NO-stimulated SGC activity revealed significant increases in cGMP formation in the cytosolic fractions from different tissues. The kidney cortex demonstrated an 87-fold increase in cGMP formation (56 ± 53 to 4899 ± 686 pmol cGMP/min per mg) (Figure 7A) after stimulation with S-nitroso glutathione, and the renal medulla demonstrated a 65-fold increase (33 ± 18 to 2133 ± 445 pmol cGMP/min per mg). We observed a 232-fold increase in the liver (8 ± 6 to 1856 ± 866 pmol cGMP/min per mg) and a 59-fold increase in the lung (112 ± 101 to 6659 ± 650 pmol cGMP/min per mg). The stimulation factors thus ranged between 56- and 232-fold, demonstrating that the observed rates of cGMP formation were substantially and significantly enhanced by NO-dependent ac-

Figure 5. Renal $\beta_1$ sGC immunoperoxidase labeling in the medulla. (a) Vascular bundle at the transition between the outer and inner stripes of the outer medulla. Media cells and pericytes of the descending vasa recta (arrows pointing down) and interstitial fibroblasts show marked immunostaining; no particular staining is observed in the ascending vasa recta (arrows pointing up). (b) Ultrastructural $\beta_1$ sGC immunoperoxidase labeling in a descending vasa rectum (inner stripe). Significant staining of the cross-sectional profiles of circumferentially arranged pericytes forming the muscle wall should be noted. (c) $\beta_1$ sGC immunoperoxidase labeling of a single pericyte from a descending vasa rectum (outer medulla). The adjacent endothelium (arrowheads) is negative. Magnifications: ×980 in a; ×3200 in b; ×7400 in c.
tivation of sGC and that, in absolute terms, the lung exhibited the highest levels of cGMP generation, as expected.

Incubation of extracts from freshly prepared mesangial cells with the NO donor SNAP led to a 39-fold increase in cGMP levels (153 ± 46 to 5922 ± 1168 pmol/well) (Figure 7B), incubation of extracts from renal medullary interstitial cells with SNAP led to a 52-fold increase (27 ± 4 to 1396 ± 366 pmol/well), and incubation of extracts from isolated hepatic Ito cells with SNAP led to a 47-fold increase (26 ± 7 to 1218 ± 177 pmol/well). The cGMP accumulation induced by the NO donor was almost completely inhibited when cells were stimulated in the presence of the specific sGC inhibitor 1H-(1,2,4)oxadiazole[4,3-a]quinoxalin-1-one. Incubation of extracts from cultured podocytes with various concentrations of the NO donor SNAP, however, never produced an increase in cGMP levels, compared with control values; in contrast, ANP produced a significant 39-fold increase (21 ± 17 to 819 ± 112 pmol/well) (Figure 7C).

Western Blot Analyses

Qualitative immunoblotting was performed with anti-β1 sGC antibody, using tissue extracts from kidney cortex, kidney medulla, liver, lung, and muscle and extracts of isolated Ito cells, mesangial cells, and interstitial cells that had been tested in the cGMP assays described above. A principal band was identified, with an apparent molecular mass of approximately 70 kD (Figure 8). Lower-molecular mass bands were attributable to degradation. The weak signal in extracts from interstitial cells may be attributable to the origin of these cells from the renal medulla, where the number of sGC-immunoreactive fibroblasts is smaller than in the cortex. The immunohistochemical signal obtained with antibody against β1 sGC was also weaker.

RT-PCR Analysis

We performed a quantitative analysis of β1 sGC mRNA in extracts from renal cortex, to verify the histochemical results regarding sGC mRNA expression. Approximately equal amounts of sGC cDNA and internal standard were amplified at a concentration of 0.02 pg of standard DNA (Figure 9A). As a result, a value of 66 pg sGC mRNA/µg total renal cortical mRNA was calculated. The presence of mRNA for both α1 and β1 subunits was further assessed in tissue extracts by using RT-PCR, with GAPDH as a reference standard. Significant bands for both subunits were observed for RNA extracts obtained from kidney cortex, isolated glomeruli, and liver (Figure 9B to E).

Discussion

The results of our histochemical investigations assign sGC expression precisely to vascular and interstitial cell types in kidney and liver. A newly generated, highly specific antibody to the β1 subunit of sGC allowed us to substantially extend previous data on sGC localization (17,31). sGC requires the coexpression of one α and one β subunit for catalytic action (3,27). Because we were limited to detection of only the β1 subunit with reliable immunohistochemical specificity, we supplemented our data with results from in situ hybridization and RT-PCR assays with specific probes for both of the “universal” subunits (α1 and β1) and with findings from functional assays on cultivated cells that were selected according to their histochemical identification in situ.

The renal distribution of sGC revealed vascular and interstitial components. This study demonstrates, for the first time, the presence of sGC in all contractile cells of the kidney, with different expression levels. In glomeruli, the smooth muscle-
derived cells of the intra- and extraglomerular mesangium were sGC-immunoreactive. RT-PCR data for isolated glomeruli support the presence of mRNA coding for \( \alpha_1 \) and \( \beta_1 \) sGC. The intraglomerular mesangial cells are thought to maintain mechanical strength by anchoring the capillary loops in the glomerular tuft (32); receptors for vasoactive hormones related to the contractility of these cells have been identified (for review, see reference 33). Local effects of the NO-sGC-cGMP pathway may counteract these effects.

In vivo, NO may be derived from constitutive NOS activity of the adjacent glomerular capillary endothelium or may diffuse from the nearby macula densa (8,10). Significant sGC staining, as detected, in the mesangial angles anchoring the glomerular basement membrane (32) suggests a particular role for cGMP in maintaining

Figure 7. Stimulation of sGC in cytosolic fractions from various tissues (A) and isolated cells (B and C), using S-nitrosoglutathione (A) or S-nitroso-N-acetylpenicillamine (SNAP) (B and C) as a NO donor. (A) Enzyme activity in the cytosolic fractions from various tissues was determined in the absence (□) or presence (■) of 300 \( \mu \)M S-nitrosoglutathione. Data are the means ± SD of three representative experiments. (B) Enzyme activity in the cytosolic fractions from various isolated cells was determined in the absence (□) or presence of 100 \( \mu \)M SNAP (■) or 100 \( \mu \)M SNAP added with the inhibitor 1H-(1,2,4)oxadiazole[4,3-a]quinazolin-1-one (10 \( \mu \)M) (■). (C) Enzyme activity in the cytosolic fractions from immortalized cultured mouse podocytes was determined in the absence (Co) or presence of 100 \( \mu \)M SNAP or 100 nM atrial natriuretic peptide (ANP). In B and C, each column represents the mean ± SD of six experiments, performed in triplicate.

Figure 8. Detection of \( \beta_1 \) sGC in various tissue (A) and isolated cell (B) lysates by Western blot analysis. The smaller bands observed in addition to the dominant 70-kD protein are degradation products. Signal generation was performed by using a chemiluminescence kit.
adequate contractile tone at these sites. Stimulation with a NO agonist was highly effective in increasing cGMP accumulation in mesangial cells \textit{in vitro}, which confirms earlier data (34) and supports the hypothesis that cGMP may play an important role in these cells.

The histochemical presence of $\beta_1$ sGC in mesangial cells and not in podocytes, as observed in this study, is in contrast to previous findings reporting $\alpha_1$ sGC immunoreactivity exclusively in podocytes (31). However, because those earlier attempts to localize sGC did not reveal a plausible overall distribution pattern for the enzyme and failed to demonstrate colocalization of its subunits, the significance of those experiments must be reconsidered in light of the findings presented here. Our \textit{in vitro} data demonstrated, in fact, that addition of a NO donor to cultured immortalized mouse podocytes failed to stimulate sGC, whereas the same cells demonstrated a response after stimulation with ANP; these results highlight the absence of sGC but confirm earlier findings on the presence of the particulate form of the enzyme in these cells in rats. Of course, discrepancies between the two species with respect to sGC expression cannot be entirely ruled out by the reported assay results.

Marked expression of $\beta_1$ sGC in cells of the extraglomerular mesangium may have functional implications. Because the mechanical integrity of the glomerular hilus is thought to be supported by these cells forming a "closure device" for the entrance into the glomerulus (35), NO-dependent regulation of their contractility, mediated by the nearby constitutive NOS in the macula densa, seems plausible (36). Extraglomerular mesangial cells are thought to transmit stimuli from the macula densa to the glomerular vasculature and the renin-containing granular cells, and the NO-sGC-cGMP pathway may represent one of the principal components in this information transfer, possibly in conjunction with structurally established intercellular communication via gap junctional coupling (9,36,37).

The findings of significant sGC immunoreactivity and transcript levels in the glomerular arteriolar walls, including the granular cells, confirm functional observations regarding the local effects of NO on these vascular cells (7–9,38). Adjustment of tubuloglomerular feedback action in response to changes in tubular NaCl load and macula densa constitutive NOS activity (8,38) may thus be related to the local abundance of sGC. The presence of sGC in the cytosol directly surrounding the renin-containing granules of the renin-producing cells supports a stimulatory role for cGMP (7,9). Prominent sGC immunoreactivity in the muscular wall of the efferent arterioles further suggests that local release of NO in this vascular portion may have important vasodilatory effects. The particularly high sGC signal in juxtamedullary efferent arterioles suggests a NO-dependent dilatory mechanism, which may determine renal medullary perfusion. Such an effect would be supported by the continuous strong sGC immunostaining in the myocytes constituting the wall of the descending vasa recta and in the pericytes of the terminal portions of these vessels within the vascular bundles. These data may represent the morphologic equivalent of the otherwise well established effects of NO on renal medullary circulation (10,11). The overall weaker $\beta_1$
sGC immunostaining of larger renal cortical arteries and arterioles, compared with small resistance vessels, suggests preferential responsiveness to NO in the latter.

Significant expression of sGC was also observed in interstitial fibroblasts in the renal cortex and, to a lesser extent, in the medulla; in situ hybridization results and the in vitro data on NO-dependent cGMP formation, using freshly isolated medullary interstitial cells, demonstrated the immunohistochemical findings, confirming that this cell type has a particular role in the NO-sGC-cGMP signaling pathway in renal parenchyma. These data agree with an earlier report on mRNA expression of α1 and β1 sGC subunits in long-term cultivated rat medullary interstitial cells and on their NO-specific responsiveness (39). In situ, a proportion of the cortical interstitial fibroblasts were previously identified as the main source of erythropoietin (40). These cells also express high levels of NADPH oxidase (41), and they are involved in the synthesis of extracellular adenosine; however, it remains to be established whether these products functionally interact and what role local cGMP release could play.

The complete absence of tubular histochemical labeling in this study is in contrast to previous PCR and immunohistochemical data that reported sGC mRNA in a variety of tubular epithelia and β2 sGC immunoreactivity in collecting ducts (12,13,31). The discrepancy between these results may be attributable to sensitivity or specificity problems; however, anti-β1 sGC immunoreactivity was reliably detected in a variety of renal and hepatic cell types, so that tubular expression, at least of β1 sGC, must be at a low level. Also, we never observed coincident histochemical localization of NOS and sGC, which suggests that NO, as a signal molecule for cGMP generation, acts through paracrine diffusion to its target, rather than by intracellular signaling.

Our results on sGC in the Ito cells of the liver agree with functional concepts of these cells. Ito cells have been described as liver-specific pericytes that are located in the space of Disse and possess long cell processes extending between the sinusoid endothelia and the parenchymal cells (42). They are related to renal cortical interstitial cells, inasmuch as the two cell types share a number of properties. In addition to the common expression of sGC, they are the principal sources of erythropoietin (40). In situ hybridization results and the in vitro data, we thus provide a more solid, extended pattern of sGC distribution, with the use of improved histochemical and in vitro methods. Significant expression was localized to vascular wall cells in both organs, with particularly high intensity in the glomerular arterioles and descending vasa recta of the kidney and in perisinusoidal Ito cells. These sites are crucial for the local adjustment of vascular tone and thus of regional end-organ perfusion. Common properties of Ito cells and renal cortical fibroblasts have been emphasized by the results presented here, which may facilitate an understanding of the functional mechanisms that are active in these cell types. The localization of sGC in renin-producing granular cells corroborates functional concepts of the role of cGMP in renin release. Significant mesangial expression of sGC and effective stimulation of cultured mesangial cells by NO suggest a prominent role for cGMP in adjustment of the contractile tone of the glomerular tuft, with possible implications for diseases of the glomeruli.

Acknowledgments

This work was supported by funds from the Deutsche Forschungsgemeinschaft (Grant Ba700/14-1).

References


