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Nitric oxide down-regulates connective tissue growth factor in rat mesangial cells

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Background. Nitric oxide (NO) exerts complex regulatory actions on mesangial cell (MC) biology, such as inhibition of proliferation, adhesion or contractility and induction of apoptosis. In our previous studies the NO-donor S-nitroso-glutathione (GSNO) was found to be a potent inhibitor of MC growth. This effect was mediated at least in part by inhibitory effects of GSNO on the transcription factor early growth response gene-1 (Egr-1) [10]. We therefore were interested in the regulation of gene expression in MC after treatment with NO.

Methods. To identify the genes that are regulated by NO in MC, gene expression was analyzed by representational difference analysis. Expression of connective tissue growth factor (CTGF) was studied by Northern and Western blot analyses.

Results. Cultured rat MCs treated with GSNO for 8 hours were compared with unstimulated MCs and the CTGF mRNA was found to be down-regulated. The down-regulation was dose-dependent and transient, with a maximum inhibition seen after 6 hours. In parallel, down-regulation of CTGF protein by GSNO was observed by Western blot analysis. Other NO-donors such as S-nitroso-N-acetyl-D,L-penicillamine and spermine-NO showed similar effects. The induction of the inducible NO-synthase by TNF-α, IL-1β and LPS provoked a transient down-regulation of CTGF mRNA, an effect that could be partially overcome by pretreatment with the NOS-inhibitor N^{ω}-nitro-L-arginine methyl ester. The observed NO-effect could be simulated by treatment with the stable cGMP analog 8br-cGMP, and was abolished by blocking the guanylyl cyclase with the inhibitor NS2028.

Conclusion. NO acts as a strong repressor of CTGF expression in cultured rat MC. Thus, in addition to its antiproliferative effects, NO potentially exerts antifibrotic activity by down-regulation of CTGF.

Nitric oxide (NO) is a small gaseous molecule produced in vivo by NO synthases (NOS) from the substrate L-arginine. Three different NOS isoforms have been identified: the constitutive, calmodulin-dependent neuronal NOS (nNOS; NOS 1) and endothelial NOS (eNOS; NOS 3), and the inducible, calmodulin-independent NOS (iNOS; NOS 2), which can be expressed in several cell types, among them macrophages and mesangial cells.

In the healthy human kidney nNOS is localized in the macula densa and the efferent arterioles, eNOS in the glomerular endothelium and the afferent and efferent arterioles, and an expression of iNOS is not or only barely found [1–3]. The inhibition of NOS by N^{G}-monomethyl-L-arginine (L-NMMA) or N^{ω}-nitro-L-arginine methyl ester (L-NAME) in healthy rats causes glomerular hypertension, glomerulosclerosis, matrix expansion and increased intraglomerular platelet aggregation [4, 5]. Whereas NOS inhibition ameliorates the course of glomerulonephritis and arthritis in MRP *lpr/lpr* mice, a model of lupus nephritis [6], positive effects of NO have been described in a variety of other models of inflammatory kidney diseases. In rat nephrotoxic serum nephritis (anti-glomerular basement membrane nephritis) the inhibition of NO production by L-NMMA leads to increased proteinuria, systemic and glomerular hypertension and worsening of the disease process [7]. NO is also protective in the subtotal nephrectomy model, where L-arginine supplementation prevents the development of glomerulosclerosis [8].

Glomerular mesangial cells (MC), a key cell type in the orchestration of glomerular inflammation and ultimately fibrosis, are capable of producing high amounts of NO by an iNOS and are therefore a source as well as a main target of NO in inflammatory glomerular diseases. The following effects of NO on glomerular MC have been described. Coincubation experiments have shown that NO released by endothelial cells leads to cyclic 3′,5′-guanosine monophosphate (cGMP) accumulation in MC and inhibits angiotensin II-induced contraction [9]. Several studies showed that NO inhibited the proliferation of MC [9–11]. Furthermore, NO inhibits the adhesion of MC to extracellular matrix proteins [12], as well as the expression of intracellular adhesion molecule-1 (ICAM-1)

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[13] and the synthesis of the matrix proteins collagen and fibronectin [14]. Therefore, NO exerts anti-mitogenic as well as anti-fibrotic actions on glomerular MC.

To further investigate these actions, we examined modifications of gene expression patterns in cultured rat MC after stimulation with NO. The NO donor S-nitrosoglutathione (GSNO) was used as a source for NO. To identify differentially expressed genes, a polymerase chain reaction (PCR) based subtractive hybridization method was used, the representational difference analysis (RDA) [15], that was able to identify connective tissue growth factor as a gene, which is down-regulated by NO.

Connective tissue growth factor (CTGF) is a member of the CCN (Cyr 61/Cef-10, CTGF/Fisp-12, Nov) immediate early gene family [16] and is considered to be critically involved in fibrosis as a downstream mediator of transforming growth factor-beta (TGF-β) [17, 18]. Human CTGF was identified as a mitogen found in the conditioned medium of human umbilical vein endothelial cells (HUVECs) immunoreactive with polyclonal antibodies raised against a preparation of platelet-derived growth factor (PDGF) [19]. The 38 kD protein is cysteine-rich, secreted and extracellular matrix associated [20]. The biological functions of CTGF have been shown to be quite diverse. Earlier studies described mitogenic and chemotactic activities of CTGF on fibroblasts, and an important role in wound healing was suggested as well [19, 21, 22]. Furthermore, CTGF was found to be involved in the proliferation and migration of vascular endothelial cells and seems to play a central role in the growth and differentiation of chondrocytes [23, 24]. CTGF expression has been associated with various fibrotic diseases such as scleroderma, lung fibrosis or arthrosclerosis [25]. In the kidney increased CTGF expression was noted in human diabetic nephropathy [26] as well as in various animal models, like streptozotocin-induced diabetes mellitus, anti-Thy-1.1-nephritis and renal fibrosis disease models including the 5/6-nephrectomy [17, 27, 28]. In glomerular MC CTGF was found to be up-regulated in a variety of profibrotic conditions, such as, after exposure to TGF-β, high glucose or stretch [18, 27, 29].

Given the presumed critical involvement of CTGF in renal fibrosis, we performed a more detailed analysis of the nature and kinetics of the NO-induced decrease of CTGF-mRNA level revealed by RDA.

METHODS

Reagents

S-nitroso-glutathione (GSNO) was synthesized as described previously [30]. Briefly, glutathione was dissolved in 0.625 N HCl at 4°C to a final concentration of 625 mmol/L. An equimolar amount of $NaNO_2$ was added and the mixture was stirred for 40 minutes at 4°C. After the addition of 2.5 volumes of acetone, stirring was continued for another 20 minutes, followed by filtration of the precipitate. GSNO was washed once with 80% acetone, two times with 100% acetone, and finally, three times with diethyl ether, and dried under vacuum. Freshly synthesized GSNO was characterized by high-pressure liquid chromatography (HPLC) analysis and ultraviolet (UV) spectroscopy. S-nitroso-N-acetyl-dL-penicillamine (SNAP) was purchased from Alexis (Grünberg, Germany), spermine-NO, 8bromo-cGMP (8br-cGMP) and tumor necrosis factor-α (TNF-α) were from Sigma (Deisenhofen, Germany), heparin was from Roche (Mannheim, Germany), interleukin-1β (IL-1β), NS2028 and lipopolysaccharide (LPS) were from Calbiochem (Merck, Darmstadt, Germany).

Growth factors and antibodies

Recombinant human CTGF, pre-immune chicken IgY (pCIgY13) and neutralizing chicken anti-CTGF antibody (pIgY13) were provided by FibroGen Inc. (South San Francisco, CA, USA). Recombinant human CTGF was generated using a baculovirus expression system and purified by heparin-Sepharose affinity chromatography as described previously [21]. Peak fractions containing recombinant human CTGF (rhCTGF) were determined by immunoblotting and Coomassie staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels. Neutralizing anti-CTGF antibody was raised in chicken by immunization with purified baculovirus-derived, full-length rhCTGF protein as previously described, and was subsequently affinity purified through a rhCTGF-Sepharose column.

Mesangial cell culture

Glomeruli from kidneys of male Sprague-Dawley rats (200 g) were isolated and glomerular outgrowth and subsequent subculturing of MC was performed as described previously [31]. MC were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated (56°C, 30 min) FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L glutamine, and 5 μg/mL insulin in a 95% air-5% CO_2 humidified atmosphere at 37°C. MC were used for experiments between passages 8 and 20. The cell viability was measured by the trypan blue-exclusion test, if indicated.

RNA extraction

Mesangial cells were grown in 10 cm dishes until subconfluency. After stimulation with GSNO/GSH, SNAP, Spermine-NO, cytokines or 8br-cGMP, cells were washed once in cold phosphate-buffered saline (PBS; 4°C) and total RNA was extracted by the method of Chomczynski and Sacchi [32]. Poly-A^+ RNA isolation from total RNA was achieved by use of oligo-dT coated polystyrene latex particles (Qiagen, Hilden, Germany). The quality

of mRNA preparations was confirmed visually after electrophoresis in a 1% agarose-formaldehyde gel.

Representational difference analysis

The representational difference analysis of cDNA (RDA) as described by Hubank and Schatz [15] was performed with the Clontech PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. For this purpose, poly-A^+ RNA was extracted from GSNO-treated and control MC and reverse transcribed into cDNA. The cDNA that contains the specific (differentially expressed) transcripts was termed "tester" and the reference cDNA "driver." The tester and driver cDNAs were digested by Rsa I, a four-base-cutting restriction enzyme that yields blunt ends. The tester cDNA was then subdivided into two portions, and each was ligated with a different cDNA adaptor. The two adaptors had stretches of identical sequence to allow annealing of PCR primers once the recessed ends have been filled in. Two hybridizations were then performed. In the first, an excess of driver was added to each sample of tester. The samples were then heat denatured and allowed to anneal. The concentration of high- and low-abundance sequences was equalized among the single strand molecules ligated to an adaptor because reannealing is faster for the more abundant molecules due to the second-order kinetics of hybridization. At the same time, these molecules were significantly enriched for differentially expressed sequences. During the second hybridization, the two primary hybridization samples and again an excess of driver were mixed together without denaturing. Now, only the remaining equalized and subtracted single-strand tester cDNAs could reassociate and form hybrid double-strand tester molecules with different ends, which correspond to the sequences of the different adaptors. The entire population of molecules was then subjected to PCR to amplify the desired differentially expressed sequences. Only the molecules, which had two different adaptors, could be amplified exponentially. These were the equalized, differentially expressed sequences. A secondary PCR amplification was performed using nested primers to further reduce any background PCR products and enrich for differentially expressed sequences.

Cloning of PCR fragments

The PCR products resulting after the subtraction procedure were blunt-end cloned into pCR-Script SK(+) vector (Stratagene, La Jolla, CA, USA) and transfected in *E. coli* XL1-Blue MRF' supercompetent bacteria (Stratagene).

Dot blot analysis

To identify differentially expressed RNAs, equal amounts of cloned cDNAs were dotted twice onto nylon membranes (Amersham Life Science, Little Chalfont, UK) as recommended by the company (Clontech). The membranes were air dried and hybridized with $\alpha[^{32}P]$dCTP labeled forward- and reverse-subtracted cDNA probes of the GSNO-treated and untreated mRNA populations. Blots were exposed to Kodak Biomax MS or MR films (Eastman Kodak Company, Rochester, NY, USA) at −80°C. Differentially expressed RNAs were used for further tests in Northern blot.

Northern blot analysis

For RNA analysis 10 μg of total RNA from GSNO or GSH stimulated MC were separated by electrophoresis in a 1% agarose-formaldehyde gel. The RNA was blotted from the gel onto nylon membranes (Amersham) and baked at 80°C for two hours. Membranes were prehybridized for 30 minutes at 68°C in ExpressHyb hybridization solution (Clontech). cDNA hybridization probes were labeled with $\alpha[^{32}P]$dCTP using a random primer labeling kit (Amersham). Hybridization was at 68°C in fresh ExpressHyb hybridization solution containing radiolabeled cDNA probes at a concentration of 2×10^6 cpm/mL for one hour. Next, membranes were washed several times with 2 × standard sodium citrate (SSC)/0.1% SDS, until the wash solution did not ray more than the background. Blots were exposed to Kodak Biomax MS or MR films (Eastman Kodak Co.) at −80°C.

Western blot analysis

Rat MC were cultured in DMEM medium containing 10% serum with or without 5 U/mL heparin to stabilize CTGF protein. Subconfluent cells were not stimulated or stimulated with 500 μmol/L GSNO or GSH once for eight hours or repeatedly at 0 and 12 hours for a total of 24 hours. The supernatants were harvested and ethylenediaminetetraacetic acid (EDTA; 1 mmol/L) and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF; 1 mmol/L), aprotinin (2 μg/mL), leupeptin (2 μg/mL) and pepstatin (0.5 μg/mL) were added. For some experiments heparin binding proteins were extracted from conditioned medium with heparin-sepharose CL-6B beads (Amersham-Pharmacia, Roosendaal, The Netherlands) for two hours at 4°C. Bound proteins were eluted by boiling in 2 × SDS sample buffer and resolved on a 8% SDS polyacrylamide gel and subsequently transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were blocked in phosphate-buffered saline (PBS) containing 0.5% Tween 20 and 5% bovine serum albumin (BSA) and incubated with chicken anti-hCTGF antibody (pIgY13) at 0.5 μg/mL in blocking buffer, followed by horseradish peroxidase (HRPO)-conjugated rabbit anti-chicken antibody (Zymed, San Francisco, CA, USA) in blocking buffer. Immobilized antibodies were visualized with the enhanced chemiluminescence system (ECL; NEN Life Science, Za-

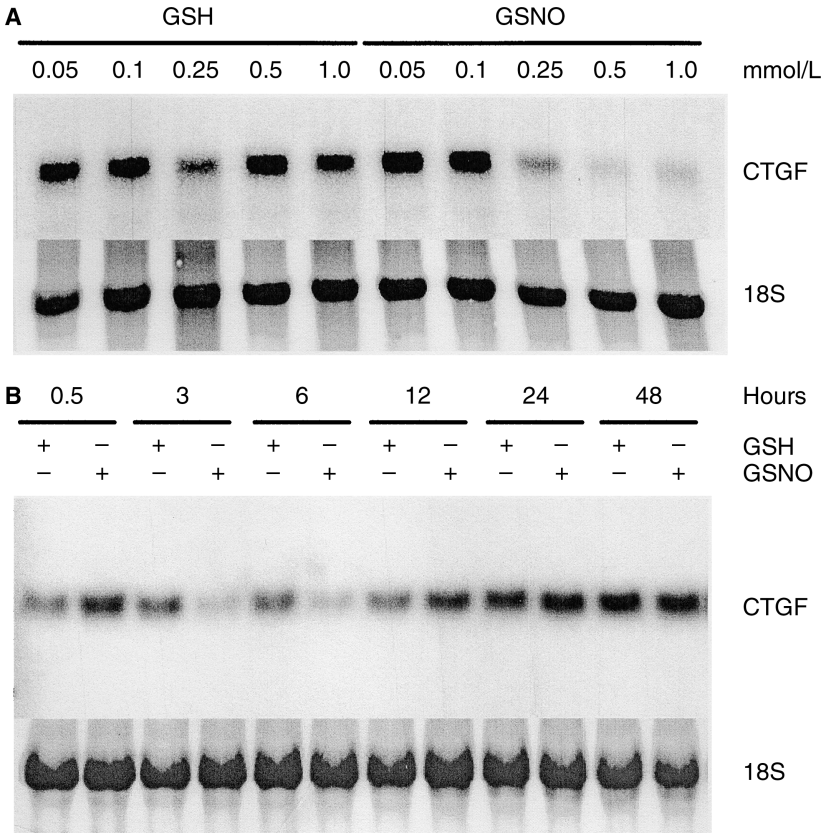

Fig. 1. Dose-dependent and transient down-regulation of CTGF by GSNO. MC were grown in medium containing 10% FCS until subconfluency and treated with increasing concentrations of GSNO (50 μmol/L, 100 μmol/L, 250 μmol/L, 500 μmol/L, 1 mmol/L) for 8 hours (*A*) or with 500 μmol/L GSNO for 0.5, 3, 6, 12, 24 and 48 hours (*B*). Control cells were treated with equimolar amounts of GSH for the same time intervals. RNA was extracted and 10 μg total RNA per lane were size fractionated on a 1% agarose gel. Blots were stained with methylene blue to control for equal loading and probed with a cDNA for CTGF radiolabeled by random priming. Abbreviations are in the **Appendix**.

ventem, Belgium) according to the manufacturer's instructions and exposure to X-Omat blue XB-1 films (Kodak, NEN Life Science).

RESULTS

Identification of CTGF as a NO-regulated gene in glomerular mesangial cells

To identify genes that are differentially expressed after NO induction, the representational difference analysis (RDA) was used, which is a polymerase chain reaction (PCR)-based subtractive hybridization method [15]. RDA has advantages over conventional subtractive hybridization methods, because the procedure is less time consuming and allows the identification of low abundance genes. For this purpose MC were stimulated for eight hours with 500 μmol/L GSNO, a concentration which inhibits MC proliferation but is not toxic and does not induce apoptosis of MC cultured in the presence of 10% fetal calf serum (FCS) [10], and compared them with untreated cells as control. Poly A^+-RNA from control and GSNO-treated cells was isolated, reverse transcribed into cDNA and subtracted as described earlier in this article. After the RDA procedure the PCR amplification products were cloned, representing potentially GSNO-regulated genes. The differential expression of the identified mRNAs was confirmed by dot blot and Northern blot analysis. Of the 51 identified clones, 12 (24%) were confirmed to be differentially expressed. One of the identified genes was CTGF, which was down-regulated by GSNO.

Down-regulation of CTGF mRNA by GSNO is dose- and time-dependent

To investigate whether the down-regulation of CTGF mRNA by GSNO is time- and dose-dependent, increasing concentrations of GSNO (50 μmol/L, 100 μmol/L, 250 μmol/L, 500 μmol/L and 1 mmol/L) and harvested cells were tested after different time points after stimulation (0.5, 3, 6, 12, 24 and 48 hours). Control MC were treated with an equimolar amount of glutathione sulfhydryl (GSH). The expression of CTGF mRNA decreased with increasing concentrations of GSNO, beginning at 250 μmol/L and reaching a minimum of CTGF transcript levels at the highest tested GSNO concentration of 1 mmol/L (Fig. 1A). GSH alone had no effect on CTGF expression. Furthermore, MC treated with 500 μmol/L GSNO and harvested after different time points showed a transient down-regulation of CTGF mRNA as early as three hours after GSNO-treatment with an expression remaining low after six hours. After 12 hours CTGF mRNA levels had returned to baseline. GSH again did not significantly affect CTGF-expression (Fig. 1B). The data were confirmed in four independent experiments.

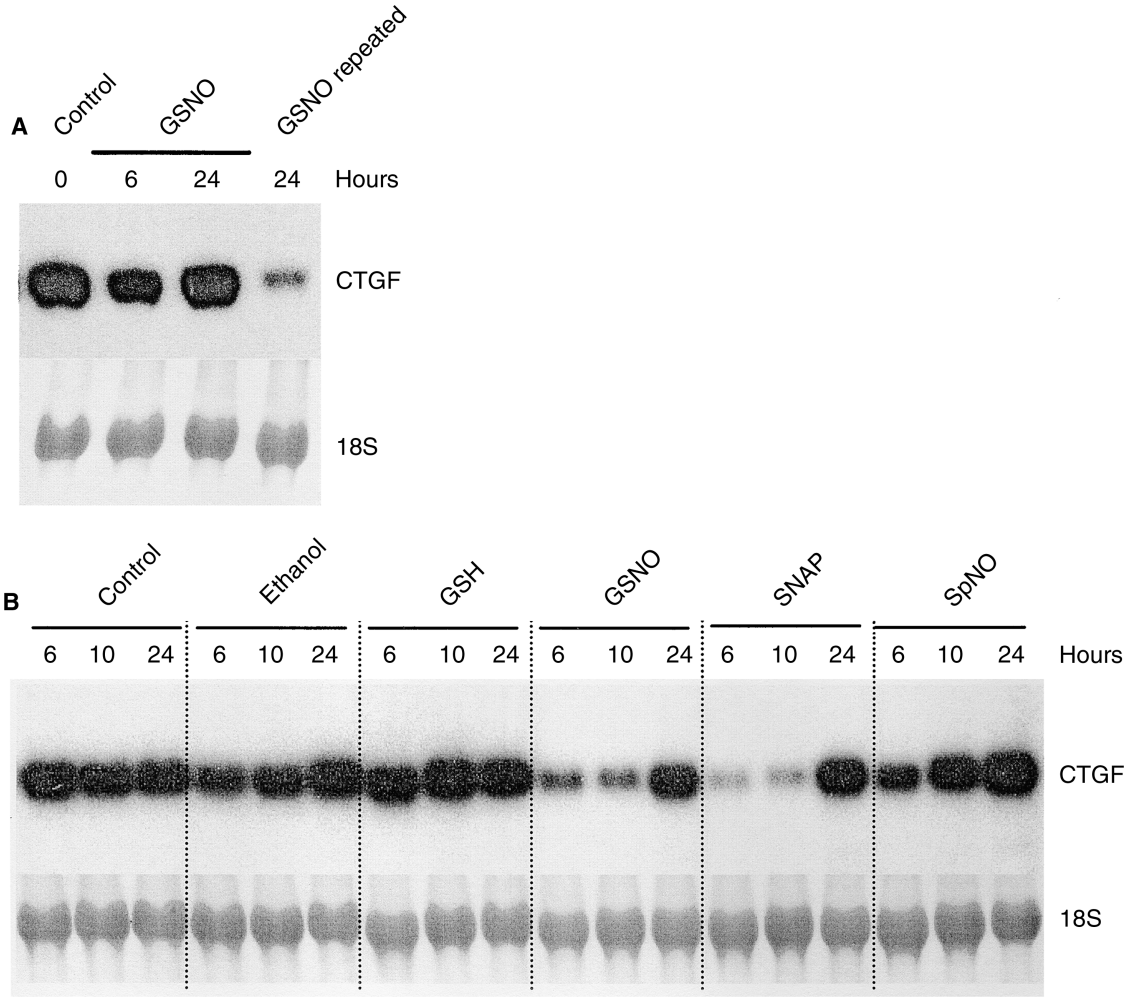

Fig. 2. Repeated GSNO-treatment leads to prolonged down-regulation of CTGF mRNA and CTGF mRNA is down-regulated by different NO-donors. (*A*) MC were grown in medium containing 10% FCS until subconfluency and not treated (lane 1), treated once (lanes 2 and 3) or repeatedly every 9 hours (lane 4) with 500 μmol/L GSNO for 6 or 24 hours. MC were grown in medium containing 10% FCS until subconfluency and treated with 500 μmol/L GSNO, 500 μmol/L SNAP or 250 μmol/L SpNO, respectively, for 6, 10, or 24 hours. Control cells were not treated, treated with 500 μmol/L GSH or with ethanol, the solvent for SNAP (*B*). RNA was extracted and 10 μg total RNA per lane was size fractionated on a 1% agarose gel. Blots were stained with methylene blue to control for equal loading and probed with a cDNA for CTGF radiolabeled by random priming. Abbreviations are in the **Appendix**.

Since CTGF expression had returned to control levels after 12 hours of GSNO stimulation, we tested whether this was due to subsiding NO release from GSNO by repeatedly adding GSNO to cultured rat MC. Repeated addition of GSNO at 0, 9 and 18 hours led to a prolonged inhibition of CTGF mRNA expression for 24 hours (Fig. 2A). The cell viability was confirmed by trypan blue-exclusion tests. We therefore concluded that the continuous presence of NO leads to sustained down-regulation of CTGF.

To test whether the effect was specific for GSNO, the question of whether other NO-donors like spermine-NO (SpNO) or SNAP also would affect CTGF expression was addressed. MC were treated with 500 μmol/L of GSNO and SNAP and with 250 μmol/L of SpNO for 6, 10 and 24 hours. As controls MC were treated with the appropriate amount of GSH, with the appropriate volume of ethanol, the dissolving agent of SNAP or left untreated. Northern blot analysis showed that all three NO donors were able to down-regulate CTGF mRNA (Fig. 2B). However, there were differences in the kinetics of down-regulation, most likely reflecting differences in the kinetics of NO release from the different NO donors.

Induction of mesangial cell iNOS leads to down-regulation of CTGF mRNA

Since external application of NO donors is a very artificial situation, we investigated whether NO release by induction of mesangial iNOS could down-regulate CTGF mRNA. MC were stimulated with 25 ng/mL TNF-α, 250

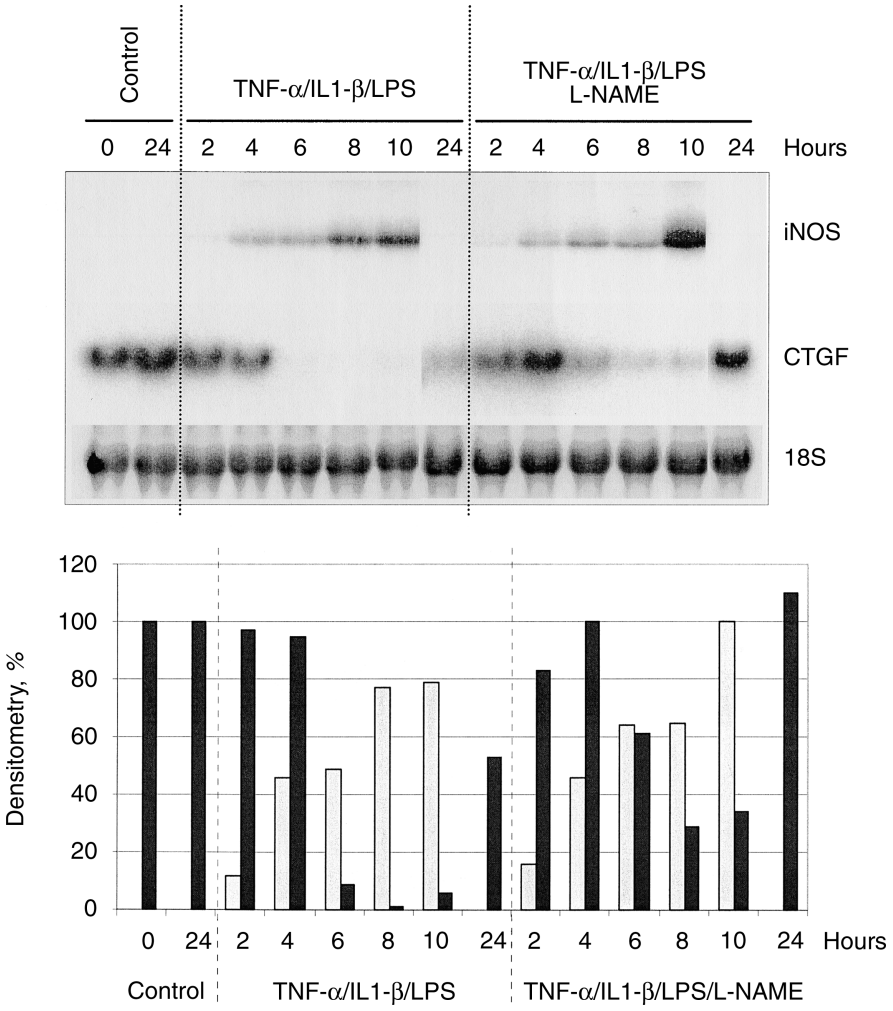

Fig. 3. Induction of iNOS leads to down-regulation of CTGF mRNA. MC were grown in medium containing 10% FCS until subconfluency, serum starved for 48 hours in medium containing 0.4% FCS and treated with 25 ng/mL TNF-α, 250 U/mL IL-1β and 10 μg/mL LPS for 2, 4, 6, 8, 10 and 24 hours (lanes 3 to 14). L-NAME 1 mmol/L was added for the same time intervals to inhibit the induced NO-production (lanes 9 to 14) and in part attenuated the down-regulation of CTGF mRNA. Serum-starved control cells were not treated and harvested after 0 and 24 hours (lanes 1 and 2). RNA was extracted and 10 μg total RNA per lane was size fractionated on a 1% agarose gel. Blots were stained with methylene blue to control for equal loading and probed with cDNAs for iNOS (▒) and CTGF (■) radiolabeled by random priming.

U/mL IL-1β and 10 μg/mL LPS, a mixture that has been shown to induce iNOS [33], for 2, 4, 6, 8, 10 and 24 hours. To verify that the observed effects were due to the release of NO and not to direct cytokine actions, MC were additionally treated with 1 mmol/L L-NAME, a structural analog of L-arginine, which inhibits or impairs the NO production by NOS. Stimulation of MC with TNF-α, IL-1β and LPS increased iNOS mRNA expression; the effect was first evident after two hours and maximum induction was seen after 10 hours. Accordingly, down-regulation of CTGF mRNA was observed beginning four to six hours after stimulation and reaching a maximum between six and ten hours. Inhibition of NO production by L-NAME led to a significant, but not complete attenuation of CTGF down-regulation (Fig. 3). Since L-NAME is a competitor to L-arginine and not a direct NOS inhibitor, a complete inhibition of the observed NO effect was not expected.

Effect of NO on CTGF gene regulation is mediated by cGMP

Nitric oxide activates the soluble form of guanylyl cyclase to generate cGMP. We therefore tested whether treatment of MC with the stable cGMP-analog 8br-cGMP (1 mmol/L) was able to down-regulate CTGF mRNA. Northern blot analysis showed that 8br-cGMP had comparable effects on CTGF mRNA expression as GSNO (Fig. 4A). Additionally, the inhibition of the guanylyl cyclase, which leads to a suppression of cGMP production by this enzyme, abolished the effect of GSNO on CTGF gene regulation. MC were treated with 5 μmol/L of the guanylyl cyclase inhibitor NS2028 six hours prior to the addition of 500 μmol/L GSNO for a further six hours. As shown by Northern blot analysis, blocking of cGMP generation could overcome the down-regulating effect of GSNO on CTGF mRNA expression (Fig. 4B). This suggests that the observed effects of GSNO on CTGF are mediated by generation of cGMP.

CTGF protein in the supernatant of MC is down-regulated after treatment with GSNO

To investigate whether the observed effect of NO on CTGF mRNA expression could be seen at the protein level, Western blot analysis was performed (Fig. 5). CTGF is a secreted protein that binds to the extracellular

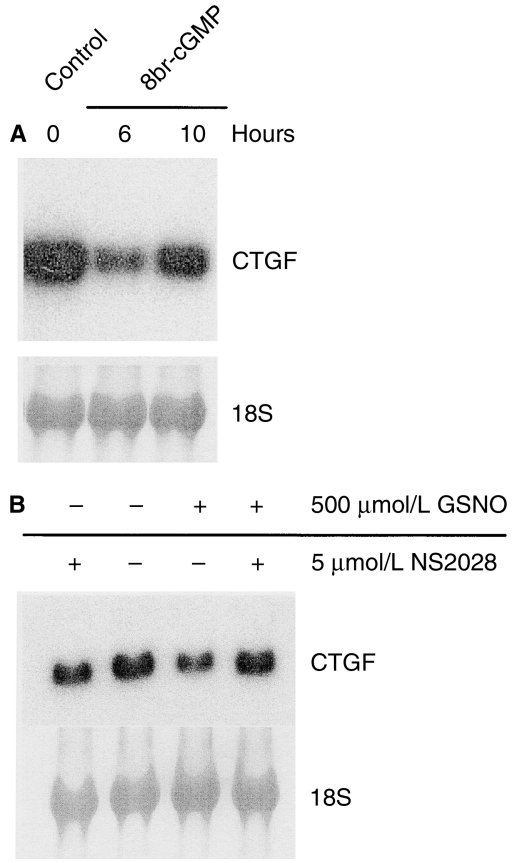

Fig. 4. The down-regulation of CTGF mRNA is mediated by cGMP. MC were grown in medium containing 10% FCS until subconfluency and treated with 1 mmol/L 8br-cGMP for 6 and 10 hours (*A*), or with 5 μmol/L of the guanylyl cyclase inhibitor NS2028 for 6 hours followed by the addition of 500 μmol/L GSNO for 6 hours (*B*). Control cells were treated with an equal amount of the inhibitor NS2028 alone or left untreated. RNA was extracted and 10 μg total RNA per lane was size fractionated on a 1% agarose gel. Blots were stained with methylene blue to control for equal loading and probed with cDNA for CTGF radiolabeled by random priming.

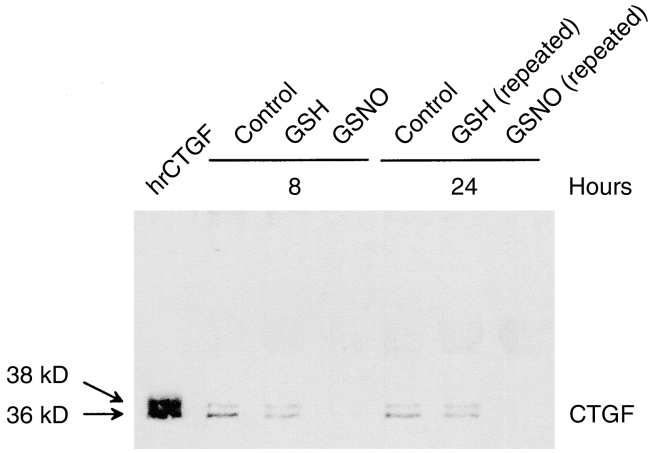

Fig. 5. CTGF protein is down-regulated by GSNO. MC were grown in medium containing 10% FCS and 5 U/mL heparin until subconfluency and treated with 500 μmol/L GSNO once for 8 hours and twice at 0 and 12 hours, for a total of 24 hours. Control cells were not treated or treated with 500 μmol/L GSH in the same manner. Proteins in the supernatants were harvested and heparin-binding proteins were extracted with heparin-sepharose CL-6B beads. Bound proteins were eluted and resolved on an 8% SDS polyacrylamide gel. Blots were stained with chicken anti-hCTGF antibody, followed by HRPO-conjugated rabbit anti-chicken antibody. Human recombinant CTGF (hrCTGF) was loaded (lane 1) to confirm the specific signal for CTGF obtained with the chicken anti-hCTGF antibody.

matrix, but is also found in the culture medium. Although the half-life of the protein is not exactly known, it seems to be short, complicating the detection of the protein. Since heparin is able to stabilize CTGF protein, it was added to the culture medium. MC were treated once with 500 μmol/L GSNO for eight hours or repeatedly at 0 and 12 hours for 24 hours, and supernatants were collected. CTGF protein was purified from equal amounts of supernatants by heparin sepharose beads. Heparin bound protein was eluted by boiling in SDS sample buffer, resolved on SDS-PAGE, and Western blot analysis was performed. Strong down-regulation of CTGF protein was evident after eight hours as well as after 24 hours of treatment with GSNO.

DISCUSSION

Mesangial cells are a source of as well as the main target cells of NO. They are key cells in glomerular inflammatory processes. In addition to their capacity of antigen presentation, phagocytosis, clearance of macromolecules and regulation of glomerular filtration, MC are able to proliferate and to produce and deposit extracellular matrix molecules, which can lead to sclerosis and fibrosis [34]. In vitro studies have shown that NO inhibits MC proliferation and reduces matrix production and adhesion [10, 12, 14]. Since it is known that NO, in addition to exerting regulatory functions on glomerular hemodynamics, can lead to direct alteration of gene expression, we tried to identify the genes regulated by NO in glomerular MC. For this purpose, the RDA method was employed, which is a PCR-based subtractive hybridization method [15].

A transient down-regulation of CTGF was observed by GSNO with levels coming back to baseline by 12 to 24 hours, whereas GSH had no influence on gene expression. This transient down-regulation most likely is due to the release kinetic of GSNO. Since NO itself is an unstable radical with a short half-life of a few seconds, the duration of the NO effect elicited by a certain NO-donor depends on the release kinetics of the specific substance. These release kinetics of NO-donors are dependent on various conditions such as pH, temperature, buffer composition and others. Moreover, half-lives vary with the method used for their analysis. Taking these restrictions into account, the half-life of GSNO is estimated to be approximately ten hours [35]. To test whether the short half-life of GSNO is responsible for

the observed transient down-regulation of CTGF, we repeatedly added GSNO to the cultured MC. As expected, down-regulation of CTGF was maintained for more than 24 hours, whereas the expression of CTGF after a single administration of GSNO had returned to baseline by 12 to 24 hours. MC could not be stimulated for prolonged periods of time, since apoptotic and toxic events occurred after 48 hours. To confirm that our cells were still alive after 24 hours of repeated GSNO-treatment, a trypan blue-exclusion test was performed and untreated cells were compared with stimulated cells. No difference between the repeatedly treated and untreated cells was observed. The NO-donors Sp-NO and SNAP had similar effects on CTGF expression as GSNO, which indicates that the down-regulation is not only GSNO-mediated, but is indeed NO-mediated. SNAP showed an even stronger down-regulation of CTGF mRNA with a similar time course as GSNO. The observed shorter down-regulation of CTGF by Sp-NO than that seen with GSNO or SNAP is in agreement with its faster release kinetic (as compared to GSNO and SNAP).

Since exogenous addition of NO is a very artificial situation, we also tested whether induction of endogenous MC iNOS would be sufficient to reproduce the inhibitory effects of NO (donors) on CTGF mRNA by stimulating MC with TNF-α, IL-1β and LPS. A strong induction of iNOS was detected by these cytokines combined with bacterial endotoxin and concomitant down-regulation of CTGF mRNA was seen. We observed a time lag of only two hours between the first appearance of iNOS mRNA and the decrease of CTGF mRNA. To prove that the down-regulation of CTGF was indeed a consequence of increase in NO-production by iNOS and not a direct effect of the cytokine mix, the competitive NOS-inhibitor L-NAME was included in a control experiment. The down-regulation of CTGF was significantly attenuated, although not entirely prevented. Because L-NAME is only a competitor to the substrate L-arginine and not a direct inhibitor of the enzyme, a high excess of competitor would be necessary to inhibit NO-production completely. To achieve this end, 1 mmol/L L-NAME did not seem to be enough; however, higher concentrations of L-NAME would change the pH of the culture medium and thus influence the experimental conditions by itself. Other groups reported similar competition rates when using L-NAME as an inhibitor. Dulak et al stimulated the iNOS of vascular smooth muscle cells with IL-1β and showed an up-regulation of vascular endothelial growth factor (VEGF) by NO. Additional supplementation with 2 to 5 mmol/L L-NAME decreased the IL-1β up-regulated VEGF synthesis by only 30 to 40% [36]. Gavin et al, who studied the VEGF mRNA increase in rat skeletal muscle in response to a single acute exercise bout, could attenuate this exercise-induced increase in VEGF mRNA by approximately 50% by treatment with 30 and 300 mg/kg L-NAME [37]. The incomplete L-NAME-mediated competition of CTGF–down-regulation after generation of the endogenous NO also could be explained by additional effects of the cytokine mix other than stimulation of iNOS.

Some actions of NO are mediated by generation of cGMP. Addition of the stable cGMP analog 8br-cGMP led to a comparable down-regulation of CTGF mRNA after six hours as the addition of NO-donors, indicating that CTGF down-regulation could be accounted for by an increase in cellular cGMP-concentration. To confirm these data, we blocked the generation of cellular cGMP by NS2028, an inhibitor of the guanylyl cyclase. NS2028 alone led to a small decrease of CTGF mRNA level, but as expected, the down-regulating effect of GSNO on CTGF expression was nearly completely abolished by the inhibition of the guanylyl cyclase, validating that the cGMP signaling pathway is used for the down-regulation of CTGF.

We demonstrated that GSNO not only down-regulates CTGF mRNA, but also CTGF protein. As described in the literature, the protein detected in our culture supernatant as well as the recombinant human CTGF separated at two bands of around 36 and 38 kD [18]. Both molecular weight forms of CTGF followed the same expression pattern and showed the same down-regulation after GSNO treatment. CTGF protein levels were significantly decreased by GSNO, rendering NO one of the strongest inhibitors of CTGF expression. For a better detection of the protein in some experiments our culture medium was supplemented with heparin, because heparin binds to CTGF and stabilizes the protein [20]. Heparin is known to be anti-mitogenic [38], but the concentration used was very low and had no evident antiproliferative effect on MC. Furthermore, we checked whether heparin itself could have an influence on CTGF mRNA expression in the absence and presence of GSNO. There was no difference in the expression pattern of CTGF with and without heparin before and after GSNO stimulation (data not shown).

Taken together, our findings identify NO as a potent inhibitor of CTGF-expression in MC. CTGF expression is increased by various stimuli (TGF-β1, TGF-β2, TGF-α, BMP2, EGF, dexamethasone, high glucose, conditioned monocyte medium, and mechanical strain), but to date only two inhibitors of CTGF expression have been identified: TNF-α and cAMP [18]. The inducing effect of dexamethasone on CTGF expression in mouse fibroblasts was attenuated by TNF-α [39]. TNF-α also has been shown to down-regulate CTGF-expression in bovine endothelial cells, fibroblasts and smooth muscle cells [40]. Interestingly, TNF-α can induce iNOS expression, and therefore it is possible that the observed CTGF down-regulation by TNF-α is mediated at least in part by the induction of an iNOS and subsequent generation

of NO. Another inhibitory effect has been described in normal rat kidney fibroblasts, where raised cAMP levels or cAMP analogs inhibited the induction of CTGF by TGF-β [41]. In this context it should be mentioned again that our findings indicate the ability of cGMP, which is raised after NO generation, to down-regulate CTGF mRNA in MC.

Nitric oxide is able to regulate not only CTGF, but also various other genes, many of which are compounds of the extracellular matrix or are associated with it. In dermal fibroblasts it was shown that the NO-donor sodium nitroprusside (SNP) suppresses collagen production [42]. This inhibition was dependent on the generation of cGMP. Chronic inhibition of NOS by L-NAME in mice induces an early activation of the collagen I gene in afferent arterioles and glomeruli, and leads to deposition of collagen I and the development of renal vascular fibrosis [43], demonstrating the antifibrotic properties of NO. A marked increase in both collagen types I and III was shown in vascular smooth muscle cells (VSMC) after inhibition of NO production [44]. In this context it should be mentioned that the TGF-β induced collagen synthesis is mediated by CTGF [41]. Not only collagen types I and III, but also fibronectin steady-state mRNA levels were reduced by NO in fibroblasts [45]. In MC the synthesis of fibronectin was shown to be suppressed by NO via generation of cGMP [46]. The secreted protein acidic and rich in cysteine (SPARC), acting as an autocrine and paracrine inhibitor of PDGF-mediated proliferation in glomerular MC [47], is also extracellular matrix-associated and was shown to be down-regulated by NO [48]. Taken together, these findings suggest that NO can exhibit antifibrotic actions by suppressing proteins of the extracellular matrix. Our finding that NO also suppresses CTGF, a central regulatory molecule in the pathogenesis of fibrotic diseases, further supports this antifibrotic potential of NO.

Connective tissue growth factor has been detected in many human tissues and in a variety of human biological fluids [49], and overexpression of CTGF has been explored in various human diseases and animal models [50, 51]. Increased expression of CTGF has been described in scleroderma, inflammatory bowel diseases, lung fibrosis and in the fibrous cap of atherosclerotic lesions [25, 49]. In the human kidney increased CTGF expression was found in mesangioproliferative and extracapillary lesions of rapidly progressive glomerulonephritides, IgA nephropathy, segmental glomerulosclerosis and diabetic nephropathy [26]. Interstitial CTGF overexpression was mainly found in regions with chronic interstitial damages [26]. CTGF was shown to be overexpressed in animal models of renal fibrosis like the streptozotocin-induced diabetes mellitus, anti-Thy-1.1-nephritis and 5/6-nephrectomy [17, 27, 28].

A common feature of all these diseases is the formation of fibrosis and sclerosis, where CTGF seems to play a key role. The ability of NO to down-regulate CTGF suggests that NO could play an important role in preventing or ameliorating fibrotic processes, when delivered to the right place in the appropriate concentration. Our findings could have important implications for understanding the pathogenesis as well as for the treatment of renal fibrosis, where regulated production of NO by intrinsic cells, such as MC, or controlled exogenous delivery of NO could ameliorate or prevent glomerular or interstitial fibrosis, scarring and ultimately loss of function. Moreover, our findings may add to the understanding of other fibrotic diseases, like atherosclerosis, where endothelial dysfunction with a decrease in NO-production could have a direct impact on CTGF-expression and consecutive deposition of fibrotic material.

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APPENDIX

Abbreviations used in this study are: 8br-cGMP, 8-bromo-cyclic guanosine-3′,S-monophosphate; CTGF, connective tissue growth factor; ECL, enhanced chemiluminescence; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; Egr-1, early growth response gene-1; GBM, glomerular basement membrane; GSH, glutathione sulfhydryl; GSNO, S-nitrosoglutathione; HSP, horseradish peroxidase; HUVECs, human umbilical vein endothelial cells; ICAM, intracellular adhesion molecule; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NMMA, N^{G}-monomethyl-L-arginine; LPS, lipopolysaccharide; MC, mesangial cells; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonyl fluoride; RDA, representational difference analysis; SNAP, S-nitroso-N-acetyl-D,L-penicillamine; SNP, sodium nitroprusside; SPARC, secreted protein acidic and rich in cysteine; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells.

REFERENCES

1. Bachmann S, Bosse HM, Mundel P: Topography of nitric oxide synthesis by localizing constitutive NO synthases in mammalian kidney. *Am J Physiol* 268:F885–F898, 1995
2. Romagnani P, Pupilli C, Lasagni L, *et al*: Inducible nitric oxide synthase expression in vascular and glomerular structures of human chronic allograft nephropathy. *J Pathol* 187:345–350, 1999
3. Furusu A, Miyazaki M, Abe K, *et al*: Expression of endothelial and inducible nitric oxide synthase in human glomerulonephritis. *Kidney Int* 53:1760–1768, 1998
4. Baylis C, Mitruka B, Deng A: Chronic blockade of nitric oxide synthesis in the rat produces systemic hypertension and glomerular damage. *J Clin Invest* 90:278–281, 1992

5. Ribeiro MO, Antunes E, De Nucci G, *et al*: Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. *Hypertension* 20:298–303, 1992
6. Weinberg JB, Granger DL, Pisetsky DS, *et al*: The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: Increased nitric oxide production and nitric oxide synthase expression in MRL-*lpr/lpr* mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered N^G-monomethyl-L-arginine. *J Exp Med* 179:651–660, 1994
7. Ferrario R, Takahashi K, Fogo A, *et al*: Consequences of acute nitric oxide synthesis inhibition in experimental glomerulonephritis. *J Am Soc Nephrol* 4:1847–1854, 1994
8. Reyes AA, Purkerson ML, Karl I, Klahr S: Dietary supplementation with L-arginine ameliorates the progression of renal disease in rats with subtotal nephrectomy. *Am J Kidney Dis* 20:168–176, 1992
9. Raij L, Shultz PJ: Endothelium-derived relaxing factor, nitric oxide: Effects on and production by mesangial cells and the glomerulus. *J Am Soc Nephrol* 3:1435–1441, 1993
10. Rupprecht HD, Akagi Y, Keil A, Hofer G: Nitric oxide inhibits growth of glomerular mesangial cells: Role of the transcription factor EGR-1. *Kidney Int* 57:70–82, 2000
11. Garg UC, Hassid A: Inhibition of rat mesangial cell mitogenesis by nitric oxide-generating vasodilators. *Am J Physiol* 257:F60–F66, 1989
12. Yao J, Schoecklmann HO, Prols F, *et al*: Exogenous nitric oxide inhibits mesangial cell adhesion to extracellular matrix components. *Kidney Int* 53:598–608, 1998
13. Ikeda M, Ikeda U, Takahashi M, *et al*: Nitric oxide inhibits intracellular adhesion molecule-1 expression in rat mesangial cells. *J Am Soc Nephrol* 7:2213–2218, 1996
14. Trachtman H, Futterweit S, Singhal P: Nitric oxide modulates the synthesis of extracellular matrix proteins in cultured rat mesangial cells. *Biochem Biophys Res Commun* 207:120–125, 1995
15. Hubank M, Schatz DG: Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res* 22:5640–5648, 1994
16. Brigstock DR: The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocr Rev* 20: 189–206, 1999
17. Clarkson MR, Gupta S, Murphy M, *et al*: Connective tissue growth factor: A potential stimulus for glomerulosclerosis and tubulointerstitial fibrosis in progressive renal disease. *Curr Opin Nephrol Hypertens* 8:543–548, 1999
18. Gupta S, Clarkson MR, Duggan J, Brady HR: Connective tissue growth factor: Potential role in glomerulosclerosis and tubulointerstitial fibrosis. *Kidney Int* 58:1389–1399, 2000
19. Bradham DM, Igarashi A, Potter RL, Grotendorst GR: Connective tissue growth factor: A cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol* 114:1285–1294, 1991
20. Lau LF, Lam SC: The CCN family of angiogenic regulators: The integrin connection. *Exp Cell Res* 248:44–57, 1999
21. Frazier K, Williams S, Kothapalli D, *et al*: Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J Invest Dermatol* 107:404–411, 1996
22. Igarashi A, Okochi H, Bradham DM, Grotendorst GR: Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol Biol Cell* 4:637–645, 1993
23. Shimo T, Nakanishi T, Nishida T, *et al*: Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells in vitro, and angiogenesis in vivo. *J Biochem (Tokyo)* 126:137–145, 1999
24. Nakanishi T, Nishida T, Shimo T, *et al*: Effects of CTGF/Hcs24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. *Endocrinology* 141:264–273, 2000
25. Clarkson MR, Gupta S, Murphy M, *et al*: Connective tissue growth factor: A potential stimulus for glomerulosclerosis and tubulointerstitial fibrosis in progressive renal disease. *Curr Opin Nephrol Hypertens* 8:543–548, 1999
26. Ito Y, Aten J, Bende RJ, *et al*: Expression of connective tissue growth factor in human renal fibrosis. *Kidney Int* 53:853–861, 1998
27. Riser BL, Denichilo M, Cortes P, *et al:* Regulation of connective tissue growth factor activity in cultured rat mesangial cells and its expression in experimental diabetic glomerulosclerosis. *J Am Soc Nephrol* 11:25–38, 2000
28. Ito Y, Goldschmeding R, Bende RJ, et al: Kinetics of connective tissue growth factor expression during experimental proliferative glomerulonephritis. *J Am Soc Nephrol* 12:472–484, 2001
29. Murphy M, Godson C, Cannon S, *et al*: Suppression subtractive hybridization identifies high glucose levels as a stimulus for expression of connective tissue growth factor and other genes in human mesangial cells. *J Biol Chem* 274:5830–5834, 1999
30. Hart TW: Some observations concerning the S-nitroso- and S-phenylsulfonyl derivatives of L-cysteine and glutathione. *Tetrahydron Lett* 26:2013–2016, 1985
31. Rupprecht HD, Dann P, Sukhatme VP, *et al:* Effect of vasoactive agents on induction of Egr-1 in rat mesangial cells: Correlation with mitogenicity. *Am J Physiol* 263:F623–F636, 1992
32. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
33. Pfeilschifter J, Kunz D, Muhl H: Nitric oxide: An inflammatory mediator of glomerular mesangial cells. *Nephron* 64:518–525, 1993
34. Couser WG, Johnson RJ: Mechanisms of progressive renal disease in glomerulonephritis. *Am J Kidney Dis* 23:193–198, 1994
35. Arnelle DR, Stamler JS: NO+, NO, and NO− donation by S-nitrosothiols: Implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. *Arch Biochem Biophys* 318:279–285, 1995
36. Dulak J, Jozkowicz A, Dembinska-Kiec A, *et al:* Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 20: 659–666, 2000
37. Gavin TP, Spector DA, Wagner H, *et al:* Nitric oxide synthase inhibition attenuates the skeletal muscle VEGF mRNA response to exercise. *J Appl Physiol* 88:1192–1198, 2000
38. Wang ZQ, Liang KH, Pahl MV, Vaziri ND: Effect of heparin on mesangial cell growth and gene expression of matrix proteins. *Nephrol Dial Transplant* 13:3052–3057, 1998
39. Dammeier J, Beer HD, Brauchle M, Werner S: Dexamethasone is a novel potent inducer of connective tissue growth factor expression. Implications for glucocorticoid therapy. *J Biol Chem* 273: 18185–18190, 1998
40. Lin J, Liliensiek B, Kanitz M, *et al*: Molecular cloning of genes differentially regulated by TNF-alpha in bovine aortic endothelial cells, fibroblasts and smooth muscle cells. *Cardiovasc Res* 38:802–813, 1998
41. Duncan MR, Frazier KS, Abramson S, *et al*: Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: Down-regulation by cAMP. *FASEB J* 13:1774–1786, 1999
42. Chu AJ, Prasad JK: Up-regulation by human recombinant transforming growth factor beta-1 of collagen production in cultured dermal fibroblasts is mediated by the inhibition of nitric oxide signaling. *J Am Coll Surg* 188:271–280, 1999
43. Boffa JJ, Tharaux PL, Placier S, *et al:* Angiotensin II activates collagen type I gene in the renal vasculature of transgenic mice during inhibition of nitric oxide synthesis: Evidence for an endothelin-mediated mechanism. *Circulation* 100:1901–1908, 1999
44. Myers PR, Tanner MA: Vascular endothelial cell regulation of extracellular matrix collagen: Role of nitric oxide. *Arterioscler Thromb Vasc Biol* 18:717–722, 1998
45. Kim NN, Villegas S, Summerour SR, Villarreal FJ: Regulation of cardiac fibroblast extracellular matrix production by bradykinin and nitric oxide. *J Mol Cell Cardiol* 31:457–466, 1999
46. Studer RK, DeRubertis FR, Craven PA: Nitric oxide suppresses increases in mesangial cell protein kinase C, transforming growth factor beta, and fibronectin synthesis induced by thromboxane. *J Am Soc Nephrol* 7:999–1005, 1996
47. Pichler RH, Bassuk JA, Hugo C, *et al*: SPARC is expressed by

mesangial cells in experimental mesangial proliferative nephritis and inhibits platelet-derived-growth-factor-mediated mesangial cell proliferation in vitro. *Am J Pathol* 148:1153–1167, 1996

48. Walpen S, Beck KF, Eberhardt W, *et al:* Downregulation of SPARC expression is mediated by nitric oxide in rat mesangial cells and during endotoxemia in the rat. *J Am Soc Nephrol* 11:468–476, 2000
49. Oemar BS, Werner A, Garnier JM, *et al*: Human connective tissue growth factor is expressed in advanced atherosclerotic lesions. *Circulation* 95:831–839, 1997
50. Yang DH, Kim HS, Wilson EM, *et al*: Identification of glycosylated 38-kDa connective tissue growth factor (IGFBP-related protein 2) and proteolytic fragments in human biological fluids, and up-regulation of IGFBP-rP2 expression by TGF-beta in Hs578T human breast cancer cells. *J Clin Endocrinol Metab* 83:2593–2596, 1998
51. Tamatani T, Kobayashi H, Tezuka K, *et al*: Establishment of the enzyme-linked immunosorbent assay for connective tissue growth factor (CTGF) and its detection in the sera of biliary atresia. *Biochem Biophys Res Commun* 251:748–752, 1998