View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Kidney International, Vol. 57 (2000), pp. 59–69

Nitric oxide modulates expression of matrix metalloproteinase-9 in rat mesangial cells

Wolfgang Eberhardt, Thomas Beeg, Karl-Friedrich Beck, Sebastian Walpen, Stefan Gauer, Hansjosef Böhles, and Josef Pfeilschifter

Zentrum der Pharmakologie, Zentrum der Kinderheilkunde, and Zentrum der Inneren Medizin, Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany

Nitric oxide modulates expression of matrix metalloproteinase-9 in rat mesangial cells.

Background. High-output levels of nitric oxide (NO) are produced by rat mesangial cells (MCs) in response to proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) by the inducible isoform of NO synthase (iNOS). We tested modulatory effects of NO on the expression and activities of matrix metalloproteinases-9 and -2 (MMP-9 and MMP-2), respectively. Temporal and spatial expression of these MMPs and their specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), seems to be critical in the extensive extracellular matrix (ECM) remodeling that accompanies sclerotic processes of the mesangium.

Methods and Results. Using the NO donors S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) and DETA-NONOate, we found strong inhibitory effects of NO mainly on the IL-1β–induced MMP-9 mRNA levels. NO on its own had only weak effects on the expression of MMP-9 and MMP-2. The addition of the NOS inhibitor N^G-monomethyl L-arginine (L-NMMA) dose dependently increased steady-state mRNA levels of cytokine-induced MMP-9, suggesting that endogenously produced NO exerts tonic inhibition of MMP-9 expression. MMP-9 activity in conditioned media from MCs costimulated with IL-1β and NO donor contained less gelatinolytic activity than media of cells treated with IL-1β alone. Exogenously added NO did not alter gelatinolytic activity of MMP-9 in cell-free zymographs. The expression levels of TIMP-1 were affected by NO similarly to the expression of MMP-9.

Conclusion. We conclude that NO modulates cytokine-mediated expression of MMP-9 and TIMP-1 in rat MCs in culture. Our results provide evidence that NO-mediated attenuation of MMP-9 gelatinolytic activity is primarily due to a reduced expression of MMP-9 mRNA, and not the result of direct inhibition of enzymatic activity.

Deposition of extracellular matrix (ECM) in the mesangium represents a tightly regulated balance between synthesis and degradative processes of matrix proteins predominantly regulated through the action of matrix metalloproteinases (MMPs). These neutral proteases belong to a unique family of metal-dependent enzymes that have in common the ability to degrade specifically components of connective tissue matrices, including collagens, laminins, gelatin, and proteoglycans.

Although MMPs are important participants in many physiologic processes such as wound healing, angiogenesis, or tissue remodeling, their altered expression is associated with severe pathologic conditions, most prominently rheumatoid arthritis [1]. In the kidney, dysregulation of homeostatic ECM turnover is known to cause impairment of glomerular filtration. The accumulation of ECM proteins, for example, is an important feature of many progressive renal diseases such as diabetic nephropathy and glomerulosclerosis [2].

The regulation of MMPs occurs on different levels, including gene expression, processing of the inactive proenzymes by removal of a 10 kD fragment and by inhibition of the active enzymes by their endogenous inhibitors delineated as tissue inhibitors of matrix metalloproteinases (TIMPs). Increased expression of MMPs, particularly MMP-2 and MMP-9, which are collectively denominated as gelatinases, by proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and γ-interferon has been described for different cell types, including articular chondrocytes [3], synovial cells [4], and glomerular mesangial cells (MCs) [5]. MCs have certain macrophage-like properties and crucially contribute to inflammatory processes of the renal glomerulus. Upon stimulation, MCs release biologically active substances, particularly IL-1β and TNF-α, cytokines that help to perpetuate the formation of other inflammatory mediators, such as eicosanoids, growth factors, or nitric oxide (NO) [6]. Previously, we have reported that inflammatory cytokines induce inducible NO synthase (iNOS) in glomerular MCs mainly on the transcriptional level [7–9]. Furthermore, recent evidence has been obtained that NO itself can modulate gene expression of inflammatory mediators such as IL-8, macrophage-

Key words: cytokines, inflammation, glomerular mesangial cells, interleukin-1β, tissue inhibitor.

Received for publication February 9, 1999
and in revised form June 2, 1999
Accepted for publication August 20, 1999

© 2000 by the International Society of Nephrology

induced protein 1α (MIP 1α) [10] and iNOS [11]. In this study, we have investigated the possible modulatory effects of NO on cytokine-induced gelatinase expression and the corresponding enzyme activities in rat MCs.

METHODS

Cell culture

Rat glomerular MCs were cultured as described previously [12]. MCs were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol glutamin, 5 ng/mL insulin, 100 U/mL penicillin, and 100 μg/mL streptomycin. Serum-free preincubations were performed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1 mg/mL of fatty acid-free bovine serum albumin for 24 hours before cytokine treatment. For the experiments, 3.0 to 5.0 $\times$ 10^6 cells per 10 cm culture dish were used. MCs were used between passages 8 and 19. All supplements were purchased from GIBCO/BRL, Life Technologies (Eggenstein, Germany). The NO donors S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) and DETA NONOate and the NOS inhibitor N^G-monomethyl L-arginine (L-NMMA) were from Alexis Biochemicals (Grünberg, Germany). N^2, 2′-O-Dibutyryl-guanosine3′:5′-cyclic monophosphate was from Sigma-Aldrich (Deisenhofen, Germany). The determination of cell numbers was done by the use of a Neubauer chamber. The amount of dead cells was determined by trypan-blue exclusion.

Cell cytotoxicity was measured by the use of a Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). For determination of the gelatinolytic activity of cellular supernatants, 1.0 to 1.5 $\times$ 10^6 MCs were grown on six-well plates, and experiments were performed in triplicates.

Nitrite measurements in mesangial cell supernatants

Nitrite contents of cellular supernatants were measured as a readout for NOS activity. Confluent MCs (1.0 to 1.5 $\times$ 10^6 cells) on six-well plates were incubated in DMEM without FCS and were stimulated with or without agents for the indicated time periods. After the incubations, 100 μL of supernatants were mixed with 100 μL of Griess reagent (Merck, Darmstadt, Germany). The absorbance at 540 nm with a reference wavelength at 595 nm was measured, and nitrite concentration was determined using a calibration curve with sodium nitrite standards.

cDNA clones and plasmids

A cDNA insert of 0.7 kb for rat 92 kD type IV collagenase was generated by reverse transcription from mRNA of MCs stimulated with IL-1β using internal primers from the complete sequence of rat mRNA (deposited in the Genbank/EMBL Databases under accession No. U36476). The following primers were used: 5′-CTT AGA TCA TTC TTC AGT GCC-3′ (sense) and 5-′GAT CCA CCT TCT GAG ACT TCA-3′ (antisense). The blunt-ended polymerase chain reaction (PCR) fragment was cloned into the EcoRV cloning site of pBluescript-II KS^+ (Stratagene GmbH, Heidelberg, Germany) to generate "pKS-MMP-9 rat." A cDNA insert of 1.2 kb of rat 72 kD type collagenase was generated using internal primers from the complete sequence of rat MMP-2 mRNA (accession No. X71466) and cloned into EcoRV-cut pBluescript-II KS^+ to generate "pKS-MMP-2 rat." The following primers were used: 5′-ATT ATC CCA TGA TGA CAT CAA-3′ (sense) and 5′-AGC AGC CCA GCC AGT CTG AT-3′ (antisense). A 0.52 kb cDNA insert for rat TIMP-1 was generated using internal primers of a partial coding sequence of TIMP-1 mRNA (accession No. L29512). The primers were 5′-CAG ACG GCG TTC TGC AAC TCG (sense) and 5′-AGA CCC AAG GGA TTG CCA GGT-3′ (antisense). PCR fragments were then cloned into EcoRV-cut pBluescript-II KS^+ to generate "pKS-TIMP-1 rat."

Northern blot analysis

Total cellular RNA was extracted from MCs using the Trizol reagent (GIBCO/BRL, Life Technologies). After ultraviolet cross-linking, 20 μg of total RNA were successively hybridized to the ^{32}P-labeled SacI/KpnI-digested cDNA clones described earlier in this article, thereby liberating inserts of corresponding fragment sizes. cDNA probes (2 $\times$ 10^6 cpm/mL) were radioactively labeled with α^{32}PdCTP (Amersham Buchler GmbH & CoKG, Braunschweig, Germany) by random priming. To correct for variations in RNA amounts, blots were finally rehybridized with a ^{32}P-labeled SacI/KpnI insert from the GAPDH cDNA clone. Membranes used for RNA transfers were obtained from NEN™ Life Science Products (Boston, MA, USA). Northern blots were maximally stripped and rehybridized three times. Specific signals were quantitated densitometrically using an automated detector system BAS 1500 from Fujifilm (Raytest, Straubenhardt, Germany).

SDS-PAGE zymography

Gelatinolytic activity of proteins from cellular supernatants was assessed by electrophoresis in the presence of sodium dodecyl sulfate (SDS) in 10% polyacrylamide gel electrophoresis (PAGE) containing 0.1% gelatin B (Sigma-Aldrich). To ensure that the supernatants were derived from equal cell numbers, for each experimental condition, cell counts were obtained separately. In general, 10 to 20 μL of culture media were loaded on gels directly after the addition of 2 $\times$ sample buffer containing 4% SDS, 0.005% bromphenol blue, and 20% glycerol. Denaturated proteins were renatured by exchanging SDS with Triton in two 15-minute incubations

with 2.5% Triton X-100 after gel runs. The gels were subsequently incubated overnight at 37°C in developing buffer containing 0.2 mol NaCl, 5 mmol $CaCl_2$, and 0.02% Brij 35 (Sigma). At the end of incubations, gels were stained with staining solution containing 30% methanol, 10% acetic acid, and 0.5% Coomassie G250 (Sigma-Aldrich). Destaining was performed in the same solution without Coomassie for several hours. Proteins with gelatinolytic activity were visualized as areas of lytic activity on an otherwise blue gel. Migration properties of proteins were determined by comparison with that of a prestained, full-range rainbow protein marker (Amersham). Photographs of the gels were scanned by an imaging densitometer system from Bio-Rad Laboratories (München, Germany). Metal dependence of gelatinolytic activities was confirmed by incubation of duplicate gels in the presence of 0.01 mol/L ethylenediaminetetraacetic acid (EDTA) in the developing buffer, which resulted in the disappearance of lytic bands.

Cell-free incubation experiments

In these experiments, the effects of exogenously given NO on MMP activities were tested in the conditioned culture medium harvested from untreated or IL-1β–stimulated MCs, respectively. Incubations were carried out in a total volume of 100 μL using final concentrations of NO donors between 250 and 500 μmol/L. At the end of each incubation, samples were mixed with 2 × sample buffer and were loaded on gels for SDS-PAGE zymography, as described earlier in this article.

Statistical analysis

Results are expressed as means ± SD. The data are presented as x-fold induction compared with control conditions or compared with IL-1β–stimulated values (N). Statistical analysis was performed using the Student's t-test. P values < 0.05 or < 0.01 were considered significant.

RESULTS

Effects of exogenous nitric oxide on mRNA levels of metalloproteinases-9 and -2

The family of MMPs includes the two genetically distinct 92 and 72 kD type IV collagenases MMP-9 and MMP-2, respectively, which are expressed in MCs [13–15]. IL-1β strongly induced MMP-9 mRNA level in MCs with a maximal accumulation observed between 36 and 48 hours of stimulation (Fig. 1A), thus confirming previous observations [15]. The data for the 36-hour coincubation of cells with IL-1β and NO donor are shown in more detail in Figure 1B.

Using the NO donor SNAP (500 μmol/L) in time kinetic experiments, we found that the NO donor on its own had no discernible effect on basal MMP-9 mRNA levels (Fig. 1 A, B). However, when given in combination with IL-1β, we observed a strong inhibitory effect of SNAP on the steady-state level of MMP-9 mRNA (6.9-fold induction with IL-1β vs. 1.9-fold induction after 36 hours of coincubation with IL-1β and SNAP; Fig. 1 A, B). Similar results were obtained with the NO-donor DETA NONOate (data not shown).

Although both type IV collagenases have similar substrate specificities, their expression pattern can markedly differ. Therefore, we determined in parallel steady-state mRNA levels using a MMP-2–specific cDNA probe. Compared with MMP-9, MMP-2 shows a higher constitutive expression as we found a corresponding band already under nonstimulated conditions (Fig. 1A). Although the treatment with IL-1β led to an increased accumulation of MMP-2 mRNA (1.7 ± 0.2-fold induction at 36 hours; mean ± SD, $N = 4$), it was less pronounced than the increase in MMP-9 mRNA, suggesting that MMP-2 expression is close to a maximum already under control conditions. A high level of constitutive expression of MMP-2 in glomerular mesangial cells has also been reported by Harendza et al [16]. In some experiments, SNAP on its own exerted weak inhibitory effects on basal MMP-2 mRNA levels (0.8 ± 0.3-fold induction; mean ± SD, $N = 4$), which, however, did not reach statistical significance. In contrast, SNAP significantly reduced the IL-1β–caused increase in the MMP-2 mRNA level (1.7 ± 0.2, $N = 4$, without SNAP vs. 0.6 ± 0.4, $N = 4$, in the presence of SNAP; mean ± SD). This inhibitory effect of NO was maximal between 24 and 36 hours of coincubation and was somewhat weaker at later time points (Fig. 1A). Similar results were obtained with DETA NONOate (data not shown).

Exogenous nitric oxide down-regulates the interleukin-1β–induced mRNA level of TIMP-1

We next checked mRNA levels of TIMP-1, an endogenous MMP-9 inhibitor that acts through the binding to the latent proenzyme, thereby preventing its activation [17]. Interestingly, looking at long-term kinetic experiments, we found that TIMP-1 followed a similar expression pattern as MMP-9 (Fig. 1A). IL-1β strongly increased TIMP-1 mRNA levels up to fivefold as compared with the basal expression levels. IL-1β–induced mRNA levels peaked after 36 hours of stimulation (Fig. 1B). Whereas the NO donor on its own caused a moderate 1.8-fold increase of basal TIMP-1 mRNA levels, there was a marked attenuation of TIMP-1 mRNA levels in cytokine-stimulated cells, which was most prominent after 36-hour coincubation with SNAP (Fig. 1B).

To test whether inhibitory effects on cytokine-induced MMP-9 and MMP-2 mRNA levels were paralleled by decreased activities of corresponding enzymes, we performed SDS-PAGE zymography. Assessing equal amounts of conditioned media from the cells used for RNA isolation, we found that gelatinolytic activity of a band migrating

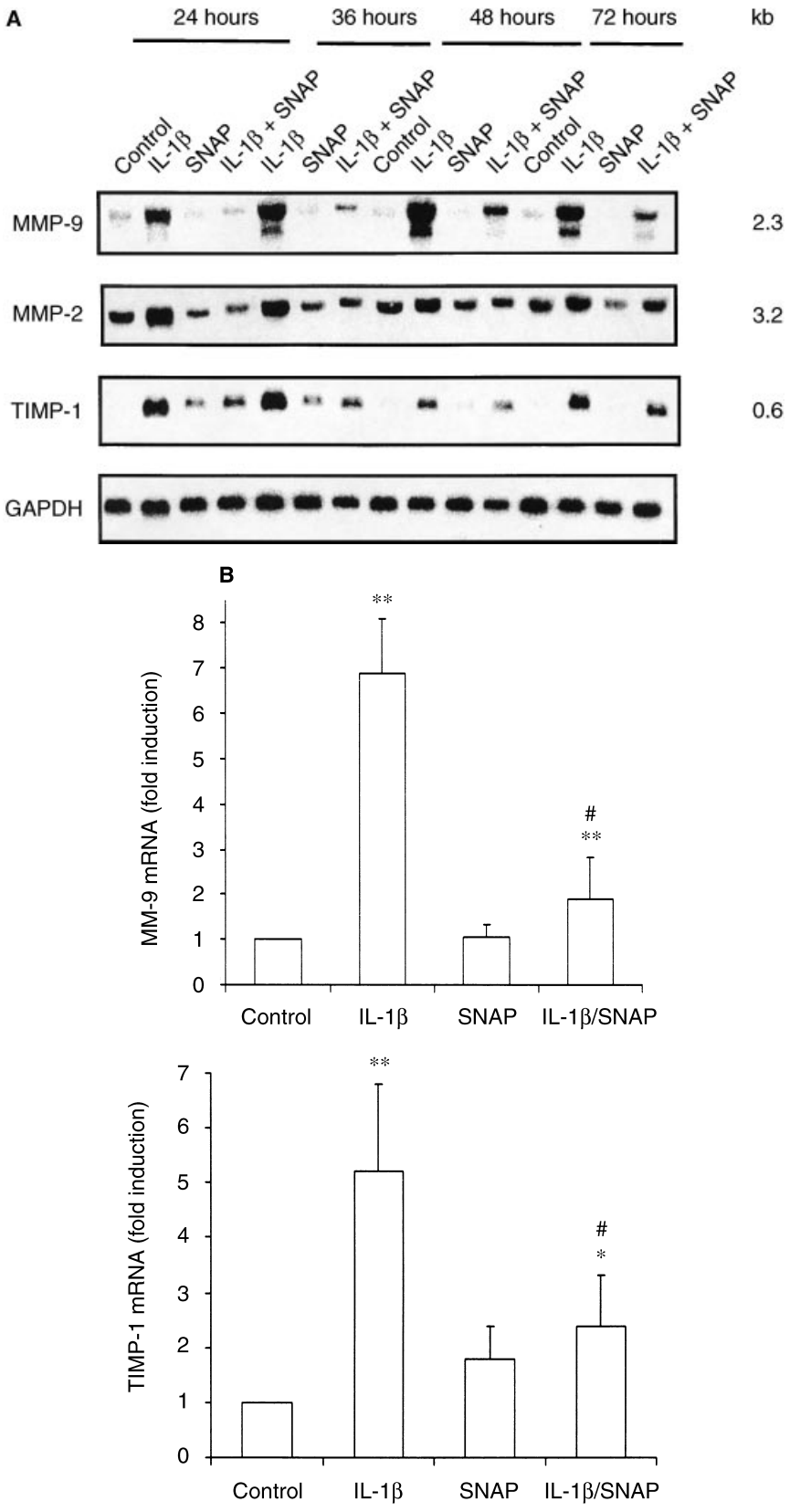


Fig. 1. (*A*) Effects of exogenous nitric oxide (NO) on expression and activity of matrix metalloproteinases (MMPs) in rat mesangial cells. Time course of steady-state level of mRNA of MMP-9, MMP-2, and tissue inhibitor of metalloproteinase-1 (TIMP-1) in mesangial cells (MCs) following treatment with interleukin-1β (IL-1β, 2 nmol/L) with or without S-Nitroso-N-acetyl-D,L-penicillamine (SNAP, 500 μmol/L). Twenty micrograms of total cellular RNA were hybridized successively to a ^{32}P-labeled cDNA inserts from KS-MMP-9, KS-MMP-2, and KS-TIMP-1, respectively. The size of the corresponding mRNA is indicated at the right end of the figure. Equivalence of loading in different lanes was ascertained by rehybridization to a GAPDH probe. Similar results were obtained in two additional independent experiments. (*B*) Statistical analysis of MMP-9 and TIMP-1 mRNA levels after 36 hours of stimulation. Data represent means ± SD ($N = 3$). $^{*}P \leq 0.05$; $^{**}P \leq 0.01$ compared with control or to IL-1β-stimulated conditions (#). (*C*) To test for corresponding lytic activities of gelatinases from the experiment shown in (A), 10 μL of cellular supernatants from corresponding culture plates were assayed by SDS-PAGE zymography. The zymogen is displayed as a negative to clarify lytical intensities of MMP-9. (*D*) Time course induction of nitrite production in supernatants of MCs upon stimulation with IL-1β (2 nmol/L). The results indicate nitrite levels after the indicated time periods and are expressed as means ± SD of four experiments. $^{**}P \leq 0.01$ compared with control.

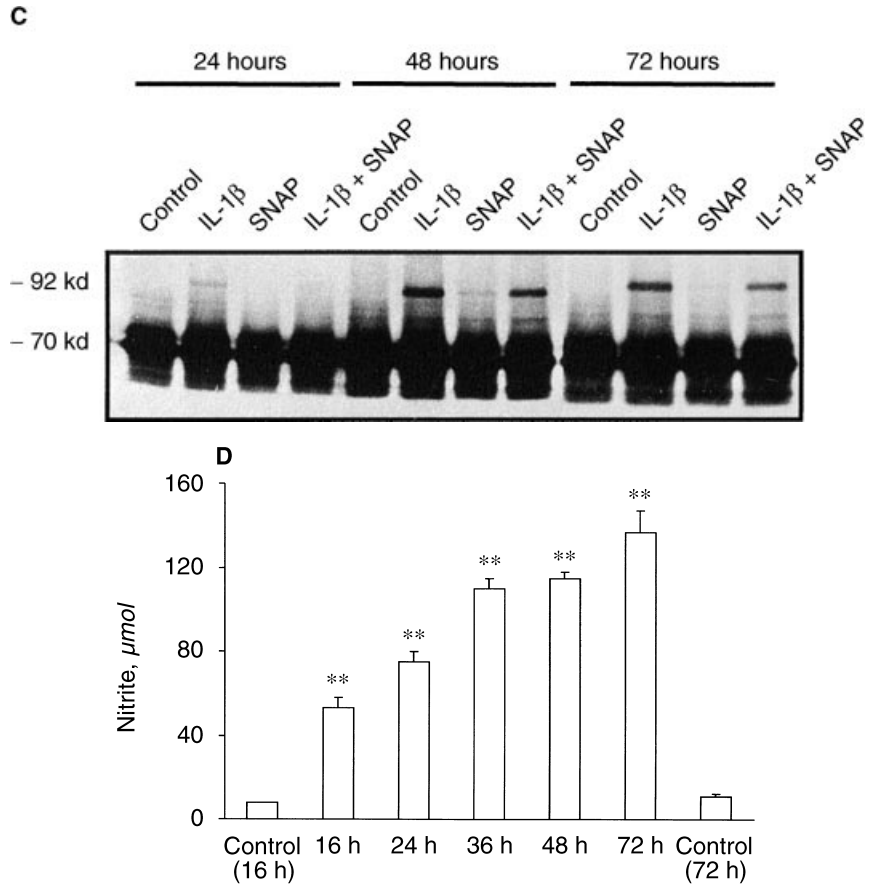


Fig. 1. (Continued)

at 92 kD tightly correlated with the changes in the steady-state levels of MMP-9 mRNA (Fig. 1C). As seen for the MMP-9 mRNA, a maximum of lytic activity was found after 48 hours of cytokine treatment. Two constitutive lytical bands migrating at 72 and 66 kD, respectively, were only marginally influenced by SNAP. Gelatinolytic activities of all bands were not observed in the presence of EDTA, indicating that they represent metalloproteinase activities (data not shown).

To determine whether the NO concentrations that we used were comparable to those endogenously produced through the induction of iNOS, the nitrite release of MCs was measured following a time course induction with IL-1β. Figure 1D shows the average of triplicate measurements of total nitrite in cell supernatants. A marked and steadily increasing nitrite production was observed between 16 and 72 hours of stimulation. The amounts of NO produced by cytokine-stimulated MCs are thus well in the range released by the exogenous NO donors used in our experiments.

Dose-dependent inhibition of IL-1β–induced MMP-9 and TIMP-1 mRNA level by SNAP

To test for dose dependence of the NO donor SNAP, MCs were stimulated for 24 hours with IL-1β plus increasing concentrations of SNAP. As shown in Figure 2, none of the concentrations used alone had strong effects on the basal expressions of MMP-9 and TIMP-1, respectively. However, the steady-state level of both mRNAs induced by IL-1β was reduced up to 20% already at a concentration of 100 μmol/L SNAP. Increased inhibition was reached by increasing SNAP concentration from 250 to 500 μmol/L to 1.0 mmol/L. Maximal inhibition was obtained using 1.0 mmol/L of SNAP (90% inhibition). For further experiments, we chose 250 and 500 μmol/L of SNAP. At these concentrations, none of the NO donors used exerted any detectable effects on cell viability (data not shown).

Inhibition of NOS strongly increases levels of MMP-9 and TIMP-1 mRNA

To determine whether the observed inhibitory effects of NO on MMP-9 mRNA levels are of physiologic relevance, cells were coincubated with IL-1β in the presence of L-NMMA, a potent enzymatic inhibitor of NO synthesis. As we have shown previously, IL-1β is a strong activator of iNOS expression in rat MCs and leads to generation of high amounts of NO by MCs [7, 9, 18]. As shown in Figure 3, IL-1β–mediated induction of MMP-9 and

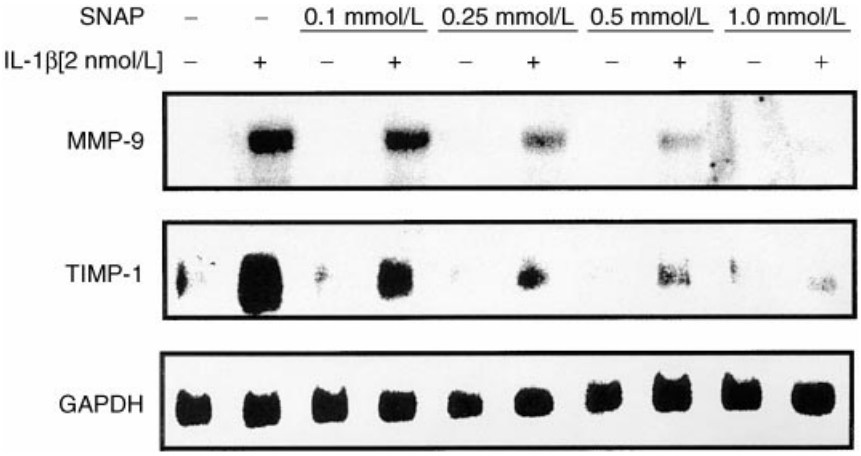


Fig. 2. Expression of MMP-9 and TIMP-1 mRNA following treatment of MCs with IL-1β and different concentrations of SNAP. MCs were treated for 24 hours with the indicated concentrations of SNAP in the absence (−) or presence of 2 nmol/L of IL-1β (+). The same blot was successively hybridized with the indicated ^{32}P-labeled cDNA inserts.

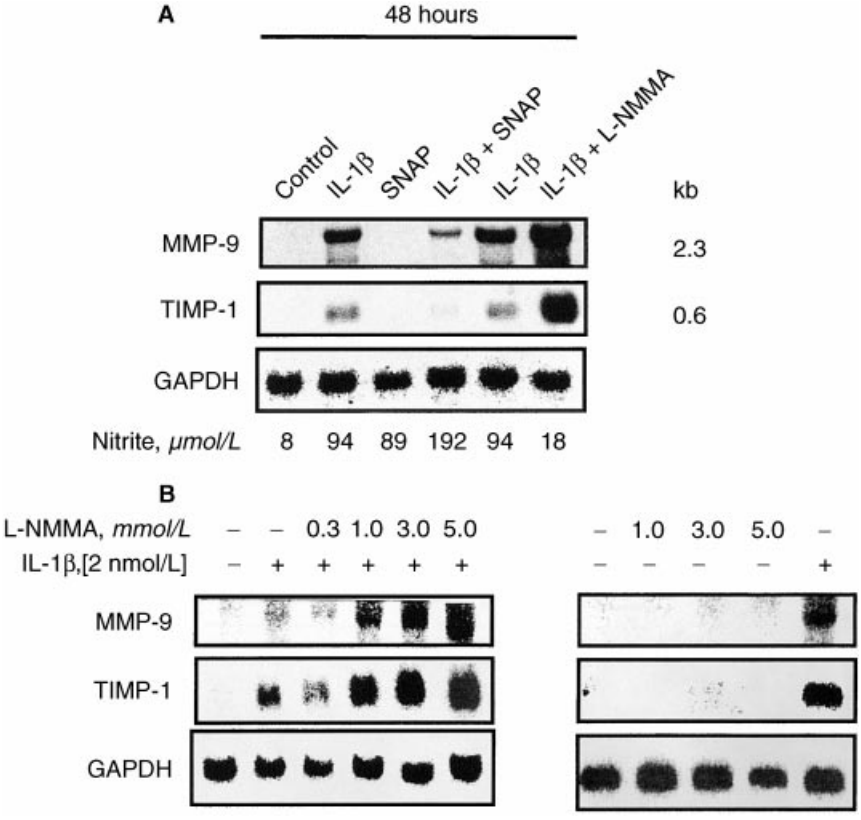
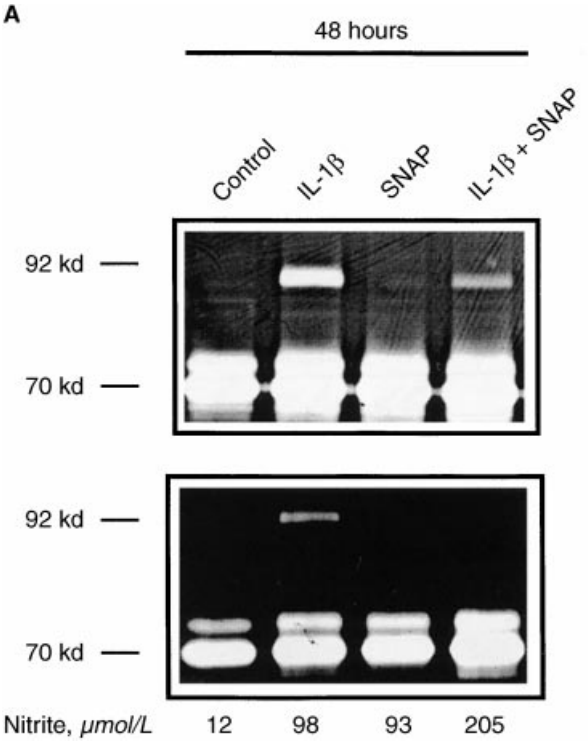


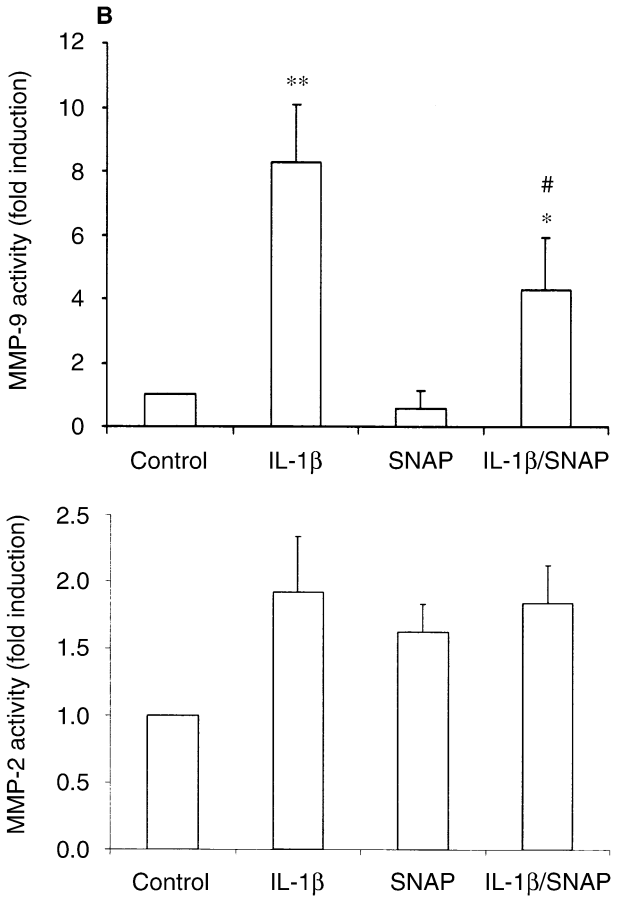
Fig. 3. L-NMMA increases IL-1β–induced MMP-9 and TIMP-1 mRNA level. Quiescent MCs were treated with vehicle (control), IL-1β (2 nmol/L), and SNAP (500 μmol/L) and L-NMMA (3 mmol/L), as indicated (*A*). After 48 hours of stimulation, cells were harvested for total RNA preparation. Twenty micrograms of RNA were hybridized successively to cDNA inserts coding for rat MMP-9 and rat TIMP-1. To correct for variations in RNA loading, the blot was rehybridized with a GAPDH probe. Similar results were obtained in two additional independent experiments. Concentrations of nitrite in cellular supernatants are given at the bottom of the gel. Dose-dependent enhancement of IL-1β–induced MMP-9 and TIMP-1 mRNA accumulation by L-NMMA (*B*). MCs were treated for 48 hours with the indicated concentrations of L-NMMA in the absence (−) or presence of 2 nmol of IL-1β (+).

TIMP-1 mRNA level was substantially amplified by treatment of cells with L-NMMA. The increase of IL-1β–induced MMP-9 and TIMP-1 mRNA level caused by L-NMMA occurred in a dose-dependent manner (Fig. 3B). Maximal stimulatory effects were reached above 1.0 mmol/L of L-NMMA. L-NMMA at any of the tested concentrations did not have any effects on the mRNA level when given alone (Fig. 3B).

Nitric oxide modulation of MMP-9 activity correlates with the alterations of the MMP-9 mRNA level

Next we tested whether the modulation of the cytokine-induced MMP-9 mRNA level by NO also affected gelatinolytic content in conditioned media from MCs. Figure 4A shows a representative zymographic analysis of serum-free conditioned medium obtained from control and stimulated MCs. To exclude that differences





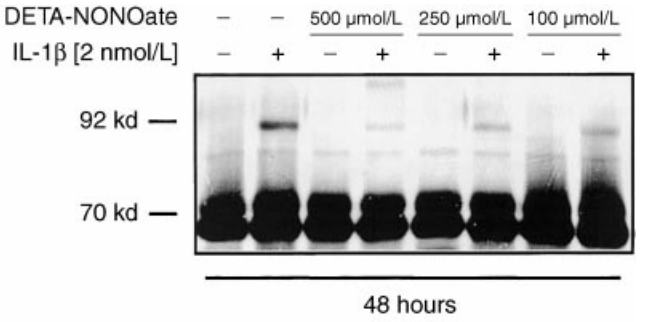


Fig. 5. Gelatinolytic activity of MMP-9 and MMP-2 in conditioned media from MCs treated with DETA NONOate and IL-1β. MCs were incubated with the indicated substances for 48 hours. Aliquots of 20 μL of cell supernatants were collected and directly subjected to gel electrophoresis. The data shown are representative of three independent experiments giving similar results.

in gelatolytic contents were due to differences in cell numbers, we also determined total cell numbers under each of the experimental condition tested; no significant changes in cell numbers were observed (data not shown). Under control conditions, we observed two major lytical bands migrating at 72 and 66 kD, respectively. The upper band at 72 kD corresponded to latent MMP-2 (also designated progelatinase-A), whereas the lower band at 66 kD represented the activated form of MMP-2 [19]. Both isoforms, independent of their activation status, are able to degrade gelatin in the zymogen assay [20]. Stimulation of MCs with 2 nmol/L of IL-1β caused an 8.3-fold induction of a band migrating at 92 kD with strong gelatinolytic activity (Fig. 4A). Under the conditions tested, the lytic activity of MMP-2 was much more intensive then that of MMP-9. However, as gelatin is not the only substrate of either MMP enzyme, gelatinolytic activity measured in this assay did not necessarily reflect activities of MMP-2 and MMP-9 toward relevant substrates *in vivo.* Conditioned medium from cells treated with SNAP alone did not display any MMP-9 activity, thus confirming the findings made by Northern blot analysis. Supernatants from MCs treated with IL-1β plus SNAP showed

Fig. 4. Gelatinolytic activity of MMP-9 in conditioned media from cells treated with SNAP and IL-1β. MCs were incubated with the indicated substances (IL-1β, 2 nmol/L, SNAP, 500 μmol/L). Aliquots of 10 μL of cell culture supernatants were collected after 48 hours of stimulation and were directly subjected to gel electrophoresis (*A*). Migration properties were determined using standard molecular weight markers. The lower panel displays a short-term exposed image of the same gel to clarify lytical intensities of MMP-2 gelatinase activities. The data shown are representative of three independent experiments. Measurement of nitrite levels given at the bottom of the gel was done by the Griess assay. (*B*) Statistical analysis of MMP-9 and MMP-2 gelatinolytic activities after 48 hours of stimulation, as was assessed by SDS-PAGE zymography. Data represent means ± SD ($N = 4$). *$P \leq 0.05$; **$P \leq 0.01$ compared with control or to IL-1β–treated conditions (#).

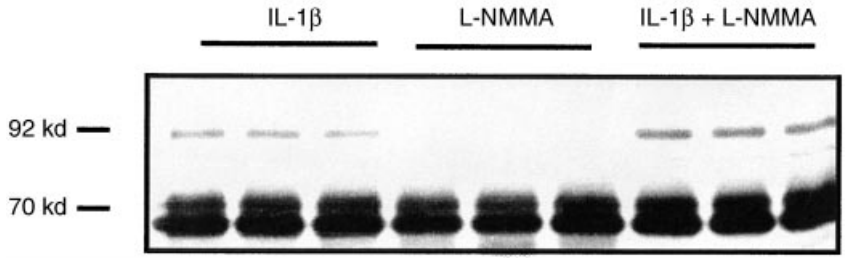


Fig. 6. Gelatinolytic activity of MMP-9 in conditioned media from MCs is increased if cells have been cotreated with L-NMMA. MCs were incubated with the indicated substances for 48 hours (IL-1β, 2 nmol/L; L-NMMA, 3 mmol/L). Lytical activity of an IL-1β–inducible band migrating at 92 kD was determined using standard molecular weight markers. Similar results were obtained in three separate experiments. The zymogram is displayed as a negative to clarify lytical intensities of MMP-9 gelatinase activity.

a marked reduction of 51% in the lytic activity of MMP-9 when compared with cells treated with IL-1β alone (Fig. 4B). In some experiments, lytic activities of bands migrating at 72 and 66 kD were weakly enhanced by IL-1β treatment but remained unaffected by NO (Fig. 4A, lower panel). As shown in Figure 5, similar results were obtained with MCs treated with DETA-NO. DETA-NO treatment led to a reduced gelatinolytic content of MMP-9 in cellular supernatants in doses ranging from 100 to 500 μmol/L.

In the next step, we tested MMP gelatinolytic activities in conditioned media from cells treated with IL-1β in the presence of 1 mmol/L L-NMMA. In analogy to the data obtained for MMP-9 mRNA level, gelatinase activity evoked by IL-1β treatment was amplified by 2.1 ± 0.8-fold ($N = 3$) used in cells treated with L-NMMA (Fig. 6). Densitometric analysis of lytical activities showed that the intensity of MMP-2 bands at 66 and 72 kD were not significantly altered by L-NMMA.

Effect of exogenously added NO on MMP-9 activity *in vitro*

To evaluate whether NO directly is able to alter MMP-9 activity, conditioned media obtained from MCs either untreated or stimulated for 48 hours with IL-1β were treated with SNAP or alternatively with DETA-NONOate (both at 500 μmol/L) for three hours or in overnight incubations (Fig. 7). None of the conditions tested had any discernible effects on activation of cytokine-induced MMP-9 zymogen activity. Furthermore, none of the NO donors tested had altered constitutive lytic activities of MMP-2. From these results, we conclude that NO-mediated alteration of zymogen activity is predominantly due to decreased MMP-9 expression but not to a direct inhibition of MMP-9 enzyme activity. Thus far, we have not been able to check the expression levels of MMP-9 protein, as none of the commercially available mouse or human anti–MMP-9 antibodies tested in Western blot or ELISA showed any cross-reactivity with the rat MMP-9 antigen.

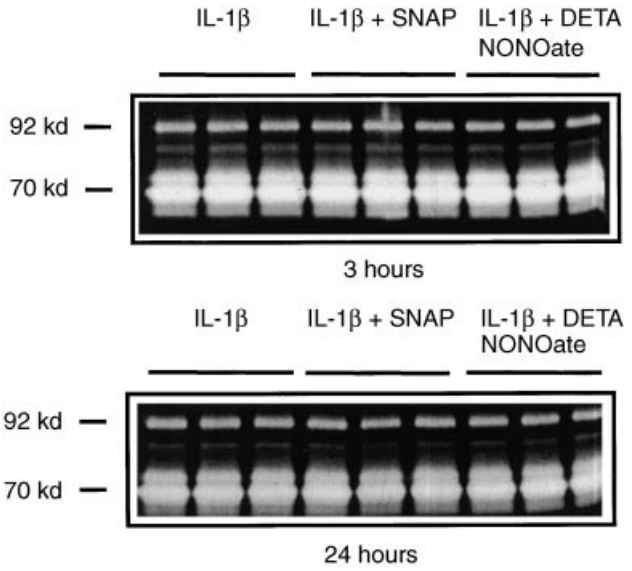


Fig. 7. Exogenous NO has no effect on IL-1β–induced gelatinolytic MMP-9 and MMP-2 activities in cell-free incubations. MCs were incubated with vehicle (control) or with IL-1β (2 nmol/L) for 48 hours. One hundred microliters of cell culture supernatants were collected after 48 hours of stimulation and were exposed to the indicated NO donors (SNAP, 500 μmol; DETA NONOate, 500 μmol/L) for the indicated time points. Subsequently, 20 μL of treated supernatant were assayed by SDS-PAGE zymography. The results are shown as triplicates.

Nitric oxide-mediated effects on MMP-9 and TIMP-1 mRNA are independent from changes in cGMP

A wide spectrum of physiologic actions of NO can be explained by the activation of soluble guanylate cyclase most prominently through a direct binding to the enzyme's prosthetic heme group. To see whether inhibition of MMP-9 and TIMP-1 expression by NO is mediated by activation of soluble guanylate cyclase and subsequent cGMP formation, we tested whether exogenously applied cGMP analogues were able to mimic the inhibitory effects of NO. Coincubation of cells with the membrane-permeable analogue dibutyryl cGMP did not alter the IL-1β–induced mRNA levels of either MMP-9 or TIMP-1 (Fig. 8). These findings suggest that inhibition of cytokine-induced MMP-9 and TIMP-1 by NO is independent of alterations of cGMP level in MCs.

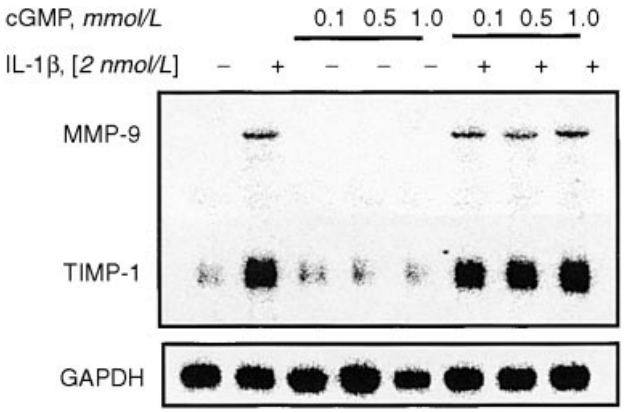


Fig. 8. Suppression of IL-1β–induced MMP-9 and TIMP-1 expression by NO is not mediated by cGMP. MCs were stimulated for 24 hours with the indicated concentrations of dibutyryl cGMP in the absence (−) or presence (+) of 2 nmol/L of IL-1β. Total cellular RNA was simultaneously hybridized to ^{32}P-labeled MMP-9 and TIMP-1 probes, as described in the **Methods** section. To assess for variations in RNA loading, blots were stripped and rehybridized to a GAPDH cDNA probe.

DISCUSSION

In this study, we evaluated modulatory effects of NO on gelatinase expression in rat MCs. These types of metalloproteinases seem to be of high impact for disturbances in the turnover and remodeling of glomerular ECM. Furthermore, MMPs are discussed as central mediators of the response of intrinsic MCs toward inflammatory stimuli [17]. Upon treatment with diverse stimuli, MCs have been shown to synthesize and secrete a variety of serine and metalloproteinases, including the 72 and 92 kD gelatinases MMP-2 and MMP-9, respectively [2, 13]. Whereas MMP-2 in most nontumor and nontransformed cell lines is constitutively expressed, MMP-9 is strongly induced by cytokines and phorbol esters [21]. Previously, we have shown that cultured MCs produce high levels of NO upon stimulation with proinflammatory cytokines such as IL-1β and TNF-α due to increased expression of iNOS [7–9]. This study provides strong evidence for a modulatory role of NO on cytokine-induced MMP-9 and to a lesser extent also on MMP-2 gene expression. MMP-9 mRNA levels were detected only in low amounts under control conditions but were strongly increased upon stimulation with IL-1β. Exogenously added NO, having weak effects on its own, was able to significantly reduce IL-1β–induced MMP-9 and MMP-2 mRNA levels. Furthermore, we demonstrate that inhibition of endogenous NO production by L-NMMA leads to a concomitant, dose-dependent potentiation of cytokine-induced MMP-9 and TIMP-1 mRNA levels.

In MCs, NO acts in a positive feedback loop, regulating its own synthesis by amplifying cytokine-induced iNOS gene expression [11]. The negative effects of NO on cytokine-induced MMP-9 expression reported here may prevent in a protective manner the MC from excessive matrix degradation provoked by cytokines. NO effects on metalloproteinases have been described for several cell types and were shown to differ substantially, depending on the cell type examined. A direct stimulatory effect of NO on MMP-2 activity was shown in human and bovine cartilage and chondrocytes [3], and also in rat MCs [22]. Tamura et al demonstrated that the IL-1β–induced increase in MMP-2 and MMP-9 activity is mediated by NO in rabbit chondrocytes [23]. In contrast, suppressive effects of NO on cytokine-induced proteolytic activity of MMP has been reported for bovine articular cartilage [24]. However, all of these studies exclusively refer to NO-mediated actions on enzymatic activity but did not investigate modulatory effects on mRNA levels of the corresponding genes. In our hands, NO donors had no direct effects on MMP-9 activity, thus suggesting that NO-mediated effects on gelatinase activity primarily result from alterations in MMP-9 mRNA levels. Possible reasons for these disparate effects of endogenously produced NO on metalloproteinase activity and expression may be due to differences in the experimental setup and especially to different amounts of reactive oxygen derivates produced simultaneously with NO. In rat fibroblasts, simultaneous generation of both NO and superoxide caused inhibition of MMP-9 activity, whereas NO by itself had no effects [25]. In MCs, the ratio between NO and superoxide is critical for the induction of apoptosis [26]. Finally, the local balance between NO and superoxide formation may determine the extent of ECM degradation during inflammatory processes.

One further key aspect of MMP regulation is the inhibition of the activated enzymes by their specific inhibitors, the TIMPs. MMP-9 activity is regulated in particular by TIMP-1 through a direct protein–protein interaction [1].

When looking for NO-mediated effects on cytokine-induced mRNA levels, we found that TIMP-1 at certain time points (24 to 72 hours) follows a coordinate expression pattern with MMP-9. A similar regulation of MMPs and their specific TIMPs has been shown for MMP-1 and TIMP-1 [27, 28] and is thought to be a common mechanism for protecting tissues from overwhelming proteolysis by fine tuning of local proteolytic activity [20]. Interestingly, following short-time kinetic experiments (8 to 16 hours), we found that TIMP-1 mRNA is already detectable after eight hours of cytokine treatment, whereas MMP-9 mRNA is not detectable by Northern blotting before 24 hours of treatment with IL-1β (data not shown). Moreover, we observed that NO modulation of the IL-1β–induced TIMP-1 mRNA level seems to be biphasic. Up to 16 hours, coincubation of IL-1β with NO donors strongly increases the steady-state mRNA level of TIMP-1 (data not shown). Appar-

ently, MMP-9 and TIMP-1 are differentially regulated by NO and do not follow a similar time course that may result in a final decrease of net protease activity. A differential regulation of glomerular MMP-9 and TIMP-1 has also been shown in a rat model of obesity [29]. Cellular supply with TIMPs in excess over the MMP's expression may prevent excessive matrix degradation caused by proinflammatory cytokines, and may thus be of considerable physiologic importance. The molecular mechanisms underlying NO-triggered gene expression are still poorly understood. NO can directly influence gene transcription by alteration of transcription factor activity most probably by interfering with the cellular redox state. Prominent candidates include activated protein-1 (AP-1) and nuclear factor-κB (NF-κB), both of which play a crucial role in cytokine-mediated MMP-9 expression in rat MCs [15]. We found that NO preferentially modifies IL-1β–activated MMP-9 mRNA level while having no effect on its own. Obviously, NO specifically interferes with the cytokine-induced signaling pathway in rat MCs, which involves activation of NF-κB [30, 31], and this activation of NO is not mimicked by cGMP. Moreover, alteration of MMP-9 mRNA stability in MCs by NO is a further possibility that needs to be evaluated in future experiments.

In conclusion, our study provides convincing evidence for a modulatory role of NO on cytokine-induced MMP-9 and TIMP-1 expression in rat MCs. Under physiologic conditions, a constant release of low amounts of NO produced by the constitutive NO synthase of the adjacent endothelium may suppress the expression of MMP-9, and thus guarantee a balanced homeostasis of glomerular architecture. By contrast, under inflammatory conditions when iNOS is expressed by invading macrophages and by resident MC, the NO production markedly exceeds the concentrations produced by the constitutive NOS, and thus other regulatory pathways become predominant. Depending on the conditions of the microenvironment, excessive matrix deposition and/or decreased matrix degradation may become obvious. In this context, it should be mentioned that a number of glomerular diseases such as diabetic nephropathy are characterized by an accumulation of matrix proteins that is possibly the result of an inappropriate rate of intrinsic MMP synthesis or activity. An increasing number of gelatinase-secreting MCs coinciding with the development of mesangial injury has been reported during induction of anti-Thy 1.1 glomerulonephritis [13]. This model system of glomerulonephritis is characterized by high-output NO formation during a first phase of mesangiolysis followed by a second phase of MC proliferation lacking detectable NO production. This second phase is also accompanied by increased matrix production [32, 33]. Under many pathologic conditions, the enhanced proliferation and excessive matrix production seem to play an important role for sclerotic processes within the glomerulus. The ultimative effect of NO-mediated down-regulation of MMP-9 and TIMP-1 expression is virtually time dependent and therefore may have protective as well as destructive consequences, depending on the time point of its occurrence. Further investigation of these critical time periods in experimental models of glomerular diseases is necessary to more precisely define the therapeutic consequences of strategies based on the inhibition of NO production.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB553) with a grant from the commission of the European Communities (Biomed 2, PL 950979) and by a grant from the Paul and Ursula Klein-Stiftung (Frankfurt, Germany). We thank Dr. Ch. Rordorf (Novartis Pharma, Inc., Basel, Switzerland) for providing human recombinant IL-1β and Ms. Martina Apel and Ms. Ute Schmidt for their valuable technical assistance. We also thank Dr. Victoria Cattell for helpful discussion.

Reprint requests to Josef Pfeilschifter, M.D., Professor of Pharmacology, Zentrum der Pharmakologie, Klinikum der Johann Wolfgang Goethe Universität Frankfurt, Theodor-Stern Kai 7, D-60590 Frankfurt am Main, Germany.
E-mail: Pfeilschifter@em.uni-frankfurt.de

APPENDIX

Abbreviations used in this article are: ECM, extracellular matrix; FCS, fetal calf serum; IL-1β, interleukin-1β, iNOS, inducible nitric oxide synthase; L-NMMA, N^G-monomethyl L-arginine; MC, mesangial cell; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; SNAP, S-Nitroso-N-acetyl-D,L-penicillamine; TIMP, tissue inhibitors of metalloproteinase; TNFα, tumor necrosis factor-α.

REFERENCES

1. Cawston T: Matrix metalloproteinases and TIMPs: Properties and implications for the rheumatic diseases. *Mol Med Today* 4:130–137, 1998
2. Johnson RJ, Lovett D, Lehrer RI, Couser WG, Klebanoff SJ: Role of oxidants and proteases in glomerular injury. *Kidney Int* 45:352–359, 1994
3. Murrell GA, Jang D, Williams RJ: Nitric oxide activates metalloprotease enzymes in articular cartilage. *Biochem Biophys Res Commun* 206:15–21, 1995
4. Woessner JF: Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 5:2145–2154, 1991
5. 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
6. Pfeilschifter J, Schalkwijk C, Briner VA, Van den Bosch H: Cytokine-stimulated secretion of group II phospholipase A2 by rat mesangial cells: Its contribution to arachidonic acid release and prostaglandin synthesis by cultured rat glomerular cells. *J Clin Invest* 92:2516–2523, 1993
7. Pfeilschifter J, Schwarzenbach H: Interleukin 1 and tumor necrosis factor stimulate cGMP formation in rat renal mesangial cells. *FEBS Lett* 273:185–187, 1990
8. Pfeilschifter J, Rob P, Mulsch A, Fandrey J, Vosbeck K, Busse R: Interleukin 1 β and tumour necrosis factor α induce a macrophage-type of nitric oxide synthase in rat renal mesangial cells. *Eur J Biochem* 203:251–255, 1992
9. Kunz D, Mühl H, Walker G, Pfeilschifter J: Two distinct signaling pathways trigger the expression of inducible nitric oxide syn-

thase in rat renal mesangial cells. *Proc Natl Acad Sci USA* 91:5387–5391, 1994
10. Mühl H, Dinarello CA: Macrophage inflammatory protein-1α production in lipopolysaccharide-stimulated human adherent blood mononuclear cells is inhibited by the nitric oxide synthase inhibitor N(G)-monomethyl-L-arginine. *J Immunol* 159:5063–5069, 1997
11. Mühl H, Pfeilschifter J: Amplification of nitric oxide synthase expression by nitric oxide in interleukin 1 β-stimulated rat mesangial cells. *J Clin Invest* 95:1941–1946, 1995
12. Pfeilschifter J, Vosbeck K: Transforming growth factor β2 inhibits interleukin 1β- and tumour necrosis factor α induction of nitric oxide synthase in rat renal mesangial cells. *Biochem Biophys Res Commun* 175:372–379, 1991
13. Davies M, Martin J, Thomas GJ, Lovett DH: Proteinases and glomerular matrix turnover. *Kidney Int* 41:671–678, 1992
14. Marti HP, Lee L, Kashgarian M, Lovett DH: Transforming growth factor-β1 stimulates glomerular mesangial cell synthesis of the 72-kD type IV collagenase. *Am J Pathol* 144:82–94, 1994
15. Yokoo T, Kitamura M: Dual regulation of IL-1β-mediated matrix metalloproteinase-9 expression in mesangial cells by NF-κB and AP-1. *Am J Physiol* 270:F123–F130, 1996
16. Harendza S, Pollock AS, Mertens PR, Lovett DH: Tissue-specific enhancer-promoter interactions regulate high level constitutive expression of matrix metalloproteinase 2 by glomerular mesangial cells. *J Biol Chem* 270:18786–18796, 1995
17. Baricos WH, Shah SV: Proteolytic enzymes as mediators of glomerular injury. *Kidney Int* 40:161–173, 1981
18. Mühl H, Kunz D, Rob P, Pfeilschifter J: Cyclosporin derivatives inhibit interleukin 1β induction of nitric oxide synthase in renal mesangial cells. *Eur J Pharmacol* 249:95–100, 1993
19. Ward RV, Hembry RM, Reynolds JJ, Murphy G: The purification of tissue inhibitor of metalloproteinases-2 from its 72 kDa progelatinase complex: Demonstration of the biochemical similarities of tissue inhibitor of metalloproteinases-2 and tissue inhibitor of metalloproteinases-1. *Biochem J* 278:179–187, 1991
20. Hanemaaijer R, Koolwijk P, Le Clercq L, De Vree WJ, Van Hinsbergh VW: Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells: Effects of tumour necrosis factor β, interleukin 1 and phorbol ester. *Biochem J* 296:803–809, 1993
21. Okada Y, Gonoji Y, Naka K, Tomita K, Nakanishi I, Iwata K, Yamashita K, Hayakawa T: Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT 1080 humanfibrosarcoma cells: Purification and activation of the precursor and enzymic properties. *J Biol Chem* 267:21712–21719, 1992
22. Trachtman H, Futterweit S, Garg P, Reddy K, Singhal PC: Nitric oxide stimulates the activity of a 72-kDa neutral matrix metalloproteinase in cultured rat mesangial cells. *Biochem Biophys Res Commun* 218:704–708, 1996
23. Tamura T, Nakanishi T, Kimura Y, Hattori T, Sasaki K, Norimatsu H, Takahashi K, Takigawa M: Nitric oxide mediates interleukin-1-induced matrix degradation and basic fibroblast growth factor release in cultured rabbit articular chondrocytes: A possible mechanism of pathological neovascularization in arthritis. *Endocrinology* 137:3729–3737, 1996
24. Stefanovic-Racic M, Morales TI, Taskiran D, McIntyre LA, Evans CH: The role of nitric oxide in proteoglycan turnover by bovine articular cartilage organ cultures. *J Immunol* 156:1213–1220, 1996
25. Owens MW, Milligan SA, Jourd'Heuil D, Grisham MB: Effects of reactive metabolites of oxygen and nitrogen on gelatinase A activity. *Am J Physiol* 273:L445–L450, 1997
26. Sandau K, Pfeilschifter J, Brüne B: The balance between nitric oxide and superoxide determines apoptotic and necrotic death of rat mesangial cells. *J Immunol* 158:4938–4946, 1997
27. Murphy G, Reynolds JJ, Werb Z: Biosynthesis of tissue inhibitor of metalloproteinases by human fibroblasts in culture: Stimulation by 12-O-tetradecanoylphorbol 13-acetate and interleukin 1 in parallel with collagenase. *J Biol Chem* 260:3079–3083, 1985
28. Colige AC, Lambert CA, Nusgens BV, Lapiere CM: Effect of cell-cell and cell-matrix interactions on the response of fibroblasts to epidermal growth factor in vitro: Expression of collagen type I, collagenase, stromelysin and tissue inhibitor of metalloproteinases. *Biochem J* 285:215–221, 1992
29. Schaefer L, Han X, August C, Matzkies F, Lorenz T, Schaefer RM: Differential regulation of glomerular gelatinase B (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in obese Zucker rats. *Diabetologia* 40:1035–1043, 1997
30. Eberhardt W, Plüss C, Hummel R, Pfeilschifter J: Molecular mechanisms of inducible nitric oxide synthase gene expression by IL-1β and cAMP in rat mesangial cells. *J Immunol* 160:4961–4969, 1998
31. Satriano J, Schlondorff D: Activation and attenuation of transcription factor NF-κB in mouse glomerular mesangial cells in response to tumor necrosis factor-α immunoglobulin G, and adenosine 3′:5′-cyclic monophosphate: Evidence for involvement of reactive oxygen species. *J Clin Invest* 94:1629–1636, 1994
32. Cattell V, Lianos E, Largen P, Cook T: Glomerular NO synthase activity in mesangial cell immune injury. *Exp Nephrol* 1:36–40, 1993
33. Cook HT, Ebrahim H, Jansen AS, Foster GR, Largen P, Cattell V: Expression of the gene for inducible nitric oxide synthase in experimental glomerulonephritis in the rat. *Clin Exp Immunol* 97:315–320, 1994