Differential regulation of vascular endothelial growth factor and its receptor *fms*-like-tyrosine kinase is mediated by nitric oxide in rat renal mesangial cells

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Under conditions associated with local and systemic inflammation, mesangial cells and invading immune cells are likely to be responsible for the release of large amounts of nitric oxide (NO) in the glomerulus. To further define the mechanisms of NO action in the glomerulus, we attempted to identify genes which are regulated by NO in rat glomerular mesangial cells. We identified vascular endothelial growth factor (VEGF) and its receptor *fms*-like tyrosine kinase (FLT-1) to be under the regulatory control of exogenously applied NO in these cells. Using *S*-nitroso-glutathione (GSNO) as an NO-donating agent, VEGF expression was strongly induced, whereas expression of its FLT-1 receptor simultaneously decreased. Expressional regulation of VEGF and FLT-1 mRNA was transient and occurred rapidly within 1–3 h after GSNO treatment. Expression of a second VEGF-specific receptor, fetal liver kinase-1 (FLK-1/KDR), could not be detected. The inflammatory cytokine interleukin-1β mediated a moderate increase in VEGF expression after 24 h and had no influence on FLT-1 expression. In contrast, platelet-derived growth factor–BB and basic fibroblast growth factor had no effect on VEGF expression, but strongly induced FLT-1 mRNA levels. Obviously, there is a differential regulation of VEGF and its receptor FLT-1 by NO, cytokines and growth factors in rat mesangial cells.

Key words: gene expression, glomerulus, inflammation.

INTRODUCTION

Within the last decade, a key player during the process of acute and chronic inflammation has become evident, i.e. nitric oxide (NO). High amounts of NO are released from the inducible NO synthase (iNOS) isoform in response to inflammatory stimuli from a variety of cell types [1]. Mesangial cells and invading immune cells are capable of expressing iNOS upon stimulation with tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and bacterial lipopolysaccharide (LPS), and, thus are likely to be responsible for the release of large amounts of NO during TNF-α, IL-1β and LPS-triggered inflammatory conditions in the glomerulus [2–5]. Increasing evidence indicates that NO contributes to the pathomechanisms of septic shock [6] and the progression towards renal failure during several forms of glomerulonephritis [7,8].

Vascular endothelial growth factor (VEGF), which is a potent mitogen for endothelial cells [9,10], has been reported to be expressed in several tissues, including kidney [11,12]. Besides its mitogenic properties, VEGF is able to promote angiogenesis [13], induce proteases [14,15] and increase vascular leakage [16,17]. Furthermore, VEGF has been shown to be an essential growth factor for kidney development and glomerulogenesis [18]. *In vivo* studies have revealed expression of VEGF in glomerular podocytes and proximal and distal tubular and collecting duct cells within the kidney [12,19,20]. In the kidney, the VEGF-specific receptors *fms*-like-tyrosine kinase (FLT-1) and fetal liver kinase-1 (FLK-1/KDR) are differentially expressed, with FLT-1 being the most abundant VEGF-specific receptor [20]. However, VEGF has been reported to be expressed only in damaged tubular epithelia during glomerulonephritis [19]. Furthermore, the number of VEGF-expressing cells is even reduced in focal or global glomerular sclerosis, amyloidosis and diabetic and crescentic glomerulonephritis [21]. In the Thy-1-induced model of glomerulonephritis, VEGF is involved in endothelial cell proliferation and therefore mediation of angiogenic effects [22]. In contrast to the *in vivo* situation, where glomerular mesangial cells are not positive for VEGF expression in the limited number of glomerulopathies investigated so far [19], mesangial cells have been reported to express VEGF and its receptors *in vitro*. Cultured mesangial cells produce VEGF after application of mechanical stretch [23] or addition of fetal bovine serum, PMA, transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF) respectively [24-26]. Additionally, due to its ability to increase dramatically vascular permeability, VEGF contributes to the relaxing capacity of the renal vasculature [27].

Here we demonstrate a rapid expressional regulation of VEGF and its receptor FLT-1 by NO, thus revealing VEGF and FLT-1 as novel NO-regulated genes in rat mesangial cells.

EXPERIMENTAL

Cell culture and treatment of mesangial cells

Rat glomerular mesangial cells were cultured and cloned as described previously [28]. Cells were grown in RPMI 1640 supplemented with 10 % (v/v) fetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 μg/ml). For the experiments, passages 10–18 were used. For the induction experiments, cells were grown to confluency without changing the medium

Abbreviations used: bFGF, basic fibroblast growth factor, ECL, enhanced chemiluminescence; FCS, fetal calf serum; FLK-1/KDR, fetal liver kinase-1; FLT-1, *fms*-like tyrosine kinase; GSNO, *S*-nitroso-glutathione; IL-1β, interleukin 1β; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; PDGF-BB, platelet-derived growth factor-BB; TGF-β1, transforming growth factor-β1; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor.

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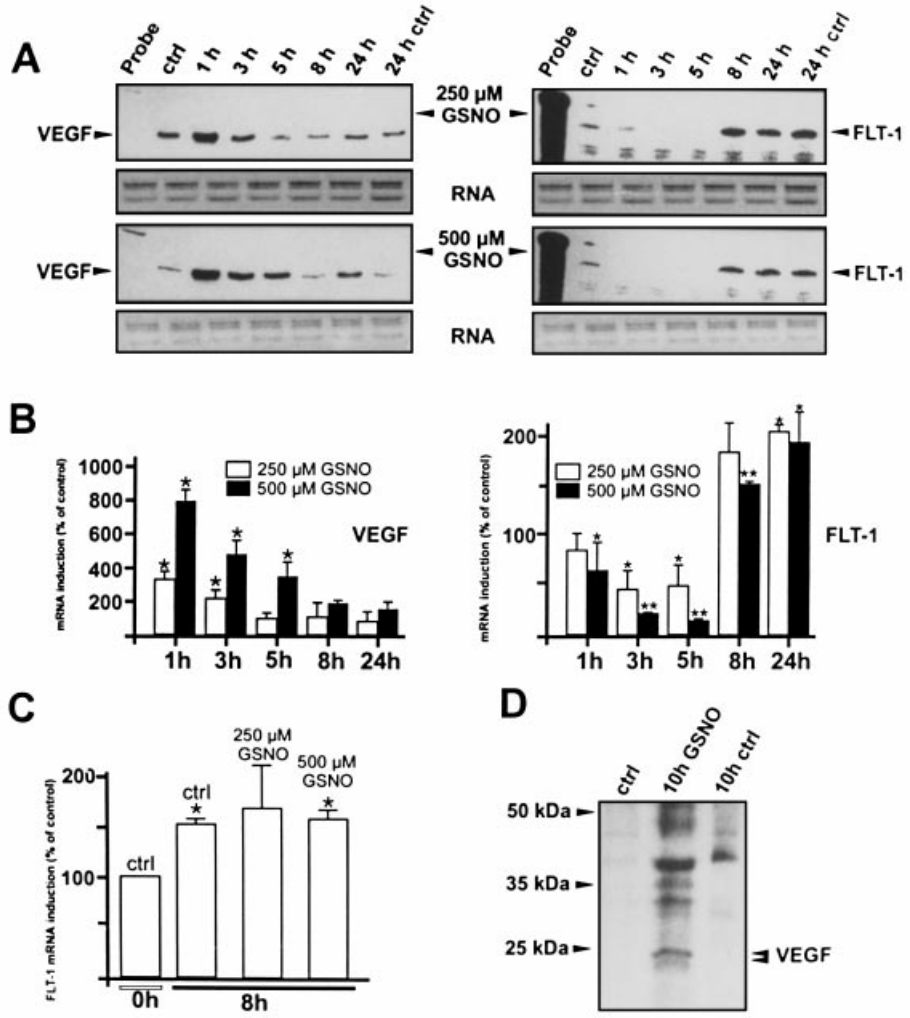

Figure 1 Modulation of VEGF and FLT-1 expression by NO in cultured rat mesangial cells

(**A**) RNase protection assay demonstrating the regulation of VEGF and FLT-1 mRNA expression by the NO-donating agent GSNO. Cells were rendered quiescent by serum starvation and stimulated with 250 μM or 500 μM GSNO for the indicated time periods. Samples of 20 μg of the same batch of total cellular RNA were analysed for VEGF and FLT-1 mRNA expression and results for one representative experiment are shown; 1000 c.p.m. of the hybridization probe was used as a size marker. Ethidium bromide stains of 2 μg of the RNA analysed in each case are shown below the protection assay panels. The concentration-dependent GSNO-induced increase (for VEGF) and decrease (for FLT-1) in mRNA levels as assessed by PhosphoImager (Fuji) analysis of the radiolabelled gels from three independent experiments are shown schematically in (**B**). Results are expressed as a percentage of unstimulated control (100%). Mean percentage change in VEGF (left-hand panel) and FLT-1 (right-hand panel) mRNA levels $\pm$ S.D. are shown ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$ compared with control. (**C**) Increase in FLT-1 mRNA levels after 8 h is not dependent on exogenously added NO. To investigate effects of medium replacement on FLT-1 mRNA expression during the stimulation experiments, 20 μg of total cellular RNA was analysed by RNase protection assay for FLT-1 mRNA after 8 h in control cells (ctrl, without GSNO) and GSNO-treated cells (250 μM, 500 μM GSNO). Results are expressed as a percentage of unstimulated control (100%). Mean percentage changes in FLT-1 mRNA levels $\pm$ S.D. ($n = 3$) are shown. $^*P < 0.05$ compared with control. (**D**) GSNO-stimulated expression of VEGF protein. Medium (10 ml) was conditioned for 10 h by quiescent mesangial cells in the presence or absence of 500 μM GSNO. Heparin-binding proteins were precipitated from the conditioned medium using heparin–Sepharose beads and analysed by immunoblotting for the presence of VEGF protein. Non-conditioned medium (ctrl) and medium after 10 h incubation without GSNO-treatment (10 h ctrl) were used as controls. Two VEGF proteins of 22 and 24 kDa, which were detected in conditioned medium from GSNO-stimulated cells (10 h GSNO) are indicated with arrows. These proteins were not present in conditioned medium from serum-starved cells (10 h ctrl) and non-conditioned medium (ctrl).

and rendered quiescent by a 24 h incubation in RPMI 1640 without serum. Cells were then incubated for various periods in fresh RPMI 1640 containing 2 nM IL-1β, 2 nM TNF-α, 10 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml platelet-derived growth factor-BB (PDGF-BB), 250 μM *S*-nitroso-glutathione (GSNO), 500 μM GSNO, 1 mM cAMP, 1 mM cGMP, 2 μg/ml actinomycin D or 20 μg/ml cycloheximide respectively. Cells and supernatants were harvested at different time points after addition of these agents and used for RNA and protein isolation respectively. Each experiment was repeated twice. FCS and RPMI were purchased from Gibco Life Technologies, Inc. (Eggenstein, Germany), growth factors and cytokines were from Boehringer Mannheim Biochemicals (Mannheim, Germany), cAMP, cGMP, actinomycin D and cycloheximide were from Sigma Biochemicals (Deisenhofen, Germany).

RNA isolation and RNase protection analysis

RNA isolation was performed as described [29]. Total RNA (20 μg) from the different experimental time points of the cell-culture experiments were used for RNase protection assays. RNase protection assays were carried out as described [30].

Briefly, DNA probes were cloned into the transcription vector pBluescript II KS (+) and linearized. An antisense transcript was synthesized *in vitro* using T3 or T7 RNA polymerase and [α-^{32}P]UTP (800 Ci/mmol). RNA samples were hybridized at 42 °C overnight with 100000 c.p.m. of the labelled antisense transcript. Hybrids were digested with RNases A and T1 for 1 h at 30 °C. Under these conditions, every single mismatch is recognized by the RNases. Protected fragments were separated on 5% acrylamide/8 M urea gels and analysed using a PhosphoImager (Fuji). To permit appropriate statistical analysis, all protection assays were carried out with at least three different sets of RNA from independent cell-culture experiments.

Probe DNAs

The rat VEGF cDNA probe was cloned by PCR using 5′-ATG GAC GTC TAC CAG CGC AGC-3′ as a 5′-primer and 5′-CTG CTA TGC TGC AGG AAG CTC-3′ as a 3′-primer. The amplified cDNA fragment corresponds to nucleotides 131–380 of the published sequence [31]. Furthermore, we cloned cDNA fragments representing rat VEGF-specific receptor tyrosine kinases FLT-1 and FLK-1/KDR by PCR using 5′-CCC GGT TTG CTG AAC TTG TGG-3′ as a 5′-primer and 5′-GGC ATT TGG TGA AAG CTC CTC-3′as a 3′-primer for FLT-1, and 5′-GAT GTT GAA AGA GGG AGC AAC-3′ as a 5′-primer and 5′-ACA TAG TCT TTC CCA GAG CGG-3′ as a 3′-primer for FLK-1/KDR. The cDNA fragments correspond to nucleotides 3669–3941 for FLT-1 and 2839–3090 for FLK-1/KDR of the published sequences [32,33].

Detection of VEGF-specific proteins in conditioned cell-culture supernatants

Glomerular mesangial cells were grown to confluency in RPMI 1640 with 10% (v/v) FCS without changing the medium. Cells were rendered quiescent by a 24 h incubation in serum-free RPMI 1640. Subsequently, cells were treated for 10 h with 500 μM GSNO. After 10 h the conditioned medium from three Petri dishes was centrifuged for 10 min at 350 ***g*** to remove cell debris. PMSF (1 mM) and leupeptin (15 μg/ml) were added. Heparin-binding proteins were precipitated from the supernatant with 120 μl of heparin–Sepharose (1:1 slurry) overnight at 4 °C. Heparin beads were precipitated by centrifugation and washed three times in 20 mM Tris/HCl, pH 7.4/0.3 M NaCl. Heparin–Sepharose-bound proteins were extracted by a 5 min incubation in Laemmli sample buffer at 95 °C and separated by SDS gel electrophoresis. After transfer to a PVDF membrane, VEGF proteins were detected using a polyclonal antiserum directed against VEGF (Santa Cruz Biotechnology, New York, NY, U.S.A.). A secondary antibody coupled to horseradish peroxidase and the enhanced chemiluminescence (ECL) detection system were used to visualize VEGF protein. PMSF and leupeptin were supplied by Sigma Biochemicals (Deisenhofen, Germany), the antibody coupled to horseradish peroxidase was from Biomol (Hamburg, Germany), and the ECL detection system was obtained from Amersham (Braunschweig, Germany).

GSNO synthesis

GSNO was synthesized as described previously [34]. Briefly, glutathione was dissolved in 0.625 M HCl at 0 °C to a final concentration of 625 mM. An equimolar amount of $NaNO_2$ was added and the mixture was stirred at 0 °C for 40 min. After the addition of 2.5 vol. of acetone stirring was continued for another 20 min, followed by filtration of the precipitate. GSNO was washed once with 80% acetone, two times with 100% acetone and finally three times with diethylether, and was dried under vacuum. GSNO was characterized by UV spectroscopy.

Statistical analysis

Results are given as means ± S.D. The data are presented either as *x*-fold induction or as percentage change compared with the control (100%). For each reagent, under each set of conditions, results were analysed by Student's *t*-test using the software SigmaPlot (Jandel Scientific, Erkrath, Germany).

RESULTS

Modulation of VEGF and FLT-1 expression by GSNO

We have previously demonstrated a substantial release of NO by rat glomerular mesangial cells after stimulation with TNF-α or IL-1β [2,3]. Because of the potency of the generation of large amounts of NO *in vitro*, NO release from mesangial cells has been suggested to contribute to the tissue injury seen in certain types of glomerulonephritis [7,8,35]. Therefore, we are interested in NO-mediated gene expression in mesangial cells to determine potential players that might be involved in the progress of inflammatory processes in the kidney. Using cultured mesangial cells as an *in vitro* system, we found expression of VEGF and its receptor tyrosine kinase FLT-1 to be under control of exogenously added NO. Expression of the second VEGF-specific receptor FLK-1/KDR could not be detected by RNase protection analysis in these cells (results not shown). As shown in Figure 1(A), low basal levels of VEGF and FLT-1 mRNA were detected in quiescent mesangial cells. Upon addition of increasing amounts of the NO-donor GSNO (250 μM, 500 μM), a strong induction of VEGF mRNA was observed with a maximal stimulation of VEGF expression at 500 μM of GSNO. In

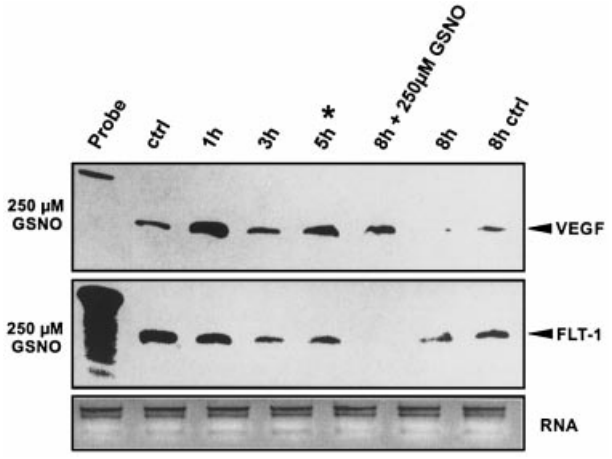

Figure 2 GSNO-stimulated cells are sensitive to an additional NO stimulus

Serum-starved mesangial cells were stimulated for different time periods with 250 μM GSNO. At the 5 h experimental time point, one part of the cells was lysed to isolate total RNA (marked with an asterisk), whereas the second part of cells received a second addition of 250 μM GSNO. At the 8 h time point, we therefore obtained an additional set of total RNA from cells that had received a second NO-stimulus (8 h + 250 μM GSNO). A 20 μg amount of the same batch of total RNA was analysed by RNase protection analysis for the expression of VEGF (upper panel) and FLT-1 (middle panel) and results for one representative experiment are shown. An ethidium bromide stain of 2 μg of analysed RNA is also shown (lower panel). Note that VEGF mRNA levels remained high after the second NO stimulus (8 h + 250 μM GSNO) compared with cells that had received only a single GSNO treatment. In contrast, FLT-1 mRNA expression is further decreased after 8 h (8 h + 250 μM GSNO).

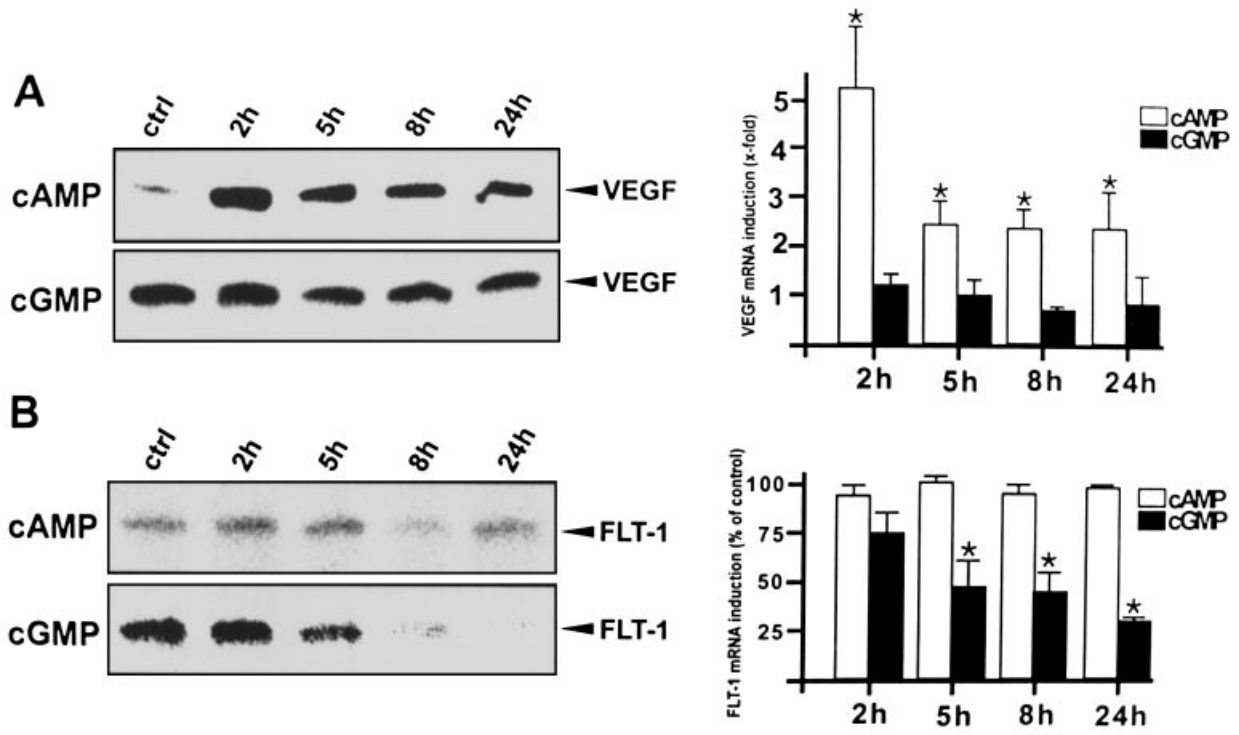

Figure 3 Regulation of VEGF and FLT-1 mRNA expression by cGMP or cAMP

Serum-starved mesangial cells were stimulated for 2, 5, 8 or 24 h with 1 mM cGMP or 1 mM cAMP respectively. Total cellular RNA (20 μg) was analysed by RNase protection analysis for the expression of VEGF (**A**) and FLT-1 (**B**) mRNA and the results from one representative experiment are shown. The degree of mRNA induction of the radiolabelled gels from three independent experiments as assessed by PhosphoImager (Fuji) analysis is shown schematically (right-hand panels). Results are expressed as *x*-fold-induction (for VEGF, **A**) or as a percentage (for FLT-1, **B**) of unstimulated control. Mean changes in VEGF (**A**) and FLT-1 (**B**) mRNA levels $\pm$ S.D. ($n = 3$) are shown. $^*P < 0.05$ compared with control. VEGF or FLT-1 mRNA levels and the cyclic nucleotide used are indicated on the Figure.

contrast, FLT-1 mRNA levels simultaneously decreased in a dose-dependent manner after addition of GSNO. Note that the short exposure time for both FLT-1 autoradiographs (Figure 1A, right-hand panels) has been chosen to illustrate the concentration-dependent effects of GSNO on FLT-1 mRNA expression. A longer exposure time did in fact lead to detection of FLT-1 mRNA signals for the 1, 3 and 5 h experimental time points (Figure 1B, right-hand panel). The effect of NO on VEGF and FLT-1 expression was transient.

The increase in FLT-1 mRNA levels at 8 h after addition of GSNO, as shown in Figures 1(A) and 1(B), was not due to a stimulatory effect of NO. In all experiments, we observed an increase in FLT-1 expression after 8 h in the presence or absence of GSNO (Figure 1C; see also Figure 5B, 8 h ctrl). Thus, increased FLT-1 mRNA levels were caused by adding fresh medium at the beginning of the experiments.

VEGF represents a dimer consisting of two subunits with a molecular mass of 46 kDa. To determine whether the observed induction of VEGF mRNA expression correlates with the induction of immunoreactive protein, conditioned medium was prepared from GSNO-treated cells and non-treated mesangial cells and analysed for the presence of VEGF proteins. Because of the kinetics of VEGF induction at the mRNA level, the conditioned medium was harvested after 10 h. Heparin-binding proteins were enriched by their capacity to bind to heparin–Sepharose and subsequently analysed for the presence of VEGF protein by immunoblot. As shown in Figure 1(D), two major forms of VEGF with estimated molecular masses of about 22–24 kDa were detected in conditioned medium from GSNO-stimulated cells. The size of these proteins correlated with the known molecular sizes of the VEGF gene products. To exclude the possibility that the VEGF-specific proteins in conditioned medium from GSNO-treated cells result from basal expression and secretion of VEGF from unstimulated cells, conditioned medium from untreated cells at 0 h and 10 h was used as a control. These results demonstrate that induction of VEGF mRNA by GSNO also correlates with induction of VEGF protein. FLT-1 protein could not be determined, as an antibody against the rat protein is currently not commercially available.

Transient regulation of VEGF and FLT-1 expression can be extended by a second NO stimulus

As a next step we tested the potency of GSNO to prolong the transient effects on VEGF and FLT-1 mRNA levels observed after addition of GSNO to mesangial cells. Cells were stimulated with 250 μM GSNO to induce VEGF mRNA expression and reduce FLT-1 expression levels. As shown in Figure 2, VEGF mRNA levels increased and FLT-1 mRNA declined within 5 h after addition of GSNO, thus confirming the results obtained from our previous experiments. After 5 h of treatment with 250 μM GSNO, we repeated the addition of 250 μM GSNO to the cells. For VEGF expression we could observe clearly increased mRNA levels 3 h after the additional NO-stimulus compared with VEGF mRNA levels 8 h after the first addition of GSNO. In contrast, FLT-1 mRNA levels were further decreased 3 h after

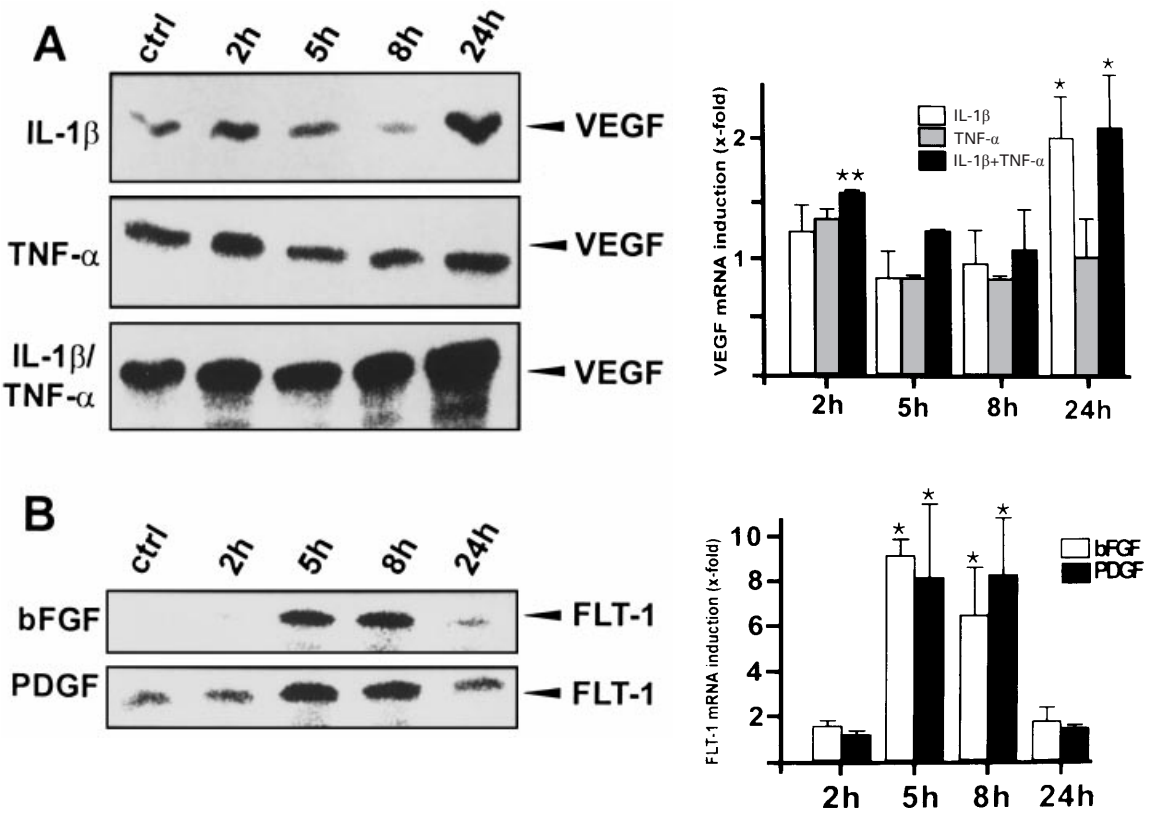

Figure 4 Regulation of VEGF and FLT-1 mRNA levels by growth factors and inflammatory cytokines

Serum-starved cells were stimulated for different time periods with 2 nM IL-1β, 2 nM TNF-α, a combination of IL-1β + TNF-α, 10 ng/ml bFGF or 10 ng/ml PDGF-BB as indicated. Total RNA (20 μg) was analysed by RNase protection analysis for the expression of VEGF (**A**) and FLT-1 (**B**) and the results from one representative experiment are shown. The changes in VEGF and FLT-1, as assessed by PhosphoImager (Fuji) analysis of the radiolabelled gels from three independent experiments, are shown schematically (right-hand panels). Results are expressed as *x*-fold-induction of unstimulated control. Mean changes in VEGF (**A**) and FLT-1 (**B**) mRNA levels $\pm$ S.D. ($n = 3$) are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

the second GSNO treatment compared with the increasing mRNA levels after 8 h. These results suggest that NO-induced mesangial cells were still sensitive to a second NO stimulus.

NO-mediated FLT-1, but not VEGF, expression is dependent on activation of soluble guanylate cyclase

NO activates soluble guanylate cyclase [36,37], leading to an increase in the intracellular levels of cGMP, which is the second messenger for most of the physiological actions of NO. To examine whether the NO-induced modulation in VEGF and FLT-1 expression is secondary to activation of soluble guanylate cyclase and hence cGMP, we treated cells with the membrane-permeable cGMP analogue 8-bromo-cGMP (1 mM). We did not observe an increase in VEGF mRNA levels, which remained at basal levels during 24 h of treatment (Figure 3A). Thus, the expression of VEGF caused by NO is most likely independent of the formation of intracellular cGMP. In contrast, 8-bromo-cGMP clearly reduced FLT-1 mRNA levels, indicating that activation of the soluble guanylate cyclase is involved in signal transduction processes that regulate NO-dependent FLT-1 expression (Figure 3B). In addition to the signalling pathways activated by growth factors and cytokines, the activation of adenylate cyclase, leading to an increase in intracellular cAMP, has been described as a second independent signalling pathway for iNOS and related enzyme induction in rat glomerular mesangial cells [38–40]. As shown in Figure 3(A), VEGF mRNA levels are strongly induced by 1 mM N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate, whereas FLT-1 expression is not influenced by this cAMP analogue (Figure 3B).

Induction of VEGF and FLT-1 expression by growth factors and inflammatory cytokines

We tested the potency of growth factors and inflammatory cytokines in stimulating VEGF and FLT-1 expression, as there are few results available concerning the regulation of these genes in glomerular mesangial cells. As shown in Figure 4(A), the inflammatory cytokine IL-1β induced VEGF mRNA levels only about 2-fold, whereas TNF-α had almost no effect. Treatment of cells with both cytokines did not result in an additive or synergistic induction of VEGF expression. Thus the observed induction of VEGF mRNA following the addition of both cytokines is most likely to represent the stimulatory potency of IL-1β alone. Compared with the rapid and strong NO-mediated induction of VEGF expression (Figure 1A), we observed delayed and weak VEGF mRNA levels after cytokine stimulation (IL-1β, IL-1β + TNF-α), reaching a maximum after 24 h. Basal expression levels of FLT-1 mRNA are not influenced at all by these cytokines (results not shown). In contrast, the growth factors bFGF and PDGF-BB strongly induced FLT-1 mRNA expression (Figure 4B), whereas VEGF mRNA levels remained unchanged (results not shown). These results suggest that VEGF and its

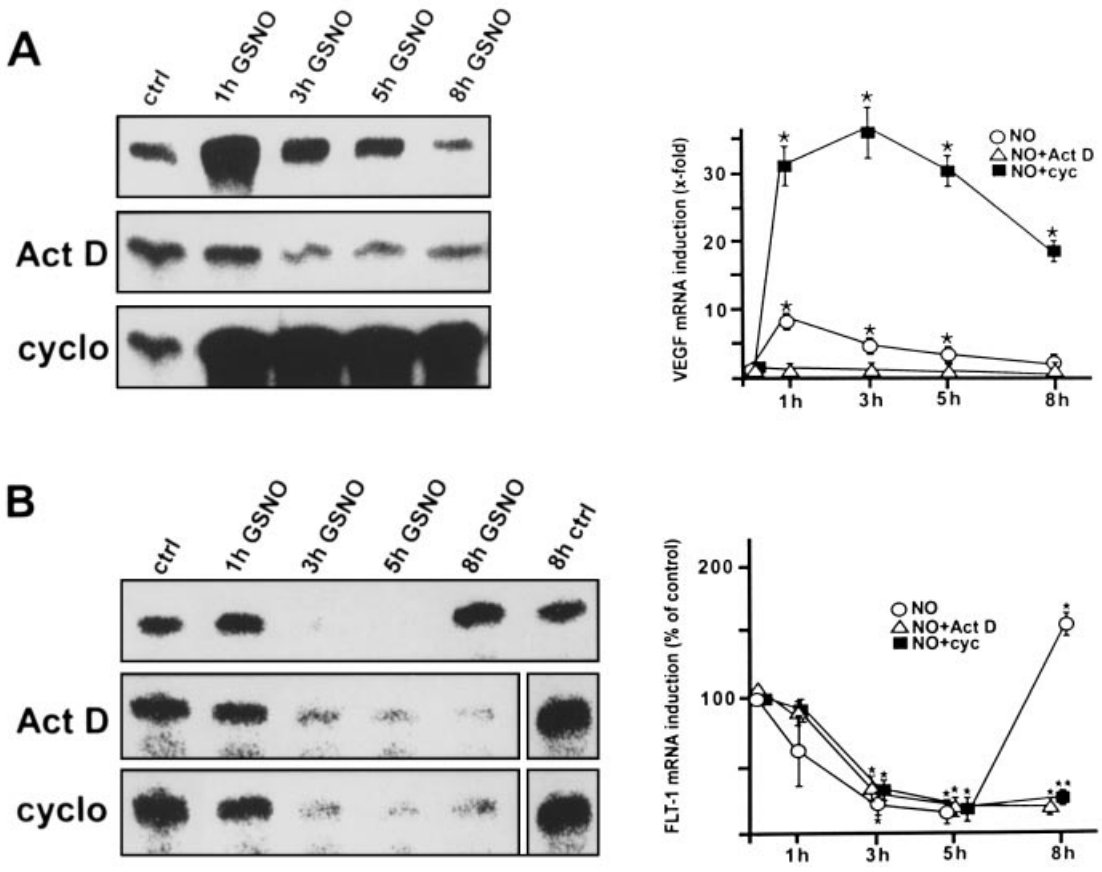

Figure 5 Rapid NO-induced up-regulation of VEGF mRNA levels is dependent on *de novo* RNA synthesis

Serum-starved mesangial cells were treated with 500 μM GSNO in the presence or absence of 2 μg/ml actinomycin D (Act D) or 20 μg/ml cycloheximide (cyclo) as indicated. A 20 μg amount of the same batches of total RNA from these cells was analysed for VEGF (**A**) and FLT-1 (**B**) expression by RNase protection analysis and the results from one representative experiment are shown. The degree of VEGF and FLT-1 induction, as assessed by PhosphoImager (Fuji) analysis of the radiolabelled gels from three independent experiments, is shown schematically (right-hand panels). Results are expressed as *x*-fold-induction of unstimulated control. Mean changes in VEGF (**A**) and FLT-1 (**B**) mRNA levels $\pm$ S.D. ($n = 3$) are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

receptor FLT-1 are likely to be subjected to different regulatory mechanisms in glomerular mesangial cells.

NO-mediated induction of VEGF mRNA is dependent on *de novo* RNA synthesis

As a last step, we addressed further the molecular mechanisms by which NO alters VEGF and FLT-1 mRNA levels. Changes either in transcription or in mRNA stability may be responsible for the observed changes in mRNA levels seen in Figure 1(A) and Figure 2. We analysed the effect of the transcription inhibitor actinomycin D (2 μg/ml) and cycloheximide (20 μg/ml), a potent inhibitor of *de novo* protein synthesis, on NO-regulated VEGF and FLT mRNA levels. Cells were incubated with either actinomycin D or cycloheximide 45 min before addition of exogenously added GSNO (500 μM). As shown in Figure 5(A), VEGF mRNA expression was induced by GSNO (8-fold). Addition of actinomycin D completely inhibited the NO-induced increase in VEGF mRNA levels, suggesting that *de novo* RNA synthesis is required for NO-mediated VEGF induction. Interestingly, cycloheximide did not inhibit GSNO-mediated induction of VEGF mRNA, but resulted in a large super-induction (30-fold) of VEGF mRNA expression in response to GSNO. The induction of VEGF gene expression in the presence of cycloheximide revealed that VEGF gene activation is independent of *de novo* protein synthesis. Therefore the gene encoding VEGF represents a member of the family of primary response genes. As expected, the NO-mediated decrease in FLT-1 mRNA expression was not affected by incubation with either actinomycin D or cycloheximide (Figure 5B). Remarkably, the NO-independent increase in FLT-1 mRNA levels that we observed in all experiments is inhibited by actinomycin D or cycloheximide, indicating that the increase in FLT-1 mRNA levels is dependent on *de novo* RNA and protein synthesis.

DISCUSSION

The mesangium represents a highly specialized pericapillary tissue that is involved in the most pathological processes in the renal glomerulus. Lying central in the morphological architecture of the glomerulus, mesangial cells are likely to play a very prominent role within these processes. An important feature of the intrinsic mesangial cell is the secretion of a variety of mediators, which is initiated by cross-communication with invading immune cells [41]. Accordingly, we could demonstrate the induction of iNOS by the inflammatory cytokines IL-1β and TNF-α in rat glomerular mesangial cells, resulting in the formation of large amounts of NO *in vitro* [2,3]. A potential role for

NO as a novel mediator, serving the communication between cells by triggering cellular gene expression, has become more evident in recent years [42,43]. For this reason, we focused on the identification of novel NO-regulated genes in mesangial cells. We have discovered that the expression of VEGF and its receptor tyrosine kinase FLT-1 is under regulatory control by exogenously applied NO, and, therefore, that VEGF and its receptor FLT-1 are NO-regulated genes in mesangial cells. It is remarkable that NO led to differential regulation of both genes: as VEGF expression increased after NO-stimulation, FLT-1 mRNA levels clearly declined to levels hardly detectable by RNase protection analysis. To complete the study concerning NO-mediated regulation of VEGF and its receptors, we looked for expression of FLK-1/KDR, the second VEGF-specific receptor tyrosine kinase. Remarkably, we could not detect any expression of the FLK-1/KDR receptor in rat mesangial cells *in vitro*. This was in part unexpected, since expression of the FLK-1/KDR has been described for rat mesangial cells [26]. The FLT-1/KDR signal described by Takahashi et al. [26] was obtained using the reverse transcriptase PCR method, indicating that FLT-1/KDR mRNA levels might be below the detection limits of RNase protection analysis. Moreover, expression patterns of the VEGF/FLT-1 system are also regulated by inflammatory cytokines and growth factors in cultured mesangial cells. Interestingly, our results suggest differential regulation of VEGF and FLT-1, as VEGF is induced by the inflammatory cytokine IL-1β, and FLT-1 is not. Remarkably, IL-1β is much less potent in inducing VEGF expression compared with exogenously added NO. The delayed IL-1β-induced VEGF expression observed after 24 h might, at least partially, be due to the preceding production of NO by IL-1β-induced iNOS. This enzyme is known to release large amounts of NO in mesangial cells after IL-1β stimulation [2–5]. However, IL-1β-mediated up-regulation of VEGF expression is in line with observations from several cell lines, including keratinocytes, aortic smooth muscle cells or synovial fibroblasts [44–46]. In contrast, serum growth factors bFGF and PDGF-BB were able to induce FLT-1 expression but did not influence VEGF expression. Up-regulation of FLT-1 by PDGF, which has been reported for rat mesangial cells [26], is in accordance our own observations. However, serum-mediated up-regulation of VEGF expression in mesangial cells [24,25] is likely not to be mediated by PDGF, since PDGF did not induce VEGF in these cells.

Most physiological effects of NO appear to be mediated via activation of its intracellular receptor guanylyl cyclase [47], followed by an increase in cGMP. Accordingly, mesangial cells have been shown to increase intracellular cGMP levels after LPS- and TNF-α-stimulated induction of iNOS [2–5]. The physiological relevance of this NO-activated cGMP signalling pathway in mesangial cells is supported by the observed down-regulation of FLT-1 after addition of a membrane-permeant cGMP analogue. However, NO is likely to use a second signalling pathway in mesangial and other cells that is independent of the classical cGMP pathway [48]. The presence of an NO signalling pathway that is independent of soluble guanylate cyclase activity is indicated in this study, as NO-triggered induction of VEGF mRNA and protein could not be mimicked by addition of the cGMP analogue. Previous observations support the presence of a cGMP-independent pathway in mesangial cells, since an NO-mediated positive feedback loop amplifying IL-1β-stimulated iNOS transcription could not be compensated for by cGMP [49]. In contrast to VEGF regulation in mesangial cells, NO-triggered expression of VEGF in hepatocarcinoma cells is directly coupled to activation of soluble guanylate cyclase [42], suggesting different cell-type-specific signalling pathways for NO-mediated VEGF induction. Recently, we reported an increase in intracellular cAMP as a second independent signalling pathway for iNOS and related enzyme induction in rat glomerular mesangial cells [38–40]; this pathway is also likely to also be involved in VEGF induction. This is consistent with the presence of activator protein-1 and -2 sites in the VEGF gene promoter, since phorbol esters and forskolin induce VEGF mRNA expression in ovarian bovine granulosa cells and mesangial cells [24,25,50].

Until now, there has been only a small amount of data available concerning the regulation of the VEGF system in diseased kidney. For the undiseased organ, VEGF expression is described for glomerular podocytes and the proximal and distal tubular and collecting duct cells [12,19,20]. Furthermore, FLT-1 is the most abundant VEGF receptor expressed in the kidney [20], thus being in accordance with FLT-1 expression in mesangial cells. Besides its crucial role for kidney development and glomerulogenesis, the presence of VEGF and FLT-1 in the adult kidney suggests a function that is independent of angiogenesis. Since chronic inflammatory diseases of the kidney, including glomerulonephritis, diabetic nephropathy, global glomerular sclerosis and amyloidosis, are characterized by a reduced number of VEGF-expressing cells [19,20], a role for NO-induced VEGF expression in mesangial cells has to be discussed for the situation of acute inflammation or septic shock that is associated with the presence of large amounts of NO in the glomerulus [51]. NO-mediated down-regulation of FLT-1 suggests a simultaneous desensitization of the mesangial cells to the NO-mediated release of the VEGF ligand. As FLT-1 receptor proteins were found predominantly in, and were also restricted to, endothelial cells in adult kidney [20], it is tempting to speculate that mesangial cell-derived VEGF plays a role in regulation of vascular leakage and microcirculation during acute inflammation or septic shock. Further experiments are in progress to evaluate this hypothesis.

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