Tumor Necrosis Factor α Activates Soluble Guanylate Cyclase in Bovine Glomerular Mesangial Cells Via an L-Arginine-dependent Mechanism

By Philip A. Marsden and Barbara J. Ballermann

Summary

Endothelium-derived nitric oxide (NO) causes vasodilatation by activating soluble guanylate cyclase, and glomerular mesangial cells respond to NO with elevations of intracellular guanosine 3',5'-cyclic monophosphate (cGMP). We explored whether mesangial cells can be stimulated to produce NO and whether NO modulates mesangial cell function in an autocrine or paracrine fashion. Tumor necrosis factor alpha (TNF-α) raised mesangial cell cGMP levels in a time- and concentration-dependent manner (threshold dose 1 ng/ml, IC₅₀ 13.8 ng/ml, maximal response 100 ng/ml). TNF-α-induced increases in mesangial cGMP content were evident at 8 h and maximal at 18-24 h. The TNF-α-induced stimulation of mesangial cell cGMP production was abrogated by actinomycin D or cycloheximide suggesting dependence on new RNA or protein synthesis. Hemoglobin and methylene blue, both known to inhibit NO action, dramatically reduced TNF-α-induced mesangial cell cGMP production. Superoxide dismutase, known to potentiate NO action, augmented the TNF-α-induced effect. N⁶-monomethyl-L-arginine (L-NMMA) decreased cGMP levels in TNF-α-treated, but not vehicle-treated mesangial cells in a concentration-dependent manner (IC₅₀ 53 μM). L-arginine had no effect on cGMP levels in control or TNF-α-treated mesangial cells but reversed L-NMMA-induced inhibition. Interleukin 1β and lipopolysaccharide (LPS), but not interferon γ, also increased mesangial cell cGMP content. Transforming growth factor β, blunted the mesangial cell response to TNF-α. TNF-α-induced L-arginine-dependent increases in cGMP were also evident in bovine renal artery vascular smooth muscle cells, COS-1 cells, and 1502 human fibroblasts.

These findings suggest that TNF-α induces expression in mesangial cell of an enzyme(s) involved in the formation of L-arginine-derived NO. Moreover, the data indicate that NO acts in an autocrine and paracrine fashion to activate mesangial cell soluble guanylate cyclase. Cytokine-induced formation of NO in mesangial and vascular smooth muscle cells may be implicated in the pathogenesis of septic shock.

Tumor necrosis factor α (TNF-α), also known as cachectin, has been implicated as a primary mediator in the pathogenesis of septic shock, LPS-induced fever, and wasting associated with chronic disease or disseminated malignancy (1-4). Animals infused with TNF-α developed changes in systemic hemodynamics, including profound reductions in peripheral vascular resistance and organ failure characteristic of gram-negative septicemia or LPS administration (4). Furthermore, animals passively immunized against endogenous TNF-α did not develop septic shock when challenged with a standard LD₅₀ dose of live Escherichia coli and failed to develop a systemic hemodynamic response to LPS administration (5, 6). Such observations suggested that TNF-α mediates many of the pathophysiologic sequelae of gram-negative sepsis. However, little is known about the mechanisms involved in TNF-α-induced hemodynamic changes. Pathways involving stimulation of phospholipase A₂ (7-9), GTPase activity (10), and ADP-ribosylation (11) in the cellular action of TNF-α have been reported.

Synthesis of nitric oxide (NO) is a newly discovered biochemical process in mammals. NO, derived from the guanido nitrogens of L-arginine, has been characterized as endothelium-
derived relaxing factor (EDRF) released from the vascular endothelium (12-15) and a mediator of macrophage-induced cytotoxicity (16, 17). NO generation is constitutively expressed in endothelial cells, yet in macrophages, NO generation requires treatment of cells with cytokines, such as IFN-γ or LPS. In the renal glomerulus, tone of the mesangial cell, a specialized vascular smooth muscle cell, regulates glomerular filtration by altering glomerular capillary surface area. It was therefore of interest to determine whether cytokine-activated mesangial cells, like activated macrophages, express NO synthase. Given that glomerular mesangial cells (GMC) respond to endothelial-derived NO with elevations of intracellular guanosine 3′, 5′-cyclic monophosphate (cGMP) (18, 19), we explored whether GMC produce NO and whether NO modulates GMC function in an autocrine or paracrine fashion.

Materials and Methods

Materials. Cell culture media and balanced salt solutions were purchased from Gibco Laboratories, Grand Island, NY; low endotoxin defined supplemented bovine calf serum (SCS) from HyClone Laboratories, Logan, UT; cGMP RIA kits from Biomedial Technologies, Stoughton, MA; cell culture plates from Costar, Cambridge, MA; Millicell culture inserts from Millipore Products Division, Bedford, MA; N4-monomethyl-L-arginine (l-NMMA) from Calbiochem-Behring, San Diego, CA; glass coverslips from Belco Biotechnology, Vineland, NJ; human recombinant TNF-α (rHuTNF-α, specific activity 5.0 × 108 U/mg) and rHuIFN-γ (specific activity, 3.4 × 107 U/mg) were gifts of Genentech (South San Francisco, CA); rHuE-β (specific activity, 3 × 107 U/mg) was a gift of The Upjohn Co., Kalamazoo, MI; porcine platelet-derived TGF-β1, from R & D Systems, Minneapolis, MN; [3H]-guanosine 3′, 5′-cyclic monophosphate (specific activity 33.3 Ci/mmol) from New England Nuclear, Boston, MA; t-arginine, d-arginine, LPS (E. coli serotype 026:B6, phenol extracted), 3-isobutyl-1-methyl-xanthine (IBMX), bovine liver superoxide dismutase, bovine red cell hemoglobin, methylene blue, sulfanilamide, naphthylethylenediamine HCl, cGMP, and all other reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Cell Isolation and Culture. Calf kidneys were used to initiate bovine glomerular mesangial cell cultures (GMC). GMC clones were established as described (18) and used at passages 10 to 15. Individual cell clones were characterized morphologically and by expression of angiotensin II receptors as well as the absence of factor VIII-related antigen expression or uptake of fluorescent acetylated LDL. Cells were fed every 48 h with RPMI 1640 medium supplemented with 1-glutamine, 15% low endotoxin supplemented bovine calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin. Bovine renal artery vascular smooth muscle cells (VSMC) were isolated from the renal artery of the same calf using published methods (20). Individual cell clones of VSMC were established to obtain pure cell populations then cultured under conditions identical to GMC. Cells were used at passages 5 to 8. COS-1 a monkey-derived kidney cell line (ATCC CRL 1650) and 1502 (ATCC CRL 1502) a normal human skin fibroblast cell line were obtained from American Type Culture Collection, Rockville, MD.

cGMP Assay. Bovine GMC were plated on 12-mm glass coverslips in 24-well plates. Replicate plated coverslips of GMC contained 0.9–1.2 × 104 cells/coverslip as determined by Coulter counter (Coulter Electronics, Hialeah, FL). GMC were kept in serum-free medium for 48 h before assay. Cell monolayers were gently washed with 1 ml of a physiologic salt solution composed of 130 mM NaCl, 5 mM KCl, 10 mM D-glucose, 1 mM MgCl2, 1.5 mM CaCl2, 25 mM Hepes (pH 7.4). Additions to the incubation buffer had no effect on pH of the medium. Coverslips of GMC were removed from 24-well plates, placed in 12-well plates, and allowed to equilibrate for 1 h at 37°C in 1 ml of incubation buffer. IBMX (1 mM) was added 5 or 10 min, as indicated, before termination of the assay. To terminate the assay, GMC coverslips were placed into 0.5 ml of ice-cold 15% trichloroacetic acid. Samples were then transferred to 12 × 75-mm glass tubes, extracted four times with 2 ml of water-saturated ether, vacuum lyophilized, and stored at -20°C for determination of cGMP. GMC cell-associated cGMP was determined by radioimmunoassay. To determine the effect of rHuTNF-α and l-NMMA on other cell types, cells were grown on 18-mm glass coverslips in 12-well tissue culture plates. To ensure that intracellular l-arginine levels were not rate-limiting, 100 μM l-arginine was included in the assay buffer. For the co-incubation assay, GMC grown on 12-mm glass coverslips were placed inverted, by use of sterile technique, on 30-mm Millipore Millicell culture inserts. Inserts were placed in 6-well culture plates with GMC, treated with TNF-α or vehicle, or no cells. Cells were allowed to equilibrate for 1 h at 37°C in 3 ml of incubation buffer and additions were made directly to the co-incubation assay. In this coculture system, cell monolayers are spatially separated but both are exposed to added agents. The ability to grow and treat monolayers individually, to bring them together only for the period of co-incubation and separate the monolayers to determine reporter GMC-associated cGMP levels allowed the determination of whether TNF-α-treated donor monolayers released soluble mediators that modulated cGMP production in reporter monolayers. To exclude cGMP uptake from the extracellular medium we determined whether GMC demonstrated specific uptake of [3H]cGMP. [3H]-cGMP (1 pmol/ml) was added 5, 10, 20, or 60 min to GMC maintained for 1 h in incubation buffer at 37°C, in both the presence and absence of 1 nmol/ml unlabeled cGMP. To terminate uptake, GMC were rapidly washed four times with 1 ml volumes of ice-cold 150 mM NaCl, 0.2% BSA (Fraction V). Cell-associated [3H]-cGMP, extracted as described above, was determined by scintillation counting.

Nitrite and Nitrate Analysis. Nitrite and nitrate in tissue culture medium were determined as described (21). Cells were treated for 48 h with concentrations of rHuTNF-α over the concentration range 0.1–250 ng/ml in phenol red- and serum-free culture medium (DMEM). After centrifugation at 3,000 g for 15 min, nitrate in the culture supernatant was reduced by using nitrate reductase (22). Nitrite concentration of the resulting supernatant was taken to reflect total nitrite/nitrate in the medium. To 150 μl of cell supernatant, 900 μl of the Griess reagent was added. The Griess reagent (final concentrations in the reaction mixture: 0.75% sulfanilamide in 0.5 N HCl/0.075% N-(1-naphthylethylenediamine diHCl in H2O) reacts with nitrite to form a chromophore absorbing at 543 nm, which is quantitated spectrophotometrically. Using known concentrations of NaNO2, a standard curve can be constructed over the linear range of the assay (0–50 μM nitrite). Nonconditioned medium had trace levels of nitrite measuring 0.40 ± 0.01 μM (n = 3, duplicate determinations).

Data Analysis. Unless otherwise indicated, data are expressed as the mean ± SE obtained in at least three separate experiments. Comparisons were made with analysis of variance followed by Dunnett’s modification of the t-test whenever comparisons were made with a common control and the unpaired two-tailed Student’s t test for other comparisons. The level of statistically significant difference was defined as p < 0.05.
Results

The Effect of Tumor Necrosis Factor on Mesangial Cell Cyclic GMP Content. Addition of 100 ng/ml rHuTNF-α for 24 h increased bovine mesangial cell-associated cGMP from baseline values of 0.12 ± 0.01 to 0.41 ± 0.01 pmol/10^6 cells (n = 3, triplicate determinations, p < 0.01). Addition of the phosphodiesterase inhibitor, IBMX (1 mM, 10 min), potentiated the TNF-α-induced increase in GMC cGMP, with values increasing from 0.32 ± 0.06 to 8.97 ± 1.05 pmol/10^6 cells (n = 3, triplicate determinations, p < 0.001). Levels of cGMP in medium conditioned for 24 h by vehicle-treated GMC averaged 0.06 ± 0.01 pmol/ml. After 24 h of TNF-α (100 ng/ml) cGMP levels in GMC-conditioned medium were 0.15 ± 0.01 pmol/ml (n = 3) (p < 0.001).

Fig. 1A demonstrates the time-dependent increase in GMC cGMP after addition of 100 ng/ml rHuTNF-α. TNF-α-induced increases in cGMP levels were evident at 8 h and maximal at 18–24 h (n = 4, triplicate determinations). Shown in Fig. 1B, rHuTNF-α produced a concentration-dependent increase in the cGMP content of GMC treated for 24 h with TNF-α doses ranging from 0.1 to 250 ng/ml (threshold 1.0 ng/ml, maximal response 100 ng/ml, n = 3, triplicate determinations). Half-maximal responses were estimated from log-logit transformation of the data and averaged 13.8 ± 3.7 ng/ml (n = 6, triplicate determinations). Though not shown, treatment of GMC with doses of rHuTNF-α ranging from 0.1 to 100 ng/ml for 8 h led to a similar dose-dependent elevation in GMC cGMP content with half-maximal responses seen at rHuTNF-α concentrations averaging 21.4 ± 5.3 ng/ml (n = 3, triplicate determinations).

The effects of several pharmacologic agents on basal and TNF-α-stimulated GMC cGMP production is shown in Fig. 2. GMC were treated for 10 min with the noted agents and IBMX (1 mM) for 5 min before determination of cGMP content. Hemoglobin (oxygenated, 10 μM) markedly lowered TNF-α–induced mesangial cell cGMP accumulation on average by 81.9 ± 4.9% (n = 3). Methylene blue (10 μM), similarly lowered TNF-α–induced increases in mesangial cell cGMP on average by 79.6 ± 3.7% (n = 3). Superoxide dismutase (SOD, 100 U/ml) significantly increased TNF-α–stimulated mesangial cell cGMP levels by an average 74.0 ± 22.2% (n = 3). Basal mesangial cell–associated cGMP levels tended to be lower in the presence of hemoglobin or methylene blue and higher in the presence of SOD, compared with control cells. However, these changes failed to reach statistical significance.

To determine whether de novo RNA or protein synthesis was required for TNF-α–induced increases in mesangial cell–associated cGMP, cells were treated with actinomycin D (0.2 μg/ml) or cycloheximide (0.2 μg/ml) for 24 h in the presence and absence of rHuTNF-α (100 ng/ml). As shown in Table 1, cycloheximide and actinomycin D abrogated TNF-α–induced increases in bovine GMC cGMP levels.

TNF-induced increases in Mesangial Cell cGMP Are t-Arginine-dependent. t-NMMA decreased mesangial cell–associated cGMP levels in TNF-α–treated, but not vehicle-treated GMC in a concentration-dependent manner over the range from 1 to 2,500 μM (Fig. 3 A). Half-maximal responses were estimated from log-logit transformation of the data and averaged 53.0 ± 2.6 μM (n = 3, triplicate determinations). Maximum inhibition of TNF-α–induced increases in mesangial cell–associated cGMP levels were observed at 2,500 μM and averaged 84.3 ± 5.4% inhibition (Fig. 3 A). t-NMMA at a concentration of 250 μM induced a response representing on average 95.3% of the maximal t-NMMA–induced inhibition. Shown in Fig. 3 B, addition of t-arginine at concentrations ranging from 0.01 to 10 mM reversed the t-NMMA–induced (250 μM) inhibition of TNF-α–induced GMC cGMP accumulation.
Effects of agents on mesangial cell cGMP content. Mesangial cell-associated cGMP levels 24 h after addition of vehicle (hatched bar) or rHuTNF-α (100 ng/ml) (solid bars). Cells were pretreated for 10 min before assay termination with vehicle (control), hemoglobin (oxygenated, 10 μM), methylene blue (10 μM), or superoxide dismutase (100 U/ml). IBMX (1 mM) was added 5 min before assay termination. Columns represent mean ± SE of three experiments, triplicate determinations. *p < 0.05 vs. TNF-α-treated GMC.

Table 1. Effect of Actinomycin D and Cycloheximide on TNF-α–induced Increases in GMC cGMP Levels

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>Actinomycin D</th>
<th>Cycloheximide</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHuTNF-α</td>
<td>0.42 ± 0.05</td>
<td>0.33 ± 0.01</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>rHuTNF-α*</td>
<td>3.15 ± 0.43</td>
<td>0.28 ± 0.01*</td>
<td>0.43 ± 0.05*</td>
</tr>
</tbody>
</table>

Effect of 24-h treatment with actinomycin D (0.2 μg/ml) or cycloheximide (0.2 μg/ml) on cyclic cGMP content of mesangial cells treated with vehicle or rHuTNF-α (100 ng/ml) for 24 h. Cells were treated with 1 mM IBMX 10 min before extraction of cGMP. Data points represent mean ± SE, n = 3, triplicate determinations. *p < 0.01 vs. TNF-α-treated cGMP levels.

Effects of LPS, IL1β, IFN-γ, and TGF-β1 on GMC GMP Levels. It was of interest to determine whether the addition of microbial products or cytokines other than rHuTNF-α could modulate levels of GMC cGMP. Shown in Fig. 4 are the effects of rHuTNF-α (25 ng/ml), rHuIL1β (25 ng/ml), LPS (1 μg/ml), and rHuIFN-γ (200 U/ml) on mesangial cell-associated cGMP as a function of time (n = 3, triplicate determinations). As shown, rHuIFN-γ had no effect on GMC cGMP levels. In separate studies, purified human IFN-γ also failed to modulate GMP cGMP content (data not shown). Addition of rHuIL1β or LPS for 8 h increased mesangial cell-associated cGMP levels. Of interest, rHuIL1β- and rHuTNF-α–induced increases in mesangial cell cGMP that were significantly greater than those observed after treatment of GMC with LPS (p < 0.05).

Figure 2. Effects of agents on mesangial cell cGMP content. Mesangial cell-associated cGMP levels 24 h after addition of vehicle (hatched bar) or rHuTNF-α (100 ng/ml) (solid bar). Cells were pretreated for 10 min before assay termination with vehicle (control), hemoglobin (oxygenated, 10 μM), methylene blue (10 μM), or superoxide dismutase (100 U/ml). IBMX (1 mM) was added 5 min before assay termination. Columns represent mean ± SE of three experiments, triplicate determinations. *p < 0.05 vs. TNF-α–treated GMC.

Figure 3. Effect of L-NMMA and L-arginine on TNF-α–induced increases in mesangial cell cGMP. Cells were treated for 24 h with 100 ng/ml rHuTNF-α. The cGMP content of mesangial cells treated for 24 h with vehicle averaged 0.33 ± 0.09 pmol/10⁶ cells. L-NMMA, L-arginine, and IBMX (1 mM) were added 10, 7.5, and 5 min before termination of the assay, respectively. (A) Concentration-response relationship for L-NMMA–induced decreases in the cGMP content of rHuTNF-α–treated mesangial cells. (B) Concentration-response relationship for L-arginine–stimulated reversal of L-NMMA–induced inhibition of mesangial cell cGMP content. In the absence of L-NMMA cGMP levels in TNF-α–treated mesangial cells averaged 2.89 ± 0.50 pmol/10⁶ cells. Data points represent mean ± SE, n = 3, triplicate determinations. Where error bars are not evident, SE was smaller than data point.
We further determined the effect of treatment for 24 h with rHuIFN-γ, LPS, and rHuIL-1β in the absence or presence of rHuTNF-α. TNF-α was added at threshold (1 ng/ml) and maximal doses (100 ng/ml). IFN-γ (200 U/ml) had no significant effect on mesangial cell-associated cGMP levels in either the presence or absence of TNF-α (data not shown). As was seen at 8 h, treatment of GMC with LPS (1 μg/ml) for 24 h increased GMC cGMP levels (0.40 ± 0.05 and 0.90 ± 0.12 pmol/10^6 cells for control and LPS-treated GMC, respectively, n = 3, p < 0.05). Furthermore, LPS potentiated in a synergistic fashion the effect of a threshold dose of TNF-α (0.84 ± 0.10 and 2.77 ± 0.46 pmol/10^6 cells for GMC treated with TNF-α or TNF-α and LPS, respectively, n = 3, p < 0.05). In contrast, LPS did not significantly increase levels of cGMP in GMC treated concurrently with a maximal dose of TNF-α (data not shown, n = 3). IL-1β (25 ng/ml) increased mesangial cell-associated cGMP levels at 24 h to values that were not significantly different from the effect of a maximal dose of TNF-α. Moreover, IL-1β increased levels of cGMP in GMC treated with threshold or maximal doses of rHuTNF-α in a manner that was additive when compared with the observed effect of GMC treated with TNF-α alone (n = 3). For example, levels of cGMP in control GMC averaged 0.40 ± 0.05 pmol/10^6 cells, whereas cGMP levels in TNF-α (100 ng/ml), IL-1β (25 ng/ml), and TNF-α/IL-1β-treated GMC were 6.75 ± 0.42, 6.34 ± 0.65, and 14.21 ± 1.72 pmol/10^6 cells, respectively. TGF-β1 (10 ng/ml, 24 h) had no significant effect on GMC cGMP levels (0.36 ± 0.05 vs. 0.37 ± 0.05 pmol/10^6 cells, for vehicle- and TGF-β1-treated cells, n = 3). However, levels of cGMP in GMC treated concurrently with TNF-α (100 ng/ml, 24 h) and TGF-β1 (10 ng/ml, 24 h) were lower than cGMP levels in TNF-α (100 ng/ml, 24 h) treated GMC (3.16 ± 0.21 vs. 7.15 ± 0.20 pmol/10^6 cell, n = 3, p < 0.01).

Effect of TNF on Cyclic GMP Production by Various Cell Types. Given that rHuTNF-α increased GMP levels in GMC via an L-arginine-dependent mechanism, it was of interest to determine whether TNF-α had a similar effect on other cell types. Fig. 5 demonstrates the effect of TNF-α treatment (100 ng/ml, 24 h) on levels of cGMP in bovine renal artery VSMC, a human skin fibroblast cell line (1502) and COS 1 cells. To ensure that intracellular L-arginine levels were not rate-limiting, 100 μM L-arginine was included in the assay buffer. TNF-α treatment for 24 h increased levels of intracellular cGMP in the three cell types studied (5 min, 1 mM IBMX, n = 3, triplicate determinations). Compared with the large increase in cell-associated cGMP observed in GMC after addition of 100 ng/ml rHuTNF-α for 24 h, an average 8.23 ± 1.07-fold increase (5 min, 1 mM IBMX), TNF-α-induced increases in VSMC, fibroblasts, and COS 1 cGMP levels were significantly lower than those observed in GMC.
were 2.79 ± 0.05, 1.76 ± 0.28, and 2.28 ± 0.30-fold above baseline values, respectively. The fold increase in GMC cGMP content in response to TNF-α treatment was significantly greater than the increase observed with these other cell types (each case p < 0.05 vs. TNF-α-treated GMC). l-NMMA (500 μM, 10 min) decreased levels of cGMP in TNF-α-treated VSMC, skin fibroblasts, and COS 1 cells. Whereas l-NMMA failed to decrease levels of cGMP in vehicle-treated VSMC or GMC, as also shown earlier, l-NMMA decreased levels of cGMP in vehicle-treated fibroblasts and COS 1 cells.

**GMC Treated with TNF Release a Soluble Factor that Increases cGMP in Adjacent Cells.** The finding of TNF-α-stimulated l-arginine-dependent cGMP production by GMC suggested that TNF-α induces the production of NO by GMC. It was therefore of interest to determine whether donor monolayers of TNF-α-treated GMC released a factor(s) that could augment cGMP production in reporter monolayers. Untreated GMC, serving as reporter monolayers, were grown on coverslips and placed inverted in the upper chamber of Millicell culture inserts. Inserts were then placed in wells that contained confluent donor monolayers of GMC: vehicle- or TNF-α-treated. Levels of cGMP in these reporter monolayers were greater in the presence of TNF-α-treated GMC than in the presence of vehicle-treated GMC, but only in the presence of SOD (200 U/ml) (Table 2). Levels of cGMP in GMC reporter monolayers placed on control GMC monolayers were not different than cGMP levels in GMC reporter monolayers studied in the absence of a donor cell type. It is unlikely that uptake of extracellular cGMP, released from donor monolayers, mediated the observed increase in reporter monolayer cGMP content. GMC uptake of [3H]cGMP averaged 0.7 ± 0.1, 1.4 ± 0.2, 2.5 ± 0.3, and 6.7 ± 0.3% of added counts at 5, 10, 20, and 60 min, respectively (n = 3, triplicate determinations). Assuming that incorporated tracer was not metabolized, levels of GMC [3H]cGMP at 60 min would correspond to 0.03 pmol cGMP/10^6 cells. Given that levels of cGMP in the medium of GMC were ~0.1 pmol/ml and that uptake from medium containing 1 pmol/ml [3H]cGMP accounted for <10% of cell-associated cGMP, it is unlikely that uptake of cGMP accounts for the increased levels of cGMP in reporter monolayers incubated with TNF-α-treated donor monolayers. GMC uptake of [3H]cGMP was competitively inhibited by unlabeled cGMP, with uptake at 60 min averaging 1.672 ± 76 cpm/10^6 cells and 521 ± 30 cpm/10^6 cells in the absence and presence of 1 nmol/ml unlabeled cGMP, respectively (n = 3, triplicate determinations, p < 0.05). Moreover, uptake was significantly lower at 4°C and not modified by treatment of GMC with TNF-α for 1 or 24 h (data not shown). Interestingly, IBMX (1 mM, 1 h) significantly inhibited uptake of extracellular [3H]cGMP (1,419 ± 50 vs. 165 ± 5 cpm/10^6 cells measured at 60 min, p < 0.01). Therefore, though GMC uptake of [3H]cGMP is inhibited by unlabeled cGMP, temperature-sensitive and blocked by IBMX, the magnitude of this uptake is insufficient to account for the results of the coincubation assay. The observation that cGMP levels are increased in GMC cocultured with TNF-treated donor GMC monolayers, but only in the presence of SOD, is consistent with the hypothesis that TNF-treated GMC release NO.

**TNF Increases Nitrite/Nitrate in the Medium of Glomerular Mesangial Cells.** Fig. 6 demonstrates TNF-α induced increases in the content of nitrite/nitrate (NO_2^-/NO_3^-) in the medium of GMC. Measured at 48 h, rHuTNF-α at concentrations ranging from 0.1 to 250 ng/ml increased the NO_2^-/NO_3^- content of the culture medium in a dose-dependent manner (n = 4, duplicate determinations).

### Table 2. Effect of Donor Monolayers on Cyclic GMP Content of Responder Cell Monolayer

<table>
<thead>
<tr>
<th>Donor cell</th>
<th>Control</th>
<th>Superoxide dismutase</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>0.31 ± 0.03</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>Vehicle-treated GMC</td>
<td>0.30 ± 0.01</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>rHuTNF-α-treated GMC</td>
<td>0.29 ± 0.01</td>
<td>0.77 ± 0.04*</td>
</tr>
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</table>

Effect of donor monolayers on cGMP content of responder cell monolayer. Confluent monolayers of responder cells, glomerular mesangial cells, GMC, grown on glass coverslips, were placed inverted in 30-mm Millicell culture inserts on confluent donor monolayers. Donor monolayers: none, vehicle-treated GMC, rHuTNF-α-treated (100 ng/ml, 24 h) GMC were grown on 6-well plates. The co-incubation assay was treated with IBMX (1 mM, 10 min) and where indicated superoxide dismutase (200 U/ml, 10 min). Cell-associated cGMP levels were determined in responder monolayers. Data points represent mean ± SE, n = 4, duplicate determinations.

* p < 0.01 vs. control GMC cGMP levels.

![Figure 6. Determination of the NO_2^-/NO_3^- content in the medium of mesangial cells treated with increasing concentrations of rHuTNF-α for 48 h. Data points represent mean ± SE, n = 3, duplicate determinations.](image-url)
Discussion

Biosynthesis of NO represents a complex enzymatic process that converts guanido nitrogens of L-arginine to NO via an NADPH-dependent citrulline-forming enzyme (23, 24). NO synthase activity is constitutively expressed in endothelial cells (25), neutrophils (26), the adrenal gland (27), and cerebellar cells (28, 29) though basal rates of EDNO release can be augmented by agonists that increase cytosolic calcium concentration. In macrophages, NO generation is induced by IFN-α alone or in combination with bacterial LPS or muramyl dipeptides, the combination of IFN-γ with TNF-α or TNF-β, and IFN-α or IFN-β with LPS (21, 30–32). The enzyme activity in macrophages is cytosolic and dependent upon the cofactors NADPH, reduced bioprotein (33), and FAD flavoprotein (34). But unlike the constitutive enzyme of endothelial cells, macrophage enzyme activity does not appear to be dependent on calcium. Evidence now indicates that NO synthase can also be induced in some tumor cells (35, 36) and Kupffer cells (37). Given that NO generation may be widely expressed it was of interest to determine whether NO secretion can be induced to produce NO and whether mesangial-derived NO modulates GMC function.

TNF-α increased mesangial-associated cGMP content in a time- and concentration-dependent manner. Increases in GMC cGMP levels were evident as early as 8 h and maximal at 18–24 h (Fig. 1). TNF-α-induced increases in mesangial cGMP levels were observed in both the presence and absence of IBMX, an inhibitor of phosphodiesterase activity, suggesting enhanced production of cGMP as opposed to inhibition of cGMP degradation. Levels of cGMP were higher in the medium of GMC treated for 24 h with TNF-α compared with vehicle-treated GMC. We attribute the small increase in medium cGMP to the high capacity of cell-associated 5′-nucleotidase activity.

The pharmacologic manipulations used provide evidence that the active agent released by TNF-α-activated GMC was related to NO. TNF-α-induced mesangial cell cGMP accumulation was inhibited by methylene blue and hemoglobin and potentiated by superoxide dismutase (SOD) (Fig. 2). Methylene blue mediates its effects through inhibition of soluble guanylate cyclase, thereby inhibiting NO action on the target enzyme. EDNO action is also potently inhibited by hemoglobin. NO binds to this and other heme-containing proteins, including soluble guanylate cyclase, forming nitrosyl-heme adducts (38). Hemoglobin, a molecule whose size precludes access to the intracellular compartment, inhibited TNF-α-stimulated increases in GMC cGMP content, indicating that the mediator(s) implicated in the activation of mesangial cell guanylate cyclase had free access to the extracellular compartment. SOD catalyzes the reaction 2O₂⁻ + 2H⁺ → O₂ + H₂O₂. The pharmacologic half-life of NO is markedly shortened in the presence of superoxide radical (O₂⁻·), a mechanism possibly involving the production of nitrogen oxides from NO. Although cGMP levels in vehicle-treated GMC tended to be lower in the presence of hemoglobin or methylene blue and higher in the presence of SOD these changes failed to reach statistical significance. The finding in the current study that TNF-α-induced elevations of mesangial cell cGMP content were blunted by hemoglobin and methylene blue and potentiated by SOD is consistent with the view that TNF-α-activation of GMC soluble guanylate cyclase is mediated by NO.

NO is synthesized from the semi-essential amino acid L-arginine. L-NMMA serves as an enantiomerically specific competitive inhibitor of NO synthesis. L-NMMA is an inhibitor of macrophage cytotoxicity and NO₂⁻ or NO₃⁻ production (16, 21). Systemic infusion of L-NMMA induces a potentiator pressor response and local infusion of L-NMMA increases vascular resistance (39–41). Both of these L-NMMA-induced effects could be reversed with L- but not D-arginine. In isolated vascular smooth muscle rings, L-NMMA induces a small but significant endothelium-dependent vasoconstriction and blunts the vasodilatory response to acetylcholine (42). These observations have been taken to suggest that L-NMMA inhibits basal release of EDNO in vivo and in vitro. Activity of NO synthase in endothelial, macrophage, cerebellar, and adrenal homogenates is inhibited by L-NMMA in a concentration-dependent manner (27, 29, 33, 43). The L-arginine dependence of NO synthesis in other cell types suggested that TNF-α-induced increases in GMC cGMP content may be L-arginine dependent. In the current study we have demonstrated that TNF-α-induced elevations of GMC cGMP content are dependent upon L-arginine in that L-NMMA decreased cGMP levels in a TNF-α-treated GMC in a concentration-dependent manner (Fig. 3). L-NMMA-inhibition of TNF-α-induced expression could be reversed by L-arginine in a competitive manner. Competitive reversal by L-arginine was enantiomerically specific because D-arginine was without effect. It was of interest that L-arginine had no effect on rates of cGMP production by TNF-α-treated GMC, suggesting that enzyme activity was not limited by the cellular content of free L-arginine, even when GMC had been maintained for 1 h in a physiologic salt solution devoid of L-arginine. Furthermore, L-NMMA had no effect on cGMP levels of vehicle-treated GMC, suggesting that basal rates of mesangial cGMP production were not dependent upon L-arginine. Consistent with the hypothesis that TNF-α induces the formation of NO in GMC is the demonstration of nitrogen oxides (NO₂⁻ and NO₃⁻) in the conditioned medium of TNF-α-treated GMC (Fig. 6).

The most reasonable interpretation of our observations is that TNF-α induces in GMC the expression of an enzyme(s) involved in the formation of L-arginine–derived NO. Our findings that inhibitors of new RNA synthesis or protein synthesis abrogate TNF-α-induced increases in GMC cGMP content are taken to represent an effect of TNF-α on transcription of a gene whose protein product is rate-limiting for L-arginine–dependent NO synthesis, possibly NO synthase. Expression of enzyme activity is minimal in vehicle-treated GMC in that cGMP production was not blunted by L-NMMA though cells could respond to sodium nitroprusside, an exogenous activator of mesangial cell soluble guanylate cyclase, with elevation of cGMP content (data not shown).

Macrophages in culture treated with IFN-γ and LPS syn-
thesize nitrogen oxides (NO, NO$_2^-$, and NO$_3^-$) and l-citrulline from l-arginine (17, 33, 44). This enzymatic activity is dependent upon activation of macrophages, given that it is absent from unstimulated cells. Furthermore, induction of enzymatic activity requires new RNA and protein synthesis. In many regards, therefore, TNF-α–treated GMC resemble activated macrophages. However, the present study suggests that macrophages and mesangial cells differ with respect to the cytokines capable of activating the NO synthetic activity. We found that TNF-α induced NO production by mesangial cells, whereas previous studies showed that TNF-α had minimal effect on nitrogen oxide(s) production by macrophages, although TNF-α augmented IFN-γ-induced nitrogen oxide(s) production (31, 32). Furthermore, whereas IFN-γ apparently augmented NO production by macrophages, in this study rHuIFN-γ was without effect in unstimulated and in TNF-α–stimulated mesangial cells. IFN-γ induces the expression of MHC class I and class II antigens in rat GMC (45), indicating a functional response in this cell type. However, the possibility that bovine GMC fail to respond to rHuIFN-γ cannot be excluded. Responses by macrophages and GMC to IL-1β also appear to differ. IL-1β does not have a significant effect on macrophage nitrogen oxide production and does not act as a cofactor when added in the presence of IFN-γ (32, 46). By contrast, IL-1β increased mesangial cell–associated cGMP levels at 24 h to values that were not significantly different from the effect of maximal TNF-α doses, and addition of IL-1β to GMC treated with maximal doses of TNF-α increased cGMP levels above cGMP levels achieved with TNF-α alone. Increases in the cGMP content of IL-1β-treated GMC are of interest in that IL-1β treatment of vascular smooth muscle strips impairs contractile responses in an endothelium-independent manner (47). Recent observations suggest that IL-1 activates guanylate cyclase in rat vascular smooth muscle rings and cultured VSMC, an effect that is blocked by hemoglobin and methylene blue (48).

LPS toxicity is thought to be mediated in large part by TNF-α, and LPS stimulates TNF-α production by macrophages and by rat mesangial cells (49). Therefore, it was of interest that LPS could also induce NO-dependent cGMP production by mesangial cells. Prolonged exposure of rat aorta to low levels of endotoxin in vitro results in impaired contractility that may be mediated by cytokines derived from the vasculature (50) and preliminary observations suggest that this effect that can be reversed with L-NMMA (51). Thus, this study demonstrates that TNF-α, IL-1β, and LPS increase cGMP levels in GMC. Furthermore, cytokine-induced responses of GMC differ from those reported for macrophages. Whether the apparent differences result from differences in the cytokine receptors expressed on the two cell types remains to be determined. It is of interest that TGF-β blocked the ability of IFN-γ to induce release of nitrogen oxides from mouse peritoneal macrophages (52). We also demonstrated that TGF-β, though not modulating GMC cGMP levels by itself, blunted the TNF-α response.

To extend our observations we determined the effect of TNF-α on the cGMP content of bovine renal artery VSMC, a human skin fibroblast cell line (1502) and renal-derived COS 1 cells. In each of the cell types examined, TNF-α increased cGMP production and L-NMMA decreased cGMP levels in TNF-α–treated cells, suggesting that cell types other than GMC respond to TNF-α with induction of NO synthase activity. In contrast to the lack of effect of L-NMMA on basal GMC or renal artery VSMC cGMP production, L-NMMA decreased basal levels of cGMP in 1502 fibroblasts and COS 1 cells. We interpret these observations to suggest that basal rates of guanylate cyclase activity in COS 1 cells and 1502 fibroblasts are l-arginine dependent.

Current evidence suggests that NO synthesized by donor cell types, such as endothelial cells, can stimulate soluble guanylate cyclase in adjacent responder cell types, such as VSMC or GMC (15, 24). For instance, within the renal glomerulus, glomerular endothelial cells (GEN) can serve as a source of EDNO, to which GMC can respond (18). We therefore determined whether TNF-α-treated GMC release a factor to which adjacent cells can respond. Levels of cGMP were higher in reporter GMC monolayers cocultured in Millicell culture systems with TNF-α–treated GMC compared to vehicle-treated GMC, but only in the presence of SOD. Though the uptake of extracellular cGMP is unlikely to account for this observation in that GMC exhibit a low capacity uptake process for cGMP, we cannot exclude a minor contribution of extracellular cGMP. The low capacity for cellular uptake of cGMP is consistent with the weak biologic effects of cGMP on intact tissue where lipid soluble analogues, namely 8'-bromo-cGMP and dibutyryl-cGMP, are more effective. Furthermore, the observed requirement for SOD in the coculture experiments suggests that the biologic activity of NO was required for this response. These data indicate that TNF-α–treated GMC exert a paracrine effect on the cGMP content of adjacent cells. Our model suggesting a role for mesangial–derived NO in the autocrine and paracrine modulation of cell function, in this case activation of soluble guanylate cyclase, would be similar to the proposed role for NO in activated macrophages wherein NO has been suggested as the mediator of l-arginine–dependent autocrine and paracrine inhibition of cellular metabolism (17, 21, 44).

The results of the current study suggest that TNF-α induces the expression in GMC of an enzyme(s) involved in the formation of l-arginine derived NO. Moreover, NO acts in an autocrine and paracrine fashion to activate GMC soluble guanylate cyclase. Since elevations in cGMP inhibit mesangial cell contraction (19), the study suggests that NO produced by TNF-α–activated GMC may regulate mesangial cell contractility in vivo under pathophysiologic conditions. Furthermore, the data suggest that TNF-α induces the expression of NO synthase in vascular smooth muscle cells and fibroblasts. As a possible mechanism for TNF-α–induced hemodynamic changes we propose that TNF-α increases NO synthase activity within vascular tissues which, under basal conditions, is otherwise poorly expressed. Consistent with this hypothesis, Kilbourn et al. demonstrated that L-NMMA inhibited TNF-induced hypotension (53). Recently, Beasley et al. in this laboratory (48), also suggested that II-1–induced hypotension could be mediated, in part, by the production of NO in vascular smooth muscle cells. This study suggests...
that NO produced by TNF-α-stimulated mesangial cells may
have an autocrine and paracrine action within the renal
glomerulus and in the postglomerular circulation, namely
stimulation of soluble guanylate cyclase, yet the renal response
to LPS or TNF-α is intense vasoconstriction and reduction
of glomerular filtration rate (54, 55). Though these studies
do not address whether TNF-α induces NO synthase activity
in vivo, it is possible that production of NO within the
microvasculature of the kidney serves to offset LPS- or
TNF-α–induced vasoconstriction.

We thank Babette Radner for excellent technical assistance. We thank Dr. C. B. Carpenter for kindly re-
viewing the manuscript.

Dr. Philip A. Marsden is a Medical Research Council of Canada fellowship recipient. This work was
supported by National Institutes of Health DK-39249.

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Received for publication 14 May 1990 and in revised form 20 August 1990.

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