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NITRIC OXIDE MODULATES THE SYNTHESIS OF EXTRACELLULAR MATRIX PROTEINS IN CULTURED RAT MESANGIAL CELLS

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SUMMARY. Nitric oxide (NO) is an important effector molecule of the inflammatory response. It is synthesized by mesangial cells and has been proposed to contribute to glomerular injury in various disease states. We studied whether NO modulates extracellular matrix production in cultured rat mesangial cells. Stimulation of rat mesangial cell NO release with γ-interferon and lipopolysaccharide resulted in reduced production of collagen (by 35%) fibronectin (by 48%) ($P<0.05$). In contrast, laminin synthesis was enhanced two-fold by the same maneuver ($P<0.05$). These changes were reversed by the addition of L-NAME, a selective inhibitor of inducible nitric oxide synthase. This is the first demonstration that NO regulates the synthesis of extracellular matrix by mesangial cells. The results indicate that increased renal production of NO in glomerular diseases may attenuate the production and accumulation of matrix proteins and limit the severity of glomerulosclerosis.

Nitric oxide (NO) is a newly discovered, short-lived messenger molecule that participates in neurotransmission, the immune response and blood pressure homeostasis (1,2). NO serves many functions within the kidney including regulation of afferent arteriolar tone (3), tubular Na^+ handling (4,5) and proliferation of mesangial cells (6). Disturbances in NO metabolism occur in a variety of experimental renal diseases including streptozocin-induced diabetic nephropathy (7,8), reduced renal mass (9), obstructive uropathy (10,11) and chronic puromycin aminonucleoside nephropathy (11).

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The chronic accumulation of extracellular matrix (ECM) proteins in glomeruli and tubulointerstitial regions of the kidney is critical in the progressive loss of function in renal diseases (12). The fibrotic process is tightly regulated by many factors including cytokines and circulating hormones (13). The role of NO in the control of ECM production in the kidney has never been examined. Therefore, we conducted the following studies to ascertain the effect of NO on the synthesis of collagen, fibronectin and laminin by rat mesangial cells *in vitro*.

METHODS

Cells: Rat mesangial cells were obtained from primary glomerular explants and used between passages 6-10 (14). On phase-contrast microscopy, the identity of mesangial cells was confirmed by their elongated, stellate or fusiform appearance; in addition, there was no inhibitory effect of puromycin aminonucleoside or D-valine on cell growth (14). Cells were plated at a density of 250,000/ml in 12 well plates and incubated at 37°C in a humidified atmosphere of 10% CO_2-90% air. Cells were maintained in Dulbecco's modified Eagle (DME) medium, supplemented with 100 μg/ml streptomycin, 100 μg/ml penicillin and 10% fetal bovine serum.

Experimental conditions: After the cells reached confluence (usually within 3-5 days, the plates were randomly assigned to one of the following four experimental conditions: (1) Control: DME without additives; (n=6) (2) L-NAME: DME supplemented with 1 mM L-NAME (N^G-nitro-L-arginine methylester) (n=6); (3) IFN/LPS: DME + γ-interferon, 50 U/ml + lipopolysaccharide, 10 μg/ml (n=6); (4) IFN/LPS + L-NAME: DME + γ-interferon, 50 U/ml + lipopolysaccharide, 10μg/ml + 1 mM L-NAME (n=6). Cells were maintained for an additional 24 h in the various test media.

Nitrite assay: Nitrite production by rat mesangial cells was determined with the Greiss assay (15). Briefly, 125 μl of the Greiss reagent containing 1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride and 2.5% phosphoric acid was added to 125 μl of conditioned media. Samples were incubated at 25°C for 10 min and absorbance was measured at 550 nm.

Collagen production: Collagen production was evaluated using the technique of Peterkofsky and Diegelman (16) as modified by Berg et al (17). Ascorbate (50 μg/ml) was added to the test media to stimulate collagen production. After 18 h incubation, fresh medium containing L-[2,3 ^{3}H] proline (5 μCi/ml) was added to the wells for the final 6 h. Cells and media were then harvested, homogenized and proteins were precipitated with 100% TCA and dissolved in 0.2 N NaOH. Samples were neutralized with 1 M HEPES buffer and then digested with purified bacterial collagenase, type III and percentage collagen production was calculated using the formula of Breul et al (18). The results are expressed as a percentage of total protein synthesis.

Dot blot analysis: At the end of the 24 h experimental period, cells were scraped into 2 M urea-Tris buffer and dissolved overnight. An aliquot was removed for protein determination. Samples were spun at 10,000 rpm for 10 min and 250 μl of supernatant was applied to nitrocellulose paper in each well of the dot blot apparatus. Samples and standards were incubated with the primary antibody (1:1000 dilution) in 100 mM Tris-saline-0.1% Tween 20 buffer (TTB), pH 7.5, for 1 h. After rinsing and exposure to biotinylated secondary antibody in TTB for 30 min, vectastain ABC reagent was added and color was developed with a substrate solution containing TTB:methanol (5:1), 0.15% H_2O_2, 0.05% 4-chloro-naphthol. Dot blot analysis was performed twice (n=6, for each experimental condition), once for fibronectin and once for laminin.

Protein assay: The protein contents of the cell homogenates were determined using a Coomassie blue reagent (BioRad, Richmond, CA).

Reagents: Cell culture media, fetal bovine serum, penicillin and streptomycin were purchased from Gibco (Grand Island, NY). Chemical reagents including L-NAME, LPS (*Escherichia coli*, serotype O26:B6) were from Sigma Chemical Company (St. Louis, MO). The γ-IFN was from Genzyme (Boston, MA) and the purified bacterial collagenase, type III was from Advanced BioFactures (Lynbrook, NY). The primary antibodies to collagen (goat) fibronectin (goat) and laminin (rabbit) were purchased from Southern Biotechnology Associates, Inc., Birmingham, AL or Sigma Chemical Co. The secondary antibodies and Vectastain ABC reagent kit were from Vector Laboratories, Burlingame, CA).

Statistical methods: Results are presented as mean ± SEM. The means between the experimental groups were compared using analysis of variance; post hoc inter-group comparisons were made using the Bonferroni correction.

RESULTS

Nitrite production: Basal NO synthesis by mesangial cells during the 24 h incubation was minimal, 0.11 ± 0.02 nmol per aliquot of conditioned control media, and addition of L-NAME lowered it below the detectable limit of the assay. Stimulation of mesangial cells with IFN (50 U/ml) and LPS (10 μg/ml) enhanced NO production nearly six-fold to 0.63 ± 0.05 nmol/sample ($P < 0.01$). The cytokine-induced increase in NO production was reduced to 0.20 ± 0.02 nmol/sample by L-NAME ($P < 0.01$); however, NO synthesis under these conditions still exceeded the level in unstimulated mesangial cells ($P < 0.05$).

Collagen production: Incubation of mesangial cells with IFN/LPS resulted in a 35% reduction in net collagen production, expressed as a percentage of total protein synthesis, compared to control conditions ($P < 0.05$). Although the inducible nitric oxide synthase (iNOS) inhibitor, L-NAME (19), had no effect on collagen production in control media, addition of L-NAME reversed the effect of IFN/LPS (Figure 1).

Fibronectin synthesis: Exposure of the mesangial cells to media containing IFN/LPS yielded a 48% decrease in fibronectin synthesis compared to control media ($P < 0.05$). This change was significantly greater than the 15% lowering in total protein content in mesangial cells incubated with IFN/LPS. The reduction in fibronectin synthesis was reversed by the addition of L-NAME. L-NAME alone had no effect on fibronectin synthesis (Figure 2).

Laminin synthesis: In contrast to the inhibitory effect of IFN/LPS on collagen and fibronectin synthesis by rat mesangial cells, stimulation of NO release resulted in a near doubling of laminin synthesis from 204 ± 17 (control) to 395 ± 59 ng/mg protein

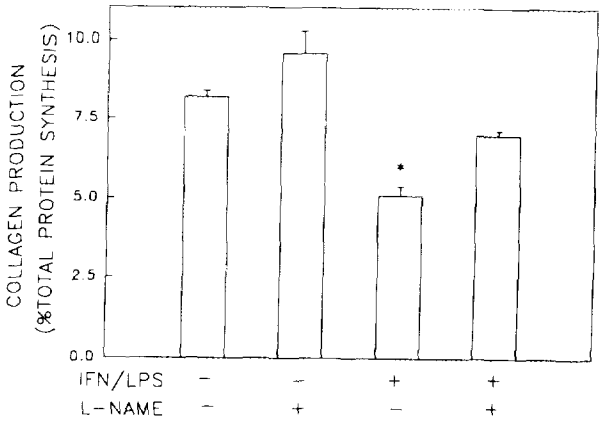

Figure 1. Net collagen production, expressed as a percent total protein synthesis, in rat mesangial cells. IFN, γ-interferon, 50 U/ml; LPS, lipopolysaccharide, 10 μg/ml; L-NAME, N^G-nitro-L-arginine methylester, 1 mM. n=6, each experimental condition. *$P<0.05$ *versus* other conditions.

(IFN/LPS) ($P<0.05$). This change occurred in the face of a 33% lowering of total protein content in mesangial cells exposed to IFN/LPS. The effect of IFN/LPS was completely reversed by the addition of L-NAME to the culture media. L-NAME alone did not alter laminin synthesis by the mesangial cells (Figure 3).

DISCUSSION

There is intense interest in the role of NO in the pathogenesis of renal disease because it participates in the regulation of glomerular perfusion and tubular function (3,4,5). Thus, it has been suggested that NO may mediate the glomerular hyperfiltration

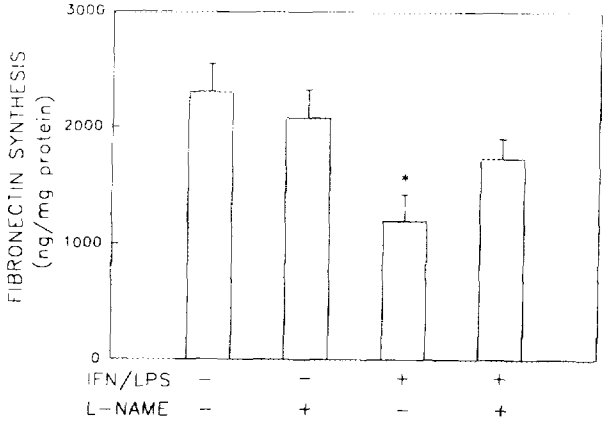

Figure 2. Fibronectin synthesis, ng/mg protein, in rat mesangial cells. IFN, γ-interferon, 50 U/ml; LPS, lipopolysaccharide, 10 μg/ml; L-NAME, N^G-nitro-L-arginine methylester

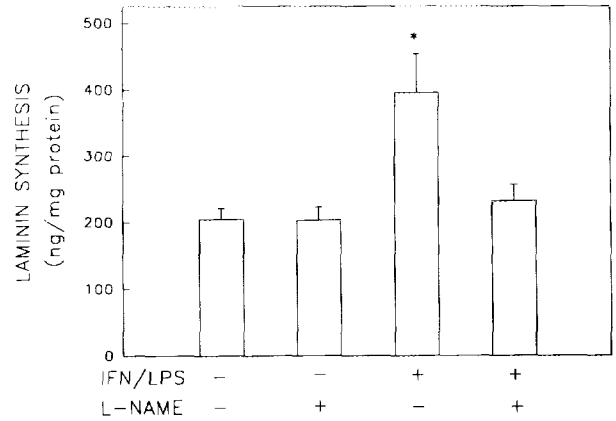

Figure 3. Laminin synthesis, ng/mg protein, in rat mesangial cells. IFN, γ-interferon, 50 U/ml; LPS, lipopolysaccharide, 10 μg/ml; L-NAME, N^G-nitro-L-arginine methylester, 1 mM. n=6, each experimental condition. * $P<0.05$ *versus* other conditions.

that characterizes animals with diabetic nephropathy or reduced renal mass (7,8,9). In addition, as a cytotoxic effector of the immune system, glomerular production by native or infiltrating cells may mediate inflammation in experimental glomerulonephritis (20,21). Our findings are the first to document an effect of NO on ECM protein synthesis by rat mesangial cells *in vitro*. There have been no previous studies that addressed this issue.

Glomerular mesangial cells contain the enzyme iNOS and are fully capable of synthesizing NO in response to inflammatory cytokines (22). Our data demonstrate that NO directly modulates the production of ECM proteins by mesangial cells. The inhibition of collagen and fibronectin together with stimulation of laminin synthesis indicates that individual components of the ECM are regulated independently in response to NO. There is precedent for disparate control of ECM proteins by one regulatory factor. Thus, like NO, insulin inhibits fibronectin synthesis but stimulates laminin production by rat mesangial cells *in vitro* (23).

It is known that NO has an inhibitory effect on total protein synthesis (24). However, the reduction in collagen and fibronectin synthesis was disproportionately larger than the decrease in total protein synthesis. Moreover, the increase in laminin occurred in the face of diminished protein synthesis.

These findings suggest that NO release by mesangial cells modulates the synthesis of ECM proteins in glomerular disease. TGF-β and PDGF inhibition of mesangial cell NO synthesis may contribute to the fibrotic and proliferative response to these cytokines that are upregulated in experimental glomerulonephritis (25,26). Furthermore, reduced

NO release as a result of endothelial cell damage may potentiate sclerosis in these conditions. Enhanced NO formation in diabetic nephropathy may be an adaptive response that limits ECM deposition and fibrosis following glomerular injury and attenuates progressive glomerulosclerosis. Additional studies are required to determine whether NO synthesis by renal tubular epithelial cells also acts to reduce ECM synthesis and to attenuate tubulointerstitial fibrosis. Thus, NO may have opposing effects on renal cells -- direct cytotoxicity and antifibrosis -- and the sum of these actions may determine the outcome following glomerulonephritis.

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