Posttranscriptional regulation of human iNOS by the NO/cGMP pathway

DOLORES PÉREZ-SALA, EVA CERNUDA-MOROLLÓN, MANUELA DÍAZ-CAZORLA, FERNANDO RODRÍGUEZ-PASCUAL, AND SANTIAGO LAMAS

Department of Estructura y Función de Proteínas, Centro de Investigaciones Biológicas, Madrid, and Instituto “Reina Sofía” de Investigaciones Nefrológicas, CSIC, 28006 Madrid, Spain

Received 17 March 2000; accepted in final form 1 November 2000

Pérez-Sala, Dolores, Eva Cernuda-Morollón, Manuela Díaz-Cazorla, Fernando Rodríguez-Pascual, and Santiago Lamas. Posttranscriptional regulation of human iNOS by the NO/cGMP pathway. Am J Physiol Renal Physiol 280: F466–F473, 2001.—Nitric oxide (NO) and cGMP may exert positive or negative effects on inducible NO synthase (iNOS) expression. We have explored the influence of the NO/cGMP pathway on iNOS levels in human mesangial cells. Inhibition of NOS activity during an 8-h stimulation with IL-1β plus tumor necrosis factor (TNF)-α reduced iNOS levels, while NO donors amplified iNOS induction threefold. However, time-course studies revealed a subsequent inhibitory effect of NO donors on iNOS protein and mRNA levels. This suggests that NO may contribute both to iNOS induction and downregulation. Soluble guanylyl cyclase (sGC) activation may be involved in these effects. Inhibition of sGC attenuated IL-1β/TNF-α-elicited iNOS induction and reduced NO-driven amplification. Interestingly, cGMP analogs also modulated iNOS protein and mRNA levels in a biphasic manner. Inhibition of transcription unveiled a negative posttranscriptional modulation of the iNOS transcript by NO and cGMP at late times of induction. Supplementation with 8-bromo-cGMP (8-BrcGMP) reduced iNOS mRNA stability by 50%. These observations evidence a complex feedback regulation of iNOS expression, in which posttranscriptional mechanisms may play an important role.

Inflammatory mediators; human mesangial cells; mRNA stability; nitric oxide; inducible nitric oxide synthase; guanosine 3',5'-cyclic monophosphate

GLOMERULAR MESANGIAL CELLS are specialized smooth muscle cells that contribute to the structural support of the glomerulus and to the control of glomerular filtration rate. They also play an important role in the pathological processes of the glomerulus, particularly in glomerulonephritis and glomerulosclerosis (24). On exposure to proinflammatory agents, these cells become activated and express increased levels of adhesion molecules, like intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, and generate cytokines, prostaglandins, and nitric oxide (NO) (24). The increased generation of NO associated with glomerular inflammation is due in part to the expression of the inducible form of nitric oxide synthase (iNOS).

The induction of iNOS in mesangial cells has been documented in vitro (36) as well as in human glomerulonephritis (9). The role of NO in glomerular disease is not fully elucidated (see Ref. 34 for review). NO may exert protective effects by inhibiting the increased production of extracellular matrix, which is associated with the progression of glomerulonephritis (17). However, high levels of NO can be deleterious due to cytotoxic effects (13). The existence of glomerular mechanisms to terminate the production of NO is suggested by the increased expression of negative regulators of iNOS expression like interleukin (IL)-13, IL-4, and transforming growth factor-β (TGF-β) in glomerulonephritis (2, 16, 20, 28). In addition, NO itself has been proposed to participate in the negative control mechanisms regulating its own synthesis (5, 12, 29).

NO has been reported to modulate both the activity and expression of iNOS. A negative feedback of NO on NOS enzymatic activity has been previously attributed to the ability of NO to interact with the heme component of the enzyme (1, 12). In addition, NO has been shown to modulate iNOS expression. However, the nature of the effects encountered appears to depend on the experimental system under study. In smooth muscle and rat mesangial cells stimulated with IL-1, NO has been proposed to play a positive role in iNOS induction (3, 25). In contrast, NO donors have been reported to limit iNOS induction in glial cells (29) and in macrophages (30). Generation of cGMP by activation of soluble guanylyl cyclase (sGC) has been implicated in mediating some of the effects of NO donors on gene expression (8, 33, 38, 41). With regard to iNOS, cGMP-elevating agents have been reported to exert either positive or negative effects on iNOS induction in a cell type-dependent manner. Whereas atrial natriuretic factor and cell-permeable cGMP analogs amplify iNOS induction in smooth muscle cells (14), the same agents inhibit iNOS expression in murine macrophages (15).

In this work, we have used human mesangial cells to explore the role of NO and cGMP in the modulation of iNOS expression. We have observed that both NO donors and cGMP analogs can exert either stimulatory...
or inhibitory effects on iNOS expression in a time-dependent fashion.

MATERIALS AND METHODS

**Materials.** Recombinant human TNF-α was a generous gift of Dr. J. M. Redondo (Centro de Biología Molecular, CSIC). Recombinant human IL-1β (5 × 10^5 U/mg) was from Boehringer Mannheim. Recombinant human TGF-β1 was from R&D Systems Europe (Abingdon, UK). Cell culture media and fetal bovine serum were from Bio-Whittaker. α-[32P]dCTP (3,000 Ci/mmmol) was from Amersham (Buckinghamshire, UK). Antibodies for immunoblotting were monoclonal anti-iNOS (Transduction Laboratories), polyclonal anti-TGF-β1 (Promega, Madison, WI), and peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulins (Dako, Glostrup, Denmark). 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) was from Alexis (Läufelfingen, Switzerland). 3-(5-hydroxymethyl-2'-furyl)-1-benzylindazol (YC-1) was from Calbiochem (San Diego, CA). All other reagents used were of the highest purity available from Sigma (St. Louis, MO).

**Cell culture.** Human mesangial cells (HMC) were obtained and characterized as previously described (36). For experiments, cells from three independent preparations between passages 5 and 15 were used. Confluent HMC were incubated in RPMI without phenol red with the indicated agents in the absence of serum. Cell viability, as evaluated by trypan blue exclusion, was above 90% under all experimental conditions studied.

**Nitrite determination.** The accumulation of nitrite in the cell culture supernatant of HMC was taken as an index of iNOS activity. After treatment with the various agents, nitrite was measured in the supernatants of HMC by the Griess reaction as previously described (36), by using sodium nitrite as a standard.

**RNA isolation and Northern blot analysis.** Total cellular RNA was isolated from HMC by using the guanidinium thiocyanate-penton-chloroform method (4). Ten micrograms of total RNA were separated on 1% agarose/0.68 M formaldehyde gels, transferred to MSI magnagraph membranes (Westborough, MA), and ultraviolet (UV) cross-linked with a UV Stratalinker 1800 (Stratagene, La Jolla, CA). For analysis of iNOS mRNA expression, a 2.1-kb fragment from the human iNOS cDNA, a gift of Dr. D. A. Geller (10), was labeled with α-[32P]dCTP by using the kit Rediprime for random primer labeling from Amersham. A 2.4-kb fragment of human TGF-β1 (gift of Dr. J. Eigo, Fundación Jiménez Díaz, Madrid, Spain) labeled as above, was used to assess TGF-β1 mRNA levels. Hybridization was performed at 42°C for 16 h. Membranes were then washed at final stringency conditions of 1× standard sodium citrate (SSC), 0.5% SDS at 42°C, and exposed to X-OMAT S Kodak film at −80°C. To ensure even loading of the samples, blots were stripped and rehybridized with a probe for the 28 S RNA gene (pTRI RNA 28S) from Ambion (Austin, TX). Densitometric analysis was performed by using an Agfa StudioStar TPO scanner with the public domain software NIH IMAGE 1.60b5. Results were calculated as the ratio of iNOS mRNA to 28 S rRNA expression.

**Determination of iNOS mRNA half-life.** HMC were stimulated with IL-1β/tumor necrosis factor (TNF-α) for 4 h in the presence or absence of 8-bromo-cGMP (8-BrcGMP). For determination of iNOS mRNA half-life, actinomycin D (10 μg/ml) was subsequently added and cells were then harvested for RNA isolation and Northern blot analysis every 2 h for 8 h as described above.

**SDS-PAGE and immunoblotting.** For analysis of iNOS protein expression, HMC were harvested by treatment with trypsin-EDTA. Cell extracts were obtained and analyzed by Western blot as described (6, 37). Blots were probed with anti-iNOS antibody (1:1,000 dilution) followed by secondary antibody at 1:2,000 dilution, and the iNOS band was visualized by using an enhanced chemiluminiscence (ECL) detection system from Amersham. Levels of iNOS protein were estimated by densitometry of the ECL exposures as previously described (6), and the values obtained were corrected by the band intensities of the Coomassie staining of membranes after the blotting procedure.

**Data analysis.** Unless otherwise indicated, data are expressed as means ± 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-tail Student’s t-test for other comparisons. The level of statistically significant difference was defined as P < 0.05.

**RESULTS**

**Characterization of iNOS induction in HMC.** Induction of iNOS activity in HMC occurs in response to the stimulation with combinations of cytokines or of cytokines plus bacterial lipopolysaccharide (LPS) (36). Under our experimental conditions, levels of iNOS protein assessed by Western blot showed a good correlation with the induction of iNOS activity as estimated from the accumulation of nitrite in the cell culture supernatant (Fig. 1A). In subsequent experiments HMC were stimulated with a combination of IL-1β/TNF-α. This cytokine combination elicited a submaximal induction of iNOS protein (Fig. 1A, bottom). Levels of iNOS mRNA were undetectable in unstimulated cells (Fig. 1B). In cells stimulated with IL-1β/TNF-α, iNOS mRNA could be detected after a 4-h treatment and reached maximal levels between 8 and 12 h. After 12 h, iNOS mRNA levels started to decline but remained elevated above basal levels up to at least 24 h (Fig. 1B).

**Effect of NOS inhibitors and NO donors on iNOS expression in HMC.** To assess the importance of NO generation for iNOS induction, we stimulated HMC with IL-1β/TNF-α in the presence of the NOS inhibitors nitro-L-arginine methyl ester (L-NAME) and N-nitro-L-arginine (L-NMMA) or the NO donors sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP). At 500 μM, L-NAME reduced cytokine-induced NO generation by 88% (Fig. 2A). This effect was associated with a 56 ± 3% reduction of iNOS protein levels (mean ± SE of three experiments), assessed after an 8-h treatment (Fig. 2B). A similar effect was observed when L-NMMA was used as NOS inhibitor. Supplementation of HMC with a single dose of SNP at the time of stimulation with cytokines resulted in an accumulation of nitrite in the cell culture supernatant that was more than additive with respect to the amounts generated by cells treated only with cytokines or with SNP (Fig. 2A). This suggested a positive role of SNP on iNOS induction. In fact, both SNP and SNAP clearly amplified iNOS protein expression at...
8 h (3.2 ± 0.6-fold amplification in the case of SNP, n = 3) (Fig. 2B). None of the NO donors or NOS inhibitors elicited iNOS expression per se (not shown).

We next studied the temporal pattern of IL-1β/TNF-α-elicited iNOS induction in the absence and presence of SNP. As it is shown in Fig. 3A, coincubation with SNP potently amplified iNOS protein abundance up to 24-h treatment. However, a reduction in iNOS protein levels was observed at later time points (53 ± 18% inhibition after 48-h treatment, n = 3). A similar effect was observed at the mRNA level (Fig. 3B). Quantitation of these results by densitometric analysis revealed a 2.6-fold amplification of iNOS mRNA expression at 4 h in SNP-supplemented cells and a 44% inhibition after 12-h treatment, with respect to cells treated with cytokines alone (Fig. 3B, bottom). Thus in cells supplemented with SNP, both the onset and the decline of iNOS protein and mRNA expression took place at earlier time points.

**Effect of ODQ and cGMP analogs on iNOS expression elicited by IL-1β/TNF-α.** It is well known that IL-1β/TNF-α-induced NO generation activates sGC in glo-
merular mesangial cells (GMC) (23, 32). Therefore, we explored the potential involvement of sGC activation and cGMP generation in the amplifying effects of endogenous and exogenous NO. Treatment of HMC with IL-1β/TNF-α for 8 h in the presence of the sGC inhibitor ODQ at 1 μM resulted in lower levels of iNOS protein (41 ± 5% reduction, n = 3) (Fig. 4A). This suggests that the integrity of the NO/cGMP pathway is important for iNOS induction. ODQ also reduced iNOS induction in SNP-supplemented cells (40.5 ± 7.2% reduction, n = 3). In keeping with this, the cell permeable analogs of cGMP, 8-BrcGMP, and dibutiryl-cGMP, amplified IL-1β/TNF-α-elicited iNOS induction in a dose-dependent manner (Fig. 4B). A clear amplification of iNOS induction was observed even with the lowest dose of 8-BrcGMP employed (3.4 ± 0.7-fold potentiation of iNOS protein levels in cells stimulated for 8 h in the presence of 10 μM 8-BrcGMP with respect to the levels found in cells treated with cytokines alone, n = 3). This effect was associated with a twofold increase in nitrite formation (control: 2 ± 0.4; cytokines: 7 ± 0.9; cytokines plus 10 μM 8-BrcGMP: 13.3 ± 0.6 pmol of nitrite/10⁶ cells, P < 0.05 vs. cytokines; n = 7). An amplification of iNOS activity was also evident when HMC were stimulated with cytokines in the presence of the NO-independent activator of sGC, YC-1 (26) at 20 μM for 24 h (2.2 ± 0.3 amplification of nitrite accumulation, n = 4). None of the cGMP analogs elicited iNOS expression per se. These results suggest that cGMP generation may contribute to the stimulatory effect of NO donors on iNOS expression. To study the predominance of the effects of NO and cGMP, the potentiating effect of 8-BrcGMP was assessed in the presence of L-NAME and/or ODQ. Neither inhibitor reduced the amplifying effect of 8-br-cGMP on iNOS protein expression (Fig. 4C) thus suggesting that cGMP supplementation could overcome the effect of the inhibition of iNOS and of sGC.

We next explored the time course of the effect of cGMP analogs on cytokine-elicited iNOS expression. In cells supplemented with 10 μM 8-BrcGMP, iNOS protein levels were clearly higher up to 12-h treatment (Fig. 5A). At later times, however, either no amplification or a reduction of iNOS protein levels was observed (32 ± 4% inhibition at 24 h, n = 3). This modulatory effect of 8-BrcGMP on iNOS expression was also evident when explored at the mRNA level (Fig. 5B). Whereas iNOS transcript levels were fourfold higher in 8-BrcGMP-supplemented cells after a 4-h treatment, a
27% reduction was observed at 8 h with respect to cells treated with cytokines alone. The densitometric quantitation of these results is shown (bottom). These results suggest the involvement of the NO/cGMP pathway in the limitation of iNOS induction.

Effect of donors and inhibitors of NO/cGMP generation on the decay of iNOS mRNA. To gain insight into the mechanisms underlying the diverse effects of NO and cGMP donors on cytokine-elicited iNOS induction, we evaluated their potential influence on iNOS mRNA stability. For this purpose, HMC stimulated for 4 h with IL-1β/TNF-α alone or in combination with various modulators of the NO/cGMP pathway, were treated with actinomycin D to inhibit further transcription, and 8 h later the amount of iNOS transcript remaining was assessed by Northern blot. As it can be observed in Fig. 6, inhibition of the NO/cGMP pathway with either l-NAME or ODQ resulted in a higher proportion of iNOS mRNA remaining after actinomycin D treatment than that observed in cytokine-treated cells in the absence of inhibitors. In contrast, supplementation with 8-Br-cGMP accelerated iNOS mRNA decline. These observations suggest that the NO/cGMP pathway may exert a negative regulation of iNOS expression at a posttranscriptional level.

8-Br-cGMP reduces iNOS mRNA stability. To characterize the effect of cGMP donors, the half-life of iNOS mRNA from HMC that had been treated with IL-1β/ TNF-α alone or in combination with 8-Br-cGMP for 4 h, was determined after the addition of actinomycin D (Fig. 7). We observed that 8-Br-cGMP significantly shortened iNOS mRNA half-life, from 7.9 ± 1.0 to 4.0 ± 0.3 h (P < 0.05 by t-test). Thus 8-Br-cGMP can exert a negative effect on cytokine-elicited iNOS mRNA levels by acting at a posttranscriptional level. The half-life of iNOS mRNA as herein determined is within experimental error of the values previously reported by us (37) and in agreement with those measured by other authors in different experimental systems (7, 11), although in a number of studies a shorter half-life for the iNOS transcript has been observed (19, 42). From these and other studies it appears that the stability of iNOS mRNA may be cell type, stimulus, and/or time dependent.

DISCUSSION

The NO/cGMP pathway has been reported to modulate the steady-state levels of various transcripts in different cell types. Among the gene products modulated by NO/cGMP are TNF-α, cyclooxygenase-2 (COX-
The mechanisms by which NO brings about these effects may be multiple. NO and cGMP have been reported to modulate gene expression by posttranscriptional mechanisms. In smooth muscle cells, NO donors and cGMP analogs have been shown to reduce the stability of the transcripts of sGC (8) and cGMP-dependent kinase-I (38). In the case of human iNOS, a number of recent reports emphasize the importance of posttranscriptional mechanisms in determining the extent and duration of iNOS induction (27, 35). However, as far as we know, an effect of NO or cGMP analogs on iNOS mRNA stability has not been reported previously. The reduction of iNOS mRNA half-life in the presence of cGMP analogs, described in our study, suggests the existence of a novel level of regulation of iNOS expression by the NO/cGMP pathway. iNOS mRNA destabilization by cGMP could contribute to the limitation of iNOS induction observed at late treatment times in cells supplemented with NO or cGMP donors. The events leading to a reduction of the iNOS transcript half-life in cGMP-supplemented cells are presently under study. NO and cGMP have been reported to influence the synthesis of cytokines that in turn may be able to reduce iNOS mRNA stability, like TGF-β and TNF-α (14, 39, 43). Results from our laboratory indicate that cGMP supplementation may induce an amplification of cytokine-elicited TGF-β1 transcript and immunoreactive TGF-β in HMC-conditioned media (Pérez-Sala D, Cernuda-Morollón E, Díaz-Cazorla M, and Lamas S, unpublished observations). Thus it could be hypothesized that TGF-β may contribute to the increased degradation of the iNOS transcript observed in the presence of cGMP analogs.

The events underlying the early amplifying effect of NO donors and cGMP analogs on iNOS protein and mRNA levels remain to be elucidated. The results herein presented do not exclude the participation of transcriptional mechanisms in these effects. NO donors have been previously reported to augment the rate of transcription of iNOS in rat mesangial cells stimulated with IL-1β (25). Potential targets of NO and cGMP are the transcription factors activator protein-1 (AP-1) and nuclear factor (NF)-κB, which play an important role in the induction of iNOS in response to cytokine stimulation (22, 40). NO-releasing agents and cGMP analogs have been shown to increase AP-1-DNA binding and transcriptional activity in rat embryo fibroblasts (33). However, we have not observed changes in AP-1 DNA binding activity of nuclear extracts from HMC supplemented with 8-BrcGMP during cytokine stimulation (results not shown). NO- and cGMP-elevating agents have also been reported to modulate the activity of NF-κB either in a positive (18, 21) or in a negative manner (15, 31). We have recently described that NO donors can exert both types of effects on IkBα expression in HMC, leading to increased nuclear translocation of NF-κB at the initial stages of stimulation with IL-1β/TNF-α and decreased nuclear levels of NF-κB at later times, and this is associated with a biphasic modulation of COX-2 expression (6). Thus the modulation of IkBα levels may also play a role in the

**Fig. 7.** Effect of 8-BrcGMP on iNOS mRNA stability in HMC. The expression of iNOS mRNA was induced in HMC by treatment with the cytokine combination IL-1β/TNF-α (Ck) for 4 h, in the presence or absence of 10 μM 8-BrcGMP, as indicated. After induction, transcription was inhibited by addition of 10 μg/ml Act D, and levels of iNOS mRNA and 28S rRNA were assessed every 2 h by Northern blot. Autoradiographic exposures shown are representative of 3 experiments. *Bottom*: the ratios of iNOS mRNA to 28S rRNA levels for every time point after Act D addition, obtained by densitometric analysis, are expressed in arbitrary units as percentage of the values obtained at time 0 for every experimental condition. Results are average values of 3 independent experiments ± SE. Ck, ○; Ck+8-BrcGMP, ●. Where error bars are not visible they are smaller than symbols.
regulation of iNOS expression by NO. In contrast, 8-BrcGMP did not elicit detectable changes in cytokine-induced NF-κB nuclear translocation or in 1kBa expression (Pérez-Sala D, Cernuda-Morollón E, Díaz-Cazorla M, and Lamas S, unpublished results). These and previous observations (3, 6, 30, 31) suggest that NO can modulate NF-κB activation by cGMP-independent mechanisms.

In summary, our results suggest the existence of autocrine pathways by which NO and/or cGMP generation appear to control iNOS expression. This control involves positive and negative feedback mechanisms that determine the amplitude of iNOS induction. These and previous observations illustrate a multifunctional role of the NO/cGMP pathway in HMC because it may contribute both to the onset and to the limitation of the response of HMC to proinflammatory stimuli.

We are indebted to the transplant coordination team from Hospital 12 de Octubre, Madrid, Spain, for supplying us with kidneys unsuitable for transplantation.

This work was supported by grants from CICYT (SAF97–0035 and SAF2000–0149), Comunidad Autónoma de Madrid (CAM) Grant 08.4/0032/1998, and a grant-in-aid from the Spanish Society of Nephrology. M. Díaz-Cazorla and E. Cernuda-Morollón were recipients of a fellowship from Instituto “Reina Sofia” de Investigaciones Nefrológicas (Spain).

REFERENCES


38. **Soff GA, Cornwell TL, Cundiff DL, Gately S,** and **Lincoln TM.** Smooth muscle cell expression of type I cyclic GMP-dependent protein kinase is suppressed by continuous exposure to nitrovasodilators, theophylline, cyclic GMP, and cyclic AMP. *J Clin Invest* 100: 2580–2587, 1997.


