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Two distinct signaling pathways trigger the expression of inducible nitric oxide synthase in rat renal mesangial cells

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ABSTRACT **The expression of nitric oxide synthase (NOS; EC 1.14.13.39) is induced in rat glomerular mesangial cells by exposure to the inflammatory cytokine interleukin 1β (IL-1β) or cAMP-elevating agents. Stimulation with IL-1β alone leads to an approximately 40-fold increase in NOS activity and nitrite synthesis, whereas the elevation of cAMP with forskolin, cholera toxin, salbutamol, or dibutyryl-cAMP for 24 h resulted in a 2- to 12-fold increase in NOS activity. Moreover, the combinations of IL-1β with each of the cAMP-elevating agents greatly enhanced NOS activity in a synergistic fashion. Northern-blot analysis demonstrated a single band of ≈4.5 kb for the NOS mRNA in rat mesangial cells. IL-1β increased NOS mRNA levels in a dose- and time-dependent fashion with a peak of NOS mRNA at 24 h. Dibutyryl-cAMP also increased NOS mRNA levels in mesangial cells in a dose- and time-dependent manner. Furthermore, combination of IL-1β and forskolin revealed a strong synergy with maximal mRNA levels 12 h after stimulation. Nuclear run-on transcription experiments suggest that IL-1β and cAMP synergistically interact to increase NOS gene expression at the transcriptional level. Furthermore, message stability studies established that NOS mRNA induced by cAMP has a longer half-life than the IL-1β-induced message. Moreover, cAMP exposure markedly prolonged the half-life of NOS mRNA from 1 h to 3 h. These data suggest that the level of NOS mRNA is controlled by at least two different signaling pathways, one involving cAMP and the other being triggered by cytokines such as IL-1β. The two pathways act synergistically and thus potently up-regulate the expression of inducible NOS in rat mesangial cells.**

In recent years, nitric oxide (NO) has been identified as a pleiotropic intercellular messenger molecule regulating a variety of diverse cellular functions in many tissues (1, 2).

Initial reports characterized NO as an endothelium-derived relaxing factor important for the maintenance of the vascular tone and the regulation of blood pressure (3, 4). In the brain, NO participates in the actions of excitatory neurotransmitters such as glutamate (1, 5). In macrophages, NO is a cytotoxic mediator and contributes to the antimicrobial and tumoricidal activity of these cells (2, 6). Moreover, NO has been reported to be linked to pathophysiological effects. It may, for instance, account for the destruction of pancreatic islet cells during the development of type I diabetes (7).

The generation of NO from L-arginine is catalyzed by NO synthase (NOS; EC 1.14.13.39). Molecular cloning and sequencing analyses revealed the existence of at least three main types of NOS isoforms (1, 2). The earlier report described the molecular cloning of a constitutive brain NOS, a 150 kDa-protein, displaying recognition sites for the three redox cofactors NADPH, FAD, and FMN. The cDNA was cloned from rat cerebellum and identifies a 10-kb mRNA on Northern-blot analysis (8). Recently, the molecular cloning of a distinct constitutive NOS isoform, a 133-kDa protein, from endothelial cells was reported (9). The brain and endothelial enzymes are 60% identical at the amino acid level. A third type, the macrophage NOS, has been cloned by two groups from RAW 264.7-cells and identifies an approximately 4.4-kb mRNA (10, 11). The macrophage NOS is induced by bacterial endotoxin and γ-interferon and is calcium- and calmodulin-independent. In contrast, the brain and endothelial types of NOS are constitutively expressed and are regulated by Ca^{2+} and calmodulin. Recent reports demonstrated that cytokines and endotoxin regulate the expression of the NOS gene at the transcriptional level in mouse macrophages (10, 11), in rat pancreatic islet cells (12), and in hepatocytes (13). We and others have shown that the proinflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα) induce a macrophage-type of NOS activity in glomerular mesangial cells (14–16).

In this report we present evidence obtained with rat mesangial cells that IL-1β and cAMP interact in a synergistic fashion to induce NOS gene transcription.

MATERIALS AND METHODS

Cell Culture. Rat renal mesangial cells were cultured and cloned as described previously (17). Passages 9–11 of mesangial cells were used for the experiments.

NOS Assay. NOS activity was measured as nitrite production in rat glomerular mesangial cells as described (18).

Northern Blot Analysis. Total cellular RNA was extracted from mesangial cell pellets by using the guanidinium thiocyanate/cesium chloride method (19). Samples (20 μg) of RNA were separated on 0.8% agarose gels containing 0.66 M formaldehyde prior to transfer to GeneScreen membranes (New England Nuclear). After baking at 80°C for 2 h and prehybridization for 4 h, the filters were hybridized for 16–18 h to a ^{32}P-labeled *Sma* I-digested cDNA insert from pMac-NOS (11). To correct for variations in RNA amount, the NOS probe was stripped with boiling 0.1× SSPE/1% SDS (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA) and the blots were rehybridized to either the ^{32}P-labeled *Bam*HI/*Sal* I-digested cDNA insert from clone pEX6 coding for β-actin or to the ^{32}P-labeled *Eco*RI-digested insert coding for 28S rRNA. DNA probes (≈2 × 10^6 cpm/ml) were radioactively labeled with [α-^{32}P]dATP by random priming. Hybridization reactions were performed in 50% (vol/vol) formamide/5× SSPE/10× Denhardt's solution/0.5% SDS containing salmon sperm DNA at 250 μg/ml. Filters were washed three times in 2× SSPE/0.1% SDS at room temperature for 30 min and then in 2× SSPE/2% SDS at 65°C for 30 min. Filters were exposed for 6–48 h to Kodak X-Omat XAR film.

Nuclear Run-on Transcription. For preparation of nuclei approximately 5 × 10^7 cells were lysed in ice-cold RSB (10 mM Tris·HCl, pH 7.4/5 mM KCl/3 mM $MgCl_2$) containing

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Abbreviations: NOS, nitric oxide synthase; IL-1β, interleukin 1β; TNFα, tumor necrosis factor α; Bt_2cAMP, N^6,$O^{2'}$-dibutyryl-cAMP.

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0.5% Nonidet P-40. Nuclei were isolated by spinning at 800 × *g*, washed once in ice-cold phosphate-buffered saline (PBS), and finally resuspended in 40% (vol/vol) glycerol/50 mM Hepes, pH 8.0/5 mM $MgCl_2$/2 mM dithiothreitol. For the run-on transcription assay, the nuclei suspension was mixed with 0.2 ml of 2× reaction buffer [100 mM Hepes, pH 8.0/10 mM $MgCl_2$/300 mM KCl/200 units of RNasin (Boehringer Mannheim) per ml/1 mM each ATP, GTP, and CTP/150 μCi (1 μCi = 37 kBq) of [^{32}P]UTP (3000 Ci/mmol)] and incubated for 30 min at 30°C. Transcription was stopped by adding 20 μg of DNase I, followed by 80 μg of proteinase K. The ^{32}P-labeled RNA was purified by extraction with phenol/chloroform and two sequential precipitations with ammonium acetate. Equal amounts of labeled RNA (8×10^7 dpm/ml) were hybridized in 50% formamide/5× SSC/5× Denhardt's solution/1% SDS (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0) at 42°C for 72 h. Filters contained 10 μg each of linearized plasmids immobilized on GeneScreen membranes after blotting in 12× SSPE with a dot-blot apparatus. After hybridization filters were rinsed for 30 min in 2× SSC at 60°C, for 5 min in 2× SSC containing 10 μg of RNase A per ml at 37°C, and finally for 1 h in 2× SSC at 37°C. Filters were air dried and were exposed at −70°C for 2 days.

Chemicals. Recombinant human IL-1β and salbutamol were gifts from CIBA–Geigy; forskolin and 1,9-dideoxyforskolin were from Calbiochem; and cholera toxin, actinomycin D, cycloheximide, and $N^6,O^{2'}$-dibutyryl-cAMP (Bt_2cAMP) were purchased from Sigma. The cDNA clone pMac-Nos, coding for the inducible macrophage NOS, was kindly provided by J. Cunningham (Harvard Medical School, Brigham and Women's Hospital, Boston). The cDNA clone pEX6, coding for human β-actin, was a gift from U. Aebi (Maurice E. Müller Institute, Basel). The cDNA clone coding for 28S ribosomal RNA was a gift from T. Geiger (CIBA–Geigy); the plasmid pSPTBM20 was from Boehringer Mannheim. [α-^{32}P]dATP (3000 Ci/mmol) was from Amersham. All other chemicals were from either Merck or Fluka.

RESULTS

Previous work has demonstrated that rat glomerular mesangial cells express an inducible macrophage-type of NOS after incubation with IL-1β and TNFα (14, 15). To study other signaling pathways which possibly are capable of triggering NOS activity in mesangial cells, we examined the effects of cAMP-elevating agents.

NOS activity was measured as nitrite production in culture supernatants of untreated (control) cells and cells that had been stimulated with IL-1β or cAMP-elevating agents Bt_2cAMP, cholera toxin, salbutamol, forskolin, and its analogue 1,9-dideoxyforskolin for 24 h. As shown in Table 1, unstimulated mesangial cells already have a low basal level of nitrite production. Incubation with IL-1β alone leads to a 36-fold increase in nitrite production. Among all the cAMP-elevating agents the membrane-permeant analogue of cAMP, Bt_2cAMP, was the most potent stimulus, increasing NOS activity up to 12-fold. In a further approach we used salbutamol and cholera toxin, two compounds that activate adenylate cyclase by different mechanisms. Salbutamol binds to β-adrenergic receptors on the cell surface of mesangial cells and activates adenylate cyclase through the stimulatory G protein, G_s. Cholera toxin causes ADP-ribosylation of G_s, thus altering this G protein to a state of permanent activation, leading to the subsequent stimulation of the catalytic moiety of adenylate cyclase. As shown in Table 1, these two agents enhanced nitrite production by mesangial cells by 3-fold and 5-fold, respectively. Forskolin, a compound that directly activates the catalytic subunit of adenylate cyclase, turned out to be the weakest stimulus, but still caused a 2-fold increase in nitrite generation (Table 1). The 1,9-dideoxy derivative of forskolin, which is unable to activate adenylate cyclase (20), and the membrane-permeant analogue of cGMP, Bt_2cGMP, had no effect on NOS activity in mesangial cells (Table 1).

Table 1. Effects of different cAMP-elevating agents on IL-1β-stimulated nitrite formation in mesangial cells

Addition	Nitrite, nmol/mg of protein
None	26 ± 7
IL-1β (1 nM)	956 ± 102*
Bt_2cAMP (1 mM)	312 ± 13*
Cholera toxin (125 ng/ml)	132 ± 18*
Salbutamol (1 μM)	84 ± 13*
Forskolin (10 μM)	57 ± 12*
1,9-Dideoxyforskolin (10 μM)	22 ± 5
Bt_2cGMP (1 mM)	25 ± 8
IL-1β + forskolin	2388 ± 74*‡
IL-1β + 1,9-dideoxyforskolin	854 ± 104*
IL-1β + cholera toxin	2236 ± 56*‡
IL-1β + salbutamol	2070 ± 62*‡
IL-1β + Bt_2cAMP	2288 ± 137*‡
Bt_2cAMP + actinomycin D (10 μg/ml)	35 ± 10
Bt_2cAMP + cycloheximide (10 μM)	29 ± 9

The results are 24-h nitrite production elicited by the indicated agents. All values represent the mean of four values ±SD. *, $P < 0.05$ versus control (no addition); and ‡, $P < 0.05$ versus IL-1β alone, by Student's *t* test.

Next, the cells were incubated with a combination of IL-1β and the cAMP-elevating agents in concentrations given above. There was a dramatic up-regulation of NOS activity compared with control cells, ranging from 80-fold for IL-1β plus salbutamol as the weakest and 92-fold for IL-1β plus forskolin as the most potent inducing combination (Table 1). These data suggest that IL-1β and cAMP-elevating agents, alone or in combination, are capable of up-regulating NOS activity in mesangial cells. Fig. 1 shows IL-1β dose-dependently stimulated nitrite production, reaching a plateau between 1 and 5 nM. Forskolin, at a maximally effective concentration of 10 μM, gives a moderate but significant increase in nitrite synthesis (Table 1). When IL-1β and forskolin were added simultaneously, there was a synergistic interaction, as shown in Fig. 1.

Addition of actinomycin D (10 μg/ml) or cycloheximide (10 μM) completely suppressed Bt_2cAMP-induced formation of nitrite by mesangial cells (Table 1), as has been reported for IL-1β-stimulated nitrite production (14).

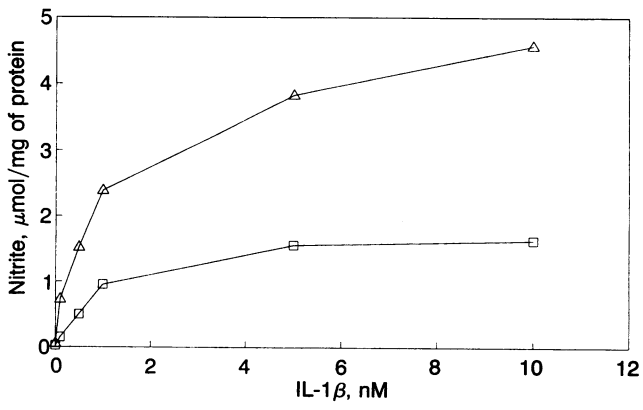


FIG. 1. Synergistic stimulation of nitrite formation in mesangial cells by IL-1β and forskolin. Confluent cells were stimulated for 24 h with the indicated concentrations of IL-1β alone (□) or together with forskolin (10 μM, △). Thereafter the medium was removed and used for nitrite determination. Results are means of four experiments; the SD was 4–32%.

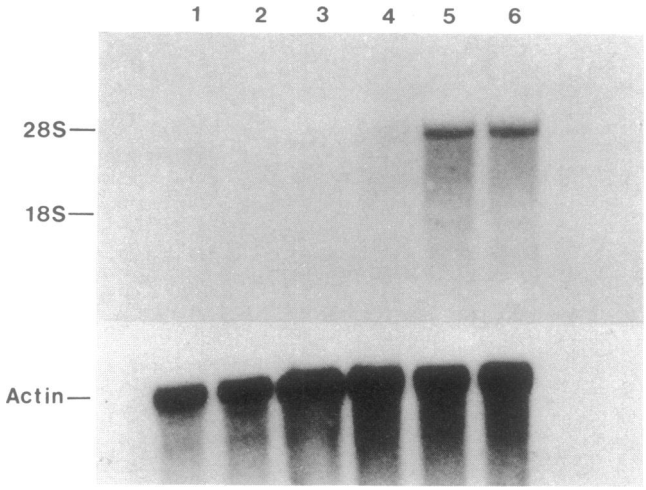


FIG. 2. Dose-dependent accumulation of NOS mRNA in IL-1β-stimulated mesangial cells. Mesangial cells were incubated with vehicle (control) (lane 1) or IL-1β at 1 pM (lane 2), 10 pM (lane 3), 100 pM (lane 4), 1 nM (lane 5), or 10 nM (lane 6) for 24 h. Total cellular RNA (20 μg) was successively hybridized to ^{32}P-labeled NOS and β-actin cDNA probes.

To evaluate further whether this increased NOS activity was due to regulation of NOS gene expression, we performed Northern blot analyses. Fig. 2 shows the dose-dependent increase of NOS mRNA steady-state levels in mesangial cells after stimulation with IL-1β. The NOS mRNA was present as a single band of ≈4.5 kb. In unstimulated mesangial cells there was no detectable NOS mRNA (Fig. 2, lane 1). As shown in Fig. 3, increases in NOS mRNA levels can be seen 8 h after stimulation with IL-1β as a faint band on the Northern blot (Fig. 3). A maximum concentration of NOS mRNA is detectable 24 h after stimulation with a dramatic decline by 48 h (Fig. 3). Bt_2cAMP, the most potent inducer of nitrite synthesis, also increased NOS mRNA levels in a dose- and time-dependent manner (Figs. 4 and 5). Increases in NOS mRNA steady-state levels can be detected 4–8 h after stimulation with Bt_2cAMP (Fig. 5). A maximal amount of NOS mRNA is seen 12 h after stimulation of cells, and significantly elevated levels are detected even after 48 h.

Stimulation of mesangial cells with a combination of IL-1β and forskolin (Fig. 6) reveals a broader induction profile

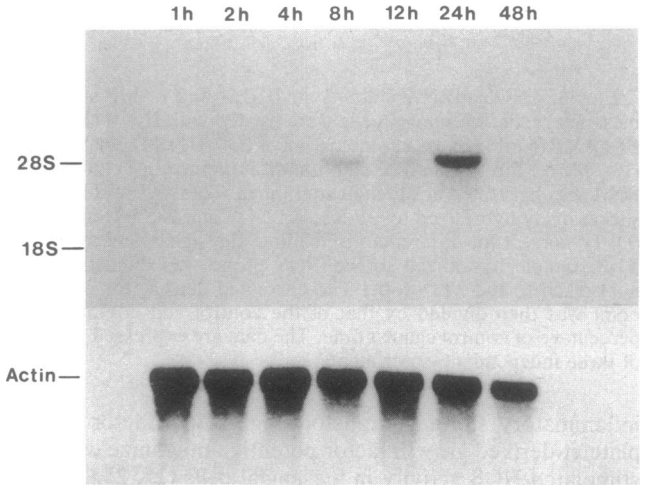


FIG. 3. Time course of induction of NOS mRNA in mesangial cells after stimulation with IL-1β. Mesangial cells were incubated with IL-1β (1 nM) for the indicated time periods. Total cellular RNA (20 μg) was hybridized successively to ^{32}P-labeled NOS and β-actin cDNA probes.

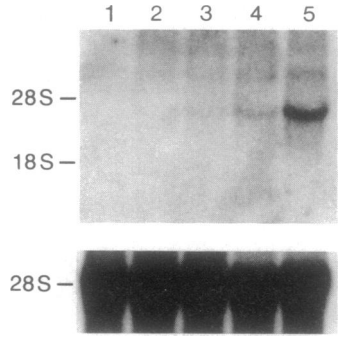


FIG. 4. Dose-dependent accumulation of NOS mRNA in Bt_2cAMP-stimulated mesangial cells. Mesangial cells were incubated with vehicle (control) (lane 1) or Bt_2cAMP at 0.5 mM (lane 2), 1 mM (lane 3), 5 mM (lane 4), or 10 mM (lane 5) for 24 h. Total cellular RNA (20 μg) was successively hybridized to ^{32}P-labeled NOS and 28S rRNA probes.

compared with IL-1β alone (Fig. 3). The NOS mRNA levels are drastically increased 2 h after stimulation, peak at 12 h, and remain at approximately 75% of this level at 24 h. In contrast to stimulation with IL-1β alone, after which the NOS mRNA levels are already gone at 48 h, there are still high amounts of mRNA detectable 48 h after stimulation with IL-1β and forskolin. This suggests that IL-1β and forskolin act synergistically and thus lead to a prolonged production and availability of NOS mRNA.

To determine specifically the molecular mechanism of the increased levels of NOS mRNA induced by IL-1β and cAMP, we directly measured the rates of transcription of the inducible NOS gene. We found that both agents, IL-1β and cAMP, increase NOS gene transcription in mesangial cells (Fig. 7). Most striking is the strong synergistic stimulation of NOS gene transcription obtained by a combination of the two compounds as shown in Fig. 7.

The effect of IL-1β and cAMP exposure on NOS mRNA stability was assessed by actinomycin D experiments. Mesangial cells were stimulated with either IL-1β or Bt_2cAMP or a combination of IL-1β plus forskolin for 15 h. Thereafter actinomycin D was added to the cells to inhibit further transcription. At various times after the addition of actinomycin D, total cellular RNA was isolated and examined by Northern blot analysis. The blots were successively hybridized to a radioactively labeled NOS cDNA and after stripping were hybridized to a radioactively labeled probe coding for 28S rRNA. To correct for differences in loading, the signal density of each RNA sample hybridized to the NOS probe

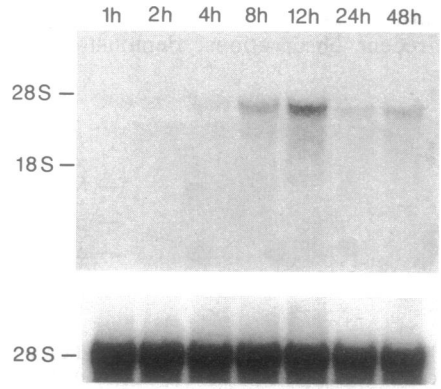


FIG. 5. Time course of induction of NOS mRNA in mesangial cells after stimulation with Bt_2cAMP. Mesangial cells were incubated with Bt_2cAMP (5 mM) for the indicated time periods. Total cellular RNA (20 μg) was successively hybridized to ^{32}P-labeled NOS and 28S rRNA probes.

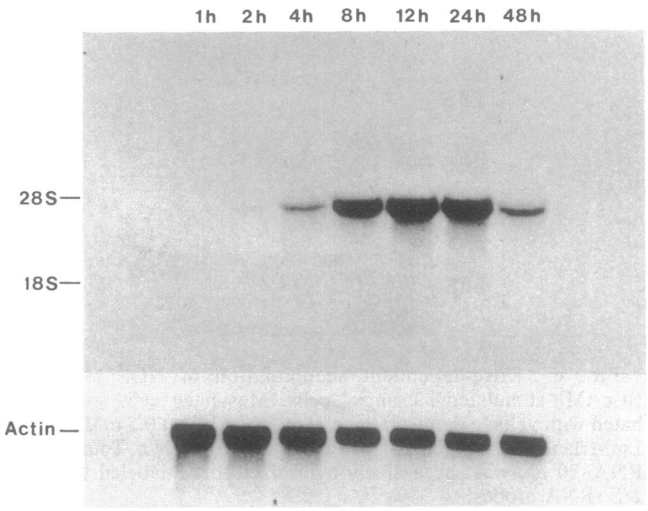


FIG. 6. Time course of induction of NOS mRNA in mesangial cells after stimulation with a combination of IL-1β and forskolin. Mesangial cells were treated with IL-1β (1 nM) plus forskolin (10 μM) for the indicated time periods. Total cellular RNA (20 μg) was successively hybridized to ^{32}P-labeled NOS and β-actin cDNA probes.

was divided by that hybridized to the 28S probe. The decay of NOS mRNA after stimulation with IL-1β corresponded to a half-life of about 1 h (Fig. 8 *A* and *D*). After costimulation with IL-1β and forskolin the mRNA half-life was increased markedly to about 3.3 h (Fig. 8 *C* and *D*). Stimulation of mesangial cells with Bt_2cAMP resulted in NOS mRNA displaying half-life of approximately 2.9 h (Fig. 8 *B* and *D*).

DISCUSSION

The physiological significance of NO biosynthesis in the kidney is only beginning to be elucidated (21). Morphologically, mesangial cells resemble smooth muscle cells and are able to contract upon stimulation with vasoactive hormones (22). The cells respond to endothelial cell-derived NO with increased levels of intracellular cGMP and relaxation (23, 24).

In addition to their contractile, smooth muscle-like properties, glomerular mesangial cells have also features in common with macrophages—i.e., they are able to phagocytose and to produce oxygen radicals, cytokines, and eicosanoids (21, 22). Formation of NO has been shown to be an important cytotoxic mechanism of activated macrophages (6). NO secreted from mesangial cells may act as a powerful vasodilator, but excessive formation of NO may also contribute to the pathological alterations seen in immune-mediated glomerulonephritis (21). The latter hypothesis gains support from our recent observations, demonstrating that anti-

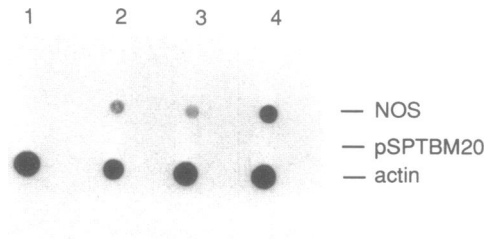
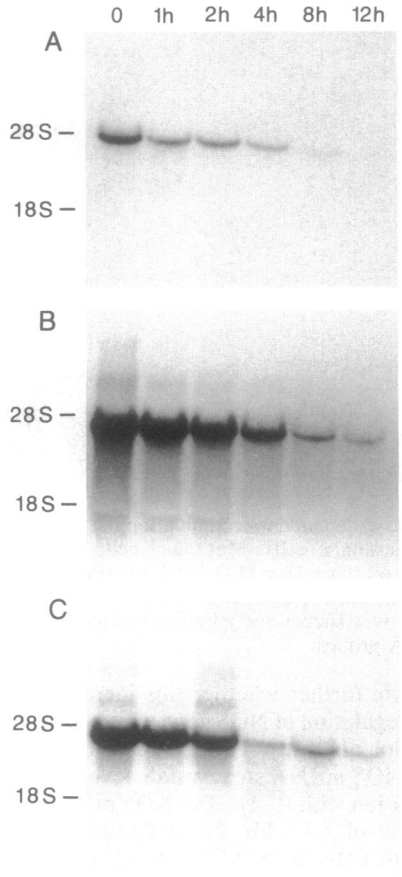


FIG. 7. NOS gene transcription in IL-1β- and cAMP-stimulated mesangial cells. Mesangial cells were unstimulated (lane 1) or stimulated with IL-1β (1 nM; lane 2), Bt_2cAMP (5 mM; lane 3), or a combination of IL-1β (1 nM) plus forskolin (10 μM; lane 4) for 16–18 h. The rate of transcription of the genes of NOS or β-actin by isolated nuclei was determined by hybridizing the elongated, labeled RNA transcripts to NOS cDNA, pSTPBM20, or actin probes immobilized onto GeneScreen membranes.



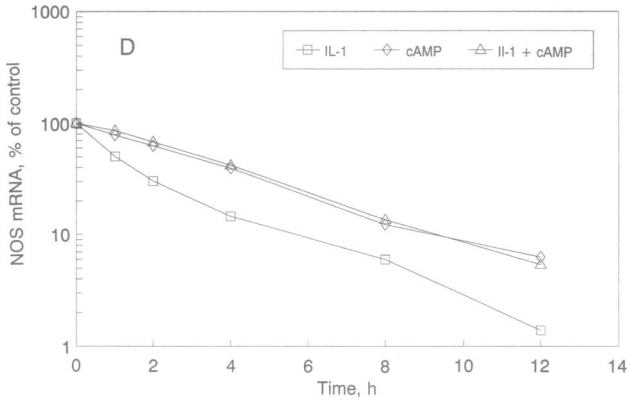


FIG. 8. NOS mRNA stability in IL-1β- and cAMP-stimulated mesangial cells. Mesangial cells were treated with IL-1β (1 nM; *A*), Bt_2cAMP (5 mM; *B*), or a combination of IL-1β (1 nM) plus forskolin (10 μM; *C*) for 15 h before addition of actinomycin D (10 μg/ml). RNA was harvested at the indicated times. Total RNA (20 μg) was successively hybridized to ^{32}P-labeled NOS and 28S rRNA probes. (*D*) To correct for differences in loading, the signal density of each RNA sample hybridized to the NOS probe was divided by that hybridized to the 28S probe. The corrected density for each time point was then divided by that of the control and is plotted as a percentage of control against time. The data are expressed as means of three independent experiments.

inflammatory steroids, transforming growth factor β_2, and platelet-derived growth factor potently antagonize cytokine-stimulated NOS activity in mesangial cells (17, 25, 26).

Previously, we and others reported that the inflammatory cytokines IL-1β and TNFα are capable of inducing NOS activity in rat mesangial cells (14–16). To identify additional second messenger pathways which might be involved in the regulation of NOS expression, we examined the effects of

cAMP-elevating agents on the induction of NOS activity in mesangial cells. cAMP has been reported to potentiate cytokine-triggered nitrite production in Kupffer cells (27) and brain microvessel endothelial cells (28). Our findings demonstrate that IL-1β as well as cAMP alone up-regulates NOS activity in mesangial cells. Among the cAMP-elevating agents, Bt_2cAMP is the most potent stimulator of NOS activity. To investigate whether IL-1β and cAMP would act transcriptionally, we performed Northern-blot analyses. The results given above demonstrate that IL-1β as well as Bt_2cAMP up-regulates NOS activity in mesangial cells by increasing steady-state NOS mRNA levels in a dose- and time-dependent manner. Furthermore, IL-1β and cAMP synergistically stimulate NOS mRNA and thus increase NOS activity in mesangial cells. NOS mRNA levels and NOS activity induced by IL-1β plus cAMP were far above the sum of the individual effects, indicating that the stimulators do not trigger NOS activity by the same mechanism. This synergistic action on NOS gene expression results in a prolonged production and thus availability of NOS mRNA. To the best of our knowledge, this is the first report demonstrating that cAMP alone is able to induce NOS expression and to synergize, in this respect, with inflammatory cytokines such as IL-1β.

To evaluate the exact molecular mechanism of IL-1β and cAMP induction of NOS mRNA, we first evaluated the effects of actinomycin D, an RNA synthesis inhibitor, on IL-1β- and Bt_2cAMP-induced NOS expression and activity. Coincubation of mesangial cells with actinomycin D abolished IL-1β- (14) and Bt_2cAMP-induced nitrite production (Table 1). These results, together with a lag period of several hours before the onset of NOS expression (Figs. 3 and 5), suggest that the accumulation of NOS mRNA during IL-1β or cAMP treatment results from activation of transcription of the gene. Nuclear run-on assays established an increased transcription of the NOS gene by IL-1β and cAMP. A strong synergistic increase in NOS transcription was observed when IL-1β and cAMP were used in combination (Fig. 7). Moreover, mesangial cells seem to use multiple mechanisms to up-regulate expression of NOS. Bt_2cAMP and forskolin caused a pronounced stabilization of NOS mRNA, as shown in Fig. 8. We cannot tell whether IL-1β also acts to stabilize NOS mRNA, since steady-state mRNA levels were undetectable prior to stimulation. The decay of NOS mRNA after exposure to IL-1β corresponded to a half-life of approximately 1 h. Coincubation with cAMP increased the mRNA half-life by a factor of 3, comparable to the value obtained with cAMP alone (Fig. 8). Thus, NOS mRNA seems to be a very labile mRNA species comparable to those mRNAs coding for many transiently expressed genes, including some cytokines, oncogenes, and transcription factors (29, 30). The presence of A+U-rich sequences in the 3'-untranslated regions of certain mRNAs may function as a destabilizing element which targets these mRNAs for rapid cytoplasmic degradation. We do not yet know if 3'-untranslated or other specific mRNA sequences are important for NOS mRNA stabilization by cAMP. Recently, we obtained evidence that pyrrolidine dithiocarbamate, an inhibitor of the nuclear factor κB activation (31), potently suppressed IL-1β-induced but not cAMP-stimulated NOS expression, indicating that different transcription factors mediate cytokine and cAMP regulation of NOS gene expression (33).

Recently Xie and colleagues (32) reported on the cloning of the 5'-flanking region of the inducible NOS gene from a mouse genomic library. The mRNA transcription start site is preceded by a TATA box and at least 22 consensus sequences for the binding of transcription factors involved in the induction of other genes by cytokines and lipopolysaccharide. However, no binding sites for AP-2 or the cAMP-regulatory element binding protein (CREB) were detected in the mouse NOS gene that could confer inducibility by cAMP. This does not necessarily exclude a transcriptional regulation of the rat NOS gene by cAMP, as there may be some interspecies variability. Alternatively cAMP may induce the production of an autocrine cytokine that synergizes with IL-1. In this way a cytokine network may play a role in regulating NOS expression in mesangial cells.

From the results presented in this paper it is becoming obvious that the induction of NOS activity in mesangial cells is regulated by multiple signaling pathways which may act alone or in combination and thus provide an excellent model system to study the cross-talk between signal transduction pathways. Further studies are needed to define the precise mechanisms by which these signals trigger NOS activity.

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