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Concomitant Transcriptional Activation of Nitric Oxide Synthase and Heme Oxygenase Genes during Nitric Oxide-Mediated Macrophage Cytostasis

Shun-ichi Kurata,*,[1] Midori Matsumoto,† and Uki Yamashita‡

*Department of Biochemical Genetics, Medical Research Institute, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113; †Department of Life Science and Technology, Tokyo Institute of Technology, Kanagawa 226; and ‡Department of Immunology and Patasitology, Hiroshima University School of Medicine, Hiroshima 734

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During *in vitro* activation of mouse peritoneal macrophages with interferon-γ (IFN-γ) and lipopolysaccharide (LPS), their synthesis of peroxynitrite and their cytostatic activity against mouse lymphocytic leukemia (L1210) cells were examined. The activation of the genes for nitric oxide synthase (iNOS) and heme oxygenase (HO-1) was also determined during the activation of the macrophages. Results showed that activation of peroxynitrite synthesis in macrophages was accompanied by the transcriptional activation of iNOS and HO-1 genes. Both genes seem to be activated simultaneously upon activation of the macrophages. Simultaneous activation of iNOS and HO-1 genes may be important because degradation of heme by HO-1 is one of the most important reaction that produces CO in higher organisms, and nitric oxide (NO) and carbon monoxide (CO) can react with heme-containing guanylate cyclase.

Key words: carbon monoxide, cytostasis, heme oxygenase, nitric oxide, nitric oxide synthase.

Macrophages can cause growth inhibition in a wide variety of microbiral targets and tumors by producing nitric oxide (NO) (*1*). NO is synthesized enzymatically from L-arginine by nitric oxide synthase (NOS) (*2, 3*) and can activate the heme-containing guanylate cyclase by increasing its affinity to GTP (*4, 5*). Since one of the important targets of NO is the heme of guanylate cyclase, heme oxygenase (HO-1), which produces carbon monoxide (CO) during degradation of heme, may be related to the activity of guanylate cyclase. Interestingly, lipopolysaccharide (LPS), an activator of NO synthesis by macrophage, also activates the HO-1 gene in rat liver cells (*6*). It is also well documented that an inducible type of NOS (iNOS) is produced by treatment of macrophages with LPS and INF-γ. In this study, we examined the transcriptional activations of the HO-1 and iNOS genes on activation of murine peritoneal macrophages with LPS and IFN-γ.

MATERIALS AND METHODS

Assay of Cytostatic Activity of Peritoneal Macrophages—Peritoneal macrophages were obtained from mice that had been treated 4 days previously with 2 ml of 4% Brewer's thioglycollate broth by peritoneal lavage with phosphate buffered saline (PBS) containing 25 mM glucose. The macrophages were resuspended in α-modification of Eagle's medium (α-MEM) and plated in 96-well plates (10^5 cells/well). After 3 h of culture, the nonadherent cells were removed by aspiration. The adherent cells were activated in α-MEM containing 10 μg/ml lipopolysaccharide (LPS) and various concentrations of interferon-γ (IFN-γ). After activation of peritoneal macrophages, L1210 cells (mouse lymphocytic leukemia cells; 5×10^4 cells/well) were added to wells of macrophage cultures with test agents [in some cases, *N*-monomethyl-L-arginine (NMA, 250 μM) and a superoxide-generating system]. After co-culture for 6 h, 2.5 μCi tritium thymidine ([^{3}H]TdR) was added, and incubation was continued for 12 h. The cells were then harvested with a cell harvester and [^{3}H]TdR incorporation was measured in a liquid scintillation counter. L1210 cell-specific [^{3}H]TdR incorporation was determined by subtracting the value for incorporation in cultures of macrophages without L1210 cells. Results are expressed as percentage cytostasis, which was calculated as follows:

$$\text{Cytostasis (\%)} = [1-\{[^3\text{H}]\text{TdR incorporation into L 1210 cultured with M}\phi\ (\text{cpm})\}/\{[^3\text{H}]\text{TdR incorporation into L 1210 cultured without M}\phi\ (\text{cpm})\}]\times100$$

Peroxynitrite Determination—The culture supernatants of macrophages stimulated with LPS and IFN-γ were collected and peroxynitrite synthesis was determined by the method of Ischiropoulos *et al.* (*7*). Briefly, mouse macrophages were cultured in medium containing LPS (10 μg/ml), IFN-γ (10 ng/ml), 1 mM 4-hydroxy-3-nitrophenylacetic acid, and 0.1 mg/ml of superoxide desmutase (SOD) for 5 h. The supernatants were acidified with H_3PO_4 (final concentration, 10%), and acetonitrile (final concentration, 20% v/v) was added. After filtration, samples were analyzed by reversed-phase HPLC, and the amounts of peroxinitrite were determined according to Ischiropoulos *et al.* (*7*).

[1] To whom correspondence should be addressed. FAX: +81-03-5803-0248

Northern Blot Analysis—Mouse peritoneal macrophages (5×10^6 cells) were incubated for 4 h in α-MEM with or without LPS (10 μg/ml), IFN-γ (10 ng/ml), and NMA (0.25 mM). Then their RNAs were extracted, separated by electrophoresis in 1% agarose gel with formaldehyde and transferred to nylon membranes. The membranes were hybridized with ^{32}P-labeled β-actin, HO-1 (*8*), and iNOS DNA probes. The membranes were then washed and exposed to X-ray film at −70°C for about 2 days.

Nuclear Run-Off Assay—In vitro nuclear transcription assay was performed by our modification (*9*) of the methods of Groudine *et al.* (*10*) and Greenberg and Ziff (*11*). Briefly, mouse peritoneal macrophages (1×10^7 cells) activated with LPS and IFN-γ were collected at 2-h intervals for 10 h and treated with 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40 (pH 7.4) to isolate the nuclei. The isolated nuclei were incubated with 200 μCi of [^{32}P]UTP at 30°C for 15 min. Then the reaction was terminated and nuclear RNA was extracted. Labeled RNAs were hybridized with 5 μg of iNOS, HO-1, β-actin, or pBR322 DNA probes on blot filters. The membranes were exposed to X-ray film at −70°C for about 7 days. The radioactivities of the hybridized slots were removed and measured in a liquid scintillation counter, and the relative transcriptional activations were calculated.

Binding of Nuclear Proteins to the NF-κB-Binding Motifs—Nuclear proteins that bound to the NF-κB-binding DNA motifs were detected with a Gibco-BRL band-shift assay kit. A 42-mer DNA (GATCCAAGGGACTTTCCATGGATCCAAGGGGACTTTCCATG) containing two NF-κB-binding DNA motifs (underlined) was end-labeled with [γ-^{32}P]ATP for binding of proteins with nuclear extracts. Nuclear extracts were prepared at 2-h intervals by the method of Dignam *et al.* (*12*) after treatment of peritoneal macrophages with LPS and INF-γ. Samples of 5 ng of end-labeled DNA fragments were incubated with 3 μg of nuclear proteins in a solution that contained 20 mM Hepes buffer (pH 7.9), 100 mM KCl, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 125 mM spermidine, and 3 μg of poly (dI-dC) for 20 min. The mixtures were then fractionated by electrophoresis in a 4% polyacrylamide gel in Tris-borate-EDTA buffer and subjected to autoradiography. For competition assays, excess amounts of unlabeled 42-mer fragments and synthetic mutant variants of the NF-κB-binding motif [underlined; TCGACAGAATTCACTTTCCGAGAGGCTCGA; Lee *et al.* (*13*)] were used. For super-shift assays, 10-fold diluted rabbit antiserum against NF-κB (p65) was also added to the binding reaction. The complexes of DNA, nuclear protein and antibody were identified by electrophoresis and autoradiographed as described previously (*14*).

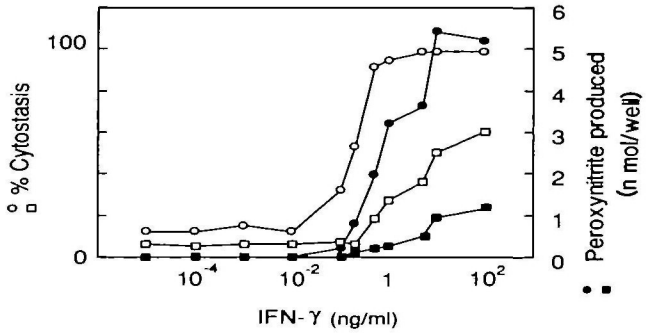

Fig. 1. **Peroxynitrite synthesis and cytostatic activity of peritoneal macrophages.** Peritoneal macrophages of C$_3$H/He mice were activated with 10 μg/ml LPS and various concentrations of IFN-γ with or without NMA and co-cultured with L1210 cells for 18 h. Cytostatic activity was determined as growth inhibition of L1210 cells and the peroxynitrite production was determined by the method of Ischropoulous *et al.* (*8*). Values represent means for 5 wells. Peroxynitrite synthesis with (■) or without (●) NMA or L1210 cell cytostasis with (□) or without (○) NMA.

RESULTS AND DISCUSSION

Cytostasis and Peroxynitrite Synthesis by Activated Peritoneal Macrophages—Mouse peritoneal macrophages were collected and activated with LPS (10 μg/ml) and various concentrations of IFN-γ, then their cytostatic activities against co-cultured L1210 cells and the activities of peroxynitrite synthesis were determined. The synthesis of peroxynitrite was found to depend on the concentration of IFN-γ and to be closely correlated with L1210 cell cytostasis in co-cultures (Fig. 1). The cytostasis was prevented by a substrate-based inhibitor of macrophage NO synthesis (NMA). These results confirm a previous report (*2*) that cytostasis of co-cultured tumor cells is required for the peroxinitrite-producing pathway in macrophages (Fig. 1).

Amounts of iNOS and HO-1 mRNAs—NO is formed from L-arginine by NOS and activates guanylate cyclase by increasing its affinity to GTP (*4, 5*). NO is known to interact with the heme of guanylate cyclase (*15*), and CO is also known to interact directly with the heme of guanylate cyclase in the same way as NO. We were, therefore, interested to investigate the transcriptional activity of HO-1 gene in this system. It is known that oxidative reagents and LPS activate the HO-1 gene in rat liver cells (*6*). Macrophages were activated with LPS and INF-γ and then their RNAs were extracted before activation (0 h) and 4 h after activation. Extracted RNAs were separated by electropho-

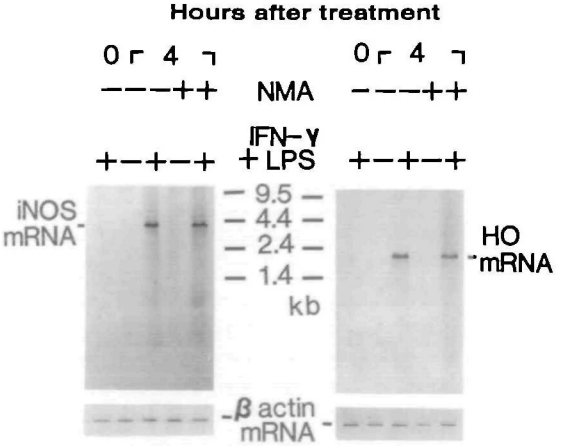

Fig. 2. **Accumulations of HO-1 and iNOS mRNAs in activated peritoneal macrophages.** Peritoneal macrophages were treated or untreated with LPS, IFN-γ, and NMA. Macrophages were collected at 0 and 4 h after activation and total RNAs were isolated. Five micrograms of RNA was separated by electrophoresis, blotted, and hybridized with labeled HO-1 and iNOS DNA probes and autoradiographed.

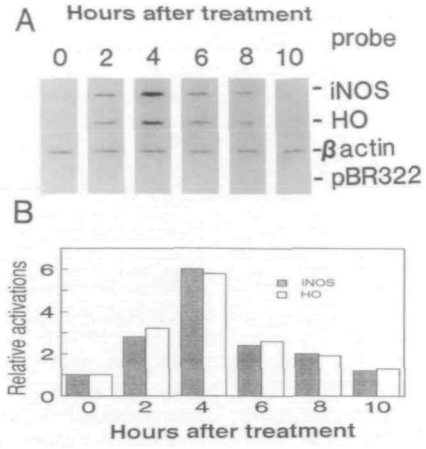

Fig. 3. **Time courses of transcriptional activations of iNOS and HO-1 genes in peritoneal macrophages.** A: Nuclear run-off assays of iNOS and HO-1 genes in peritoneal macrophages. Peritoneal macrophages (1×10^7 cells) were activated with LPS and IFN-γ, their nuclei were isolated and nuclear run-off assays were carried out at 2-h intervals. Samples of 5 μg of unlabeled DNAs (HO-1, iNOS, β-actin, and pBR322) were blotted onto filters, hybridized with labeled transcripts and autoradiographed. B: Kinetics of activation of iNOS and HO-1 genes with LPS and IFN-γ. The radioactivities of the hybridized slots in A were measured in a liquid scintillation counter. Relative activations of the iNOS and HO-1 genes were calculated by subtracting background values and normalizing to the 0 h values of the corresponding genes. Values are averages for three experiments.

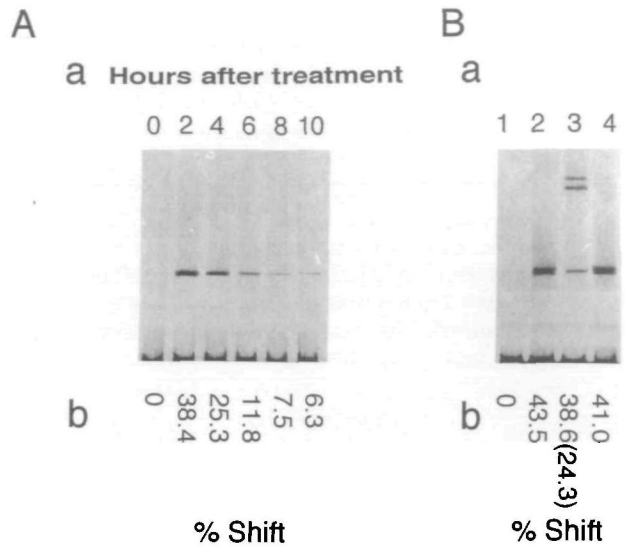

Fig. 4. **Band-shift assays of the NF-$\varkappa$B-binding motif.** A: Time course of the band-shift assay after activation of peritoneal macrophages. a: Band-shift assays: Binding of the 42-mer DNA containing two NF-$\varkappa$B-binding motifs with nuclear proteins from peritoneal macrophage treated with LPS and INF-γ. A 42-mer DNA from a Gibco-BRL kit was labeled and used for binding assays. b: Percentage shift: Percentage shifts of the complex of 42-mer DNA and nuclear proteins were calculated as follows:

$$\text{shift (\%)} = \frac{\text{cpm of shifted band}}{\text{total cpm}} \times 100.$$

B: Super-shift and competition assay of the NF-$\varkappa$B-binding motif with nuclear proteins from peritoneal macrophages 2 h after activation. a: Band-shift assays: Lane 1, band-shift assay with a 50-fold excess amount of unlabeled 42-mer NF-$\varkappa$B DNA added during the assay; lane 2, a 25-fold excess amount of an unlabeled mutant of the NF-$\varkappa$B-binding motif was added; lane 3, super-shift assay with NF-$\varkappa$B p65-specific antibodies; lane 4, band-shift assay without competitor DNA and specific antibodies. b: Percentage shift: Percentage shifts were determined as in Ab. For lane 3, total percentage shift and percentage (super-shift) are indicated.

resis and hybridized with labeled HO-1 and iNOS DNA probes (Fig. 2). Results showed that the amounts of HO-1 and iNOS mRNAs were increased by treatment with LPS and IFN-γ. Treatment with NMA (an inhibitor of NO synthesis) did not prevent the increases in HO-1 and iNOS mRNAs (Fig. 2).

Nuclear Run-Off Assays of HO-1 and iNOS Genes—Mouse peritoneal macrophages were collected and activated with LPS (10 μg/ml) and INF-γ (10 ng/ml), then their nuclei were isolated at 2-h intervals for 10 h. The isolated nuclei were incubated with labeled RNA precursor ([^{32}P]-UTP). After incubation for 15 min at 30°C, labeled RNAs were isolated and hybridized with HO-1, iNOS, β-actin, and pBR322 DNA probes. Accumulations of HO-1 and iNOS mRNAs were observed 4 h after activation with LPS and IFN-γ (*cf.* Fig. 2). In the run-off assay, the transcriptional activities of the HO-1 and iNOS genes were first observed 2 h after treatment with LPS and IFN-γ, and increased for 2 h, then decreased (Fig. 3A). The transcriptions of the HO-1 and iNOS genes were activated simultaneously, but transcription of β-actin did not change during the experiment. After the run-off assay, the hybridized slots were removed and their radioactivities were counted in a liquid scintillation counter. The patterns of changes in HO-1 and iNOS transcriptions during activation relative to the radioactivities at 0 h are shown in Fig. 3B. These results indicate that the HO-1 and iNOS genes are transcriptionally activated simultaneously and that the transcriptional activations of these genes were highest 4 h after treatment of macrophages with LPS and IFN-γ.

Binding of a Nuclear Factor from Peritoneal Macrophages Treated with LPS and INF-γ to the NF-$\varkappa$B-Binding Motif—Human HO-1 and iNOS genes both have a distal NF-$\varkappa$B binding motif in the promoter region (*16, 17*). If NF-$\varkappa$B is activated by treatment with LPS and IFN-γ, both HO-1 and iNOS genes may be activated simultaneously. We tried, therefore, to see the activation of NF-$\varkappa$B by gel-shift assay using 42-mer NF-$\varkappa$B-binding DNA fragments. To examine the binding activity of a nuclear factor in peritoneal macrophages to the NF-$\varkappa$B-binding motif, we end-labeled a 42-mer NF-$\varkappa$B-binding fragment of DNA and incubated it with nuclear proteins from activated peritoneal macrophages. The 42-mer DNA was then separated by electrophoresis and mobility shifts were examined (Fig. 4A, a). The shifted band was first observed 2 h after treatment and then decreased. The percentage shift of the shifted band seemed to relate the transcriptional activation of the HO-1 and iNOS gene (Fig. 4A, b; *cf.*, Fig. 3). The shifted band disappeared on addition of a 50-fold excess of an unlabeled 42-mer fragments but did not disappear on addition of a 25-fold excess of an unlabeled synthetic mutant of the NF-$\varkappa$B motif. The shifted bands were also super-shifted by antiserum against NF-$\varkappa$B p65 and super-shifted bands appeared (Fig. 4B, a). These results suggest that LPS and INF-γ treatment may activate NF-$\varkappa$B-like factors. And the activated NF-$\varkappa$B-like factors might be related to be transcriptional activation of HO-1 and iNOS genes. It is interesting to note that these genes have a distal

NF-κB-binding motif in their promoter regions (*16, 17*). The hemoprotein guanylate cyclase is known to be activated by NO due to the binding of NO and its heme. It is well known that CO can also react with guanylate cyclase in same way as NO, and both are known to interact with the Fe^{3+} ion of heme (*15, 18*). Thus, heme oxygenase may influence the signal transduction process involving NO and guanylate cyclase. We found that activation of NO synthesis and transcriptional activation of the iNOS gene in macrophages are associated with the transcriptional activation of the HO-1 gene. The relationship between NO and HO-1 is unknown, but it seems that production of HO-1 may be correlated with the synthesis of NO.

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