Induction of High Mobility Group-I(Y) Protein by Endotoxin and Interleukin-1β in Vascular Smooth Muscle Cells

ROLE IN ACTIVATION OF INDUCIBLE NITRIC OXIDE SYNTHASE*

Andrea Pellacani, Michael T. Chin‡‡, Philippe Wiesel, Maureen Ibanez, Anand Patel, Shaw-Fang Yet, Chung-Ming Hsieh, Joseph D. Paulauskis§§, Raymond Reeves, Mu-En Lee‡‡, and Mark A. Perrella***

From the Cardiovascular Biology Laboratory, Harvard School of Public Health, Boston, Massachusetts 02115, the ‡Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, the §Cardiovascular and **Pulmonary and Critical Care Divisions, Brigham and Women’s Hospital, Boston, Massachusetts 02115, the †Department of Environmental Health, Harvard School of Public Health, Boston, Massachusetts 02115, and the ¶Department of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164

Nonhistone chromosomal proteins of the high mobility group (HMG) affect the transcriptional regulation of certain mammalian genes. For example, HMG-I(Y) controls cytokine-mediated promoters that require transcription factors, such as nuclear factor-κB, for maximal expression. Even though a great deal is known about how HMG-I(Y) facilitates expression of other genes, less is known about the regulation of HMG-I(Y) itself, especially in cells in primary culture. Therefore we investigated the effect of endotoxin and the cytokine interleukin-1β on HMG-I(Y) expression in vascular smooth muscle cells. Induction of HMG-I(Y) peaked after 48 h of interleukin-1β stimulation (6.2-fold) in cells in primary culture, and this increase in mRNA corresponded to an increase in HMG-I(Y) protein. Moreover, immunohistochemical staining revealed a dramatic increase in HMG-I(Y) protein expression in vascular smooth muscle cells after endotoxin stimulation in vivo. This increase in HMG-I(Y) expression (both in vitro and in vivo) mirrored an up-regulation of inducible nitric oxide synthase, a cytokine-responsive gene. The functional significance of this coinduction is underscored by our finding that HMG-I(Y) potentiated the response of inducible nitric oxide synthase to nuclear factor-κB transactivation. Taken together, these studies suggest that induction of HMG-I(Y), and subsequent transactivation of iNOS, may contribute to a reduction in vascular tone during endotoxemia and other systemic inflammatory processes.

Nonhistone chromosomal proteins of the high mobility group (HMG) play a role in the transcriptional regulation of mammalian genes, the promoter/enhancer regions of which are close to AT-rich sequences (1–3). HMG proteins contribute to the regulation of gene expression by serving as architectural factors that alter chromatin structure (4). An important member of this family is HMG-I(Y). HMG-I(Y) refers to two proteins, HMG-1 and HMG-Y, that are derived by alternative splicing of the same gene (5). The biological functions of the two proteins are indistinguishable. HMG-I(Y) binds to AT-rich regions in the minor groove of DNA via motifs known as AT-hooks (6), thereby facilitating the assembly of functional nucleoprotein complexes (enhanceosomes). This assembly is promoted by modifying DNA conformation and by recruiting nuclear proteins to an enhancer (7). By binding to DNA and altering chromatin structure, HMG-I(Y) can have positive or negative effects on transcription factor binding to promoter regions (5, 8).

The role of HMG-I(Y) in enhanceosome assembly during viral induction of the interferon (IFN)-β gene has been studied extensively (7, 9–11). HMG-I(Y) binds to positive regulatory domains in the IFN-β enhancer, reverses an intrinsic bend in the DNA, and recruits transcription factors to their binding sites to have a positive effect on transcription during viral stimulation. Factors recruited by HMG-I(Y) include nuclear factor (NF)-κB, ATF-2/c-Jun, and IRF-1 (11, 12). Other genes that require NF-κB and HMG-I(Y) for full transcriptional activation include E-selectin (13, 14) and the chemokine melanoma growth stimulatory activity/growth related protein α (MGSA/GROα) (15). E-selectin and MGSA/GROα are cytokine-responsive genes critical to mediation of inflammation. E-selectin affects the interaction of leukocytes with the vascular wall during an inflammatory response (16), and MGSA/GROα is a potent chemoattractant for neutrophils (15). These observations suggest that HMG-I(Y) may be important for full induction of cytokine-driven promoters that require NF-κB for activation. Thus, the ability of HMG-I(Y) to assemble higher order transcription factor complexes may be important during systemic inflammatory responses.

Sepsis is a disease process that results from an overwhelming inflammatory response due to severe infection (17–20). During Gram-negative bacterial infection, lipopolysaccharide (LPS) or endotoxin is released from the bacterial cell wall to activate the immune cells of the host. These immune cells, which include macrophages, then release proinflammatory cytokines. When proinflammatory cytokines, such as interleukin-

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‡‡ To whom correspondence should be addressed: Harvard School of Public Health, 677 Huntington Ave., Boston, MA 02115. Tel.: 617-432-2273; Fax: 617-432-2980; E-mail: perrella@cvlab.harvard.edu.

§§ The abbreviations used are: HMG, high mobility group; NF, nuclear factor; MGSA/GROα, melanoma growth stimulatory activity/growth related protein α; NO, nitric oxide; iNOS (or NOS2), inducible isoform of NO synthase; RASMC, rat aortic smooth muscle cell; CAT, chloramphenicol acetyltransferase; LPS, lipopolysaccharide; IFN, interferon; FBS, fetal bovine serum; TBS-T, 10 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.05% Tween-20.
(IL-1β) and tumor necrosis factor-α, are released in exaggerated amounts, the result is hypotension and shock (17, 19). An important mediator of this cytokine-induced hypertensive response is the potent vasodilator nitric oxide (NO) (21, 22). Generation of NO under these circumstances is regulated by the inducible isof orm of NO synthase (iNOS or NOS2). As it does in the induction of IFN-β by viral stimulation and E-selectin and MGSAGROα by cytokine stimulation, NF-kB plays a critical role in the activation of iNOS (23). Moreover, recent experiments have emphasized the importance of NF-kB binding activity in the pathophysiology of sepsis (24). In addition to a role for HMG-I(Y) in the transcriptional regulation of genes, previous studies have shown an association between high levels of HMG-I(Y) and the neoplastic transformation of cells. HMG-I(Y) is up-regulated in malignant tumors (25–27), and its level of expression correlates with the malignant phenotype of neoplasms in humans (28–30). Little is known, however, about the regulation of HMG-I(Y) expression in nontransformed or nonmalignant cells. For example, we do not know how endotoxin and proinflammatory cytokines influence HMG-I(Y) in vascular smooth muscle cells (which are important for the regulation of vascular tone) or what the significance is of an up-regulation in HMG-I(Y)—and its potential influence on genes, such as iNOS, that regulate vascular tone—during systemic inflammatory processes.

In investigating the regulation of HMG-I(Y) in vascular smooth muscle cells in vitro and in vivo, our goals in the present study were to 1) determine whether the proinflammatory cytokine IL-1β regulated HMG-I(Y) mRNA and protein in primary cell cultures, 2) determine whether such a response also occurred in smooth muscle cells of the blood vessel wall after endotoxin stimulation in vivo, and 3) determine whether regulation of HMG-I(Y) correlated with induction of iNOS, and 4) determine whether HMG-I(Y) plays a role in the activation of iNOS gene transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Rat aortic smooth muscle cells (RASMCs) were harvested from male Sprague-Dawley rats (1 g) by an aortic isolation according to the method of Gunther et al. (31). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (J. R. H. Biosciences, Lenexa, KS) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT), penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 μM Hepes (pH 7.4) (Sigma) in a humidified incubator at 37 °C. RASMCs were passaged every 4–5 days, and experiments were performed on cells 4–6 passages from primary culture. Alveolar macrophages were harvested from the lungs of male Sprague-Dawley rats as described (32). Wright-Giemsa staining revealed that >95% of the cells were normal lung macrophages. The cells were suspended in RPMI 1640 medium (J. R. H. Biosciences) supplemented with 2% heat-inactivated fetal horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified incubator at 37 °C. Drosophila SL2 cells (ATCC, Manassas, VA) (33) were maintained at 23 °C in Schneider’s insect medium (Sigma) supplemented with 12% heat-inactivated FBS and gentamicyn (50 μg/ml). SL2 cells were passaged every 4 days.

**Northern Blot Analysis**—Total RNA was obtained from rat aortas, after removal of adventitial tissue, and from cultured RASMCs and rat alveolar macrophages by guanidium isothiocyanate extraction and centrifugation through cesium chloride (34). RNA was fractionated on a 1.3% formaldehyde-agarose gel and transferred to nitrocellulose filters. The filters were hybridized at 68 °C for 2 h with [32P]-labeled mouse HMG-I(Y) and rat iNOS (35) probes. The hybridized filters were then washed in 30 mM sodium chloride, 3 mM sodium citrate, and 0.1% SDS solution at 55 °C and autoradiographed with Kodak XAR film at ~80 °C for several days. The filters were washed in 0.1% sodium dodecyl sulfate on phosphate buffered saline (PBS) solution at 80 °C and rehybridized with a [32P]-labeled oligonucleotide probe complementary to 18 S ribosomal RNA. Images were displayed and radioactivity was measured on a PhosphoImager running the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Plasmids**—To evaluate the effect of IL-1β on HMG-I(Y) transcription in vascular smooth muscle cells, plasmid Δ180 was used (36). This construct, cloned into pCAT-Basic, contained the second transcription start site of HMG-I(Y) (36, 37). Plasmid pGL2-Control contained the firefly luciferase gene driven by an SV40 promoter and enhancer. To evaluate iNOS promoter activity in either vascular smooth muscle cells or alveolar macrophages, we inserted 1× and 3× pairs of 3′ upstream of the mouse iNOS gene and the first 31 base pairs after the transcription start site into pGL2-Basic to make iNOS(–1485/-31) and iNOS(–1485/-31 NF-κB), which contained a mutated downstream NF-κB site (~50 to ~83, GGG to CTC) (38), was used to assess the specificity of NF-κB subunit binding. Plasmid pPAC has been described elsewhere (10). Plasmid pOPRSVI-CAT contained the chloramphenicol acetyltransferase (CAT) gene driven by a Rous sarcoma virus-long terminal repeats promoter, and plasmid 4385mLIM has been described elsewhere (41). Transfections—RASMCs were transfected by a diethylaminoethyl (DEAE)-dextran method (38). In brief, 50,000 cells were plated onto 10-mm tissue culture dishes and allowed to grow for 48–72 h (until 80–90% confluent). Then, plasmids Δ180 and pGL2-Control (to correct for differences in transfection efficiency) were added (5 μg each) to the RASMCs in a solution containing 500 μg/ml of DEAE-dextran. RASMCs were transiently shocked with 10% dimethyl sulfoxide for 1 min and then allowed to recover in medium containing 10% heat-inactivated FBS. Twelve hours after transfection, RASMCs were placed in 2% FBS. RASMCs were then stimulated with vehicle or human recombinant IL-1β (10 ng/ml) (Beckton Dickinson, Los Angeles, CA) for 24 h. Cell extracts were prepared by detergent lysis (Promega), and CAT assays were performed by a modified two-phase fluor diffusion method as described (42, 43). Luciferase activity was measured with an EG&G AutoLumat LB953 luminesimeter (Gaithersburg, MD) and the Promega Luciferase Assay system to assess efficiency of transfection. The ratio of CAT to luciferase activity in each sample served as a measure of normalized CAT activity. To assess the effect of distamycin A (Sigma) on iNOS promoter induction by IL-1β, we transfected RASMCs by the DEAE-dextran method described above. Plasmid iNOS(–1485/-31) or 4385mLIM (5 μg) was transfected with pOPRSVI-CAT (to correct for differences in transfection efficiency) into the RASMCs. Twelve hours after transfection, the cells were placed in 2% FBS and incubated with the maximal stimulatory dose of IL-1β (10 ng/ml) for 24 h in the presence or absence of distamycin A (5 μg). The cells were subsequently harvested and assayed for luciferase activity. In these experiments, the ratio of luciferase to CAT activity in each sample served as a measure of normalized luciferase activity.

SL2 cells were transfected by the calcium-phosphate method according to Di Nocera and Dawid (44). In brief, SL2 cells were plated in 6-well tissue culture dishes (Costar Corp.) 24 h before transfection. Plasmids iNOS(–1485/-31) and iNOS(–1485/-31 NF-κB) were added at 1 μg/ well in 0.5 ml of DMEM, and pPAC and phsp82LacZ were added at 100 ng/well. Plasmid pPACHMGI was added at 0.1, 0.5, or 1 μg/well, alone or in combination with p50-pPAC and p65-pPAC. Forty-eight hours after the initial transfection, extracts from the SL2 cells were prepared and luciferase activity was measured as described for RASMCs. β-Galactosidase assays, to assess transfection efficiency, were performed as described elsewhere (45). The ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of normalized luciferase activity.

**Western Blot Analysis of HMG-I(Y) in RASMCs**—RASMCs were plated in 150-mm tissue culture dishes (Beckton Dickinson, Franklin Lakes, NJ). When the cells were 80–90% confluent, the 10% FBS medium was replaced with 0.4% bovine serum medium. After 48 h, vehicle or IL-1β (10 ng/ml) was added to the cells. RASMCs were then collected in phosphate-buffered saline solution with a cell scraper 48 h later. HMG-I(Y) protein was obtained from RASMCs by acidic extraction followed by trichloroacetic acid precipitation (46). The resulting protein pellet was resuspended in 30 μl of high pressure liquid chromatography-grade water and stored at –20 °C until it was assayed. SDS-polyacrylamide gel electrophoresis was performed by mixing 10 μl of sample dissolved in 4× Laemmli sample buffer (2×) with 10 μl of Laemmli sample buffer (2×). After 5 min of boiling, the samples were resolved on a 15% polyacrylamide gel and transferred to polyvinylidene difluoride (Immobilon-P, Millipore, Bedford, MA) overnight. The membrane was blocked with 10% skim milk in 10 ml Tris-HCl, 150 mM NaCl, pH 8.0, 0.05% Tween-20 (TBS-T) for 1 h at room temperature. It was then incubated with anti-HMG-I(Y) antibody N19 (Santa Cruz Biotechnology, Santa
Cruz, CA) at 0.1 μg/ml in 5% skim milk in TBS-T for 2 h at room temperature. After a wash in TBS-T, the membrane was incubated with anti-goat, horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology) under the same conditions used for the primary antibody. After a final wash in TBS-T, the blot was developed with an enhanced chemiluminescence kit (Amer sham Pharmacia Biotech). Because the extraction also led to the isolation of histone H1, under transfer conditions insufficient for removal of H1 from the gel, we used the histone H1 band to correct for differences in protein loading. After scanning, the intensities of the HMG-I(Y) band and the Coomassie Blue-stained histone H1 band were measured with the NIH Image software, and the ratio of the two values was used to represent the normalized intensity of the HMG-I(Y) band.

**Immunohistochemical Analysis of HMG-I(Y) Expression in Aortas from LPS-treated Rats**—Aortic samples were collected and frozen. Sections were cut to a thickness of 5 μm, fixed in paraformaldehyde, and washed in phosphate-buffered saline. Sections were then incubated in 10% goat serum for 20 min at room temperature. Sections were next incubated with anti-HMG-I(Y) antibody (N19, Santa Cruz Biotechnology) at 0.25 μg/ml in phosphate-buffered saline-0.4% Triton X-100 for 1 h at room temperature and then overnight at 4 °C. Control sections were incubated under the same conditions with normal goat IgG (Research & Diagnostic Systems, Minneapolis, MN) at 0.25 μg/ml. After a wash, sections were incubated with biotinylated rabbit anti-goat antibody (Vector Laboratories, Burlingame, CA) at 1.5 μg/ml in phosphate-buffered saline-0.4% Triton X-100 for 1 h at room temperature. After they had been incubated with avidin, the sections were developed with a peroxidase 3,3′-diaminobenzidine (DAB) kit (Vector Laboratories). To ensure specificity of staining, we treated a series of sections from the same animals with anti-HMG-I(Y) antibody N19 that had been preincubated for 1 h at room temperature with the synthetic peptide (40 μg/ml) used to raise it. The peptide represents amino acids 2–20 of the HMG-I(Y) sequence (ESSSSKSSQPLASKQEK-DGT, Santa Cruz Biotechnology).

**Statistical Analysis**—Data from the SL2 cell transfection experiments were subjected to analysis of variance followed by Scheffe’s test. Significance was assumed at p < 0.05.

**RESULTS**

**IL-1β Increased HMG-I(Y) mRNA in RASMCs in Primary Culture**—Because the proinflammatory cytokine IL-1β is an important downstream mediator of the vascular response to LPS, we studied the effect of IL-1β on HMG-I(Y) gene expression in vitro. Cultured RASMCs were exposed to various concentrations of the cytokine for 24 h, and then total cellular RNA was extracted for Northern blot analysis. IL-1β increased HMG-I(Y) mRNA expression in a dose-dependent fashion (Fig. 1A), with a maximal effect at 10 ng/ml. We then studied the time course of the effect of IL-1β at 10 ng/ml. In comparison with vehicle-treated RASMCs, IL-1β-treated RASMCs showed an initial increase in HMG-I(Y) mRNA after 6 h (Fig. 1B). Peak induction of HMG-I(Y) message (6.2-fold) occurred after 48 h of IL-1β stimulation, even though HMG-I(Y) mRNA levels remained significantly elevated after 72 h of IL-1β treatment. Pretreating the cells with the protein synthesis inhibitor cycloheximide completely abolished induction of HMG-I(Y) mRNA after IL-1β stimulation (Fig. 1C), indicating that this induction depended on protein synthesis de novo. To determine whether LPS could also have an effect on HMG-I(Y) in cultured smooth muscle cells, we applied LPS directly to RASMCs. LPS produced an increase in HMG-I(Y) message (data not shown), although less dramatically than IL-1β. Previous studies have also shown that the induction of iNOS in vascular smooth muscle cells is more pronounced after stimulation with proinflammatory cytokines than with LPS (47, 48).

To determine the mechanism responsible for this increase in HMG-I(Y) mRNA levels in RASMCs after IL-1β stimulation, we measured the half-life of HMG-I(Y) mRNA in the absence or presence of IL-1β. Total cellular RNA was extracted at 0, 4, 8, 10, 12, and 24 h after stimulation with vehicle or IL-1β, and the HMG-I(Y) mRNA half-life was calculated. The half-life of the message was approximately 12 h (Fig. 2A), and IL-1β did not increase HMG-I(Y) mRNA stability. Thus, the increase in HMG-I(Y) mRNA in response to IL-1β treatment could not be explained by a change in mRNA stability after cytokine stimulation. We then performed transient transfection experiments with HMG-I(Y) promoter construct Δ180 (containing the second transcription start site) (36). IL-1β increased HMG-I(Y) promoter activity in RASMCs after 24 h of stimulation (Fig. 2B), suggesting that the induction of HMG-I(Y) mRNA by

![Fig. 1. Effect of IL-1β on HMG-I(Y) mRNA levels in RASMCs in primary culture. A, induction of HMG-I(Y) mRNA by IL-1β dose response. RASMCs were treated with increasing doses of IL-1β as indicated, and total RNA was extracted after 24 h of stimulation. B, induction of HMG-I(Y) mRNA by IL-1β: time course. Cells were treated with vehicle (open bars) or IL-1β (10 ng/ml) (filled bars), and total RNA was extracted at the indicated times. C, effect of protein synthesis inhibition on induction of HMG-I(Y) mRNA by IL-1β. RASMCs were treated with cycloheximide (CHX) (10 μg/ml) for 30 min before the addition of IL-1β (10 ng/ml). Total RNA was extracted after 12 h. In all three experiments, Northern blot analysis was performed with 10 μg of total RNA per lane. Representative blots are shown. In A and B, the signal intensity of each RNA sample hybridized to the HMG-I(Y) probe was divided by that of each sample hybridized to the 18S control. Normalized signal intensities were plotted as the fold induction from the 0 ng/ml intensity (A, mean ± S.D.) or the vehicle intensity (B, mean ± S.D.). Each experiment was performed at least twice, and points of peak HMG-I(Y) induction in A and B were assessed three times.
mRNA levels, which peaked 48 h after IL-1β treatment. To see whether the increase in HMG-I(Y) protein levels was due to an increase in gene transcription, this overexpression plays a major role in the pathophysiology of endotoxaemia (21, 22).

Fig. 2. Effect of IL-1β on HMG-I(Y) mRNA half-life and promoter activity in RASMCs in primary culture. A, half-life of HMG-I(Y) mRNA. Cells were treated with vehicle (open circles) or IL-1β (10 ng/ml) (filled circles) for 24 h. After this incubation period, actinomycin D (10 μg/ml) was added to the cells, and total RNA was extracted at the indicated times. Northern blot analysis was performed with 10 μg of total RNA as described for Fig. 1. The signal intensity of each RNA sample hybridized to the HMG-I(Y) probe was divided by that hybridized to the 18 S probe. The normalized intensity was then plotted as a percentage of the 0 h value against time (in log scale). This experiment was performed three times. B, effect of IL-1β on HMG-I(Y) promoter activity. A plasmid that contains the HMG-I(Y) promoter and drives a CAT reporter, Δ180, was transfected transiently into RASMCs. Cells were then stimulated with vehicle (open bar) or IL-1β (filled bar) for 24 h, after which the cell extracts were harvested. Normalized CAT activity is shown as the fold induction from the activity of vehicle-treated cells (mean ± S.D., n = 4 in each group).

IL-1β was due to an increase in gene transcription. Analysis of a larger promoter construct (base pairs –172 to –202), containing the first three transcription start sites of HMG-I(Y), produced no further increase in reporter activity compared with plasmid Δ180 (data not shown). A promoter construct containing more of the downstream sequence (base pairs –3771 to +4928), including the fourth transcription start site of HMG-I(Y), was not responsive to IL-1β stimulation (data not shown).

IL-1β Induced HMG-I(Y) Protein Expression in RASMCs in Primary Culture—To see whether the increase in HMG-I(Y) mRNA levels, which peaked 48 h after IL-1β treatment (Fig. 1A), corresponded to an increase in HMG-I(Y) protein levels, we treated cultured RASMCs with vehicle or IL-1β for 48 h. Proteins soluble in 5% HClO4 were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with an antibody to HMG-I(Y). IL-1β induced an increase in HMG-I(Y) protein levels of 11.1-fold, as assessed by densitometry after normalization against the levels of histone H1 (Fig. 3).

Endotoxin Increased the Expression of HMG-I(Y) in Vascular Smooth Muscle Cells in Vivo—We intraperitoneally injected male Sprague-Dawley rats (220–250 g) with vehicle or bacterial LPS. Nine hours after treatment, the rats were killed, and abdominal aortas were collected for immunohistochemical analysis with an anti-HMG-I(Y) antibody. As shown in the top right panel of Fig. 4, LPS treatment induced a dramatic up-regulation of HMG-I(Y) immunoreactivity in the vascular smooth muscle and endothelial cells of the blood vessel wall. This observation extends our findings in vitro by demonstrating that HMG-I(Y) protein does indeed accumulate in response to LPS (and hence cytokine stimulation) in vivo. Also, the pattern of staining confirmed to the expected nuclear localization of the HMG-I(Y) protein. The bottom panels of Fig. 4 show aortic sections from the same animals treated with HMG-I(Y) antibody that had been preincubated with the peptide used as its immunogen. In the presence of the immunogen, one would expect HMG-I(Y) immunoreactivity to be lost in areas of specific staining. The absence of staining in the smooth muscle cells of the vessel (Fig. 4, bottom panels) shows that the staining in the top panels was specific. Staining in the endothelium was not entirely lost after preincubation with the immunogen.

Coiduction of HMG-I(Y) and iNOS by Cytokines in Vascular Smooth Muscle Cells and Macrophages—The iNOS gene is overexpressed dramatically when vascular smooth muscle cells are stimulated with proinflammatory cytokines (35, 49), and this overexpression plays a major role in the pathophysiology of endotoxaemia (21, 22). Thus, we investigated the relationship between HMG-I(Y) and iNOS gene expression in vascular smooth muscle cells in response to stimulation by IL-1β (in vitro) and endotoxin (in vivo). RASMCs in primary culture were treated with vehicle or IL-1β, and total cellular RNA was extracted 6, 12, 24, 48, and 72 h thereafter. The RNA was subjected to Northern analysis, and the blots were hybridized sequentially with HMG-I(Y) and iNOS probes. The time courses of the induction of HMG-I(Y) and iNOS mRNA by IL-1β (Fig. 5A) were remarkably similar. Both messages increased as early as 6 h after exposure to IL-1β, and peak induction occurred 48 h after exposure. To determine whether this coinduction of HMG-I(Y) and iNOS was also present in other cell types, we stimulated alveolar macrophages with LPS in vitro. HMG-I(Y) and iNOS mRNAs were both induced in alveolar macrophages after 6 h and 24 h of LPS administration (Fig. 5B). These in vitro results were confirmed fully in vivo (Fig. 6) by
Northern blot analysis of RNA extracted from rat aortas that had been harvested after 9 h of LPS treatment. HMG-I(Y) and iNOS mRNA levels increased dramatically in aortic tissue after LPS stimulation. Basal expression was low for both HMG-I(Y) and iNOS in vehicle-treated rats.

**HMG-I(Y) Promoted Transactivation of the iNOS Gene**—Because the patterns of HMG-I(Y) and iNOS mRNA induction after inflammatory cytokine stimulation were similar, we determined whether HMG-I(Y) had a causal role in transactivation of the iNOS promoter. NF-κB plays an important role in cytokine induction of iNOS (23), as it does in the induction of IFN-β, E-selectin, and MGS/GROα (10, 13, 15). We transfected the iNOS promoter construct iNOS(–1485/+31) into Drosophila SL2 cells, which were chosen because they contain far less endogenous HMG-I(Y) than do mammalian cells (10). We transfected into the SL2 cells, in conjunction with the iNOS promoter, increasing concentrations of the HMG-I(Y) expression plasmid pPACHMGI, with or without expression plasmids for the NF-κB subunits p50 and p65. HMG-I(Y) alone had no significant effect on iNOS promoter activity (Fig. 7A), whereas p50 and p65 alone produced a significant increase in promoter activity. In the presence of p50 and p65, HMG-I(Y) produced a dramatic and dose-dependent increase in iNOS promoter activity. These data suggest that increasing concentrations of HMG-I(Y) potentiate transactivation of the iNOS promoter by NF-κB. The NF-κB binding site in the downstream portion of the iNOS 5′-flanking sequence (–85 to –76) has been shown to be critical for cytokine induction of the iNOS promoter (38).

Thus, we transfected iNOS promoter constructs containing either an intact (iNOS(–1485/+31)) or a mutated downstream NF-κB site (iNOS(–1485/+31 NF-κBm)) into SL2 cells. We also transfected into the cells expression plasmids for p50 and p65, in the presence or absence of the HMG-I(Y) expression plasmid pPACHMGI, to determine whether a mutated NF-κB site alters the ability of HMG-I(Y) to transactivate iNOS. As shown in Fig. 7B, a mutated downstream NF-κB site abolishes the ability of HMG-I(Y) to potentiate iNOS transactivation by p50 and p65.

**Distamycin A Inhibited iNOS Promoter Activity and NO Accumulation Induced by IL-1β**—To determine whether HMG-I(Y) plays a role in iNOS promoter transactivation by IL-1β in vascular smooth muscle cells, we performed transfection experiments in the presence or absence of distamycin A. Distamycin A inhibited iNOS promoter activity and NO accumulation induced by IL-1β.
A is known to bind to AT-rich sequences (clusters of at least four AT base pairs) in the minor groove of DNA (3), and others have demonstrated that distamycin A interferes with the binding of HMG-I(Y) to DNA (50). After transfection into RASMCs, the promoter construct iNOS(–1485/+31) was induced markedly by IL-1β stimulation in the absence of distamycin A. However, coinoculation with distamycin A (5 µM) decreased IL-1β–induced iNOS promoter activity by 43% (Fig. 8A). To ensure that this effect of distamycin A was not related to a nonspecific inhibition of transcription initiation, we transfected a promoter construct from the CRP2/SmLIM gene (–438SmLIM) into RASMCs and exposed the cells to distamycin A or its vehicle. The CRP2/SmLIM gene is expressed in vascular smooth muscle cells (41). Distamycin A had no effect on CRP2/SmLIM promoter activity (data not shown). In addition, distamycin A did not decrease the activity of pOPRSVI-CAT or pGL2-Control, plasmids driven by the Rous sarcoma virus and the SV40 promoters respectively (data not shown). In both experiments (A and B), the amount of DNA added to each sample was normalized with vector DNA (pPAC). Transactivation of iNOS was expressed as a measurement of luciferase activity normalized against β-galactosidase activity (which was used as an internal standard). †, p < 0.05, or ††, p < 0.001 versus 0 µg of HMG-I(Y) and no (−) p50-p65. ‡, p < 0.05, or ‡‡, p < 0.001 versus the same amount of HMG-I(Y) and no (−) p50-p65. ***, p < 0.001 versus 0 µg of HMG-I(Y) and (+) p50-p65.

DISCUSSION

HMG-I(Y) mRNA and protein levels are high in rapidly dividing, undifferentiated mammalian cells (51). The level of
HMG-I(Y) expression correlates with the rate of cell proliferation, and elevated concentrations of HMG-I(Y) are characteristic of transformed cells (25, 26). In fact, HMG-I(Y) may be a marker of metastatic aggressiveness in tumors such as neoplasms of the thyroid (28) and prostate (29). Much less is known about the regulation of HMG-I(Y) under non-growth-related conditions, in vitro or in vivo. For example, although HMG-I(Y) plays a role in the regulation of cytokine-induced genes, to our knowledge there is nothing in the literature about the ability of proinflammatory cytokines to regulate HMG-I(Y) expression in cells in primary culture. We demonstrate here in cultured vascular smooth muscle cells that the proinflammatory cytokine IL-1β increases HMG-I(Y) mRNA levels in a dose- and time-dependent manner (Fig. 1) and that the induction of HMG-I(Y) message by IL-1β is not related to a prolongation in mRNA stability (Fig. 2). IL-1β does increase HMG-I(Y) promoter activity, suggesting that an increase in gene transcription contributes to the induction of HMG-I(Y) message. This increase in mRNA levels by IL-1β translates into an increase in HMG-I(Y) protein levels (Fig. 3), as demonstrated by an analysis of HMG-I(Y) protein expression in vascular smooth muscle cells.

To determine whether HMG-I(Y) is regulated in vivo by an inflammatory stimulus, we administered LPS to rats. LPS promotes a systemic inflammatory response in these animals, mimicking the pathophysiologic process of endotoxemia that occurs during sepsis in humans (17, 20). In this process, immune cells are activated and release a number of mediators and defense molecules, including proinflammatory cytokines (52). Although proinflammatory cytokines, such as IL-1β, help protect the host against infection, release of exaggerated amounts of these cytokines can also have detrimental effects. One such effect, hypotension, is the result of proinflammatory cytokines stimulating production of vasodilatory mediators in the blood vessel wall. Our interest in the work presented here was to determine the effect of an inflammatory stimulus on HMG-I(Y) expression in the vasculature. Immunohistochemical analysis of aortic tissue from rats stimulated with LPS demonstrated a dramatic increase in HMG-I(Y) staining within the blood vessel wall (Fig. 4). Also, preabsorption experiments with the immunogen that was used to generate the HMG-I(Y) antibody showed that this specific staining for HMG-I(Y) occurred within the vascular smooth muscle cells of the blood vessel wall. To our knowledge, this is the first demonstration that HMG-I(Y) can be up-regulated by an inflammatory stimulus in vivo.

NO is a vasodilatory mediator critical to the hypotension of sepsis. The enzyme responsible for NO synthesis during an inflammatory response is iNOS. Previous studies have shown that stimulation with IL-1β (in vitro) and LPS (in vivo) increases iNOS in vascular smooth muscle cells (35, 38, 49). We now demonstrate that induction of HMG-I(Y) correlates with induction of iNOS, both in vitro (Fig. 5) and in vivo (Fig. 6). Because of their similar expression patterns and the fact that HMG-I(Y) is known to facilitate transcription of cytokine-driven genes that require NF-κB for activation, we investigated the role of HMG-I(Y) in transactivation of the iNOS gene. Although HMG-I(Y) alone had no significant effect on iNOS promoter activity, increasing concentrations of an HMG-I(Y) expression plasmid in the presence of p50 and p65 (subunits of NF-κB) led to a dose-dependent increase in iNOS promoter activity (Fig. 7A). At higher concentrations, the HMG-I(Y) expression plasmid potentiated the iNOS promoter response to p50 and p65, and this potentiation required an intact NF-κB site (–85 to –76) in the downstream portion of the iNOS 5'-flanking sequence (Fig. 7B). These data demonstrate for the first time that HMG-I(Y), an architectural transcription factor, facilitates transactivation of the iNOS gene by NF-κB. Furthermore, the dose-dependent increase in iNOS promoter activity by HMG-I(Y) suggests that the induction of HMG-I(Y) by an inflammatory stimulus may have an important impact on iNOS gene regulation. This concept was confirmed by our demonstration that distamycin A (an agent that interferes with HMG-I(Y) binding to AT-rich sequences in the minor groove of DNA) suppressed IL-1β-induced iNOS promoter activity and NO accumulation in vascular smooth muscle cells (Fig. 8).

Our observation that proinflammatory cytokines are able to induce HMG-I(Y) in vascular smooth muscle cells differs from the finding of Thanos and Maniatis (9) in their study of NF-κB–dependent virus induction of the IFN-β gene in human osteosarcoma cells. In their study, viral infection did not induce transcription of the HMG-I(Y) gene. Our observation that cytokines induce HMG-I(Y) gene transcription in vascular smooth muscle cells may be related to our use of cells in primary culture and our avoidance of a tumor-derived cell line (in which HMG-I(Y) expression may have been higher at base line).

It has been suggested that the assembly of higher order nucleoprotein complexes, consisting of different families of transcription factors, may be a means of bringing together divergent signaling pathways to activate a specific gene (10). The assembly of such complexes may depend on architectural transcription factors like HMG-I(Y), which may orchestrate this process. HMG-I(Y) is important in the regulation of cytokine-driven genes, and we show in this report that HMG-I(Y) itself is up-regulated by a proinflammatory stimulus in vascular smooth muscle cells in primary culture and in aortic tissue in vivo. Moreover, our experiments show that HMG-I(Y) may play an important role in transactivation of the iNOS gene. This observation has relevance to the pathophysiology of sepsis, an intense inflammatory response that often results in hypotension and collapse of the circulatory system.

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