Down-regulation of High Mobility Group-I(Y) Protein Contributes to the Inhibition of Nitric-oxide Synthase 2 by Transforming Growth Factor-β1*

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The inducible isofom of nitric-oxide synthase (NOS2) catalyzes the production of nitric oxide (NO), which participates in the pathophysiology of systemic inflammatory diseases such as sepsis. NOS2 is transcriptionally up-regulated by endotoxin and inflammatory cytokines, and down-regulated by transforming growth factor (TGF)-β1. Recently we have shown that high mobility group (HMG)-I(Y) protein, an architectural transcription factor, contributes to NOS2 gene transactivation by inflammatory mediators. The aim of the present study was to determine whether regulation of HMG-I(Y) by TGF-β1 contributes to the TGF-β1-mediated suppression of NOS2. By Northern blot analysis, we show that TGF-β1 decreased cytokine-induced HMG-I(Y) mRNA levels in vascular smooth muscle cells and macrophages in vitro and in vivo. Western analysis confirmed the down-regulation of HMG-I(Y) protein by TGF-β1. To determine whether the down-regulation of HMG-I(Y) contributed to a decrease in NOS2 gene transactivation by TGF-β1, we performed cotransfection experiments. Overexpression of HMG-I(Y) was able to restore cytokine inducibility of the NOS2 promoter that was suppressed by TGF-β1. The effect of TGF-β1 on NOS2 gene transactivation was not related to a decrease in binding of HMG-I(Y) to the promoter of the NOS2 gene, but due to a decrease in endogenous HMG-I(Y) protein. These data provide the first evidence that cytokine-induced HMG-I(Y) can be down-regulated by TGF-β1. This down-regulation of HMG-I(Y) contributes to the TGF-β1-mediated decrease in NOS2 gene transactivation by proinflammatory stimuli.

The inducible isofom of nitric-oxide synthase (NOS2) catalyzes the production of nitric oxide (NO), which participates in the physiologic and pathophysiologic regulation of multiple organ systems (1–5). One disease process that is particularly affected by the overproduction of NO is sepsis (6, 7). In sepsis, a severe underlying infection triggers a cascade of events that may lead to intractable hypotension, multiple organ system failure, and death (8, 9). Within this cascade, inflammatory cytokines and vasoactive mediators promote the relaxation of vascular smooth muscle cells and a decrease in vascular tone. NO is a vasodilatory gas generated late in the sepsis cascade contributing to the hypotension. The overproduction of NO during sepsis is generated through the NOS2 pathway. NOS2 is a highly inducible gene regulated at the level of gene transcription (1, 10). Transactivation of the NOS2 gene is mediated by members of the nuclear factor (NF)-κB family of transcription factors, which bind to a site in the downstream portion of the NOS2 5′-flanking sequence (−85 to −76) (11). Recently, we demonstrated that high mobility group (HMG)-I(Y) protein, an architectural transcription factor that binds to an AT-rich octamer site (−61 to −54) close to the NF-κB binding site in the promoter of the NOS2 gene, acts in concert with p50 and p65 to facilitate NOS2 gene transactivation (12). Architectural transcription factors typically function by modifying the conformation of DNA, and thus provide a framework for the transcriptional machinery to operate. HMG-I(Y) does not drive transcription itself, but it facilitates the assembly of a functional nucleoprotein complex. Our studies revealed that binding of both HMG-I(Y) and NF-κB subunits in the downstream 5′-flanking sequence of the NOS2 gene is essential for the most potent activation of the promoter (12). Moreover, we also demonstrated that HMG-I(Y) itself was up-regulated (both at the mRNA and protein levels) by inflammatory cytokines (13). Taken together, these studies suggest that HMG-I(Y) contributes to the transcriptional regulation of NOS2 by inflammatory mediators.

Previously, we and others have demonstrated that transforming growth factor (TGF)-β1, a pleiotropic growth factor involved in a number of physiologic processes (14–16), inhibited NOS2 expression in vitro (17–19) and in vivo (20). The down-regulation of NOS2, but not NOS3, in a rodent model of endotoxemia suggested that NOS inhibition by TGF-β1 is more selective for the inducible isofom of NOS (NOS2) in an experimental model of sepsis (20). In vascular smooth muscle cells, inhibition of NOS2 by TGF-β1 occurs at the level of gene transcription (19). However, the mechanism by which transactivation of the NOS2 gene is inhibited by TGF-β1 has not been fully elucidated.

We have shown previously that HMG-I(Y) is involved in the induction of NOS2 by inflammatory mediators (12, 13). However, the ability of HMG-I(Y) to be regulated by inhibitors of

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† The abbreviations used are: NOS2, nitric-oxide synthase; NO, nitric oxide; TGF, transforming growth factor; HMG, high mobility group; LPS, lipopolysaccharide; IL, interleukin; IFN, interferon; NF, nuclear factor; RASMC, rat aortic smooth muscle cells; FBS, fetal bovine serum; Luc, luciferase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; bp, base pair(s).
NOS2 expression is not known. Furthermore, little is known about the down-regulation of HMG-I(Y) and its subsequent effect on NOS2 gene transcription. The present study was designed to determine (a) whether TGF-β1, an inhibitor of NOS2 expression, also down-regulates the expression of HMG-I(Y), and (b) the functional importance of this TGF-β1-mediated down-regulation of HMG-I(Y) on promoter activity of the NOS2 gene.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Bacterial lipopolysaccharide (LPS) *Escherichia coli* (serotype 026:B6), actinomycin D, and thioglycollate were all from Sigma Chemical Co. (St. Louis, MO). Recombinant human interleukin (IL)-1β (Collaborative Biomedical, Bedford, MA), human TGF-β1 (R & D Systems, Minneapolis, MN), and mouse interferon (IFN)-γ (R & D Systems) were stored at −80 °C until use.

**Cell Culture**—Rat aortic smooth muscle cells (RASMC) were harvested from male Harlan Sprague-Dawley rats (200–250 g) by enzymatic dissociation according to the method of Gunther et al. (21). The cells were cultured in Dulbecco’s modified Eagle’s medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (FBS), Hyclone, Logan, UT), penicillin (100 units/ml), streptomycin (100 µg/ml), and 25 mM HEPES (pH 7.4) (Sigma) in a humidified incubator at 80–90% confluence, 10% FBS medium was replaced with 0.4% bovine serum albumin (BSA) medium to correct for differences in protein loading. After washing, the intensities of the HMG-I(Y) band and the Cooassie Blue-stained histone H1 band were measured with IMAGE software (National Institutes of Health, and the ratio of two was used to represent the normalized intensity of the HMG-I(Y) band.

**Plasmids**—To evaluate the effect of IL-1β and TGF-β1 on HMG-I(Y) expression in vascular smooth muscle cells, plasmid Δ180 was used (27). This construct, cloned into pCMV-Basic, contained the second transcription start site of HMG-I(Y) (27, 28). Plasmid pGL2-Control contained the firefly luciferase gene driven by an SV40 promoter and enhancer.

To evaluate NOS2 promoter activity in either vascular smooth muscle cells or RAW 264.7 cells, we inserted 1458 bp of the 5′-flanking region of the mouse NOS2 gene before the first 3′UTR transcription start site into pGL2-Basic to make iNOS(−1458/+31), as described previously (29). To assess the effect of HMG-I(Y) overexpression on NOS2 after treatment with TGF-β1 in both RASMC and RAW 264.7, we constructed the HMG-I(Y) cDNA into the EcoRI site of pcDNA3 (Invitrogen). DNA mass was normalized by adding the control plasmids as needed. Luciferase activity was normalized by cotransferring pCMV-βgal plasmid (CLONTECH).

**Transfections**—RASMCS were transfected by a diethylaminoethyl (DEAE)-dextran method (29). In brief, 500,000 cells were plated onto 100-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 RASMC in a solution containing 500 µg/ml DEAE-dextran. RASMCs were subsequently shocked with 10% dimethyl sulfoxide solution for 1 min and then allowed to recover in medium containing 10% heat-inactivated FBS. 12 h after transfection, RASMCs were placed in 2% FBS. RASMCs were then stimulated with vehicle alone, IL-1β (10 ng/ml) alone, or IL-1β plus TGF-β1 (10 ng/ml) after 24 h, cell extracts were prepared for gelatinase (Promega), and CAT assays were performed as described (30, 31). Luciferase activity was measured with an EQ&G AutoLumat LB953 luminometer (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 investigate the effect of HMG-I(Y) overexpression on TGF-β1-induced inhibition of NOS2 promoter activity by inflammatory stimuli, RASMCS or RAW 264.7 were plated in 6-well cell culture plates at 100,000/well and 70,000/well, respectively. The next day, cells were transfected as described above using 1 µg/well iNOS(−1458/+31), 0.8 µg/well pCMV-βgal, and increasing amounts of HMG-I(Y)pcDNA3 (1, 2, or 3 µg/well). The corresponding empty vector was added when needed to keep the DNA amount constant throughout the experiment. 18 h after transfection, RASMC were treated with IL-1β (10 ng/ml) alone or IL-1β plus TGF-β1 (0.5 ng/ml). RAW 264.7 were treated with LPS alone (0.5 µg/ml) or LPS plus TGF-β1 (1 ng/ml). After an additional 24 h, cells were harvested by detergent lysis (Promega). In the resulting cell extract, luciferase and β-galactosidase activity was assayed as described previously (32).

**Electrophoretic Mobility Shift Assay (EMSA)**—For the preparation of the nuclear protein extract (29), RAW 264.7 cells were grown to 80% confluence then stimulated with either vehicle, LPS (1 µg/ml), or LPS (1 µg/ml) plus TGF-β1 (10 ng/ml). LPS-stimulated nuclear extract was subjected to electrophoretic mobility assay analysis using a double-stranded oligonucleotide probe encoding region −87 to −52 of the NOS2 gene flanking the E2 site (5′-GGGACTCTTTTGATGTTTACAACTGTATTTCACAAAAA). Binding reactions were performed in a 25-µl volume containing 20,000 cpm of labeled probe, 10 µg of nuclear extract, 100 ng of poly(dG·dC)·poly(dG·dC) (Sigma), 25 µM Hepes (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol, with or without oligonucleotide competitors. Reactions were incubated for 20 min at room temperature, and DNA-protein complexes were analyzed by electrophoresis on a 5% native polyacrylamide gel in 0.2% Tris borate-
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**RESULTS**

TGF-β1 Prevents the Induction of HMG-I(Y) and NOS2 mRNA by IL-1β in RASMC—Vascular smooth muscle cells are an important target of proinflammatory cytokines during sepsis, and their relaxation is an important determinant of the hypotension accompanying septic shock. Thus, we initially assessed the effect of TGF-β1 on NOS2 and HMG-I(Y) mRNA levels after IL-1β stimulation in RASMC. Fig. 1 shows that TGF-β1 not only prevented the induction of NOS2 mRNA by IL-1β (white bars), but it also prevented HMG-I(Y) induction (black bars). IL-1β induction of HMG-I(Y) mRNA was reduced by 96% after 24 h of TGF-β1 treatment.

To investigate the mechanism by which TGF-β1 decreased HMG-I(Y) mRNA levels after their induction by IL-1β, we assessed HMG-I(Y) mRNA half-life and promoter activity (Fig. 2). To evaluate HMG-I(Y) mRNA half-life, RASMC were cultured to 70% confluence. Cells were then treated with either vehicle (V), IL-1β alone (IL, 10 ng/ml), or IL-1β plus TGF-β1 (IL + T, 1 ng/ml). Total RNA was extracted 24 h after treatment, and Northern blot analysis was performed using 10 μg of total RNA per lane. After electrophoresis, the RNA was transferred to nitrocellulose filters, which were hybridized with 32P-labeled HMG-I(Y) and NOS2 probes. Filters were also hybridized with a 32P-labeled oligonucleotide probe complementary to 28 S ribosomal RNA to control for differences in loading. The signal intensities were then plotted as -fold increases compared with vehicle signal (mean ± S.E.) for HMG-I(Y) (black bars) and NOS2 (white bars). Each experiment was performed three times.

**FIG. 1.** Effect of TGF-β1 on IL-1β-induced HMG-I(Y) and NOS2 mRNA levels in RASMC. RASMC were treated with vehicle (V), IL-1β alone (IL, 10 ng/ml), or IL-1β plus TGF-β1 (IL + T, 1 ng/ml). Total RNA was extracted 24 h after treatment, and Northern blot analysis was performed using 10 μg of total RNA per lane. After electrophoresis, the RNA was transferred to nitrocellulose filters, which were hybridized with 32P-labeled HMG-I(Y) and NOS2 probes. Filters were also hybridized with a 32P-labeled oligonucleotide probe complementary to 28 S ribosomal RNA to control for differences in loading. The signal intensities were then plotted as -fold increases compared with vehicle signal (mean ± S.E.) for HMG-I(Y) (black bars) and NOS2 (white bars). Each experiment was performed three times.

**FIG. 2.** Effect of TGF-β1 on HMG-I(Y) mRNA half-life and promoter activity. A, half-life of HMG-I(Y) mRNA. Cells were treated with IL-1β alone (IL, 10 ng/ml, filled circles) or IL-1β plus TGF-β1 (IL + T, 1 ng/ml, open 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 (0, 4, 8, 12, and 24 h). Northern blot analysis was performed as described in Fig. 1. The normalized signal intensity was then plotted as a percentage of the 0-h value. The experiment was performed three times. B, effect of TGF-β1 on IL-1β-induced promoter activity of the HMG-I(Y) gene. A plasmid that contains the HMG-I(Y) promoter and drives a CAT reporter, Δ180, was transfected transiently into RASMC. Cells were then stimulated with vehicle (V), IL-1β alone (IL, 10 ng/ml), or IL-1β plus TGF-β1 (IL + T, 1 ng/ml) 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.E., n = 4 in each group).
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31) and increasing concentrations of expression
iNOS(RASMC and RAW 264.7 cells with promoter construct
uli. To test this hypothesis, we transiently cotransfected
point was used to assess the effect of TGF-
48 h of cytokine stimulation in RASMC (13), thus this time
RASMC and by LPS in RAW 264.7 cells. We have shown
significant increase in promoter activity of the
NOS2 gene, plotted as -fold increases compared with vehicle signal (mean ± S.E.) for NOS2 (black bars) and NOS2 (white bars). Each experiment was performed three times.

The effect of HMG-I(Y) overexpression on NOS2 and HMG-I(Y) gene expression was studied in RASMC and RAW 264.7 cells. HMG-I(Y) overexpression rescued the down-regulation of NOS2 gene expression induced by TGF-β1 in RASMC and RAW 264.7 cells. The rescued NOS2 expression was dose-dependent and was observed in both RASMC (Fig. 6A) and RAW 264.7 cells (Fig. 6B), respectively. As expected, when vector-transfected cells (pcDNA3) were treated with TGF-β1, NOS2 promoter activity in response to IL-β (RASMC) and LPS (RAW 264.7 cells) was significantly reduced in RASMC (Fig. 6A) and RAW 264.7 cells (Fig. 6B). HMG-I(Y)-transfected cells showed a dose-dependent recovery of NOS2 promoter activity by both IL-β (Fig. 6A) and LPS (Fig. 6B). At a dose of 2 μg/well HMG-I(Y) expression plasmid, the responsiveness of the NOS2 gene promoter activity was almost completely restored in both RASMC (89% of IL-β alone value, Fig. 6A) and RAW 264.7 cells (90% of the LPS alone value, Fig. 6B) despite the presence of TGF-β1. To determine whether HMG-I(Y) could restore other TGF-β1 down-regulated genes, we performed the same experiment using the promoter for the matrix metalloproteinase-12 gene (36). Overexpression of HMG-I(Y) did not rescue the TGF-β1-mediated down-regulation of matrix metalloproteinase-12 promoter activity (data not shown). These data suggest that recovery of NOS2 promoter activity by HMG-I(Y) was a specific effect.

Effect of TGF-β1 on HMG-I(Y) Binding to the Promoter of the NOS2 Gene—We investigated the binding of HMG-I(Y) protein to the downstream promoter sequence (region −87 to −52 of the NOS2 5'-flanking region, TGGGACTCTCCCTTTGG-GAACAGTTATGCAAAATA) of the NOS2 gene by using nuclear extracts obtained from RAW 264.7 cells stimulated with LPS in the presence or absence of TGF-β1 for 4, 24, and 48 h. Following LPS stimulation, we observed the appearance of a high molecular weight band that exhibited a clear induction at 24 and 48 h after LPS (band 1, Fig. 7A). A second band was also evident (band 2). Band 2 decreased in intensity in a time-dependent manner. The specificity of the two bands was evalu-
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Fig. 5. Effect of TGF-β1 on HMG-I(Y) protein expression after treatment with IL-1β or LPS in RASMC and RAW264.7 cells respectively. A, RASMC were treated with vehicle (V), IL-1β alone (IL, 10 ng/ml), or IL-1β plus TGF-β1 (IL+T, 1 ng/ml) for 48 h. B, RAW 264.7 cells were treated with vehicle (V), LPS alone (L, 0.5 µg/ml), or LPS plus TGF-β1 (L+T, 10 ng/ml) for 48 h. In both A and B, protein was extracted from the cells and analyzed by Western blotting. A representative blot is shown. The intensity of the band for HMG-I(Y) was divided by the intensity of the band for histone H1. Normalized signal intensities were plotted as the -fold induction from the intensity of vehicle (mean ± S.E.). Each experiment was performed twice.

FIG. 5.

The inflammatory response is a key component of host defense, but excessive activation of the immune system and subsequent release of vasoactive mediators (such as occurs in sepsis) may be fatal (8, 46). The inflammatory process must therefore be regulated tightly in vivo. The effects of proinflammatory cytokines are counterbalanced by the production of anti-inflammatory mediators. Among these anti-inflammatory factors, TGF-β1 has been shown to decrease hypotension and LPS-induced mortality in rats when administered exogenously (49). However, the receptor(s) downstream of the TGF-β1 is not known.

In this study, TGF-β1 prevented the IL-1β induction of the NOS2 gene in RAW 264.7 cells (data not shown), indicating that TGF-β1 was not affecting HMG-I(Y) DNA binding properties at these time points. In contrast, the intensity of band 1 was dramatically decreased after 48 h of treatment with LPS plus TGF-β1 compared with LPS alone. Interestingly, the Western blot analysis showed that, at the same time point (48 h), HMG-I(Y) protein content was dramatically decreased by TGF-β1 (Fig. 5B). These data suggest that the decreased intensity of band 1 after 48 h of TGF-β1 was related to a decrease in HMG-I(Y) protein not an alteration in binding affinity of the HMG-I(Y) protein.

In the TGF-β1 receptor responsible for transcriptional down-regulation of the NOS2 gene is(are) not known.

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HMG-I(Y) and NOS2 mRNA in RASMC (Fig. 1). The effect on HMG-I(Y) mRNA levels did not involve a modification of transcript stability (Fig. 2A) but was related to a decrease in HMG-I(Y) promoter activity in the presence of TGF-β1 (Fig. 2B). This effect of TGF-β1 on HMG-I(Y) mRNA levels was not restricted to vascular smooth muscle cells, because TGF-β1 down-regulated the HMG-I(Y) message (similar to the NOS2 message) in macrophages stimulated with inflammatory cytokines and endotoxin in vitro (Fig. 3) and in vivo (Fig. 4). In agreement with mRNA levels, TGF-β1 also down-regulated HMG-I(Y) protein in vascular smooth muscle cells (Fig. 5A) and macrophages (Fig. 5B) induced by IL-1β and endotoxin, respectively. These results provide the first evidence that TGF-β1 can decrease HMG-I(Y) expression driven by inflammatory stimuli.

We have suggested previously that induction of HMG-I(Y), and subsequent transactivation of the NOS2 gene, may contribute to a reduction in vascular tone during sepsis and other inflammatory disease processes (12, 13). In addition, we have shown in the present study that HMG-I(Y) and NOS2 expression are down-regulated by TGF-β1 in a comparable manner after stimulation with inflammatory mediators. Thus, we hypothesized that HMG-I(Y) content may be a limiting factor for NOS2 induction by endotoxin and proinflammatory cytokines. To test this hypothesis, we overexpressed HMG-I(Y) in vascular smooth muscle cells and macrophages in the presence of TGF-β1 to determine whether HMG-I(Y) could restore inflammatory cytokine and endotoxin inducibility of the NOS2 gene promoter. In both RASMC (Fig. 6A) and RAW 264.7 cells (Fig. 6B), overexpression of HMG-I(Y) was able to restore cytokine inducibility of the NOS2 promoter that was suppressed by TGF-β1. Taken together, these data suggest that factors (such as TGF-β1) that decrease the amount of endogenous HMG-I(Y) in cells limit the inducibility of NOS2 by inflammatory mediators.

HMG-I(Y) has the potential to be post-translationally modified. In vitro experiments have shown that phosphorylation of HMG-I(Y) can decrease its affinity for DNA (50, 51). Thus, to determine whether HMG-I(Y) binding to the promoter of the NOS2 gene was altered by TGF-β1, we performed EMSA. LPS induced the formation of a specific, high molecular weight complex in macrophages (Fig. 7, A and B). Supershift experiments using an anti-HMG-I(Y) antibody revealed the presence of HMG-I(Y) in this complex (Fig. 7C). We have shown previously that NF-κB binding to this portion of the NOS2 5′-flanking sequence (12) is important for NOS2 gene transactivation; however, a direct effect of TGF-β1 on NF-κB binding has not been demonstrated (29). Thus, we wanted to determine whether an alteration in HMG-I(Y) binding by TGF-β1 might be responsible for the suppression of NOS2 gene transactivation. TGF-β1 treatment did not modify the intensity of the specific HMG-I(Y) binding complex after 4 and 24 h (Fig. 7D, lanes 2 and 4), suggesting that DNA binding of HMG-I(Y) was not modified by TGF-β1. In contrast, the dramatic decrease in intensity of this complex after 48 h of TGF-β1 treatment (Fig. 7D, lane 6) corresponded with a decreased in HMG-I(Y) protein content as shown by Western blot analysis (Fig. 5). Taken together, these data suggest that a down-regulation in HMG-I(Y) protein, not a disruption in HMG-I(Y) binding, is accountable for the decreased nucleoprotein complex responsible for transactivation of the NOS2 gene by inflammatory mediators.

The present study provides further insight into the mechanism by which TGF-β1 is able to down-regulate NOS2 gene transactivation by inflammatory mediators. Our data underscore the importance of HMG-I(Y) in contributing to nucleoprotein complex formation for driving transcription of the NOS2 gene. Moreover, we show that inhibition of HMG-I(Y) transcription and subsequent decrease in HMG-I(Y) protein by TGF-β1 significantly decreases NOS2 promoter activity, which can be restored by exogenous administration of HMG-I(Y). These data confirm the importance of HMG-I(Y) in regulating NOS2 gene transactivation by inflammatory mediators in vascular smooth muscle cells and macrophages, cell types involved in the pathophysiology of sepsis and other systemic inflammatory diseases.

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