Negative Regulation of Inducible Nitric-oxide Synthase Expression Mediated through Transforming Growth Factor-β-dependent Modulation of Transcription Factor TCF11*

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David T. Berg, Akanksha Gupta, Mark A. Richardson, Lee A. O’Brien, David Calnek, and Brian W. Grinnell

From the Division of Biotechnology Discovery Research, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Indiana 46285-0444

Inducible nitric-oxide synthase (iNOS) plays a central role in the regulation of vascular function and response to injury. A central mediator controlling iNOS expression is transforming growth factor-β (TGF-β), which represses its expression through a mechanism that is poorly understood. We have identified a binding site in the iNOS promoter that interacts with the nuclear heterodimer TCF11/MafG using chromatin immunoprecipitation and mutation analyses. We demonstrate that binding at this site acts to repress the induction of iNOS gene expression by cytokines. We show that this repressor is induced by TGF-β1 and by Smad6-short, which enhances TGF-β signaling. In contrast, the up-regulation of TCF11/MafG binding could be suppressed by overexpression of the TGF-β inhibitor Smad7, and a small interfering RNA to TCF11 blocked the suppression of iNOS by TGF-β. The binding of TCF11/MafG to the iNOS promoter could be enhanced by phorbol 12-myristate 13-acetate and suppressed by the protein kinase C inhibitor staurosporine. Moreover, the induction of TCF11/MafG binding by TGF-β and Smad6-short could be blocked by staurosporine, and the effect of TGF-β was blocked by the selective protein kinase C inhibitor calphostin C. Consistent with the in vitro data, we found suppression of TCF11 coincident with iNOS up-regulation in a rat model of endotoxemia, and we observed a highly significant negative correlation between TCF11 and nitric oxide production. Furthermore, treatment with activated protein C, a serine protease effective in septic shock, blocked the down-regulation of TCF11 and suppressed endotoxin-induced iNOS. Overall, our results demonstrate a novel mechanism by which iNOS expression is regulated in the context of inflammatory activation.

Nitric oxide (NO) is an important regulator and mediator of numerous and diverse processes, including vascular function, neurotransmission, tumor biology, and innate immune response (reviewed in Ref. 1). The production and balance of NO from inducible nitric-oxide synthase (iNOS) play an important role in septic shock because overexpression mediates vascular collapse and cardiac dysfunction (2) and contributes to multiple organ dysfunction (reviewed in Ref. 3). Selective iNOS inhibition has been shown to attenuate the hemodynamic alterations in sepsis models (4).

During acute inflammatory insult, iNOS is synergistically induced by the TH-1 cytokines tumor necrosis factor-α, interleukin-1β, and interferon-γ (5). Following cytokine activation and iNOS induction, vascular smooth muscle cells produce high levels of NO (6). A key factor in controlling iNOS expression is transforming growth factor-β (TGF-β), which has been shown to suppress the expression of iNOS at the mRNA level in a variety of cells (6–8), and anti-TGF antibody has been shown to block the suppression of iNOS in the vasculature (9). Moreover, in an endotoxin model of septic shock, TGF-β1 treatment markedly reduced iNOS mRNA in several organs and blocked the lipopolysaccharide (LPS)-induced hypotension (10).

Although the key factors involved in the induction of iNOS have been defined (reviewed in Ref. 11), little is known regarding the suppression of iNOS expression by factors such as TGF-β. In this study, we explored the mechanism by which TGF-β suppresses the expression of iNOS in human smooth muscle cells. We show that the nuclear factor TCF11/MafG interacts with an upstream suppressor element in the iNOS promoter, resulting in the inhibition of cytokine induction of iNOS mRNA. This factor is up-regulated by TGF-β and the agonist Smad6-short but suppressed by Smad7. In a model of endotoxemia, we show a strong negative relationship between TCF11 and iNOS, consistent with the results observed in vitro. Furthermore, we demonstrate that activated protein C, an agent effective in model and clinical sepsis, increased TCF11 levels concomitant with a suppression of endotoxin-induced iNOS expression. Overall our data provide new mechanistic understanding of the regulation of iNOS, a key regulatory factor in vascular function.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Human aortic smooth muscle cells were obtained from Clonetics (Walkersville, MD) and grown as described previously (12). Because of poor transfection efficiency, these cells were grown as described previously (12). Because of poor transfection efficiency, these cells were not transduced with a retroviral vector expressing TGF-β1 but were grown in the presence of 5 μg/ml of TGF-β1 (R&D Systems) in the culture media. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: iNOS, inducible nitric-oxide synthase; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; bis-
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...tion of primary smooth muscle cells, human umbilical vein endothelial cells (Clonetics) grown in Clonetics endothelial growth media were used for small interfering (siRNA) experiments. All other reagents were of the highest quality available.

**DNA Binding Assay**—The gene locus containing the Homo sapiens NOS2 gene promoter region was X85759.1. Bioinformatics analysis of the promoter region of iNOS was performed using MatInspector (release 6.2.1) and TRANSFAC Matrix (release 3.3; TFMATRIX version 1.3). For gel shift assay, the oligonucleotide sequence containing the TCF11/MafG site was 5'-CCTAACAGATGCAGATGAGCCATGTGGCCT-3'. Confirmation of the binding was performed by electrophoretic mobility shift assays as follows. The double-stranded probe was γ-32P-labeled, and nuclear extracts were prepared as described previously (13). Reactions containing Buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 0.125 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol), poly(dI-dC), nuclear extract, and labeled oligonucleotide were incubated for 15–45 min and analyzed and quantified by a scanning phosphorimaging device as described previously (13). In some experiments, detection was done using fluorescence-labeled oligonucleotides obtained from LI-COR Biosciences (Lincoln, NE) that were 5’-end-labeled with IRDye 800 phosphoramidite. Unlabeled synthetic oligonucleotides were used for competition studies were obtained from Operon Biotechnologies, Inc. (Huntsville, AL). Binding reaction conditions were 5 fmol/μl labeled oligonucleotide, 4 units/μl poly(dI-dC), and 0.3 μg/μl nuclear extract, and gels were scanned with the LI-COR Odyssey imaging system. Competition reactions contained from 1- to 100-fold molar excess unlabeled oligonucleotides. A mutant oligonucleotide containing changes to the core TGA with CTC binding sequence was used as a control. The TCF11/MafG binding site from the BK virus P2 strain, 5’-CCTAACAGATGCAGATGAGCCATGTGGCCT-3’, was used as a control (14) and has a perfect core similarity of 1.0 to the consensus sequence. Antibody H-285, which binds to the leucine zipper of TCF11 (catalog no. sc-13031, anti-peptide 682-704, Santa Cruz Biotechnology, Inc.), was included in some experiments to inhibit the heterodimerization required for binding.

**Chromatin Immunoprecipitation Assay**—A chromatin immunoprecipitation (ChIP) assay was used to evaluate the association of TCF11 with its DNA binding site using a ChIP assay kit from Upstate (catalog no. 17-295-290, Millipore, Billerica, MA). Briefly, cells were cross-linked with 1% formaldehyde in growth medium for 8 min at 37 °C and rinsed two times with ice-cold phosphate-buffered saline. The cells were resuspended in 100 μl of SDS lysis buffer and incubated on ice for 10 min. Chromatin from the cross-linked cells was sheared by sonication with three 15-s pulses at one-third power and then incubated overnight with a TCF11-specific antibody (catalog no. sc-721, Nrf1C19) or anti-Maf antibody (catalog no. sc-479, C20) (Santa Cruz Biotechnology, Inc.). Antibody-chromatin complexes were immunoprecipitated using protein G-Sepharose saturated with salmon sperm DNA. Precipitated DNA-antibody complexes were first prepared using a Qiagen PCR purification kit (catalog no. 28106) and then analyzed by quantitative real-time PCR using the ABI 7900 HT sequence detection system. The following primers and probe specific for the TCF11 binding site in the iNOS promoter region were used for TaqMan analysis: forward primer, 5’-GAGCTCCCTGCTAGGAAAA-3’; reverse primer, 5’-CAAGCCACATGGCCTCATT-3’; and probe, 5’-ATCTCCAGATGCTG-3’.

**siRNA Inhibition of TCF11-TCF11 siRNA Transfection of Human Umbilical Vein Endothelial Cells**—Cells were seeded at 105 cells/well in 6-well collagen-coated plates (BD Biosciences) and incubated at 37 °C in 5% CO2 for 24 h. Following the incubation the monolayers were transfected with siRNA for TCF11, ON-TARGETplus SMARTpool human NFE2L1 or ON-TARGETplus siCONTROL (Dharmacon, Lafayette, CO) with Lipofectin (Invitrogen) according to the manufacturer’s protocol. The transfected cells were incubated at 37 °C in 5% CO2 for 48 h. The medium was aspirated and replaced with 1 ml/well serum-free medium, 100 μg/ml bovine albumin fraction V (Invitrogen), insulin/transferrin/selenium supplement (Invitrogen), 20 mM HEPES (Invitrogen), and 50 μg/ml gentamicin (Invitrogen) ±1 ng/ml TGF-β1 (R&D Systems), and after 4 h a cytokine mixture was added to each well for a final concentration of 10 ng/ml tumor necrosis factor-α, 10 ng/ml interleukin-1β, and 25 ng/ml interferon-γ (all R&D Systems). After 24 h, the conditioned medium was assayed for iNOS expression by NO generation as described previously (17).

**Analysis of iNOS Expression**—The level of iNOS mRNA in human smooth muscle cells was determined by ribonuclease protection assay as described previously (15). The iNOS ribonuclease probe was prepared from a PstI/EcoRV restriction digest of the cDNA for iNOS, yielding a 393-nucleotide protect fragment. As a control, an actin antisense riboprobe was synthesized from the TR1-β-actin-125 human template. Both the iNOS and actin mRNA levels were quantified using a Molecular Dynamics 445Si PhosphorImager.

Multimers of the TCF11/MafG binding site and of a mutated site converting the TGA sequence to CTC were cloned into plasmid pAT153 as described previously (13) to generate plasmid pTCF-BS, containing the intact binding site, and pTCD- mBS, containing the mutant binding site. Cells were transfected with these decoy plasmids as described previously (13), and the level of iNOS mRNA was determined as above.

In some experiments, a control pCIneo vector (Promega, Madison, WI) or the expression vectors for Smad6-short and Smad7 (16) were transfected into cells with Lipofectin, and the level of TCF11/MafG binding was determined in cell lysates 24 h later. The effect of TGF-β (1 ng/ml) on TCF11/MafG levels was determined 24 h after addition to the maintenance medium.

**Analysis of TCF11 and iNOS in Rat Endotoxemia**—Male Sprague-Dawley rats (Harlan) weighing 350–400 g were used in the study. Endotoxiaemia was induced by administration of *Escherichia coli* LPS (10 mg/kg, intravenous infusion for 30 min; lipopolysaccharide W E. coli 0127:B8; Sigma) using a jugular vein catheter. The control group received a pyrogen-free saline infusion (1 ml/kg, infusion for 30 min). In the activated protein C-treated groups, activated protein C (100 μg/kg, bolus) was administered 30 min prior to the administration of LPS and saline in the respective groups. All experimental methods were approved by the Institutional Animal Care and Use...
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RESULTS

Transcription Factor TCF11/MafG Binds to the iNOS Promoter Region—To explore the factors that might contribute to negative regulation of iNOS, we examined nuclear factor binding sites in the promoter. We identified the sequence 5′-33′TGCTGAAAG-TGAG-3′ as containing homologies to an NF-E2-like site, with TGCTG and CTCAC (reverse strand) being homologous to the T-mare and AP-1 half-sites, respectively, and interacting with nuclear factor TCF11/Maf heterodimers (18, 19). Using a DNA electrophoretic mobility shift assay, we assessed nuclear factor binding to this iNOS promoter sequence and observed a major band (Fig. 1A, lane 1) that could be competed with a cold probe for the iNOS binding site (lane 2). To determine whether this site interacted with the TCF11/Maf heterodimer, we performed competition with a TCF11/MafG binding sequence described previously as a transcriptional repressor from BK virus (14). As shown in Fig. 1, A and B, the iNOS binding site was completely competed with a probe for the TCF11/MafG binding site (lane 3) but not by a mutant site (lane 4). Furthermore, we demonstrated that the TCF11/MafG mutant probe could not compete with the TCF11/MafG wild type probe for TCF11/ MafG binding (lane 7).

As further confirmation, we utilized an anti-TCF11 antibody against the C-terminal leucine zipper region, i.e. the heterodimerization domain for TCF11/MafG (19), and we observed significant inhibition of DNA binding (Fig. 1C).

A ChiP assay was used to evaluate the association of TCF11 with the iNOS promoter in cells using both anti-TCF11 and anti-Maf antibodies. As shown in Fig. 2A, we were able to detect significant association of TCF11 with the iNOS binding site. As the data in Fig. 1C suggested that TCF11 was binding with its heterodimerization partner, MafG, we would expect to detect interaction with an anti-Maf antibody. As shown in Fig. 2B, this was observed. There was no difference in ChiP assays between no-antibody controls and nonbinding isotype antibody controls.
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FIGURE 2. Analysis of TCF11/MafG binding to the iNOS promoter by ChIP assay. A, determination of binding of TCF11 to the −344 to −309 region in the iNOS promoter using an antibody to TCF11. The values represent the percent of signal from the input DNA using quantitative real-time PCR. B, determination of binding of the heterodimerization partner to TCF11 to the −344 to −309 region in the iNOS promoter using an antibody to Maf. The data are the mean ± S.E. (n = three immunoprecipitation experiments).

FIGURE 3. Level of iNOS gene expression in cells transfected with decoy TCF11/MafG sites or mutated TCF11/MafG sites. Cells were transfected with 5 μg of control plasmid/10^6 cells, pTCF-BS containing multimers of the intact TCF11/MafG site, and pTCF-mBS containing multimers of the mutated binding site. After 96 h, cells were harvested, and iNOS and control actin mRNA levels were determined as described under “Experimental Procedures.” The inset shows ribonuclease protection assay of the level of iNOS mRNA in cells transfected with the control and pTCF-BS vectors. The data are the mean ± S.E. (n = six transfection experiments).

TCF11/MafG Is a Negative Regulator of iNOS Expression—To assess the functional effect of this binding site in iNOS, we examined the level of iNOS mRNA produced in cells following transfection of a decoy vector containing multimers of the TCF11/MafG binding sites using the method described previously (21). As shown in Fig. 3, the level of iNOS mRNA significantly increased in the presence of the transfected decoy vector but not in the presence of a decoy vector with the mutation in the TCF11/MafG binding site that could not compete at the iNOS site. As competition for the native binding sequence resulted in an increase in iNOS mRNA expression, the data suggest that TCF11/MafG binding is a negative regulator of iNOS.

TFG-β1 Induces TCF11/MafG Binding to the iNOS Promoter in a Smad-dependent Manner—The level of iNOS expression was determined in human smooth muscle cells following induction by a cytokine mixture as described under “Experimental Procedures.” The level of iNOS mRNA was significantly induced by the cytokine mixture treatment but blocked by the treatment of cells with TGF-β1 (data not shown). This inhibitory effect of TGF-β1 was concentration-dependent, resulting in inhibition of both mRNA (data not shown) and protein levels, consistent with the previous reports described above.

To determine whether the ability of TGF-β to inhibit iNOS expression might be related to the negative regulation through TCF11/MafG, we determined if TGF-β could increase binding of this factor to the iNOS promoter element. As shown in Fig. 4A, treatment of cells with TGF-β resulted in a significant increase in binding to the TCF11/MafG site, which could be competed with the cold binding site. Previous studies have shown that Smad6-short and Smad7 can agonize and antagonize TGF-β signaling in vascular cells, respectively (16). Overexpression of Smad6-short, which enhances TGF-β signaling, resulted in an increase in TCF11/MafG binding (Fig. 4B). In contrast, overexpression of Smad7, the negative regulator of TGF signaling, blocked the induction by Smad6-short. As expected, the overexpression of Smad7 alone, in the absence of induction, had no effect. Taken together, the above data suggest that TGF-β induces TCF11/MafG binding in a Smad-dependent manner, thereby suppressing iNOS expression.

 Knockdown of TCF11 Blocks the TGF-β-dependent Suppression of iNOS—To confirm the dependence of TCF11 in the negative regulation of iNOS by TGF-β, we utilized an siRNA to inhibit its expression and examined TGF-β-dependent iNOS regulation. As shown in Fig. 5, the level of iNOS induction, as measured by NO production, was increased by the cytokine mixture and, as expected, completely suppressed by TGF-β1. However, in cells pretreated with siRNA to TCF11 for 48 h to reduce TCF11 levels, we observed a complete block in ability of TGF-β to inhibit iNOS expression. These data strongly suggest that the negative regulation of iNOS by TGF-β is dependent on TCF11.

Dependence of TCF11/MafG Binding on Protein Kinase C Activation—We assessed the effect of TGF-β on both the TCF11 and MafG expression levels and observed no significant effect that would account for the increase in DNA binding activity shown in Fig. 4A. Previous studies have demonstrated that activation of the protein kinase C (PKC) pathway can negatively regulate iNOS gene expression (22, 23). Moreover, studies have shown that TGF-β can mediate its effects via PKC pathway activation (24–29). To determine whether PKC might play a role in our observations, we examined the effect of its activation and inhibition on the TCF11/MafG DNA binding. As shown in Fig. 6A, activation of PKC with phorbol 12-myristate 13-acetate (PMA) resulted in a 3-fold induction of TCF11/
MafG binding. Treatment of cells with staurosporine (ST) at concentrations known to inhibit PKC resulted in a significant reduction in the amount of basal TCF11/MafG binding (Fig. 6B). Moreover, the increase in TCF11/MafG binding following activation of the PKC pathway with PMA could be completely inhibited by treatment with ST (Fig. 6B). Moreover, we examined the effect of ST on TGF-β induction of TCF11/MafG binding, and as shown in Fig. 6A, we could completely block the increase in DNA binding. The effect of induction by Smad6-short was also inhibited by ST (data not shown). To obtain confirmation of the role of PKC, we utilized the specific inhibitor of PKC, calphostin C, which previously has been shown to block PKC activation induced by TGF-β (30, 31). As shown in Fig. 6C, treatment with calphostin C significantly reduced the effect of TGF-β. Therefore, it appears that TGF-β increases TCF11/MafG binding via a PKC-dependent pathway.

Counter-regulation of TCF11 and iNOS in Vivo—As induction of inflammatory response by LPS induces the expression of iNOS in vivo, we were interested in assessing the relationship between iNOS levels and TCF11 in tissues of animals treated with endotoxin. The rat iNOS promoter (GenBank™ gi 2588790) contains a perfect match to the TCF11 site at -408 from the TATA site (MatInspector scores: core, 1.0; and matrix, 1.0). As shown in Fig. 7, A and B, LPS treatment resulted in a significant suppression of the level of TCF11, both on the expected (100 kDa) gene product and splice variant (60 kDa). Coincident with the suppression of TCF11, we observed a significant increase in the levels of iNOS message as well as NOx (nitric oxide byproduct) (Fig. 7, C and D). Recent studies have demonstrated that the anti-thrombotic/anti-inflammatory factor activated protein C can suppress iNOS induction by LPS (32). As shown in Fig. 7, we observed a reversal of the suppression of TCF11 and a significant suppression of iNOS expression by activated protein C treatment. There was a highly significant relationship between the decrease in TCF11 and the increase in iNOS/NOx in these animal studies: TCF11 versus NOx, r =...
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Vascular injury plays a major role in the pathogenesis of multiple disorders. The response to initial injury triggers the activation of coagulation and inflammatory and repair processes aimed at maintaining vascular integrity. The production of TGF-β and iNOS expression both play critical roles in vascular function during inflammatory processes; however, an imbalance can lead to pathological consequences. As indicated above, the relative balance of iNOS and its control by TGF-β are critically important in the context of systemic inflammatory response and sepsis, and understanding the coordinate regulation may provide the basis for improved targets for developing therapeutic intervention. We have provided new mechanistic understanding for this key control point.

For over 1 decade, the role of TGF-β in the control of iNOS expression has been well described in the literature. Although Gupta and Kone (33) demonstrated the role of transcription factor USF binding to E-box elements on transrepression of basal expression and interleukin-1β induction, the factors that confer iNOS regulation by TGF-β have not been well understood. We have identified the presence of a negative regulatory element in the promoter of the iNOS gene that binds and is repressed by the TCF11/MafG heterodimer. TCF11 (NFE2L1, short isoform LCR-F1) is a ubiquitous transcription factor of the CNC-bZIP family (19, 34). It has been associated with regulation of fetal liver hematopoiesis and expression of the putative tumor suppressor gene CDC2L1 and the glutamate-cysteine ligase, and it may be involved in developmental gene expression (reviewed in Ref. 35). TCF11 forms heterodimers with the bZIP factor MafG, which has been shown in promoter assays to result in repression of promoter activity (19). Our data with iNOS are consistent with previous studies suggesting a negative regulatory role for the heterodimer.

Our data clearly demonstrate that the binding of the TCF11/MafG heterodimer is increased by treatment with TGF-β and suppressed by blocking TGF-β signaling via Smad7 overexpres-
sion. TGF-β family signaling is positively modulated by various
members of the Smad family of signal transduction proteins (revised in Ref. 36); however, Smad7 inhibits TGF-β signal transduction by preventing phosphorylation of receptor-activated Smad-short, thereby inhibiting signaling responses (37). Recent studies by Feinberg et al. (38) have shown the dependence of Smad3 on the ability of TGF-β to inhibit iNOS expression. These authors suggested that the effect was dependent on the CCAAT/enhancer-binding protein element −927 to −906, an element distal to the TCF11/MafG site. Further studies will be needed to determine whether there is cooperativity with these two elements in the control of iNOS.

Although there is little known regarding the post-translational modification of the TCF11/MafG complex for binding, our data suggest that phosphorylation may be required and can be mediated through activation of the PKC pathway. Furthermore, our data are consistent with reports that staurosporine can enhance NO production (39) and that PMA induction of PKC can inhibit NO production during the inflammatory response (22). Of interest, Geng et al. (22) demonstrated that PKC activation inhibited cytokine-induced NO synthesis in vascular smooth muscle cells and suggested that this effect was mediated by an unknown suppressor of iNOS expression. Our data would suggest that this hypothesized suppressor is TCF11/MafG.

Septic shock, characterized by hypotension, hypoperfusion, disseminated intravascular coagulation, and multiple organ dysfunction accounts for significant mortality in intensive care units (40). Multiple mechanisms, including production of large amounts of NO, have been attributed to promoting sepsis-in-
duced organ dysfunction (41). Recent data suggest that admin-
istration of activated protein C attenuates microcirculatory dysfunction (42) and organ injury in vivo (43), and recombiant human activated protein C, drotrecogin-α (activated), has been shown to reduce mortality in patients with severe sepsis at high risk of death (44). Recombinant human activated protein C has also been shown to prevent the hypotensive response in a study of human endotoxin challenge (20) and to inhibit iNOS expres-
sion in rat endotoxemia (32). The data presented here suggest that activated protein C functions to inhibit iNOS production by blocking the inhibition of TCF11, thereby allowing negative regulation of the iNOS promoter and offering a viable mecha-
nism for the suppression of shock.

The regulation of iNOS during systemic inflammatory response is likely complex, but our data provide a new understand-
ing of the important role of TGF-β in this process. The data also provide a newly defined role for the bZIP factor TCF11 in gene regulation and provide a potential new target for therapeutic intervention in treating vascular dysfunction.

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