Smad2 Mediates Transforming Growth Factor-β Induction of Endothelial Nitric Oxide Synthase Expression

Marta Saura, Carlos Zaragoza, Wangsen Cao, Clare Bao, Manuel Rodríguez-Puyol, Diego Rodríguez-Puyol, Charles J. Lowenstein

Abstract—Transforming growth factor-β (TGF-β) increases expression of endothelial nitric oxide synthase (eNOS), although the precise mechanism by which it does so is unclear. We report that Smad2, a transcription factor activated by TGF-β, mediates TGF-β induction of eNOS in endothelial cells. TGF-β induces Smad2 translocation from cytoplasm to nucleus, where it directly interacts with a specific region of the eNOS promoter. Overexpression of Smad2 increases basal levels of eNOS, and further increases TGF-β stimulation of eNOS expression. Ectopic expression of Smurf, an antagonist of Smad2, decreases Smad2 expression and blocks TGF-β induction of eNOS. Because Smad2 can interact with a variety of transcription factors, coactivators, and corepressors, Smad2 may thus act as an integrator of multiple signals in the regulation of eNOS expression. (Circ Res. 2002;91:806-813.)

Key Words: endothelial cell ■ hypoxia ■ atherosclerosis

Although endothelial nitric oxide synthase (eNOS or NOS3) was originally described as a constitutive NOS, subsequent work showed that eNOS expression is regulated by a variety of physiological and pathophysiological factors. Expression of eNOS is increased by exercise, shear stress, hypoxia, hyperthyroidism, cirrhosis, and endothelial cell proliferation. Various mediators can also increase eNOS expression, including estrogens, angiotensin II, glucose, oxidized linoleic acid, lysophosphatidylcholine, hydrogen peroxide, and transforming growth factor-β. Expression of eNOS is decreased by hypoxia, inflammation, and pulmonary hypertension. Mediators that decrease eNOS expression include oxidized LDL, lipopolysaccharide, and tumor necrosis factor-α.

Regulation of eNOS Expression

The level of eNOS expression is determined by multiple mechanisms, including posttranscriptional regulation and posttranslational regulation. Expression of eNOS is also regulated at the transcriptional level. The 5’-flanking region of the eNOS gene lacks a TATA box, but contains elements that mediate constitutive expression of genes in endothelial cells, including Sp1 sites and GATA. The 5’-flanking region also contains many putative sites for further transcriptional regulation of eNOS. However, only a few of these sites have been formally shown to regulate eNOS transcription, including PEA3 and AP-1 binding sites. Finally, a region of the eNOS promoter extending from −1269 and −935 mediates TGF-β induction of eNOS transcription.

TGF-β and Vascular Pathophysiology

TGF-β belongs to a superfamily of dimeric growth factors, including bone morphogenetic proteins and activins. TGF-β regulates diverse biological processes, including cell proliferation, differentiation, and migration. TGF-β also regulates cellular functions within the cardiovascular system, including angiogenesis, fibrosis, and production of extracellular matrix.

TGF-β Signal Transduction and Smads

After its secretion and activation, TGF-β forms a complex with type I and type II TGF-β receptors on target cells. Once bound to TGF-β, the type II TGF-β receptor phosphorylates the type I receptor, and the type I receptor in turn phosphorylates transcription factors known as Smads. Smad family members mediate TGF-β signal transduction, and include receptor Smads (Smad2 and Smad3) that associate with and are activated by TGF-β receptors, common Smads (Smad4) that interact with receptor Smads, and inhibitory Smads (Smad6 and Smad7) that block receptor Smad signaling. The TGF-β type I receptor phosphorylates Smad2, which then disassociates from the receptor, binds to Smad4, and enters the nucleus. This Smad complex then interacts...
with other transcription factors, coactivators, and corepressors to modulate gene transcription.\textsuperscript{52}

**TGF-β Regulates eNOS**

TGF-β regulates eNOS through pathways that are partially understood.\textsuperscript{45,53} Harrison and colleagues\textsuperscript{45} previously showed that TGF-β regulates eNOS expression in part by activating NF-1 to bind to the eNOS promoter region -1269 and -935. We hypothesized that the Smad signal transduction pathway also plays a role in mediating the effects of TGF-β on eNOS transcription. We now show that TGF-β induction of eNOS is mediated in part by Smad2 interacting directly with a specific region of the eNOS promoter.

**Materials and Methods**

**Materials**

Recombinant human TGF-β was purchased from R&D Systems, Oxon, UK. The human eNOS promoter was a generous gift of Dr Thomas Michel (Brigham and Women’s Hospital, Boston, Mass).\textsuperscript{39} The Smurf cDNA was a kind gift from Dr X.-H. Feng (Baylor College of Medicine, Houston, Tex).

**Cell Culture**

Bovine aortic endothelial cells (BAECs) and HUVECs were isolated using methods previously described.\textsuperscript{8} Human aortic endothelial cells (HAECs) were from Clonetics. Studies were performed on confluent monolayers at passages 2 to 7, made quiescent by serum deprivation (HAECs) were from Clonetics. Studies were performed on confluent monolayers at passages 2 to 7, made quiescent by serum deprivation.

**Immunofluorescence**

To analyze the cellular localization of Smad2, we cultured BAECs, fixed cells with 4% (v/v) paraformaldehyde, permeabilized them with Triton X-100, incubated them with antibody to Smad2 for 1 hour, washed them 3 times with phosphate buffered saline, and incubated them with antibody to mouse IgG conjugated to FITC (Jackson Immunoresearch). Cells were then photographed with a fluorescent microscope.

**Construction of Reporter Plasmids**

The 1.6-kb fragment of the eNOS 5'-flanking region was a generous gift from Dr Thomas Michel (Brigham and Women’s Hospital, Boston, Mass). An eNOS promoter reporter plasmid was constructed by inserting the 1.6-kb fragment of the human eNOS promoter upstream of the firefly luciferase gene in the plasmid pGL3, as described previously.\textsuperscript{9} This promoter was designated -1624 eNOS-Luc. Deletion mutants of the eNOS promoter were generated by polymerase chain reaction (PCR), as described previously.\textsuperscript{9}

**eNOS Promoter Activity**

Cell transfection was performed using methods modified from those previously reported.\textsuperscript{9} Photinus luciferase activity was determined using a luminometer and normalized against Renilla luciferase light units/mg protein. Renilla luciferase light units/mg protein.

**Electrophoretic Mobility Shift Assay**

Proteins were prepared from nuclear extracts as described previously.\textsuperscript{9} Nuclear extracts from HAECs were incubated with radiolabeled eNOS promoter fragment at 22°C for 30 minutes. The mixture was then electrophoresed through a nondenaturing polyacrylamide gel. The gel was dried and autoradiographed.

**Southwestern Blotting and Immunoblotting**

Immunoblotting was performed as described previously.\textsuperscript{9} For Southwestern blotting, nuclear extracts (50 μg of total protein) from BAECs were electrophoresed through a SDS-10% polyacrylamide gel, transferred to a membrane, denatured with 6 mol/L guanidine, washed, and renatured with decreasing concentrations of guanidine HCl. The membrane was then blocked with 5% dry milk and incubated with [\textsuperscript{32}P]end-labeled fragment of the eNOS promoter. The membrane was then washed and autoradiographed.

**Statistical Analysis**

Data for promoter activity were analyzed by analysis of variance and Newman-Keuls post hoc testing. Results are expressed as mean±SEM, with a value of \( P<0.05 \) considered statistically significant.

**Results**

**TGF-β Stimulates eNOS Expression**

To show that TGF-β increases eNOS expression in human endothelial cells, we treated HAECs with 2 ng/mL TGF-β, and analyzed total RNA by Northern blotting with a radiolabeled eNOS cDNA probe. Densitometry was used to assess relative changes in signal intensity. TGF-β increases steady-state eNOS mRNA levels after 4 to 8 hours of treatment (Figure 1A). TGF-β increases steady-state eNOS mRNA...
levels both in proliferating endothelial cells by about 1.7-fold (Figure 1A, top) and confluent endothelial cells by about 3.6-fold (Figure 1A, bottom). Simultaneous treatment of HAECs with TGF-β and cyclohexamide (CHX) show that the effect of TGF-β on eNOS mRNA does not require new protein synthesis (Figure 1B).

TGF-β also increases steady-state levels of eNOS protein. Lysates of HUVECs treated with TGF-β were analyzed by immunoblotting with antibody to eNOS. TGF-β increases steady-state eNOS protein levels after 8 hours of treatment and produces a maximum level of eNOS protein 16 hours after treatment, with an increase over baseline of approximately 15-fold (Figure 1C).

**TGF-β Transactivates the eNOS Promoter**

We next explored the ability of TGF-β signaling to transactivate the eNOS promoter. Endothelial cells were transfected with a reporter vector, consisting of the 1624 bp of the human eNOS promoter upstream of the AUG start site, driving expression of the Photinus luciferase gene. (Cells were also transfected with a vector constitutively expressing Renilla luciferase, as an internal control for transfection efficiency.) Transfected cells were then treated with vehicle alone or TGF-β, and the amount of luciferase activity was measured. (Promoter transactivation is calculated as Photinus luciferase/Renilla luciferase/mg cell protein.) The eNOS promoter is activated at basal levels in both BAECs and HUVECs treated with vehicle alone (Figure 2A). TGF-β increases the transactivation of the eNOS promoter in BAECs and HUVECs (Figure 2A).

To determine the region of the eNOS promoter that mediates activation by TGF-β signaling, we next transfected BAECs with various reporter constructs containing 5′ deletions in the eNOS promoter upstream of Photinus luciferase. Transfected cells were then treated with TGF-β or vehicle, and the amount of luciferase activity was measured. TGF-β transactivates the −1500 to 0 bp region of the eNOS promoter (Figure 2B). TGF-β also transactivates the −1300 to 0 and the −1000 to 0 region of the eNOS promoter. However, TGF-β cannot transactivate the −720 to 0 region of the eNOS promoter. Thus, TGF-β induction of the eNOS promoter is mediated by the region of the eNOS promoter extending from −1000 to −720 bp.

TGF-β increases eNOS expression in part by inducing NF-1 to interact with an NF-1 element in the eNOS promoter. The human eNOS promoter contains two NF-1 sites, at −1219 to −1191 and at −947 to −935. Deletion of the region from −1300 to −1000 containing the −1219 to −1191 NF-1 site in the human eNOS promoter causes a slight decrease in the responsiveness to TGF-β, and deletion of the region from −1000 to −720 containing the −947 to −935 NF-1 site abolishes the response to TGF-β (Figure 2B). However, site-directed mutagenesis of the −947 to −935 NF-1 element of the eNOS promoter has a modest effect on TGF-β transactivation of the eNOS promoter (Figure 2C).

**TGF-β Activates eNOS Promoter Binding Activity**

To further explore the role of the −1000 to −720 bp region of the eNOS promoter in mediating TGF-β regulation of the
eNOS promoter, we performed an electrophoretic mobility shift assay. HAECs were treated with TGF-β, and nuclear extracts were incubated with radiolabeled eNOS promoter probe (bp −1000 to −720). Cold eNOS competitor probe or cold NF-1 element probe or antibody to Smad2 or antibody to NF-1 was added to some mixtures (right lanes). Mixtures were fractionated by PAGE and autoradiographed. Results are representative of two separate experiments. A, Gel-shift assay. HAECs were treated with TGF-β, and nuclear extracts were incubated with radiolabeled eNOS promoter probe (bp −1000 to −720). Cold eNOS competitor probe or cold NF-1 element probe or antibody to Smad2 or antibody to NF-1 was added to some mixtures (right lanes). Mixtures were fractionated by PAGE and autoradiographed. Results are representative of two separate experiments. B, Southwestern assay shows proteins interact with eNOS promoter. BAECs were treated with TGF-β, and nuclear extracts were collected, fractionated by SDS-PAGE, and transferred to a membrane. Proteins on the membrane were renatured by sequential washes in buffer containing decreasing amounts of guanidine. The membrane was then incubated with a radiolabeled −1000 to −720 bp fragment of the eNOS promoter, washed, and autoradiographed. The eNOS promoter −1000 to −720 bp fragment binds to a polypeptide that appears in the nucleus 2 to 6 hours after TGF-β treatment (Figure 3B, left panel). This binding activity is competed by nonlabeled eNOS promoter fragment (Figure 3B, right panel). Thus TGF-β leads to the appearance in the nucleus of a polypeptide of approximately 55 kDa that interacts with a specific region of the eNOS promoter.

TGF-β Activates Smad2 in Endothelial Cells

Because Smad2 is a component of the TGF-β signaling cascade, we next tested the ability of TGF-β to activate Smad2 in endothelial cells. One hallmark of Smad2 activation is its translocation to the nucleus. We therefore treated BAECs with TGF-β, and then analyzed the localization of Smad2 in cells by immunofluorescence with an antibody to Smad2. BAECs were treated with 2 ng/mL TGF-β for 0 to 2 hours, and nuclear extracts were prepared and analyzed by immunoblotting with an antibody to Smad2. Results are representative of 2 separate experiments.

Figure 3. TGF-β induces eNOS promoter binding activity. A, Gel-shift assay. HAECs were treated with TGF-β, and nuclear extracts were incubated with radiolabeled eNOS promoter probe (bp −1000 to −720). Cold eNOS competitor probe or cold NF-1 element probe or antibody to Smad2 or antibody to NF-1 was added to some mixtures (right lanes). Mixtures were fractionated by PAGE and autoradiographed. Results are representative of two separate experiments. B, Southwestern assay shows proteins interact with eNOS promoter. BAECs were treated with TGF-β, and nuclear extracts were collected, fractionated by SDS-PAGE, and transferred to a membrane. Proteins on the membrane were renatured and hybridized with a radiolabeled fragment (−1000 to −720 bp) of the eNOS promoter probe (left). eNOS promoter fragment interacts with a 58-kDa polypeptide. Membranes containing fractionated nuclear extracts were also hybridized with both radiolabeled eNOS promoter probe and 50-fold excess nonlabeled eNOS promoter probe (right). Results are representative of 3 different experiments.

Figure 4. TGF-β activates nuclear translocation of Smad2. A, BAECs were treated with 2 ng/mL TGF-β for 0 to 2 hours, and the subcellular location of Smad2 was analyzed by immunofluorescence with an antibody to Smad2. BAECs were treated with 2 ng/mL TGF-β for 0 to 2 hours, and nuclear extracts were prepared and analyzed by immunoblotting with an antibody to Smad2. Results are representative of 2 separate experiments.
Smad2 Stimulates Basal eNOS Expression and TGF-β-Stimulated Expression

We next explored the effect of Smad2 on eNOS expression. We first examined Smad2 expression in control and transfected cells. Smad2 is absent from the nucleus of resting cells, and TGF-β increases nuclear levels of Smad2 (Figure 5A, left). Transfection of BAECs with a FLAG-Smad2 expression vector increases Smad2 expression. FLAG-Smad2 is present in the nucleus of resting cells transfected with FLAG-Smad2 expression vector, and TGF-β increases nuclear levels of FLAG-Smad2 (Figure 5A, right).

Overexpression of Smad2 increases eNOS promoter transactivation. BAECs were cotransfected with empty vector or with vector-expressing FLAG-Smad2, and also with the eNOS promoter-luciferase reporter vector. Overexpression of Smad2 leads to transactivation of the eNOS promoter even in the absence of TGF-β (Figure 5B, vehicle-treated Control versus vehicle-treated Smad2). Furthermore, TGF-β increases the absolute level of transactivation of the eNOS promoter when Smad2 is overexpressed (Figure 5B, TGF-β–treated Control versus TGF-β–treated Smads). However, TGF-β treatment increases eNOS promoter activity over the activity in vehicle-treated cells, by approximately the same percentage in control or Smad2-overexpressing cells.

Smad2 interacts with eNOS promoter. A, BAECs were transfected or not with Smad2 expression vector, and then treated or not with TGF-β. Nuclear extracts were isolated, immunodepleted with antibody to Smad2 or not, fractionated by SDS-PAGE, transferred to a membrane, and renatured. Membrane was hybridized with a radiolabeled eNOS promoter fragment (−1000 to −720) and autoradiographed. B, Duplicate samples were immunoblotted with antibody to Smad2. Results are representative of 3 different experiments.

Finally, we measured the effect of Smad2 overexpression on endogenous eNOS expression. Steady-state protein levels of eNOS are higher in BAECs transfected with a Smad2 expression vector than in nontransfected cells (Figure 5C). TGF-β treatment increases eNOS protein levels even higher in BAECs overexpressing Smad2 than in control transfected BAECs (Figure 5C). Smad2 overexpression had no effect on steady-state levels of β-tubulin, demonstrating the specificity of TGF-β and Smad2 for regulation of eNOS expression (Figure 5C). These data all suggest that Smad2 increases eNOS expression.

Smad2 Interacts With eNOS Promoter

Because Smad2 transactivates the eNOS promoter and increases eNOS expression, we hypothesized that Smad2 interacts directly with the eNOS promoter. We performed an electrophoretic mobility shift assay to test this hypothesis. The addition of antibody to Smad2 blocks the ability of nuclear extracts to interact with the eNOS promoter fragment (Figure 3A). In addition, antibody to NF-1 affects the interaction of nuclear proteins with the eNOS promoter fragment, decreasing the intensity of a lower but not an upper band (Figure 3A).

A Southwestern binding assay confirmed our hypothesis that Smad2 interacts with the eNOS promoter region from −1000 to −720. Nuclear extracts from BAECs were fractionated by SDS-PAGE, transferred to a membrane, renatured, and then hybridized with a radiolabeled eNOS promoter −1000 to −720 bp fragment. Control BAECs do not contain binding activity for the eNOS promoter fragment (Figure 6, lane 1). TGF-β treatment of BAECs induces a polypeptide in the nucleus with eNOS promoter binding activity (Figure 6, lane 2). However, immunodepletion of these extracts with antibody to Smad2 abolishes this activity (Figure 6, lane 3). Finally, overexpression of Smad2 in TGF-β-treated BAECs increases this eNOS promoter binding activity (Figure 6, lane 4). Thus, Smad2 (or a complex
containing Smad2) interacts directly with the eNOS promoter.

**Smurf Interference With Smad2 Blocks TGF-β Activation of eNOS Expression**

If Smad2 mediates TGF-β induction of eNOS expression, then interference with Smad2 should block the ability of TGF-β to activate the eNOS promoter. Smurf-2 modulates TGF-β signaling by activating the ubiquitination and destruction of Smad2. We therefore measured eNOS promoter activity in BAECs overexpressing Smurf-2. BAECs were cotransfected with the eNOS promoter reporter vector and a vector overexpressing Smurf-2. Smad2 is present in nuclear extracts of control transfected BAECs (Figure 7A). Overexpression of Smurf-2 drastically reduces Smad2 steady-state protein levels, but overexpression of a mutant Smurf-2 has no effect on Smad2 levels (Figure 7A).

We then measured the effect of Smurf-2 on eNOS promoter activity. TGF-β increases eNOS promoter transactivation in control cells, as before (Figure 7B, left). However, TGF-β has no effect on the eNOS promoter when Smurf-2 is overexpressed (Figure 7B, middle). TGF-β is able to transactivate the eNOS promoter when a mutant Smurf-2 is expressed (Figure 7B, right). These data support the hypothesis that Smad2 mediates TGF-β induction of eNOS expression in endothelial cells.

**Discussion**

The major finding of this study is that Smad2 mediates TGF-β regulation of eNOS expression. TGF-β activates Smad2 in endothelial cells, which translocates from cytoplasm to nucleus, where it interacts with the eNOS 5′-flanking region. Overexpression of Smad2 increases basal eNOS expression, and greatly increases TGF-β stimulation of eNOS expression. Finally, inhibition of Smad (by overexpression of Smurf) blocks TGF-β transactivation of the eNOS promoter.

TGF-β increases eNOS mRNA and protein levels within 4 to 8 hours of treatment (Figure 1). This rapid induction is consistent with the kinetics of phosphorylation and nuclear translocation of Smad2 after TGF-β binding to the signaling receptor complex (Figure 4). TGF-β induction of eNOS expression is independent of new protein synthesis (Figure 1B), which is also consistent with a signal transduction pathway involving a presynthesized messenger such as Smad2.

Our data show that Smad2 interacts with the eNOS promoter (Figures 3 and 6). However, there is no Smad consensus binding element within the eNOS promoter. Although consensus Smad binding sites are absent from the eNOS promoter, the DNA binding specificity of Smads can be regulated by various partners that interact with Smads, such as Sp-1 and forkhead activin signal transducer-1. Despite the lack of a Smad binding element in the eNOS 5′-flanking region, Smad2 may be directed to specific regions of the eNOS promoter by binding partners.

Prior work showed that NF-1 mediates part of the effect of TGF-β on eNOS expression. Harrison and colleagues demonstrated that a region of the bovine eNOS promoter between −1269 to −935 is responsive to TGF-β. This prior study also showed that NF-1 binds to an NF-1 element from −1034 to −1006 within the bovine eNOS promoter region, and that NF-1 mediates TGF-β regulation of eNOS. The human eNOS promoter contains two potential NF-1 binding sites: one NF-1 element from −1219 to −1191 (corresponding to the bovine eNOS promoter region, and a second NF-1 element from −947 to −935. Our data confirm the results of Harrison and colleagues that NF-1 plays a role in TGF-β regulation of eNOS. Our deletion mutation experiments show that the eNOS promoter region −1300 to −1000 containing the NF-1 site studied by Harrison and colleagues (bovine promoter −1034 to −1006, corresponding to human promoter −1219 to −1191) mediates some of the effects of TGF-β (Figure 2B). Our deletion experiments also show that the region containing the second NF-1 element, −1000 to −720, plays a more significant role in TGF-β regulation of eNOS (Figure 2B). Gel-shift experiments show that both NF-1 and Smad2 interact with this region of the eNOS promoter (Figure 3A). In addition, our point mutation experiments show that the second NF-1 site between −947 to −935 also mediates some of the effects of TGF-β (Figure 2C). Differences in the relative contributions of Smad2 and NF-1 to eNOS regulation in these two studies may be due in part to differences in bovine and human eNOS promoter sequences, and bovine and human endothelial cells. Thus, our data confirm the findings of Harrison and colleagues that NF-1 plays a role in TGF-β regulation of eNOS.

Identification of Smad2 as a regulator of eNOS expression suggests that pathways outside of the TGF-β signal transduc-
tion cascade can regulate eNOS as well. For example, shear stress activates Smad6, an inhibitory Smad, which can bind to Smad2 and prevent its transactivation of promoters.50 Shear stress can also activate MEKK-1, which in turn can activate Smad2 independent of TGF-β.59 Pathways that modulate the coactivator CREB binding protein (CBP) might also regulate eNOS transcription, because Smad2 interacts with CBP within endothelial cells.52,60 Thus, Smad2 can serve as an integrator of multiple pathways that regulate eNOS expression.

Our findings might explain one aspect of the antiatherogenic properties of TGF-β. Recent evidence suggests that TGF-β inhibits atherogenesis in animals and humans.81,62 These antiatherosclerotic effects of TGF-β may be mediated by multiple mechanisms. However, one possible pathway by which TGF-β reduces atherosclerosis is by activation of Smad2, followed by an increase in eNOS expression and NO synthesis. Increased NO, produced by higher levels of eNOS induced by Smad2, might in turn inhibit pathways leading to atherosclerosis.

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