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A far-upstream AP-1/Smad binding box regulates human NOX4 promoter activation by transforming growth factor- β

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Abstract

NADPH oxidase 4 (NOX4) is a member of the NADPH oxidase gene family that regulates cellular differentiation, innate immunity and tissue fibrosis. Transforming growth factor- β (TGF- β 1) is known to induce expression of NOX4 mRNA in mesenchymal cells. However, the mechanisms of transcriptional regulation of NOX4 are not well understood. In this study, we examined the transcriptional regulation of NOX4 in human lung fibroblasts by TGF- β 1. Five promoter-reporter constructs containing DNA fragments of 0.74 kb, 1.35 kb, 1.84 kb, 3.97 kb and 4.76 kb upstream from the transcriptional start site (TSS) of the human NOX4 gene were generated and their relative responsiveness to TGF- β 1 analyzed. TGF- β 1-induced NOX4 gene promoter activation requires a region between –3.97 kb and –4.76 kb. Bioinformatics analysis revealed a 15 bp AP-1/Smad binding element in this region. Mutation or deletion of either the AP-1 or the Smad element attenuated TGF- β 1 responsiveness of the –4.76 kb NOX4 promoter. Furthermore, insertion of this AP-1/Smad box conferred TGF- β 1 inducibility to the non-responsive –3.97 kb NOX4 promoter construct. Chromatin immunoprecipitation analysis indicated phospho-Smad3 and cJun associate with this element in a TGF- β 1-inducible manner. These results demonstrate that the AP-1/Smad box located between 3.97 kb and 4.76 kb upstream of the TSS site of the NOX4 promoter is essential for NOX4 gene transcription induced by TGF- β 1 in human lung fibroblasts. Our study provides insights into the molecular mechanisms of NOX4 gene expression, informing novel therapeutic approaches to interfere with upregulation of NOX4 in diseases characterized by activation of the TGF- β 1/NOX4 pathway.

Keywords

NADPH Oxidase-4; TGF- β 1; pulmonary fibrosis

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Introduction

The NADPH oxidase (NOX) gene family constitutes seven members that include NOX1-5 and the dual oxidases, DUOX1 and 2 (1). The primary function of NOX enzymes is to generate reactive oxygen species (ROS) which mediate host defense functions and/or signaling events that regulate a number of physiological responses in diverse cell types (2–4). Unlike other NOX family enzymes, NOX4 does not require the assembly of an active enzymatic complex by recruitment of cytoplasmic regulator proteins that are critical for the activation of other NOX family members, NOX1-3, or calcium-dependent activation (NOX5, DUOX1, 2). Thus, NOX4 is a unique member of this enzyme family in that it is regulated primarily at the level of gene expression (5,6), and has thus been termed a “constitutive” enzyme (7). Despite this distinctive property, mechanisms that regulate NOX4 gene expression are not well understood.

Since its cloning and initial characterization in the kidney (8,9), NOX4 has been shown to be ubiquitously expressed in a variety of cell types and tissues (10). In endothelial cells, NOX4 gene expression is upregulated by hypoxia (11) and hyperoxia (12), and is downregulated by cyclic strain (13) or shear stress (14,15). In smooth muscle cells, NOX4 has been reported to be induced by hypoxia (16–18) and interferon- γ (19). In fibroblasts and epithelial cells, the best characterized inducer of NOX4 is transforming growth factor- β 1 (TGF- β 1) (20–24). Depending on cell type, transcription factors that have been implicated in NOX4 gene regulation include Nrf2 (12), Nrf3 (25), Sp1/3 (26), NF- κ B (18,27), HIF-1 α (17,28), STAT1/3 (19), and E2F1 (29). Despite a central role for TGF- β 1 in NOX4 gene regulation in a variety of cell types, transcriptional mechanisms for its activation have not been well defined.

The molecular mechanism through which TGF- β regulates gene transcription involves the Smad pathway and its interaction with other pathways and transcription factors (30). Upon binding of TGF- β 1 to the Type II receptor on the plasma membrane, a cascade of phosphorylation events lead to the formation of a complex containing phosphorylated Smad2/Smad3 and Smad 4, which translocates to the nucleus to regulate target gene transcription (31).

In addition to the Smad pathway, other signaling pathways and transcription factors have also been implicated in TGF- β 1-mediated regulation of gene expression, some through physical interaction and functional cooperation with the Smad pathway (32,33). In this study, we explored the role of the canonical Smad pathway in TGF- β 1 regulation of NOX4 gene transcription. Our studies indicate a tandem AP-1/Smad box in the far upstream region, between –3.97 kb and the –4.76 kb, of the NOX4 promoter that confers responsiveness to TGF- β 1. This data provides insight into the molecular mechanisms of NOX4 gene expression and may lead to the development of more effective anti-oxidant and anti-fibrotic therapies for diseases characterized by TGF- β /NOX4 over-expression.

Materials and Methods

Cell culture

Human lung fibroblasts (IMR-90 cells) were purchased from American Type Culture Collection (Manassas, VA). Cells were routinely maintained in DMEM medium (Cellgro, Henden, VA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin in a humidified atmosphere with 5% CO₂ at 37°C. Cells were propagated using standard cell culture methods.

RNA isolation, cDNA synthesis and real-time PCR analyses

NOX4 mRNA was quantified by real-time PCR using ABI StepOnePlus real time PCR System (Applied Biosystems, Foster City, CA). Briefly, total RNA was isolated from IMR-90 cells using Amicon RNA isolation kit (Amicon-Millipore, Billerica, MA), and 0.8 µg of the total RNA was used for cDNA synthesis using SuperScript III cDNA Synthesis for qPCR Kit (Invitrogen, Carlsbad, CA). NOX4 TaqMan Gene Expression Assay (Applied Biosystem) was used for real-time PCR analysis of the target gene NOX4; β-actin was used as internal control. PCR reactions were performed for 40 cycles of 95°C for 15 sec and 60°C for 1 min. Relative NOX4 RNA levels were calculated using the Ct method with β-actin levels as reference for normalization.

NOX4 promoter-reporter construction

DNA fragments of various sizes (–741 bp, –1356 bp, –1848 bp, –3975 bp and –4760 bp) upstream of the putative transcription start site of the NOX4 gene were PCR-amplified using a BAC clone as template with corresponding primers (Table 1). The PCR fragments were purified, digested and cloned into the luciferase vector pGL3-Basic (Promega) with Mlu I and Xho I restriction sites. All constructs were confirmed by sequencing.

Site-directed mutagenesis

Mutations, deletions and insertions of the AP-1 and Smad binding sites were introduced into the wild type constructs (–4760 bp and –3975 bp constructs) using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) following manufacturer's instructions.

Transient transfection, TGF-β induction and luciferase assay

Transfections were conducted using Lipofectamine LTXplus reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Briefly, the day before transfection, IMR-90 cells were seeded into 24-well plate at a density of 4×10^4 cells/well in 0.5 ml of complete medium and cultured overnight. Lipofectamine LTXplusReagent-DNA complexes were prepared by incubating *Renilla* luciferase vector and respective NOX4 promoter-reporter constructs in 100 µl Opti-MEM medium; complexes were subsequently added to each corresponding well for transfection. After 24 h serum-starvation, transfected cells were treated with TGF-β1 (2 ng/ml) or vehicle (0.1% BSA; 4mM HCl) for 16 h; cells were then lysed, and luciferase activity measured using the Dual Luciferase Assay System (Promega, Fitchburg, WI) and the GENE 5 plate reader (BioTek Instruments, Winooski, VT),

according to manufacturer's instructions. The luciferase data of the NOX4 promoter-reporter constructs were calculated and normalized with *Renilla* luciferase activity. All transfections were carried out in triplicate.

Chromatin immunoprecipitation (ChIP) assays

IMR-90 cells maintained in complete growth medium were serum-starved for 24 h and then treated with TGF- β 1 (2ng/ml) or vehicle-only for 2h. ChIP was performed as previously described (34). GAPDH polyclonal antibody (Abcam #9485) was used as a negative control. Fold enrichment was determined using Ct of the experimental cJun (Abcam #31419) or phospho-Smad3 (Cell Signaling #9520) compared to pull-down with GAPDH control.

Statistical analyses

All the data were analyzed by unpaired Student's *t*-test. P values of less than 0.05 were considered statistically significant.

Results

TGF- β 1 induces NOX4 mRNA in a time-dependent manner without altering mRNA stability

We first determined the time-course of NOX4 mRNA induction in human lung fibroblasts (IMR-90). IMR-90 cells were treated with TGF- β 1 (2 ng/ml) or vehicle control for varying time intervals (Fig. 1A). NOX4 mRNA expression was induced almost 10-fold at 2 h and reached a maximum at 6 h, demonstrating a >20-fold induction over control cells. Extension of TGF- β 1 treatment up to 16 hr showed NOX4 mRNA expression at a level similar to 8 h treatment (data not shown). To determine if alterations in mRNA stability accounted, at least in part, for this induction by TGF- β 1, IMR-90 cells were treated with actinomycin-D prior to TGF- β 1 stimulation and NOX4 mRNA levels assessed at regular time-intervals. We observed no differences in the half-life of NOX4 mRNA under these conditions (Fig. 1B), suggesting that increases in mRNA stability is not the primary mechanism for TGF- β 1-induced NOX4 gene expression.

TGF- β 1-responsive elements of human NOX4 promoter are located within a region between -3976 bp and -4760 bp

NOX4 promoter-reporter gene constructs ranging from 4760 bp to 740 bp upstream of the transcription start site were cloned to analyze the regions of promoter responsiveness to TGF- β 1 (Fig. 2A). These constructs were transfected into IMR-90 cells and luciferase activities measured and normalized to the control *Renilla* luciferase activity. Each of the five NOX4 promoter-reporter constructs shown in Fig. 2A was tested and their responsiveness to TGF- β 1 examined. Of these, only the -4760 bp promoter fragment displayed TGF- β 1 responsive promoter activity (Fig. 3), while the other four constructs did not display any TGF- β 1 inducibility (data for the 1356 bp and 741 bp constructs are not shown). This suggested that cis-element(s) involved in TGF- β 1 inducibility resides in this 785 bp region (-3976 bp to -4760 bp) of the NOX4 gene promoter.

An AP-1/Smad binding box is responsible for TGF- β 1 inducibility of the human NOX4 promoter

A bioinformatics analysis of the 785 bp region responsive to TGF- β 1 revealed an AP-1/Smad binding box located at -4653 bp to -4667 bp of the NOX4 promoter (Fig. 2B). Of note, the AP-1 binding site and Smad binding sites are localized in tandem, separated by only one base pair. We then tested the requirement of this AP-1/Smad box in the responsiveness of the promoter to TGF- β 1 induction using site-directed mutagenesis. Mutations of AP-1 (2 bp) or Smad (3 bp) sites significantly reduced TGF- β 1-induced NOX4 promoter activity of the -4760 bp construct (Fig. 4A). Double mutations of AP-1 and Smad sites completely blocked TGF- β 1-induced NOX4 promoter activation (Fig. 4A). Furthermore, deletions of either the AP-1 or Smad site or both of the AP-1 and Smad sites resulted in a similar attenuation or blockade of TGF- β 1 inducibility of the NOX4 promoter activity (Fig. 4B).

We then tested whether insertion of these AP-1/Smad elements could confer TGF- β 1 inducibility to the non-responsive -3975 bp NOX4 promoter-reporter construct. The first element tested was a direct insertion (insertion 1) of the element; the second insertion (insertion 2) was identical to insertion 1 with the addition of flanking nucleotides. Both insertions conferred TGF- β 1 inducibility to the 3975 bp fragment (Fig. 5). Mutations of single AP-1 or Smad site or both of AP-1 and Smad sites abrogates the ability of insertion-1 to rescue TGF- β 1-induced NOX4 promoter activation (Fig. 5). Similar results were observed in insertion 2 with mutations of AP-1 and/or Smad sites (data not shown). Together, these results demonstrate that the AP-1 and Smad sites confer TGF- β 1 responsiveness to the NOX4 gene.

Smad3 and cJun associate with the AP-1/Smad box in vivo upon TGF- β 1 stimulation

Finally, we performed a ChIP assay to determine if Smad3 and AP-1 bind to the identified AP-1/Smad box in the NOX4 gene promoter. Stimulation with TGF- β 1 induced a 7-fold enrichment of cJun (an AP-1 family member) and a 40-fold enrichment of Smad3 binding to this promoter region (Fig. 6). This data further supports the functional activation a TGF- β 1 inducible AP-1/Smad binding element in the far upstream region of the human NOX4 promoter.

Discussion

In the present study, we demonstrate that TGF- β 1-responsiveness of the human NOX4 gene is located in the region between -3.97 kb and -4.76 kb upstream from the putative transcription start site (TSS) of the NOX4 promoter. Using bioinformatics analysis and site-directed mutagenesis approaches, we further identified the cis-acting elements, a 15bp AP-1/Smad binding box, responsible for TGF- β 1-induced upregulation of NOX4 promoter activity. Mutation or deletion of either the AP-1 or Smad cis-elements attenuates the activity of the NOX4 promoter in response to TGF- β 1, suggesting AP-1 and Smad in the regulation of TGF- β -mediated up-regulation of this promoter. Importantly, insertion of the AP-1/Smad box conferred TGF- β 1 inducibility to the non-responsive -3.97 kb NOX4 promoter construct. Chromatin immunoprecipitation (ChIP) studies demonstrate that the binding of

Smad3 and c-Jun to the AP-1/Smad box is significantly enriched in TGF- β 1-treated cells compared to that in untreated cells. Collectively, these results identify an AP-1/Smad box located between -3.97 kb and -4.76 kb from the TSS site of the NOX4 promoter that is responsible for the transcriptional activation of the NOX4 gene by TGF- β 1.

Our findings contrast with others which suggest that the TGF- β -inducible region is located within a -1848bp NOX4 promoter fragment (35). We did not observe any appreciable luciferase induction in our fibroblasts using our nearly equivalent -1848bp fragment. These authors observed a 2–3 fold increase in luciferase activity with TGF- β treatment of hepG2 cells (35), while we observed a much more robust induction (~15-fold) in human lung fibroblasts. It is possible that different cell lines use different Nox4 promoter regulatory sequences to induce Nox4 promoter activation in response to TGF- β 1.

NOX4 has been implicated in a number of physiological and pathological processes (1,4). TGF- β 1 is well recognized to induce NOX4 gene expression (20–24). Overexpression of TGF- β 1 and NOX4 is implicated in pathological tissue remodeling and fibrosis involving diverse organ systems, including the lung (23,36), kidney (37), heart (38,39), and liver (24,40). Additionally, the TGF- β 1/NOX4 axis may be critical in the stromal response that promotes carcinogenesis (41,42).

Despite a central role for TGF- β 1 and NOX4 in fibrosis and emerging roles in cancer biology, mechanisms regulating NOX4 induction by TGF- β 1 are not well understood. Among the NOX/DUOX homologs belonging to this gene family, NOX4 is unique in that it is primarily regulated at the level of gene transcription with the apparent dispensability of cytosolic factors and/or calcium to activate the enzyme (5–7). Thus, uncovering mechanisms that control NOX4 gene transcription are critical to understanding its regulation in health and disease. Although a number of transcription factors have been identified in regulating NOX4 gene transcription in response to various stimuli, this study is the first to implicate an AP-1/Smad binding box in TGF- β 1-induced NOX4 gene expression and provides insights into the molecular mechanisms of NOX4 gene expression. Novel therapeutic approaches to interfere with upregulation of NOX4 in diseases characterized by activation of the TGF- β 1/NOX4 pathway require further validation of a functional cooperativity between AP-1 and Smad in regulating NOX4 gene expression *in vivo*

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Highlights

- TGF- β regulates NOX4 gene expression, at least in part, at the transcriptional level
- TGF- β induces NOX4 promoter activity far upstream of its TSS (3975–4760bp)
- A 15bp AP-1/Smad binding box within this region mediates TGF- β -induced NOX4 promoter activation
- These results inform the design of therapeutics against NOX4-associated diseases

Figure 1. A, TGF- β treatment increases NOX4 mRNA level in human lung fibroblasts

Human lung fibroblasts (IMR-90) cells were serum-starved for 24 h and stimulated with TGF- β (2 ng/ml) or vehicle-only for control. The treated cells were collected at 2 h, 4 h, 6 h and 8 h; total RNA isolated and real time PCR performed for analysis of NOX4 expression. **B, NOX4 RNA stability study.** IMR-90 cells were treated with actinomycin-D prior to TGF- β 1 stimulation or vehicle-only treatment for control and NOX4 mRNA levels assessed at regular time-intervals. Data are expressed as mean $\pm$ SD (n=3 in each group).

Figure 2. Schematic representation of the human NOX4 promoter-reporter constructs and the TGF- β 1-responsive elements identified in this study

A, Map of the constructs. Various sizes of PCR fragments upstream from the putative transcription start site were cloned into pGL3-Basic luciferase vector and sequence-confirmed. **B**, TGF- β 1-responsive element identified in this study. The underlined sequences represent the binding sites of transcription factors AP-1 and Smad.

Figure 3. TGF- β induces luciferase activity of the -4760 bp NOX4 promoter-reporter construct

IMR-90 cells were co-transfected with the -4760 bp promoter construct and Renilla luciferase plasmids. After 24 h serum starvation, the transfected cells were stimulated with TGF- β (2 ng/ml) or vehicle only for 16 h. Cell lysates were collected, and luciferase activity was measured and normalized with the Renilla luciferase activity. Data are expressed as mean $\pm$ SD (n=3 in each group).

A.

B.

Figure 4. Mutation or deletion of AP-1 and Smad binding sites reduce TGF- β -induced luciferase activity of the -4760bp NOX4 promoter-reporter construct

IMR-90 cells were co-transfected with the wild type or mutant constructs (A) or deletion constructs (B) and Renilla luciferase vector. After 24 h serum starvation, the transfected cells were stimulated with TGF- β (2ng/ml) or vehicle for 16 h. Luciferase activity was measured and normalized with the Renilla luciferase activity. Data are expressed as mean $\pm$ SD (n=3 in each group for both **A** and **B**).

Figure 5. The AP-1-Smad box conferred TGF- β inducibility to the non-responsive –3975 bp NOX4 promoter-reporter construct Luciferase activity was measured in cells transfected with the TGF- β nonresponsive –3975 bp construct mutated to contain the wild-type AP-1/Smad element (insertion-1), or with the same element containing 3 bp of additional flanking sequences at both 5' and 3' ends (insertion-2), or insertion-1 with AP-1 mutation, Smad mutation or both AP-1 and Smad mutations. Data are expressed as mean $\pm$ SD (n=3 in each group).

Figure 6. Chromatin immunoprecipitation analysis of the AP-1/Smad element region

IMR-90 cells were treated with TGF- β for 2 hours and crosslinked chromatin was immunoprecipitated with either cJun antibody, a pSmad3 antibody, or a GAPDH antibody (negative control). Fold enrichment is relative to the GAPDH control immunoprecipitation. Bar graphs represent mean $\pm$ SD (n = 3 in each group).

Table 1

Primers used in this study

-741 bp construct	F: 5'-CCCACGGCTGTTCAGTGTGCAATAGGTACCTGGGTCAACTCCACACACAG-3' R: 5'-GGCTCGAGGCTGCCCCAGACGCCCCAGCGC-3'
-1356 bp construct	F: 5'-GGACGGCTCTGCCAATGAACTCTCTGCCCCC-3' R: 5'-GGCTCGAGGCTGCCCCAGACGCCCCAGCGC-3'
-1848 bp construct	F: 5'-GGACGGCTGGCTCAACGCCAACCCTCTGCCTC-3' R: 5'-GGCTCGAGGCTGCCCCAGACGCCCCAGCGC-3'
-3975 bp construct	F: 5' <u>gcgcACGCGTAGTGTCCTGGTAAAGAGAGAGGCGGTGATTGTGACACACACACAG</u> 3' R: 5' <u>gcgcCTCGAGGCTGCCCAAGACGCCCGGCTC</u>
-4760 bp construct	F: 5' GCG CAC GCG TAG GAG CTA ACC CAG CCA TGT GGG TGC ACG GTA GAC 3' R: 5' <u>gcgcCTCGAGGCTGCCCAAGACGCCCGGCTC</u> 3'
AP-1 site mutation	F: 5' GTAGAAAATGAGAAATGAGTTGTCTGGAACTTTGTC 3' R: 5' GACAAAGTTCAGACAGCAACTCATTTCTTCTAC 3'
Smad site mutation	F: 5' GAAAATGAGAAATGAGTCACTAIGTAGAACTTTGCCCCGAC 3' R: 5' GTCGGGGGACAAAGTTCTACATAGTGACTCATTTCTATTTC 3'
AP-1 site deletion	F: 5' TGTGTAGAAATGAGAACTGTCTGGAACTTTGTC R: 5' GACAAAGTTCAGACAGTTCATTTCTACACA 3'
Smad site deletion	F: 5' GAAAATGAGAAATGAGTCACTTTGCCCCGAC 3' R: 5' GTCGGGGGACAAAGTTGTGACTCATTTCTATTTC 3'
-3975 bp insertion-1	F: 5' GAAATGTCAGCAGCCACTGGAAATGAGTCACT <u>GTCTCTGGTCT</u> GTGGAAGAGACAAAGGAACAG 3' R: 5' CTGTTCTTGTCTCTTCCAGACCAAGACAGTCACTTCCAGTGGCTGTGACATTC 3'
-3975 bp insertion-2	F: 5' ATGTCAGCAGCCACTGGAAAGAAATGAGTCACT <u>GTCTCTGGAACT</u> TCTGGAAAGACAAAGGAAC 3' R: 5' GTTCTTGTCTCTTCCAGAGTTCCAGACAGTGACTCATTTCTCCAGTGGCTGTGACAT 3'
ChIP	F: 5' TGAATCAGATGATGGTCTACACTTG 3' R: 5' AGTGTCTCAAAGGGCTTAACATTCC 3'