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Original Contribution

Tumor necrosis factor α activates transcription of the NADPH oxidase organizer 1 (NOXO1) gene and upregulates superoxide production in colon epithelial cells

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ABSTRACT

NADPH oxidase 1 (Nox1) is a multicomponent enzyme consisting of $p22^{phox}$, Nox organizer 1 (NOXO1), Nox1 activator 1, and Rac1. Interleukin-1β, flagellin, interferon-γ, and tumor necrosis factor α (TNF-α) similarly induced Nox1 in a colon cancer cell line (T84), whereas only TNF-α fully induced NOXO1 and upregulated superoxide-producing activity by ninefold. This upregulation was canceled by knockdown of NOXO1 with small interfering RNAs. TNF-α rapidly phosphorylated p38 mitogen-activated protein kinase and c-Jun N-terminal kinase 1/2, followed by phosphorylation of c-Jun and c-Fos and appearance of an AP-1 binding activity within 30 min. We cloned the 5′ flank of the human NOXO1 gene (−3888 to +263 bp), and found that the region between −585 and −452 bp, which contains consensus elements of YY-1, AP-1, and Ets, and the GC-rich region encoding three putative binding sites for SP-1, was crucial for TNF-α-dependent promoter activity. Serial mutation analysis of the elements identified an AP-1 binding site (from −561 to −551 bp, AGTAAGTCATG) as a crucial element for TNF-α-stimulated transcription of the human NOXO1 gene, which was also confirmed by the AP-1 decoy experiments. Thus, TNF-α acts as a potent activator of Nox1-based oxidase in colon epithelial cells, suggesting a potential role of this oxidase in inflammation of the colon.

Introduction

Recently, several homologues of the catalytic, electron carrier component ($gp91^{phox}$) of phagocyte NADPH oxidase have been identified in diverse cell types of specific human tissues [1–3]. This NADPH oxidase (Nox)/dual oxidase (Duox) family includes seven homologues in human: Nox1, Nox2 ($gp91^{phox}$), Nox3, Nox4, Nox5, Duox1, and Duox2 [1–3]. Among these family members, Nox1 is expressed in abundance in the colon and at lower levels in uterus, prostate, and vascular smooth muscle. Although Nox1 was originally shown to have mitogenic property [4], reactive oxygen species (ROS) produced by this enzyme are now suggested to participate in the host defense [1–3,5,6] and to regulate physiological and pathological processes of vascular tissues [7,8].

Nox1 oxidase is a multicomponent enzyme similar to the phagocyte (Nox2-based) oxidase system. Nox2-based oxidase consists of a membrane-integrated flavocytochrome b_{558}, composed of Nox2 and $p22^{phox}$, and four cytosolic components ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac) that associate with the flavocytochrome to form an active enzyme [9]. Nox1 is also associated with the membrane-integrated protein $p22^{phox}$ to form a functional heterodimer [10–12] and requires at least two additional cofactors, Nox organizer 1 (NOXO1) and Nox activator 1 (NOXA1) [13–15]. Rac1 is also required for activation of the Nox1-based oxidase system [16–20].

To fully understand the role of ROS derived from Nox1-based oxidase, these partner proteins as well as Nox1 itself should be examined. In fact, interferon γ (IFN-γ) [21,22], flagellin [23], and 1α,25-dihydroxyvitamin D_3 [21] upregulate Nox1 expression in colon epithelial cell lines, such as T84 and Caco2 cells, although the increased Nox1 levels have not always been associated with upregulation of the $O_2^{\cdot-}$-producing capability of these cells. T84 cells constitutively express Nox1, $p22^{phox}$ $p67^{phox}$, NOXA1, and Rac1, whereas they express only a small amount of NOXO1. Overexpression of NOXO1 and stimulation by phorbol 12-myristate 13-acetate (PMA) were required to fully activate their $O_2^{\cdot-}$ production [23]. Primary cultures of guinea pig gastric mucosal cells also constitutively express Nox1, $p22^{phox}$, $p67^{phox}$, NOXA1, and Rac1. Treatment with *Helicobacter pylori* lipopolysaccharide not only increased Nox1 mRNA levels, but also stimulated expression of NOXO1 mRNA, leading to 10-fold

Abbreviations: Nox, NADPH oxidase; Duox, dual oxidase; ROS, reactive oxygen species; NOXO1, Nox organizer 1; NOXA1, Nox activator 1; IFN-γ, interferon γ; PMA, phorbol 12-myristate 13-acetate; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; siRNA, small interfering RNA; RT, reverse transcription.

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upregulation of $O_2^{\cdot-}$ production [17]. These results suggest that NOXO1 may be one of the late-limiting factors for $O_2^{\cdot-}$-generating capability of the Nox1-based oxidase in guinea pig gastric epithelium, human colon cancer cell lines, and possibly other types of cells. However, the mechanism for transcription of the NOXO1 gene has not yet been documented.

We show here that tumor necrosis factor α (TNF-α), a cytokine crucial for the pathogenesis of chronic inflammatory bowel diseases, acts as a potent transactivator of the human NOXO1 gene in T84 cells. We have cloned the 5′-flanking region up to −3888 bp of the human NOXO1 gene and suggest that an AP-1 binding site located between −561 and −551 bp plays a crucial role in the TNF-α-stimulated transcription of the human NOXO1 gene.

Materials and methods

Reagents

Human recombinant TNF-α and IFN-γ were purchased from R&D Systems (McKinley Place, NE, USA). Human recombinant interleukin (IL)-1β was obtained from Upstate Biotechnology (Lake Placid, NY, USA). A recombinant structural protein of flagella filament (rFliC) of *Salmonella enteritidis* was prepared as previously described [23]. Polyclonal antibodies for p44/p42 extracellular signal-regulated kinase (ERK) 1/2 and their phosphorylated forms (phosho-p44/p42 ERK1/2), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and phosphorylated JNK/SAPK (phospho-JNK/SAPK), p38 mitogen-activated protein kinase (MAPK) and phosphorylated p38 MAPK (phospho-p38), and phosphorylated c-Jun (phospho-c-Jun) were purchased from New England Biolabs (Beverly, MA, USA). Anti-c-Fos and anti-c-Jun antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An inhibitor of p38 MAPK (SB203580) and JNK inhibitor SP600125 were obtained from Sigma Chemical Company (St. Louis, MO, USA) and BioMol International (Philadelphia, PA, USA), respectively.

Real-time PCR and Northern hybridization

T84 cells were cultured in Dulbecco's modified Eagle's medium–Ham's F-12 (1:1) medium (Gibco, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Total RNA was extracted using an acid guanidium–thiocynate–phenol chloroform mixture [23]. cDNA was prepared from total RNA (0.5 μg) using oligo dT primers according to the instructions with the SuperScript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). mRNA levels of NOXO1 (ABI Part No. Hs00376039) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH: Hs99999905) were analyzed by quantitative real-time PCR using TaqMan gene expression assays and the ABI-PRISM 7500 sequence detection system. Each PCR was performed according to the protocol for TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA), and data were analyzed using SDS 2.2 software (Applied Biosystems). NOXO1 mRNA levels were normalized to the amount of GAPDH mRNA. NOXO1 mRNA levels were also measured by Northern hybridization using cDNA probes for NOXO1 and GAPDH, as previously described [17].

Immunoblot analysis

Whole-cell protein extracts were prepared from cultured cells, and NOXO1 levels were measured by immunoblot analysis using a rabbit polyclonal antibody against the 348–362 amino acid residues of human NOXO1, as described previously [22]. Hemagglutinin-tagged NOXO1 cDNA was recombined into pAdTrack-CMV vector, and the vector was transfected into T84 cells using the FuGENE transfection reagent (Roche Biochemical Laboratories, Burlington, NC, USA) [23]. NOXO1 levels in T84 cells transfected with the vector were assessed by immunoblotting using the anti-NOXO1 antibody [22]. We also prepared membrane fractions from T84 cells and measured the amount of Nox1 protein, as previously described [22,23]. To assay native and phosphorylated forms of MAP kinases and their target molecules (c-Jun and c-Fos), T84 cells were lysed in 50 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM NaF, 1 mM Na_3VO_4, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μM leupeptin. Cell lysates were centrifuged at 12,000 *g* for 10 min at 4 °C, and the supernatants (20 μg protein) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in an 8% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. After nonspecific binding sites were blocked with 4% purified milk casein, the filter was incubated for 1 h at room temperature with corresponding antibodies at a 1:1000 dilution. After being washed with phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20, bound antibodies were detected with an enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia, Piscataway, NJ, USA). After bound antibodies were removed, the membrane was reblotted with an anti-β-actin antibody, as previously described [24].

Measurement of $O_2^{\cdot-}$

The rate of PMA-stimulated $O_2^{\cdot-}$ release was spectrophotometrically measured by the superoxide dismutase-inhibitable reduction of cytochrome c and expressed as nanomoles per milligram of protein per hour as previously described [24].

RNA interference

The Sterth RNAi system (Invitrogen) was used to transiently knock down NOXO1. Small interfering RNA (siRNA) duplexes, which correspond to nucleotides 462–486 (NOXO1 siRNA-1) and 1001–1025 (NOXO1 siRNA-2) from the translation start site of human NOXO1 mRNA. These two target sites were selected to knock down NOXO1α, β, and γ. The Stealt RNAi Negative Control Medium GC Duplex (Invitrogen) was used as a negative control. T84 cells were plated at a concentration 5×10^5 cells/dish in 35-mm-diameter plastic culture dishes and transfected with Lipofectamine RNAiMAX (Invitrogen) at a final siRNA concentration of 10 nM, according to the reverse transfection method in the manufacturer's protocol. After incubation for 24 h, these cells were left untreated or treated with TNF-α (20 ng/ml) for 24 h. NOXO1 mRNA levels and amount of O_2– production by the cells were measured.

Cloning and generation of serially truncated or mutated segments of the human NOXO1 promoter

The 5′ flank of human NOXO1 gene was cloned by PCR amplification of a human genomic DNA library (Clontech, Palo Alto, CA, USA). A 3.8-kb DNA fragment of the proximal promoter region of the NOXO1 gene was amplified using the primer set 5′-*AGATCT*AGGAAGAGATGGCTCTTGGGAGCA-3′ (forward) and 5′-*AAGCTT*CATGGCTGTGGCTTCCAGG-3′ (reverse) (*Bgl*II and *Hin*dIII restriction sites, respectively, in italic). The amplified product was used as a template to generate the deletion construct consisting of the −1803 to +263 bp, −1181 to +263 bp, −585 to +263 bp, −452 to +263 bp, or -−198 to +263 bp sequence using the same reverse primer used to amplify the 3.8-kb fragment and one of the following forward primers:

5′-*AGATCT*GAGGAGGTGACCCTTTCTGAAGGAT-3′ for −1803 to +263 bp,
5′-*AGATCT*GGCAAGTGAGGAGCATACAGAT -3′ for −1181 to +263 bp,
5′-*AGATCT*AGCCTCTGCCCAGGCCATGTT -3′ for −585 to +263 bp,
5′-*AGATCT*GCCCTCTTTCTCCTACCCCAAGTCT -3′ for −452 to +263 bp,
5′-*AGATCT*TTGGGCGCACCCTGGCAGGGCAA -3′ for −198 to +263 bp.