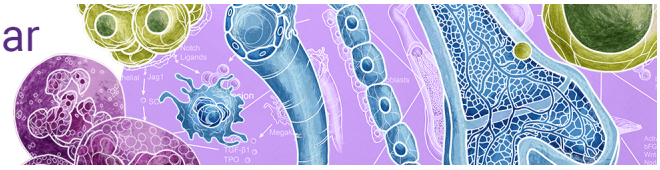

The Journal of Immunology

Cutting Edge: Direct Interaction of TLR4 with NAD(P)H Oxidase 4 Isozyme Is Essential for Lipopolysaccharide-Induced Production of Reactive Oxygen Species and Activation of NF- κB

Hye Sun Park, Hye Young Jung, Eun Young Park, Jaesang Kim, Won Jae Lee and Yun Soo Bae

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CUTTING EDGE

Cutting Edge: Direct Interaction of TLR4 with NAD(P)H Oxidase 4 Isozyme Is Essential for Lipopolysaccharide-Induced Production of Reactive Oxygen Species and Activation of NF-κB[1]

Hye Sun Park, Hye Young Jung, Eun Young Park, Jaesang Kim, Won Jae Lee, and Yun Soo Bae[2]

LPS, the primary constituent of the outer membrane of Gram-negative bacteria, is recognized by TLR4. Binding of TLR4 to LPS triggers various cell signaling pathways including NF-κB activation and reactive oxygen species (ROS) production. In this study, we present the data that LPS-induced ROS generation and NF-κB activation are mediated by a direct interaction of TLR4 with (NAD(P)H oxidase 4 (Nox) 4), a protein related to gp91^{phox} (Nox2) of phagocytic cells, in HEK293T cells. Yeast two hybrid and GST pull-down assays indicated that the COOH-terminal region of Nox4 interacted with the cytoplasmic tail of TLR4. Knockdown of Nox4 by transfection of small interference RNA specific to the Nox4 isozyme in HEK293T cells expressing TLR4 along with MD2 and CD14 resulted in inhibition of LPS-induced ROS generation and NF-κB activation. Taken together, these results indicate that direct interaction of TLR4 with Nox4 is involved in LPS-mediated ROS generation and NF-κB activation. The Journal of Immunology,* 2004, 173: 3589–3593.

In mammals, 10 members of the TLR family have been identified and shown to be involved in innate immunity and inflammation responses. TLRs recognize pathogen-associated molecular patterns and evoke various cell signaling pathways (1–3). LPS is an integral component of the outer membrane of Gram-negative bacteria. Upon binding of TLR4 to LPS, the cytoplasmic region of TLR4 recruits MyD88 linking TLR4 to IL-1R kinase (IRAK)[3] associated with TRAF6. Sequential activation of IRAK and TRAF6 results in NF-κB activation. Cells also operate the MyD88-independent pathway including protein kinase C, PI3K, and Akt for NF-κB activation in response to LPS stimulation (3–5).

NF-κB is considered to be a crucial regulator of the immune system. The activation of NF-κB leads to increased transcription of genes related to innate immunity and inflammation responses (6, 7). Several recent lines of evidence indicate that the activation of NF-κB can be controlled by reactive oxygen species (ROS) such as superoxide and H_2O_2 (6–9). ROS are produced in mammalian cells in response to the activation of various cell surface receptors and contribute to intracellular signaling processes which in turn regulate various biological activities including host defense and metabolic conversions (10). Receptor-mediated ROS production has been studied extensively in phagocytic cells (11). The enzyme NADPH oxidase in these cells is composed of at least six protein components which include two transmembrane flavocytochrome b components (gp91^{phox} and p22^{phox}) and four cytosolic components (p47^{phox}, p67^{phox}, p40^{phox}, and Rac protein) (11). Several homologues (Nox1, Nox3, Nox4, Nox5, Duox1, and Duox2) of gp91^{phox} (Nox2) have been recently identified in various nonphagocytic cells (12, 13). NAD(P)H oxidase (Nox) proteins contain binding sites for FAD, NADPH, and heme, and their NH_2-terminal portions contain a cluster of five hydrophobic segments that are predicted to form transmembrane α helices (12). The Nox protein transfers electrons from NADPH to O_2 to generate $O_2^{\cdot -}$. These superoxide free radicals are rapidly converted to H_2O_2 and O_2 in cells.

Each Nox homologue shows a distinct cellular and tissue expression pattern (12, 13). Nox1 is predominantly expressed in colon cells and involved in cell growth and angiogenesis. Nox3 and Nox5 are expressed in fetal tissues and spleen cells, respectively (13). Nox4 is expressed not only in kidney but also in placenta, pancreas, and endothelial cells (14, 15). Expression of Nox family members in different cells and tissues suggests that Nox isozymes may possess distinct functions in cells.

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[3] Abbreviations used in this paper: IRAK, IL-1R kinase; ROS, reactive oxygen species; Nox, NAD(P)H oxidase; βgal, β-galactosidase; DCF, 2′, 7′-dichlorofluorescein; DCF-DA, DCF diacetate; siRNA, small interference RNA; HA, hemagglutinin; DPI, diphenyliodonium; TIR, Toll-IL-1R.

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Several reports have shown that TLR4 is involved in LPS-induced ROS generation and that ROS are involved in TLR4-associated activation of NF-κB (8, 9). However, the mechanism by which LPS induces ROS generation is far from clear. In this study, we demonstrate that TLR4 associates with the COOH-terminal region of Nox4 in HEK293T cells. We also report a functional examination of Nox4 in LPS-mediated ROS generation and in NF-κB activation using a knockdown of Nox4 by transfection of pSUPER-Nox4 in HEK293T cells.

Materials and Methods

Cell culture and luciferase assay

HEK293T cells were maintained at 37°C under an atmosphere of 5% CO_2 in culture dishes containing DMEM (JBI, Daegu, Korea) supplemented with 10% FBS (JBI) and 1% antibiotic-antimycotic solution (Invitrogen Life Technologies, Carlsbad, CA). The human monocytic cell line U937 was cultured at a density of $1-8 \times 10^5$ cells/ml in RPMI 1640 medium supplemented with 10% FBS (JBI) and 1% antibiotic-antimycotic solution (Invitrogen Life Technologies).

HEK293T cells were grown until 50% confluent and were transfected transiently with an NF-κB-dependent luciferase reporter construct ($(\kappa B)_3$-IFN-luciferase) and constructs of pFLAG-CMV-TLR4, pFLAG-CMV-CD14, pFLAG-CMV-MD2, and pCMV-β galactosidase (βgal) plasmid using Effectine (Qiagen, Valencia, CA). U937 cells were transfected with NF-κB luciferase reporter construct and pCMV-βgal using Effectene (Qiagen). Empty vectors were used as controls. After 48 h, cells were stimulated with 1 μg/ml LPS. After 6 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to manufacturer's instructions and normalized relative to βgal activity. LPS (*Escherichia coli* 055:B5) was purchased from Sigma-Aldrich (St. Louis, MO).

Assay of intracellular H_2O_2 production

Intracellular production of H_2O_2 was assayed after stimulation of cells with LPS (1 μg/ml) for 30 min. Dishes of confluent cells were washed with HBSS and incubated for 5 min in the dark at 37°C with the same solution containing 5 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA; Molecular Probes, Eugene, OR). DCF-DA is oxidized by H_2O_2 to the highly fluorescent DCF. The cells were then examined with a laser-scanning confocal microscope (model LSM 510; Zeiss, Oberkochen, Germany) equipped with an argon laser tuned to an excitation wavelength of 488 nm, an LP505 emission filter (515–540 nm), and a Zeiss Axiovert ×100 objective lens. Images were digitized and stored at a resolution of 512 × 512 pixels. Five groups of cells were randomly selected from each sample, and the mean relative fluorescence intensity for each group of cells was measured with a Zeiss vision system (LSM510, version 2.3) and then averaged for all groups. All experiments were repeated at least five times.

Construction of small interference RNA (siRNA) for Nox4

A sequence of 19-nucleotide residues in length (GTCAACATCCAGCTG TACC) specific to the human Nox4 cDNA (nucleotide residues, 1474–1492) was selected for synthesis of a siRNA (16). pSUPER vector for siRNA was purchased from Oligoengine (Seattle, WA). The depletion of endogenous Nox4 by the siRNA was confirmed by RT-PCR.

Preparation of GST fusion proteins and GST "pull-down" assay

The plasmid pGEX4T1-TLR4-C, which encodes the GST fusion protein containing aa 676–835 of human TLR4, was introduced into *Escherichia coli* to prepare GST-TLR4 as previously described (17). For GST pull-down assays, the beads conjugated with GST-TLR4-C fusion protein were incubated with lysates of HEK293T cells transfected with pcDNA3.0-HA-Nox4-C (aa 248–575 of Nox4). The beads were then separated by centrifugation, washed three times, and subjected to immunoblot analysis with Abs to hemagglutinin (HA) (Roche, Basel, Switzerland).

Results

Interaction of TLR4 with Nox 4

To investigate the connection between LPS-dependent cell signaling and cellular redox balance, we measured the NF-κB activity and ROS generation in response to LPS in HEK293T cells. Because HEK293T cells, originated from kidney epithelial cells, do not express TLR4, the cells were transfected with TLR4 and accessory proteins MD2 and CD14. Parental HEK293T cells do not show LPS-mediated NF-κB activation, whereas stimulation of HEK293T cells expressing TLR4 along with MD2 and CD14 (TLR4/MD2/CD14) with LPS resulted in NF-κB activation. We investigated the effect of diphenyliodonium (DPI) as an NADPH oxidase inhibitor on LPS-mediated NF-κB activation. Pretreatment of HEK293T cells expressing TLR4/MD2/CD14 with DPI abolished LPS-mediated NF-κB activation (Fig. 1*A*). We next sought direct evidence of LPS-mediated ROS generation in HEK293T cells expressing TLR4/MD2/CD14. The generation of ROS in HEK293T cells was measured using an oxidation of DCF-DA to DCF with a laser-based confocal microscope. Exposure of HEK293T cells expressing TLR4/MD2/CD14 to LPS resulted in increased generation of ROS as revealed by an increase in DCF fluorescence (Fig. 1*B*). Treatment of HEK293T cells expressing TLR4/MD2/CD14 with DPI completely abolished LPS-mediated ROS generation. The result suggests that flavin-containing oxidase, NADPH oxidase, is involved in LPS-mediated ROS generation and NF-κB activation.

It is known that the COOH-terminal region of the Nox isozyme recruits several accessory proteins such as $p47^{phox}$, $p67^{phox}$, and *rac*. It is also known that the Toll-IL-1R (TIR)

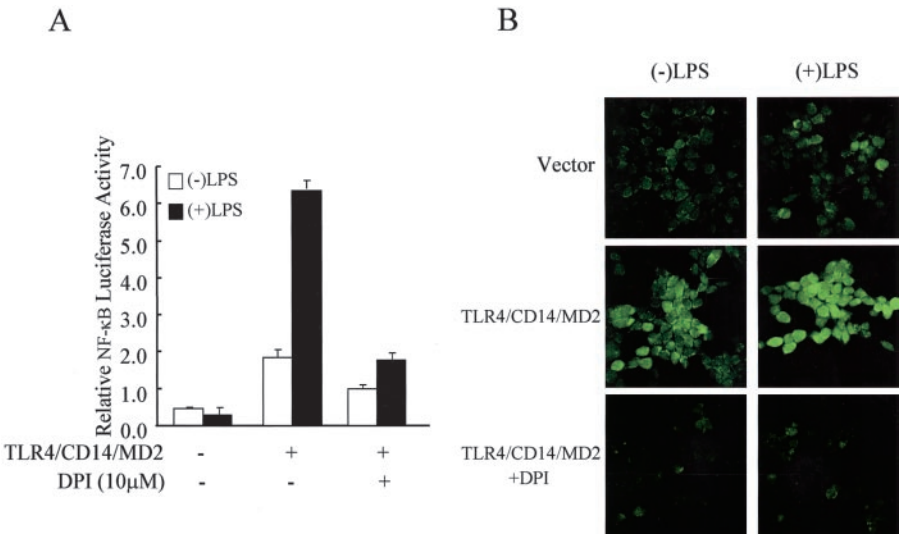

FIGURE 1. LPS-mediated H_2O_2 generation. *A*, HEK293T cells were cotransfected with an NF-κB-dependent luciferase reporter construct and expression vectors for TLR4, CD14, and MD2. Cells were pretreated with DPI (10 μM) for 30 min before treatment with LPS. Luciferase activity was measured (*A*), and the generation of H_2O_2 was then monitored by confocal microscopic analysis of DCF fluorescence (*B*). Data are means ± SE of values from three independent experiments.

domain in TLR4 interacts with various signaling molecules including MyD88 and IRAK. Thus, the COOH-terminal regions of both proteins might mediate the interaction between them directly or indirectly. To test the hypothesis that the Nox isozyme interacts with TLR4, we prepared GST fusion proteins containing the TIR domain (aa 676–835) and conjugated them to glutathione-Sepharose 4B beads. Because Nox4 is mainly expressed in HEK293T cells, we prepared a HA-tagged protein containing the COOH-terminal residues 248–575 of Nox4 (Nox4-C). Incubation of bead-conjugated GST-TIR with lysates of HEK293T cells expressing HA-tagged Nox4-C revealed a specific association of Nox4 with TIR of TLR4 (Fig. 2*A*). The COOH-terminal region of Nox4 and TIR of TLR4 could be interacting indirectly through other proteins in the complex. Therefore, we investigated whether Nox4 directly interacts with TLR4 using a yeast two-hybrid experiment (pB42AD-TIR with pLexA-Nox4-C). Yeast cells expressing pB42AD-TIR with pLexA-Nox4-C (amino acid residues, 354–579) revealed normal growth and produced blue colonies in the absence of leucine and in the presence of X-gal, indicating that the COOH-terminal region of Nox4 directly interacts with TIR of TLR4 (Fig. 2*B*).

Knockdown of Nox4 isozyme inhibited LPS-mediated NF-κB activation

To verify the role of Nox4 isozyme in LPS-mediated NF-κB activation and ROS generation in HEK293T cells, we subjected the cells to a transient transfection with pSUPER-Nox4 encoding a siRNA specific for the Nox4 gene. The cells transfected with the pSUPER-Nox4 siRNA vector exhibited a marked reduction in the abundance of the endogenous Nox4 mRNA level compared with that in cells transfected with the pSUPER vector (Fig. 3*C*). Stimulation of HEK293T cells cotransfected with TLR4/MD2/CD14 and pSUPER-Nox4 vector failed to generate ROS in response to LPS, whereas the cells expressing TLR4/MD2/CD14 transfected with pSUPER alone exhibited a marked increase of ROS in response to LPS, suggesting that Nox4 is essential for LPS-induced ROS production in HEK293 cells (Fig. 3*A*). We next asked the effect of knockdown of Nox4 isozyme on LPS-induced NF-κB activation in HEK293T cells expressing TLR4/MD2/CD14. Incubation of pSUPER-transfected HEK293T cells expressing TLR4/MD2/CD14 with LPS resulted in a marked increase in NF-κB activation. In contrast, transfection of pSUPER-Nox4 in HEK293T cells expressing TLR4/MD2/CD14 revealed a decrease in NF-κB activation by 90% in response to LPS (Fig. 3*B*). These result demonstrated that Nox4 isozyme is involved in LPS-mediated ROS generation and NF-κB activation.

Because HEK293T cells do not express TLR4 and their accessory proteins, we next confirmed the biological relevance of interaction of TLR4 with Nox4 isozyme in the U937 monocytic cell line. It is known that U937 cells express TLR4 and show NF-κB activation in response to LPS (18). Transfection

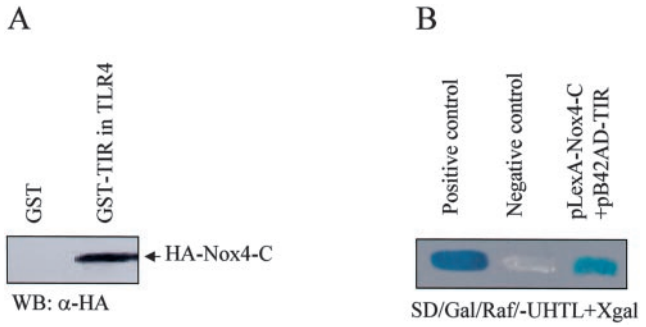

FIGURE 2. Interaction of TLR4 with Nox4. *A*, HEK293T cells were transfected with HA-tagged Nox4. Cell lysates were then prepared, incubated for 3 h with bead-conjugated GST or GST-TIR in TLR4 proteins, and then subjected to immunoblot analysis with Abs to HA. *B*, Direct interaction of TLR4-C with Nox4-C terminal was estimated by yeast two-hybrid assay (BD Clontech, Palo Alto, CA). The yeast EGY48 transformed with p80p-LacZ cells were cotransformed with a pair of pB42AD-TLR4-C (676–835) and pLexA-Nox4-C (aa 354–579). Following the selection for Trp^+ and His^+ phenotype, its Leu-dependent growth and βgal activity were tested in induction medium (SD/galactose/Raffinose). The positive control is the yeast cell line EGY48/p80p-LacZ cotransfected with pLexA53 and pB42ADT; the negative control is some yeast cell line cotransfected with pLexA/pB42AD.

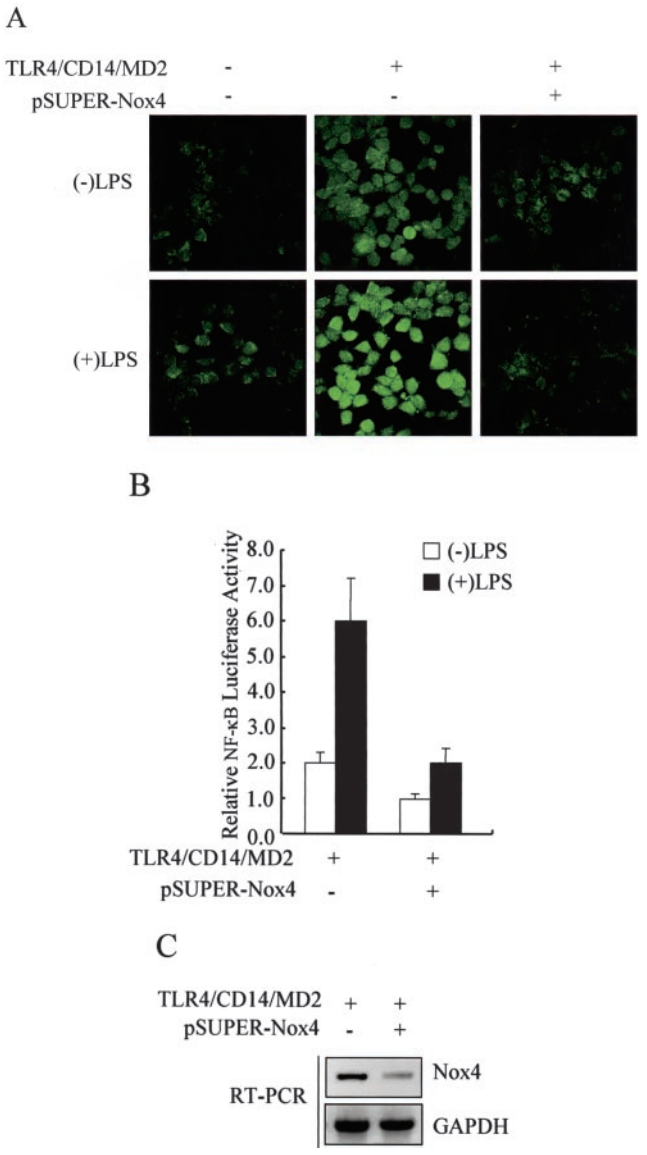

FIGURE 3. Knockdown effect of Nox4 on the generation of H_2O_2 and the activation of NF-κB in HEK293T cells. *A*, HEK293T cells were transfected with either pSUPER-Nox4 or empty vector together with expression vectors for TLR4, CD14, and MD2. After LPS (1 μg/ml) treatment, the generation of H_2O_2 was then monitored by confocal microscopic analysis of DCF fluorescence (*A*) and luciferase activity was measured (*B*). Data are means ± SE of values from three independent experiments. *C*, Total RNA was prepared and RT-PCR was performed. GAPDH serves as a loading control.

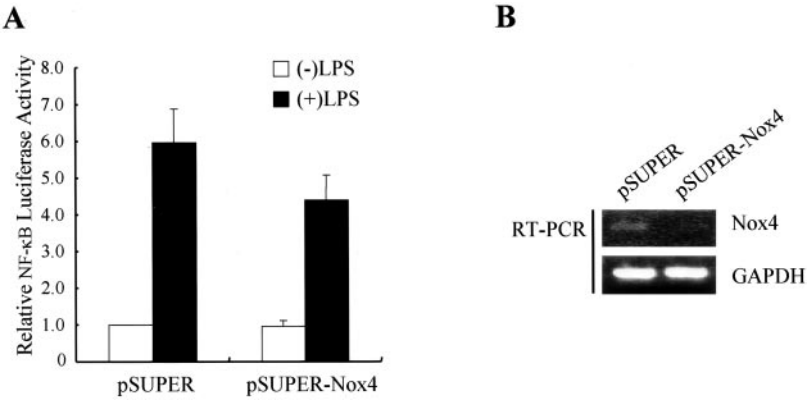

FIGURE 4. Knockdown effect of Nox4 on the activation of NF-κB in U937 cells. *A*, U937 cells were cotransfected transiently with an NF-κB-dependent luciferase reporter construct ($(\kappa B)_3$-IFN-luciferase) and pCMV-βgal plasmid with pSUPER or pSUPER-Nox4. After LPS (1 μg/ml) treatment, luciferase activity was measured. *B*, Total RNA was prepared and RT-PCR was performed. GAPDH serves as a loading control.

of pSUPER-Nox4 led to an effective knockdown (>95%) of Nox4 and resulted in 30% reduction of LPS-induced NF-κB activation compared with that by the empty pSUPER vector (Fig. 4). This is less than what was seen in HEK293T cells and suggests that other Nox isozyme might be involved in LPS-induced NF-κB activation in U937 cells. Real-time PCR experiments indicated that at least one other Nox isozyme, Nox2, is highly expressed in U937 cells (data not shown).

Discussion

Septic shocks induced by infection of Gram-negative bacteria kill over 50,000 people every year in the United States alone (19). LPS, a major component of outer membrane of Gram-negative bacteria, is the key molecule for triggering innate immunity and inflammation responses during sepsis. TLR4 recognizes LPS of Gram-negative bacteria and recruits MyD88 to mediate NF-κB activation (1–3). It is well established that NF-κB plays an important role in immune responses and inflammation processes (6–7). Various agonists stimulate the phosphorylation and ubiquitination of IκB and lead to the activation of NF-κB. Several lines of evidence indicate that NF-κB is also activated by the redox status in cells (8, 9). Exogenous addition of H_2O_2 stimulates NF-κB activation and scavenging of H_2O_2 by the addition of reducing agents abolishes activation of NF-κB. However, the molecular mechanism by which ROS activate NF-κB is still unclear.

Recently, various reports have suggested that receptor-mediated ROS generation is coupled with Nox isozymes (Nox1, Nox3, Nox4, and Nox5), novel homologues of $p91^{phox}$ of NADPH oxidase in phagocytic cells (11, 12). Nox1 mediates cell growth and angiogenesis (20, 21). Overexpression of Nox1 in quiescent cells induced cellular transformation. Previously, we reported that a sequential activation of PI3K, βPix, Rac1, and Nox1 is essential for the growth factor-induced production of H_2O_2 (17, 22). Because Nox4 expression is induced by hypoxia condition in kidney tubular epithelium, Nox4 was proposed to participate in oxygen sensing by cells (23). In addition, several lines of evidence indicated that Nox4 plays an important role in the regulation of endothelial cell growth and modulates insulin signaling in adipocytes (15, 24). However, how Nox isozymes mechanistically are linked to signaling pathways controlling these cellular behaviors is far from clear.

The TIR domain of TLR4 serves as the homodimerization domain and as the binding site for other cytosolic adaptor proteins containing the TIR domain such as MyD88, Mal, TIR domain-containing adapter inducing IFN-β, TIR domain containing adapter inducing IFN-β-related adapter molecule, and ST2 (1–3, 25, 26). These proteins are involved in TLR-mediated regulation of NF-κB activity and immune responses. Although Nox4 does not contain the TIR domain, the protein is shown here to interact with the COOH-terminal region of TLR4 (Fig. 2). Moreover, a knockdown of Nox4 by transfection of siRNA specific for Nox4 abolished LPS-mediated ROS generation and NF-κB activation (Fig. 3). These results show that interaction of TLR4 with Nox4 isozymes contributes to the production of ROS and NF-κB activation in response to LPS in HEK293T cells. To generalize the interaction of TLR4 with Nox4 in LPS signaling, we also examined the biological relevance of interaction between TLR4 and Nox4 isozyme in the U937 monocytic cell line. We saw an effective knockdown of Nox4 and a decreased level of NF-κB activation by LPS in these cells as well. The effect was not as dramatic as in HEK293T cells, but this is likely due to the expression of other Nox isozymes that compensate for the loss of Nox4. Consistently, we detected a high level of Nox2 expression by real-time PCR. In other words, pathways other than those involving Nox4 also appear to be involved in TLR4 signaling, and as far as U937 cells are concerned, Nox4 does not appear to be the dominant player. Nevertheless, the consistent effect we see with Nox4 siRNA provides clear evidence that Nox4 is a biologically relevant regulator of TLR4 signaling.

This is the first report showing a direct interaction between Nox and the receptor protein, TLR4. It is well established that NADPH oxidase activity is regulated through phosphorylation in phagocytic cells (11, 12). Phosphorylation of $p47^{phox}$ stimulates the interaction with $p67^{phox}$, and the resulting complex moves to $gp91^{phox}$ (Nox2) located in the plasma membrane to assemble the active form of NADPH oxidase. It has been reported that p41 (NoxO1) and p51 (NoxA1), homologues of $p47^{phox}$ and $p67^{phox}$ (12, 27), respectively, are required for Nox activation in nonphagocytic cells. Also, Kawahara et al. (28) reported that recombinant flagellin from *Salmonella enteritidis* stimulates ROS generation through TLR5 in T84 colon cancer cells and that cotransfection of NoxO1 and NoxA1 in the cells significantly enhanced ROS generation and IL-8 production. We also investigated the effect of NoxO1 and NoxA1 on LPS-mediated NF-κB activation in HEK293T cells. NoxO1 and NoxA1 are hardly detected in HEK293T cells. Transfection of NoxO1 and NoxA1 in HEK293T cells expressing TLR4/MD2/CD14 resulted in no enhanced activation of NF-κB

compared with the parental cells (data not shown). The result suggested that the activation of Nox4 by TLR4 is not sufficient for the supporting activity of NoxO1 and NoxA1. It is likely that the molecular mechanism of ROS generation through LPS-TLR4 in HEK293T cells is different from that through flagellin-TLR5 in T84 cells. In this report, we demonstrate that a direct interaction of TLR4 with Nox4 is essential for LPS-mediated ROS generation and NF-κB activation in HEK293T cells. How ROS signals emanating from Nox4 and TLR4 are integrated into a physiological signal that leads to the innate immunity and inflammation responses remains to be elucidated.

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