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Original Research Communication

LPS Induces Hypoxia-Inducible Factor 1 Activation in Macrophage-Differentiated Cells in a Reactive Oxygen Species–Dependent Manner

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ABSTRACT

A prominent feature of various inflamed and diseased tissue is the presence of low oxygen tension (hypoxia). Effector cells of the innate immune system must maintain their viability and physiologic functions in a hypoxic microenvironment. Monocytes circulating in the bloodstream differentiate into macrophages. During this process, cells acquire the ability to exert effects at hypoxic sites of inflammation. The transcription factor hypoxia-inducible factor 1 (HIF-1) mediates adaptive responses to reduced oxygen availability. In this study, we demonstrated that lipopolysaccharide (LPS) induces HIF-1 activation by enhancing both HIF-1α protein expression through a translation-dependent pathway and HIF-1α transcriptional activity in THP-1 human myeloid cells that have undergone macrophage differentiation but not in undifferentiated monocytic THP-1 cells. LPS-induced HIF-1 activation was blocked by treatment with antioxidant (*N*-acetylcysteine or thioredoxin-1), NADPH oxidase inhibitor (diphenyleneiodonium), indicating that reactive oxygen species generated in response to LPS are essential in this process. LPS-mediated activation of HIF-1 was independent of NF-κB activity. LPS-induced ROS generation and HIF-1 activation required the expression of Toll-like receptor 4 or myeloid differentiation factor (MyD) 88, thus providing a molecular basis for the selective activation of HIF-1 in differentiated THP-1 cells. *Antioxid. Redox Signal.* 10, 983–995.

INTRODUCTION

Differentiated macrophages act at diverse sites of inflammation, which share in common a reduction in oxygen (O_2) tension compared with normal tissue (4, 5, 34, 35, 43). Thus, effector cells of the innate immune system must maintain their viability and physiologic functions in a hypoxic microenvironment (35, 43). Hypoxic conditions have been shown to affect profoundly a broad range of myeloid cell properties *in vitro*, including expression of chemokine receptors and other cell-surface proteins, cytokine secretion, adhesion, migration, phagocytosis, and cell survival. The dependency of myeloid cells on glycolysis suggests that they are highly adapted to a hypoxic environment (30).

Hypoxia induces gene expression, leading to increased synthesis of proteins, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), glycolytic enzymes, and inflammatory mediators, which are important in the cellular and tissue adaptation to low-oxygen conditions (57). Hypoxia-inducible factor 1 (HIF-1) is a master regulator of oxygen homeostasis (58). HIF-1 is a heterodimer composed of constitutively expressed HIF-1β and inducibly expressed HIF-1α

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subunits, which bind to specific DNA sequences known as hypoxia response elements (HREs). The biologic activity of HIF-1 is determined by regulation of the synthesis, degradation, and transactivation function of the HIF-1α subunit (66).

The mechanisms regulating HIF-1α protein levels and transcriptional activity have been extensively analyzed. HIF-1α is subjected to O_2-dependent hydroxylation, which is required for binding of the von Hippel–Lindau tumor-suppressor protein (VHL), the recognition component of a ubiquitin-protein ligase that targets HIF-1α for rapid proteasomal degradation in nonhypoxic cells (21, 22). Under hypoxic conditions, the hydroxylation of proline-402 and proline-564 of HIF-1α is inhibited because of substrate (O_2) limitation, resulting in HIF-1α protein stabilization (9, 21, 22). The transcriptional activity of HIF-1α is also negatively regulated under normoxic conditions by hydroxylation of asparagine-803, which prevents interaction of HIF-1α with the coactivators p300 and CBP (32, 39). The asparaginyl and prolyl hydroxylases use O_2 and α-ketoglutarate as substrates (19, 24, 56). The iron chelator desferrioxamine (DFX) induces HIF-1α stabilization and transactivation under normoxic conditions, presumably by inhibiting the prolyl hydroxylase domain–containing proteins (PHDs) and asparaginyl hydroxylase (factor inhibiting HIF-1 [FIH-1]), which contain Fe^{2+} at their catalytic sites. In contrast, HIF-1β is constitutively expressed in most cell types.

Many physiologic stimuli can induce HIF-1α protein accumulation and modulate hypoxia-induced gene expression under nonhypoxic conditions (2, 12, 15, 25, 46, 54, 63, 64, 70). Lipopolysaccharide (LPS) is a potent inflammatory factor that has been implicated in the pathogenesis of septic shock (68). LPS induces production of proinflammatory cytokines by macrophages through the NF-κB pathway (33). In macrophages, LPS induces HIF-1α protein accumulation and HIF-1 transcriptional activity under nonhypoxic conditions (2). Conditional gene targeting in the myeloid cell lineage has demonstrated that HIF-1α plays an essential role in the inflammatory response (7). However, the mechanisms of LPS-induced HIF-1α protein expression have not been clearly defined, especially with regard to monocyte–macrophage differentiation.

In this study, we demonstrated that LPS increases HIF-1α protein expression in a dose- and time-dependent manner in macrophages that have differentiated from the monocytic cell line THP-1 but not in undifferentiated THP-1 cells. We also demonstrate that the antioxidant *N*-acetylcysteine (NAC) significantly suppresses LPS-induced HIF-1α accumulation, whereas the NF-κB inhibitors BAY11-7082 and resveratrol do not affect HIF-1α accumulation induced by LPS. Taken together, these results suggest that HIF-1 activation may participate in the acquisition of responsiveness to LPS during differentiation from monocyte to macrophage and that reactive oxygen species (ROS) generation, but not NF-κB activity, is required for HIF-1 activation induced by LPS.

MATERIALS AND METHODS

Cell culture and reagents

THP-1 human myeloid leukemia cells (a gift from Dr. Kume at Kyoto University) were maintained in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, and 100 g/ml streptomycin (60). To promote macrophage differentiation of THP-1 cells, cells were exposed to phorbol-12-myristate-13-acetate (PMA) at a final concentration of 50 n*M* for 12 h. PMA, lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, the iron chelator desferrioxamine (DFX), and the antioxidant NAC were obtained from Sigma (St. Louis, MO). Tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) were from Roche Applied Science (Tokyo, Japan). The protein synthesis inhibitor cycloheximide (CHX) and the NF-κB inhibitors (E)3-[(4-methylphenyl)sulfonyl]-2-propenenitrile (BAY11-7082) and *trans*-3,4′,5-trihydroxystilbene (resveratrol), LY294002, GF209203X, rapamycin, genistein, and PD98059 were from Calbiochem (San Diego, CA). The NOS inhibitor *N*ω-nitro-L-arginine methyl ester (L-NAME) was from Dojindo (Kumamoto, Japan). Recombinant human thioredoxin-1 (TRX-1) was prepared by following a protocol described previously (18, 55). In our pilot studies, no significant cell death was observed at a variety of time points and doses using trypan blue exclusion dye assay.

Hypoxic treatment

Tissue-culture dishes were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA), which was flushed with 1% O_2/5% CO_2/94% N_2, sealed, and placed at 37°C (20, 25).

Immunoblot assays

Whole-cell lysates were prepared by using ice-cold lysis buffer [0.1% SDS, 1% NP40, 5 m*M* EDTA, 150 m*M* NaCl, 50 m*M* Tris-Cl (pH 8.0)] containing 2 m*M* dithiothreitol, 1 m*M* $NaVO_3$, and Complete protease inhibitor (Roche Applied Science) (1, 17). Samples were centrifuged at 10,000 *g* to pellet cell debris. For HIF-1α and HIF-1β, 100-μg aliquots were fractionated by 7.5% SDS-polyacrylamide gel electrophoresis and subjected to immunoblot assay using mouse monoclonal antibodies against HIF-1α (BD Biosciences, San Jose, CA), or HIF-

TABLE 1. PRIMER SEQUENCES USED FOR RT-PCR

Primers	*Sequence*
5′ CD14	5′-CCATGGAGCGCGCGTCCTGC-3′
3′ CD14	5′-GTCTTGGATCTTAGGCAAAGC-3′
5′ TLR2	5′-GACTTCATTCCTGGCAAGT-3′
3′ TLR2	5′-CAGGTAGGTCTTGGTGTTC-3′
5′ TLR4	5′-CACCTGATGCTTCTTGCTG-3′
3′ TLR4	5′-TGCTCAGAAACTGCCAGGT-3′
5′ VEGF	5′-CCATGAACTTTCTGCTGTCTT-3′
3′ VEGF	5′-ATCGCATCAGGGGCACACAG-3′
5′ ENO1	5′-AGTCCCGGCGTTCAATGTCAT-3′
3′ ENO1	5′-GCAAACCCGCCTTCATCCC-3′
5′ GLUT1	5′-GGGCATGTGCTTCCAGTATGT-3′
3′ GLUT1	5′-ACGAGGAGCACCGTGAAGAT-3′
5′ HIF-1α	5′-GAAAGCGCAAGTCCTCAAA-3′
3′ HIF-1α	5′-CTATATGGTGATGATGTGGCACTA-3′
5′ 18S	5′-ATCCTGCCAGTAGCATATGC-3′
3′ 18S	5′-ACCCGGGTTGGTTTTGATCTG-3′

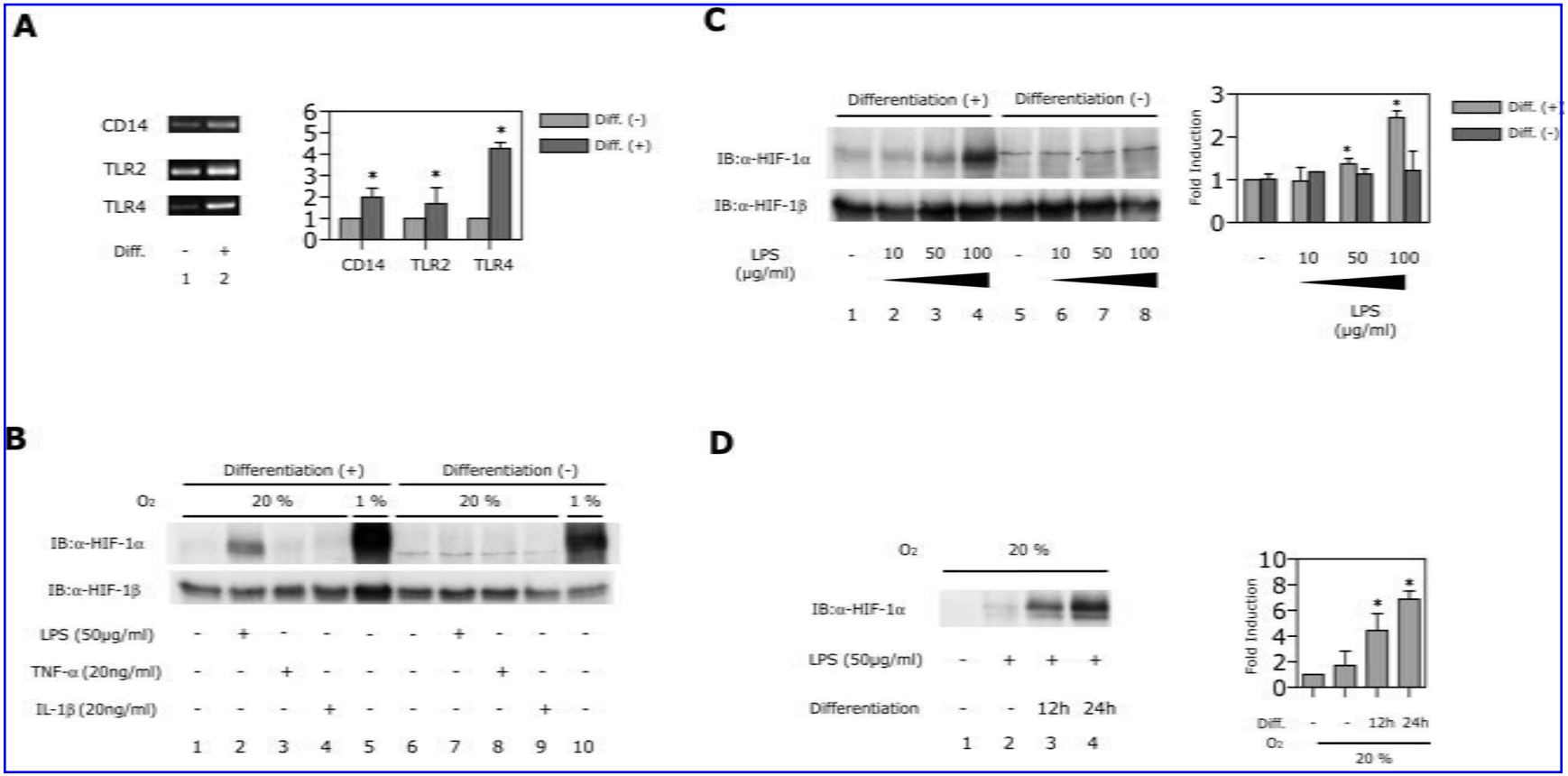

FIG. 1. Effect of LPS on HIF-1 protein levels in differentiated and undifferentiated THP-1 cells. THP-1 cells were exposed to 50 n*M* PMA for 12 h and harvested for assays. (**A**) Expression of CD14, TLR-2, and TLR-4 was analyzed by RT-PCR. (**B**) Differentiated and undifferentiated THP-1 cells were exposed to 50 μg/ml LPS (lanes 2 and 7), 20 ng/ml TNF-α (lane 3 and 8), 20 ng/ml IL-1β (lanes 4 and 9), or hypoxia (lanes 5 and 10) for 8 h and harvested for immunoblot assay for HIF-1α (*upper panel*) and HIF-1β (*lower panel*) protein expression. (**C**) Differentiated (lanes 1–4) and undifferentiated (lanes 5–8) THP-1 cells were exposed to the indicated dose of LPS for 8 h and harvested for immunoblot assay for HIF-1α (*upper panel*) and HIF-1β (*lower panel*) protein expression. (**D**) THP-1 cells were exposed to 50 n*M* PMA for 12 h (lane 3) and 24 h (lane 4) and then treated with LPS under 20% O_2 conditions or exposed to 1% O_2 conditions for 8 h continuously and harvested for immunoblot assay for HIF-1α protein expression. Experiments are repeated 3 times (**A** and **C**) or for 4 times (bar graph in B and D). Representative immunoblots are shown. Intensity of respective bands was analyzed densitometrically, and fold inductions to lane 1 are plotted accordingly as mean ± SD (**A** and **C**) or as mean (**B** and **D**). *$p < 0.05$ compared with no treatment/differentiation (−).

1β (BD Biosciences) at 1:1,000 dilution and HRP-conjugated sheep antibody against mouse IgG (Amersham Bioscience, Piscataway, NJ.) at 1:2,000 dilution. For phospho-IκBα, 20-μg aliquots were fractionated by 12% SDS-polyacrylamide gel electrophoresis and subjected to immunoblot assay using rabbit polyclonal antibodies against phospho-IκBα(Ser32/36) (Cell Signaling Technology, Beverly, MA) at 1:1,000 dilution and HRP-conjugated goat antibody against rabbit IgG (Amersham Bioscience, Piscataway, NJ) at 1:2,000 dilution. Chemiluminescent signal was developed by using ECL reagent (Amersham Bioscience).

Reverse transcription (RT)-PCR

The RT-PCR was performed as previously described (25, 62). After treatment, cells were harvested, and RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA). Then 1 μg of total RNA was subjected to first-strand cDNA synthesis by using random hexamers (Superscript II RT kit, Invitrogen). cDNAs were amplified with TaqGold polymerase (Roche Applied Science) in a thermal cycler with the specific primers (Table 1). For each primer pair, PCR was optimized for cycle number to obtain linearity between the amount of input RT product and output PCR product. Thermocycling conditions were 30 sec at 94°C, 60 sec at 57°C, and 30 sec at 72°C for 28 (CD14), 25 (VEGF, HIF-1α, TLR-2, and TLR-4), or 20 (18S rRNA) cycles preceded by 10 min at 94°C. PCR products were fractionated by 1% SeaKem GTG agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV.

Reporter gene assays

Transfection of THP-1 cells was performed by using Lipofectamine 2000 Transfection Reagent (Invitrogen) by following a protocol provided by the manufacturer. Plasmid p2.1 contains a 68-bp hypoxia response element (HRE) from the *ENO1* gene inserted upstream of an SV40 promoter in the luciferase reporter plasmid pGL2-Promoter (Promega) (59). Expression vector pGAL4/HIF-1α(531-826) was described previously (23). The reporter GAL4E1bLuc contains five copies of a GAL4 binding site upstream of a TATA sequence and firefly luciferase coding sequences. The expression vector pRc-IκBα(S32A/S36A) (50) was generously provided by Dr. Nancy R. Rice, National Cancer Institute, Frederick, MD. The pNF-κB-Luc reporter plasmid was from Stratagene, La Jolla, CA. Expression vector pFLAG-HIF-1α-DN, encoding a dominant negative form of HIF-1α fused to FLAG tag, was described previously (25, 59). The reporter gene plasmid and the control plasmid pRL-SV40 (Promega, Madison, WI), containing a SV40 promoter upstream of *Renilla reniformis* luciferase coding sequences, were premixed and used. After treatment, the cells were harvested, and the luciferase activity was determined by using the Dual-Luciferase Reporter Assay System (Promega). The ratio of firefly to *Renilla* luciferase activity was

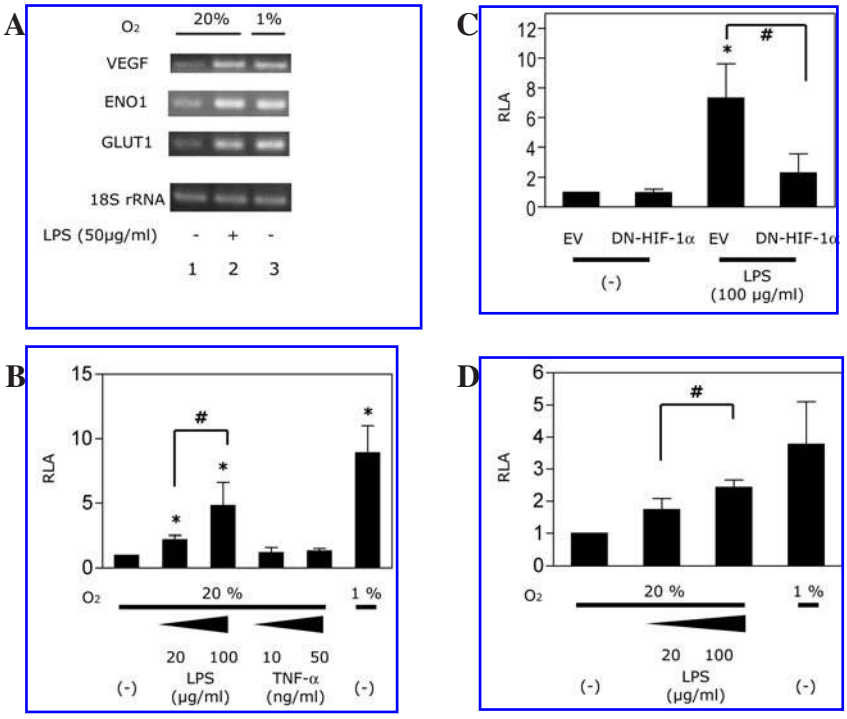

FIG. 2. Effect of LPS on HIF-1–dependent gene expression. (**A**) Differentiated THP-1 cells were exposed to LPS under 20% O_2 conditions or 1% O_2 conditions for 18 h continuously, and total RNA was isolated. Expression of mRNA of VEGF, enolase 1, Glut1, and HIF-1α, and 18S rRNA was analyzed with RT-PCR. The band density relative to lane 1 is indicated below the band. (**B–D**) THP-1 cells were transfected with the HIF-1–dependent reporter gene p2.1 encoding firefly luciferase downstream of an HRE and SV40 promoter, and pRL-SV40, encoding *Renilla* luciferase (**B**); p2.1, pCEP4, an expression vector encoding either no protein (EV) or a dominant negative form of HIF-1α (DN), and pRL-SV40 (**C**); or GAL4E1bLuc, containing five GAL4-binding sites upstream of a minimal E1b TATA sequence and the luciferase gene, pGAL4/HIF-1α(531-826), encoding a GAL4-DNA-binding domain-HIF-1α fusion protein, and pRL-SV40 (**D**). Transfected cells were exposed to 50 n*M* PMA for 24 h at 20% O_2, exposed to the indicated dose of LPS, TNF-α, or to 1% O_2, and harvested after 18 h. The ratio of firefly to *Renilla* luciferase activity was determined and normalized to the value obtained from nonhypoxic cells to obtain the relative luciferase activity (RLA). Results shown represent mean ± SD of three independent transfections. *$p < 0.05$ compared with respective control. #$p < 0.05$ between indicated groups.

determined. Normalized mean count ± SD of three independent transfections is shown as relative luciferase activity (RLA).

Gene silencing by using small interfering RNA (siRNA)

siRNAs corresponding to green fluorescent protein (cat. no. 1022064), TLR-4 (cat. no. SI00151004), and MyD88 (cat no. SI00300909) were from Qiagen Inc. (Valencia, CA). siRNAs were introduced into THP-1 cells by electroporation by using the Nucleofector (Amaxa Biosystems) by following a protocol provided by the manufacturer (44).

Lactate assay

Conditioned medium from triplicate macrophage cell cultures was harvested and assayed for lactate content by colorimetric detection (Sigma) according to the manufacturer's instructions. Sample values were calculated from a lactate standard curve and normalized to cell lysate protein content.

Intracellular reactive oxygen species measurement

Intracellular ROS production was measured by following the method described previously (45). In brief, dishes of confluent

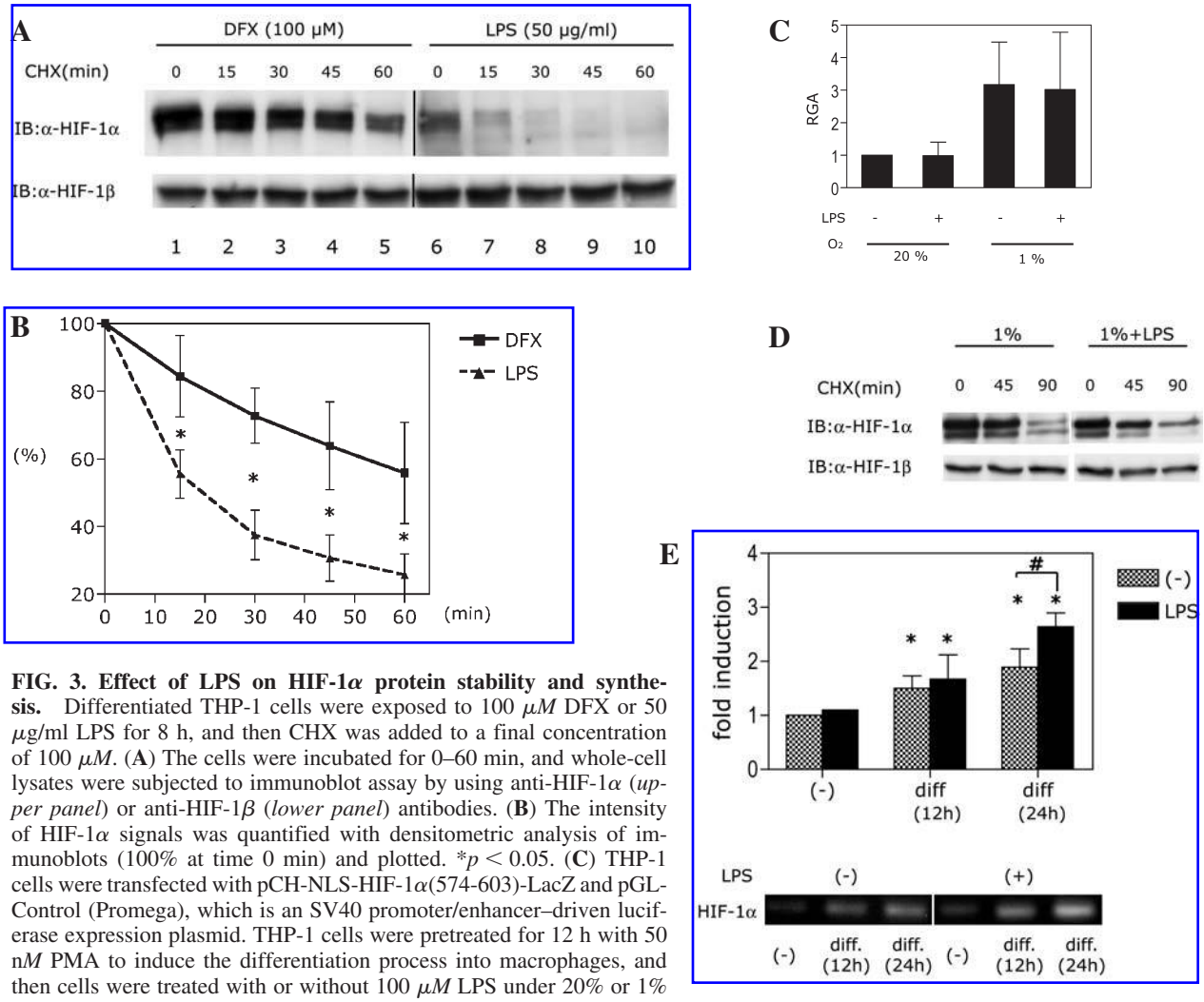

FIG. 3. Effect of LPS on HIF-1α protein stability and synthesis. Differentiated THP-1 cells were exposed to 100 μM DFX or 50 μg/ml LPS for 8 h, and then CHX was added to a final concentration of 100 μM. (**A**) The cells were incubated for 0–60 min, and whole-cell lysates were subjected to immunoblot assay by using anti-HIF-1α (*upper panel*) or anti-HIF-1β (*lower panel*) antibodies. (**B**) The intensity of HIF-1α signals was quantified with densitometric analysis of immunoblots (100% at time 0 min) and plotted. *$p < 0.05$. (**C**) THP-1 cells were transfected with pCH-NLS-HIF-1α(574-603)-LacZ and pGL-Control (Promega), which is an SV40 promoter/enhancer–driven luciferase expression plasmid. THP-1 cells were pretreated for 12 h with 50 nM PMA to induce the differentiation process into macrophages, and then cells were treated with or without 100 μM LPS under 20% or 1% O_2 conditions for 12 h and subjected to β-gal assay. Normalized β-gal activity is shown. RGA, relative galactosidase activity. Results shown represent mean ± SD of three independent transfections. (**D**) Differentiated THP-1 cells were exposed to 1% O_2 or 1% O_2 + 50 μg/ml LPS for 4 h, and then CHX was added to a final concentration of 100 μM. The cells were incubated for 0, 45, and 90 min and harvested under 1% O_2 conditions. Whole-cell lysates were subjected to immunoblot assay by using anti-HIF-1α (*upper panel*) or HIF-1β (*lower panel*) antibody. A representative immunoblot is indicated. (**E**) THP-1 cells were exposed to 50 nM PMA for 12 h or 24 h and treated with LPS for 8 h. Cells were harvested for RT-PCR to examine HIF-1α mRNA expression. A representative blot is shown as the *lower panel*. Results shown represent mean ± SD of three independent experiments. *$p < 0.05$ compared with respective control. #$p < 0.05$ between the indicated groups.

cells on a 35-mm dish at various times after stimulation with EGF were washed with modified Eagle's medium without phenol red and incubated in the dark for 5 min in Krebs–Ringer solution containing 5 m*M* 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). H_2DCF-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative H_2DCF and thereby trapped within the cells. In the presence of a proper oxidant, H_2DCF is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Culture dishes were transferred to a Zeiss Axivert 135 inverted microscope, equipped with a x20 Neoflur objective and Zeiss LSM 410 confocal attachment, and ROS generation was detected as a result of the oxidation of DCFH (excitation, 488 nm; emission, 515–540 nm).

Data analysis

All the experiments were done at least 3 times (unless mentioned otherwise), and representative blots are shown. Data were collected and expressed as mean ± standard deviation (SD). Significance test (Student's *t* test) was performed by using the Prism version 4 application.

RESULTS

LPS induced HIF-1α accumulation under nonhypoxic conditions in differentiated THP-1 cells

We analyzed the effect of LPS on HIF-1 activation under nonhypoxic conditions in undifferentiated and differentiated THP-1 cells. THP-1 cells were pretreated for 12 h with 50 n*M* PMA to induce the differentiation process into macrophages (31). The treatment induced expression of CD14, Toll-like receptor (TLR)-2, and TLR-4 mRNA in THP-1 cells (Fig. 1A). The expression of these mRNAs is characteristic of macrophages. THP-1 cells were treated with LPS or inflammatory cytokines under 20% O_2 conditions (Fig. 1B). HIF-1 protein levels were examined by immunoblot assay. Exposure of differentiated THP-1 cells to LPS for 4 h under nonhypoxic conditions induced the accumulation of HIF-1α (upper panel, lane 2), whereas TNF-α and IL-1β did not induce HIF-1α (lanes 3 and 4). In undifferentiated THP-1 cells, HIF-1α expression was not induced by LPS, TNF-α, or IL-1β (lanes 7–9). HIF-1α expression was induced in both differentiated and undifferentiated THP-1 cells under 1% O_2 conditions, but levels were higher in differentiated cells (lanes 5 and 10). In contrast, HIF-1β protein expression (lower panel) was not significantly affected by LPS, inflammatory cytokines, or hypoxia.

We next analyzed the expression of HIF-1α in THP-1 cells exposed to increasing concentrations of LPS, up to 100 μg/ml. The induction of HIF-1α accumulation by LPS was dose dependent in differentiated THP-1 cells (Fig. 1C, upper panel, lanes 1–4), but not in undifferentiated THP-1 cells (lanes 5–8). Although 50 μg/ml LPS is required to induce HIF-1α protein expression in differentiated THP-1 cells under 20% O_2 conditions, 1–10 μg/ml LPS is high enough significantly to induce HIF-1α in differentiated THP-1 cells under 5% O_2 conditions and in macrophages derived from human peripheral monocytes under 20% O_2 conditions (data not shown).

Incubation with PMA for 24 h rather than 12 h results in increased expression of macrophage-differentiation markers such as CD14, CD36, and TLRs (44). LPS induced protein expression of HIF-1α more strongly in THP-1 cells that were differentiated for 24 h as compared with 12 h under 20% O_2 conditions (Fig. 1D).

Impact of LPS on HIF-1–dependent gene expression

We used RT-PCR to analyze the expression of genes regulated by HIF-1 in differentiated THP-1 cells exposed to hypoxia or LPS (Fig. 2A). The expression of three known HIF-1 target genes, *VEGF, ENO1*, and *GLUT1*, was induced by exposure of cells to LPS (top, lane 2) or 1% O_2 (lane 3) for 18 h. No marked difference in the expression of 18S rRNA was detected (bottom panel), indicating that treatments did not affect the reverse-transcriptase reaction or PCR. These data demonstrate that LPS ex-

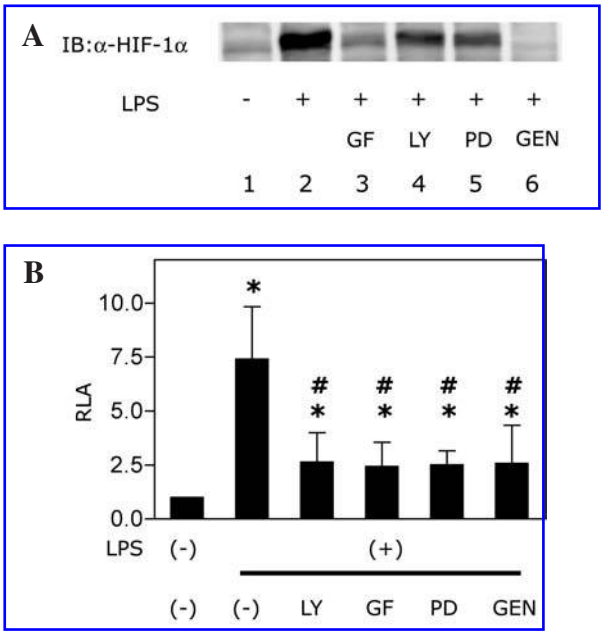

FIG. 4. Impact of kinase inhibitors on the LPS-induced expression of HIF-1α. (**A**) Differentiated THP-1 cells were exposed to vehicle or LPS for 4 h in the presence of 5 μM GF109203X (GF), 10 μM LY294002 (LY), 50 μM PD98059 (PD), or 100 μM genistein (GEN) under 20% O_2 conditions and harvested for analysis of HIF-1α. (**B**) THP-1 cells were exposed to PMA for 24 h under 20% O_2 conditions and then cotransfected with the plasmids p2.1 and pRL-SV40. After transfection, cells were treated with 100 μM LPS and each inhibitor (10 μM LY, 5 μM GF, 50 μM PD, or 100 μM GEN) and harvested after 18 h. The ratio of firefly/*Renilla* luciferase activity was determined and normalized to the value obtained from nonhypoxic cells transfected with the empty vector to obtain the relative luciferase activity (RLA). Results shown represent mean ± SD of three independent transfections. *$p < 0.05$ compared with control [(−)/(−)]. #$p < 0.05$ compared with LPS/(−).

posure induces the expression of HIF-1 target genes in differentiated THP-1 cells, which is similar to the effect of hypoxia.

The effect of LPS on HRE-dependent gene expression was investigated by using a luciferase reporter assay. As shown in Fig. 2B, LPS induced HRE-dependent gene expression in a dose-dependent manner in differentiated THP-1 cells. In contrast, TNF-α did not induce HRE-dependent gene expression. Expression of a dominant negative form of HIF-1α blocked HRE-dependent reporter gene induction, demonstrating specific involvement of HIF-1 in this induction (Fig. 2C).

We next investigated the impact of LPS on HIF-1 transcriptional activity by transfecting cells with an expression vector encoding a GAL4-HIF-1α fusion protein. Steady-state levels of the fusion protein GAL4-HIF-1α(531-826), which consists of the GAL4 DNA-binding domain fused to the HIF-1α transactivation domain (amino acid residues, 531–826), are similar under hypoxic and nonhypoxic conditions (23, 39). The GAL4-HIF-1α fusion construct can thus be used to examine the transcriptional activity of HIF-1α independent of its protein expression. Transactivation mediated by GAL4-HIF-1α(531-826)

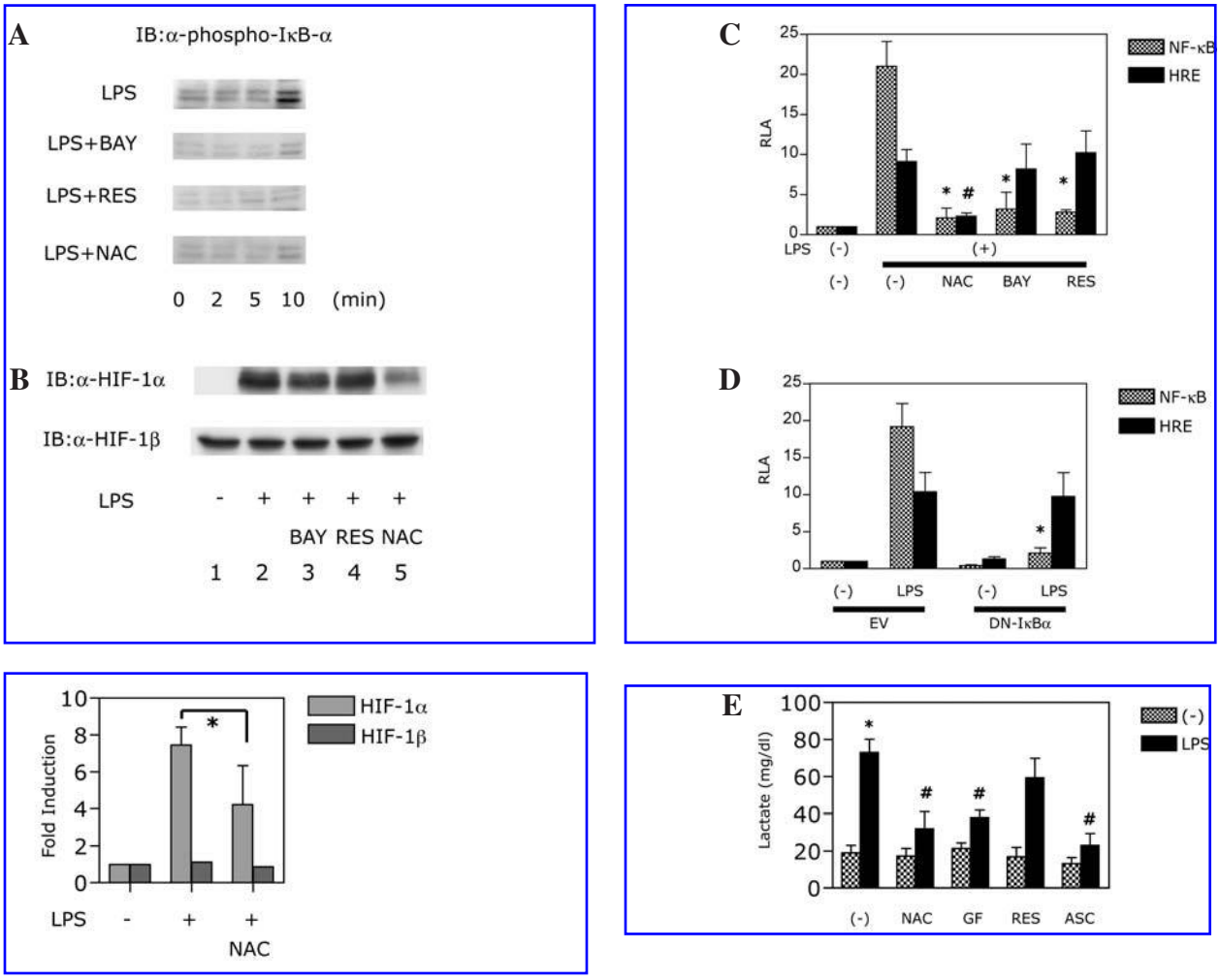

FIG. 5. Impact of NF-κB inhibitors on HIF-1 activity. (**A**) Differentiated THP-1 cells were exposed to 50 μg/ml LPS with 20 μM BAY11-7082 (BAY), 20 μM resveratrol (RES), and 10 mM *N*-acetylcysteine (NAC) for the indicated times and harvested for immunoblot assay by using anti-phospho-IκB-α antibody. (**B**) Differentiated THP-1 cells were exposed to 50 μg/ml LPS with 20 μM BAY (lane 4), 20 μM RES (lane 5), and 10 mM NAC (lane 3) for 8 h and harvested for immunoblot assay for HIF-1α (*upper panel*) and HIF-1β (*lower panel*) protein expression. Intensities of respective bands were analyzed densitometrically, and fold inductions to lane 1 are plotted accordingly as mean ($n = 4$). (**C, D**) THP-1 cells were transfected with the plasmids p2.1 (**C** and **D**) or pNF-κB-Luc (**C**), pRL-SV40 (**C** and **D**), and pRc-CMV-IκBα 32A/S36A (DN-IκBα) or pRc-CMV (EV) (**D**), and then exposed to PMA for 24 h under 20% O_2 conditions. Cells were treated with 100 μM LPS and harvested after 18 h. The ratio of firefly to *Renilla* luciferase activity was determined and normalized to the value obtained from cells transfected with an empty vector to obtain the relative luciferase activity (RLA). Results shown represent mean ± SD of three independent transfections. *$p < 0.05$ compared with LPS(+)/EV. (**E**) Lactate concentrations in supernatant of THP-1 cells were measured either under control conditions or after the treatment with LPS. When indicated, 10 mM NAC, 5 μM GF109203X (GF), 20 μM RES, or 50 μM ascorbate (ASC) was used. *$p < 0.05$ compared with control [(−)/(−)]. #$p < 0.05$ compared with LPS(+)/(−).

is increased in cells exposed to hypoxia or LPS (Fig. 2D). The result demonstrates that LPS not only promotes accumulation of HIF-1α but also enhances HIF-1α transcriptional activity.

LPS does not prolong HIF-1α protein half-life

To determine whether LPS treatment affected HIF-1α protein half-life, differentiated THP-1 cells were treated with DFX or LPS for 8 h to induce HIF-1α protein accumulation, and then CHX was added to block ongoing protein synthesis. In presence of CHX, the half-life of HIF-1α was >60 min in DFX-treated cells but <15 min in LPS-treated cells (Fig. 3A and B). We also assayed the stability of a fusion protein, consisting of a nuclear localization signal, β-galactosidase (β-gal) sequences (encoded by the *lac Z* gene), and HIF-1α residues 548–603 (which encompassed the hydroxylation site at proline-564). The NLS-LacZ-HIF1α(548–603) expression vector was transfected into THP-1 cells, and β-gal activity was analyzed by a luminometer (Fig. 3C). Hypoxic treatment induced β-gal activity, but LPS treatment did not affect β-gal activity under either 20% or 1% O_2 conditions. Moreover, LPS did not markedly affect HIF-1α half-life under 1% O_2 conditions (Fig. 3D). These results indicate that HIF-1α expression in LPS-treated cells is dependent mainly on ongoing protein synthesis. Therefore, we evaluated whether differentiation could modify the levels of HIF-1α mRNA. As seen in Fig. 3E, differentiation increased HIF-1α mRNA levels in THP-1 cells, and LPS treatment further induced HIF-1α mRNA expression. These experiments suggest that LPS-stimulated HIF-1α protein expression is due at least in part to increased HIF-1α mRNA levels.

Impact of kinase inhibitors on LPS-induced HIF-1 activation

To examine the signaling pathways leading to the induction of HIF-1α protein expression in differentiated THP-1 cells exposed to LPS, we investigated the role of protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), the ERK mitogen-activated protein kinases (MAPKs), and tyrosine kinases. Differentiated THP-1 cells were pretreated with GF209203X, LY294002, PD98059, or genistein, which are selective pharmacologic inhibitors of PKC, PI3K, MEK, or tyrosine kinases, respectively, for 30 min, treated with LPS for 4 h, and then harvested for immunoblot assays (Fig. 4A). All of the agents tested, particularly GF209203X (lane 3) and genistein (lane 6), inhibited the LPS-induced accumulation of HIF-1α protein expression in THP-1 cells.

Next, the impact of kinase inhibitors on HIF-1 transcriptional activation was investigated by using HRE-luciferase reporter gene construct. All of the kinase inhibitors significantly suppressed HIF-1 transcriptional activation elicited by LPS in differentiated THP-1 cells (Fig. 4B).

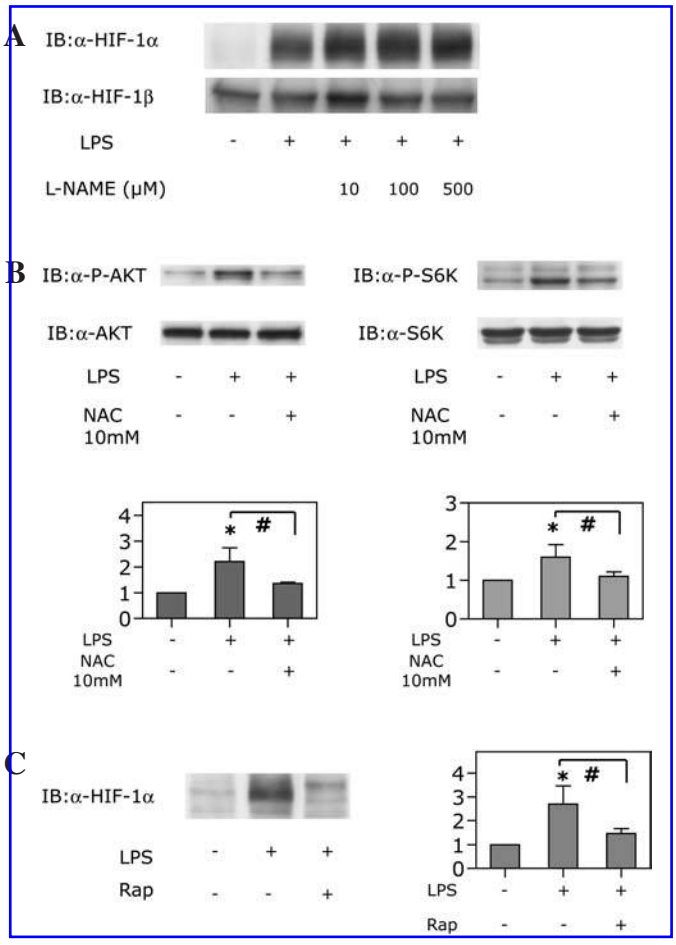

FIG. 6. Effect of NOS inhibitor on HIF-1α protein accumulation and the phosphorylation of protein kinases that regulate protein translation. Differentiated THP-1 cells were exposed to 50 μg/ml LPS with or without 10–100 μM *N*ω-nitro-L-arginine methyl ester (L-NAME) (**A**), 10 m*M* *N*-acetylcysteine (NAC) (**B**), or 200 n*M* rapamycin (**C**) for 8 h and harvested for immunoblot assays of HIF-1α and HIF-1β proteins (**A, C**). NAC-treated lysates were analyzed by using antibodies that recognize phosphorylated (*top panels*) and total (*bottom panels*) Akt (*left panels*), and S6 kinase (*right panels*) (**B**). Representative immunoblots are shown. Intensities of respective bands were analyzed densitometrically, and fold inductions to lane 1 are plotted accordingly as mean ± SD. *$p < 0.05$ compared with control [(−)/(−)]. #$p < 0.05$ between the indicated groups.

Impact of NF-κB inhibitors and N-acetylcysteine on LPS-induced HIF-1α accumulation

Because LPS is a strong NF-κB activator, and several reports indicate involvement of NF-κB in HIF-1 activation (10, 11, 38), the NF-κB inhibitors BAY11-7082 (49) and resveratrol (8, 65) were used to investigate the role of NF-κB in the LPS-induced HIF-1 activation pathway. LPS-induced NF-κB activation is regulated by IκBα protein phosphorylation on Ser32 and Ser36. To investigate whether BAY11-7082 and resveratrol treatment affected IκBα phosphorylation in THP-1 cells, we examined phosphorylation of IκBα in differentiated THP-1 cells (Fig. 5A). IκBα protein phosphorylation was induced by 10-min LPS treatment (Fig. 5A, uppermost panel). The NF-κB inhibitors BAY11-7082 and resveratrol and the potent antioxidant NAC suppressed LPS-induced IκBα phosphorylation (Fig. 5A), indicating that in THP-1 cells, the pathway from LPS to NF-κB is active. In contrast to their effect on IκBα, neither BAY11-7082 nor resveratrol affected HIF-1α accumulation induced by LPS (Fig. 5B, upper panel, lanes 3 and 4). However, NAC significantly suppressed not only IκBα phosphorylation but also HIF-1α accumulation (lane 5).

Although BAY11-7082 and resveratrol each inhibited LPS-induced NF-κB–dependent gene expression, they did not affect HIF-1–dependent gene expression. In contrast, NAC blocked both NF-κB– and HIF-1–dependent reporter gene expression (Fig. 5C). Overexpression of a degradation-resistant form of IκBα (IκBα32A/36A) significantly suppressed LPS-induced NF-κB–dependent luciferase activation but not LPS-induced HRE-luciferase activation (Fig. 5D). These results indicate that ROS generation is required, whereas NF-κB pathway activation does not contribute to HIF-1α accumulation induced by LPS in differentiated THP-1 cells. To establish a connection between effects on gene expression and cell physiology, we examined the effects of various reagents on lactate accumulation (Fig. 5E). The lactate levels in culture media increased in response to LPS exposure, and this increase was suppressed by treatment with NAC or GF209203X, which inhibited HIF-1 activation, but not by treatment with resveratrol, which had no effect on LPS-induced HIF-1 activation. The HIF-1α hydroxylase cofactor ascorbate also suppressed LPS-induced lactate accumulation.

Impact of NOS inhibition on LPS-induced HIF-1α accumulation

LPS induces generation of ROS and NO (2). Both ROS and NO are reported to be involved in the regulation of HIF-1 (3, 26). The inhibitory effect of NAC (Fig. 5B) indicates a role for ROS in the activation of HIF-1α expression in response to LPS stimulation (Fig. 5B). Because LPS induces iNOS activity and NO induces HIF-1 activation (25, 27), we pursued the possibility of involvement of NO in the response to LPS. The NOS inhibitor *N*ω-nitro-L-arginine methyl ester (L-NAME) did not decrease HIF-1α accumulation induced by LPS (Fig. 6A, up-

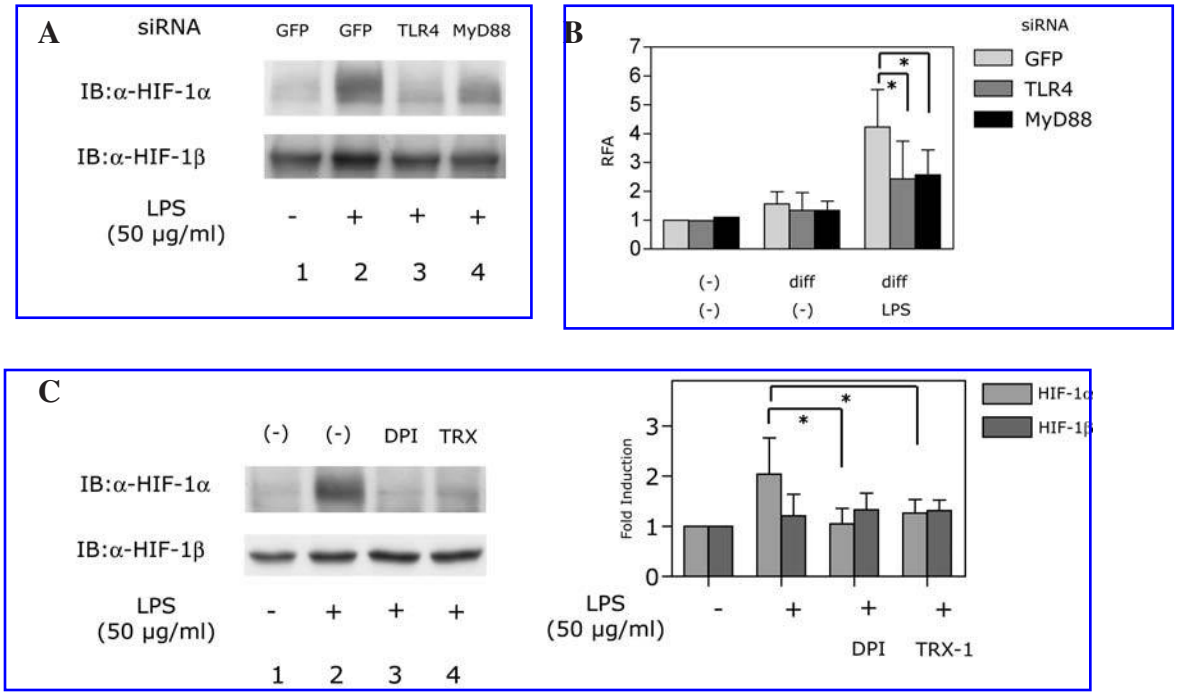

FIG. 7. Impact of TLR4 and MyD88-dependent ROS production on LPS-induced HIF-1 activation. (**A**, **B**) siRNAs against GFP, TLR4, or MyD88 were introduced into THP-1 cells. Cells were allowed to differentiate into macrophages by treatment with 50 n*M* PMA, exposed to 50 μg/ml LPS for 8 h, and harvested for immunoblot assays of HIF-1α protein. Representative immunoblots are shown. Intracellular ROS production was examined with fluorescence-based assay, described in Materials and Methods. RFA, relative fluorescence activity. *$p < 0.05$ compared with control [(−)/(−)]. #$p < 0.05$ between the indicated groups (**B**). (**C**) Cells were treated with 50 μg/ml LPS (lanes 3–4), with 10 μ*M* DPI (lane 3), or with 100 μg/ml recombinant TRX-1 (lane 5) and harvested for immunoblot assays of HIF-1α and HIF-1β protein. Intensity of respective bands was analyzed densitometrically, and fold inductions to lane 1 are plotted accordingly as mean ± SD. *$p < 0.05$ compared with control [(−)/(−)].

per panel, lanes 3–5), suggesting that NO is not required for LPS-induced HIF-1 activation in THP-1 cells.

Because Akt and S6 kinase are critical regulators of signal transduction to the translational machinery, activation of Akt and S6 kinase in response to LPS stimulation was examined by using phosphospecific antibodies. LPS induced phosphorylation of Akt and S6 kinase in a NAC-sensitive manner (Fig. 6B). Akt phosphorylates the mammalian target of rapamycin (mTOR), which in turn phosphorylates p70 S6 kinase. Exposure of cells to rapamycin, a specific inhibitor of mTOR, suppressed HIF-1α protein accumulation elicited by LPS (Fig. 6C). Taken together, these data indicate that ROS activation of the Akt-mTOR-S6 kinase pathway is required for activation of HIF-1 in THP-1 cells exposed to LPS.

LPS-induced HIF-1 activation is dependent on ROS generated downstream from TLR4 and MyD88

As demonstrated in Fig. 1A, during THP-1 differentiation, TLRs and CD14 mRNA expression are induced. Among TLRs, TLR4 expression is induced more strongly than TLR2. The evidence prompted us to investigate the involvement of TLR4 and its downstream molecule MyD88 in LPS-induced HIF-1 activation. We examined effect of interfering with TLR4 and MyD88 mRNA expression. siRNAs were introduced into THP-1 cells. The cells were differentiated by PMA treatment for 12 h and then exposed to LPS. Knockdown of TLR4 or MyD88 expression abrogated LPS-induced HIF-1α protein accumulation (Fig. 7A) and ROS generation, as measured by DCF fluorescence (Fig. 7B). Because LPS elicits ROS production by NADPH-dependent peroxidases and TRX-1 inhibits LPS-induced signal transduction, the NADPH-oxidase inhibitor DPI or TRX-1 was tested (40). DPI or TRX-1 treatment blocked LPS-induced HIF-1α protein accumulation (Fig. 7C).

DISCUSSION

HIF-1 activation plays important roles in inflammation and wound healing (43, 61, 67). In this study, we demonstrated that the potent proinflammatory agent LPS induces HIF-1α protein expression and HIF-1 transcriptional activation, leading to increased expression of HRE-driven genes specifically in macrophage-differentiated THP-1 cells.

HIF-1 transcriptional activity is dependent on HIF-1α protein expression and on the interaction of the HIF-1α transactivation domain with coactivators, two processes that are independently regulated. As shown in Fig. 4, the kinase inhibitors tested in this study had differential inhibitory effects on LPS-induced HIF-1α protein expression and transcriptional activity. PKC and tyrosine kinase inhibitors blocked both processes, whereas MAPK and PI3K inhibitors blocked transcriptional activity but had more modest effects on HIF-1α protein expression.

Steady-state levels of HIF-1α protein are determined by regulation of its stability and synthesis. HIF-1α stability is regulated by HIF-1α prolyl hydroxylases in an O_2-dependent manner (9, 19, 24, 56). In contrast, IGF-1, insulin, and the NO donor NOC18 increase HIF-1α protein synthesis, which is sensitive to kinase inhibitors (12, 25, 64). As shown in Fig. 3A, LPS treatment does not prolong HIF-1α protein half-life as effectively as DFX, which inhibits HIF-1α prolyl hydroxylase activity (9, 21, 22). Induction of HIF-1α protein expression by LPS is blocked by inhibition of PI3K, PKC, MAPK, or tyrosine kinase activity (Fig. 4). LPS induces phosphorylation of

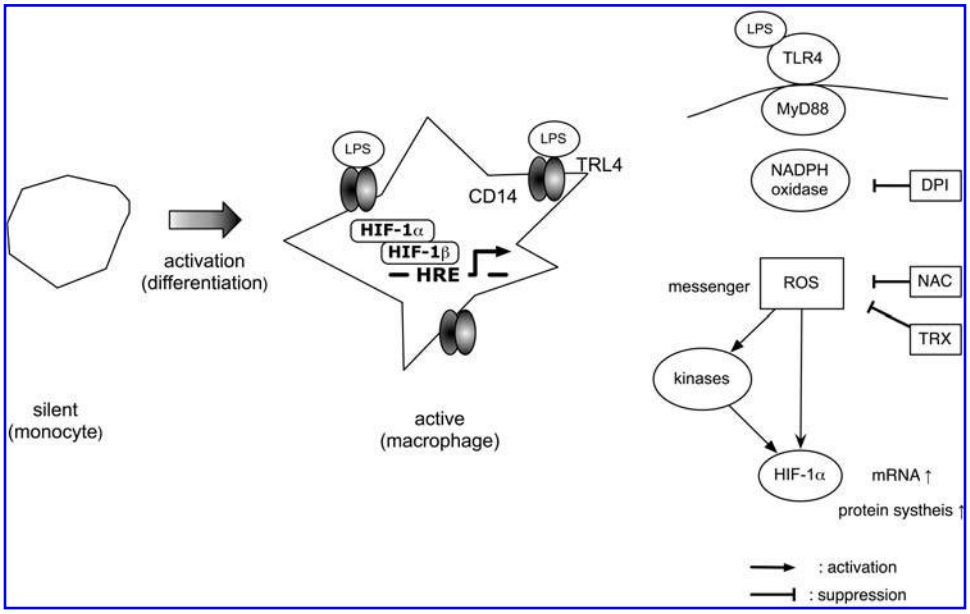

FIG. 8. Signal-transduction pathways to LPS-induced HIF-1 activation in activated THP-1 cells. In response to activation or differentiation, expression of CD14 and Toll-like receptor 4 (TLR4) is induced in macrophages. LPS induces generation of reactive oxygen species (ROS) in a MyD88- and DPI-sensitive NADPH oxidase–dependent manner. ROS activates kinases including PKC, PI3K, MAPK, or certain tyrosine kinases to induce HIF-1α mRNA accumulation and HIF-1α protein synthesis.

Akt and S6 kinase (Fig. 6B), both of which are involved in control of protein translation (16, 51). Moreover, rapamycin significantly suppresses HIF-1α accumulation (Fig. 6C). The results presented in Fig. 3C demonstrate that LPS also increases HIF-1α mRNA levels in macrophage-differentiated THP-1 cells. Taken together, these data indicate that LPS increases the rate of HIF-1α protein synthesis through increased HIF-1α mRNA levels and by activation of the Akt-mTOR-S6 kinase pathway.

Zhou *et al*. (69) reported that TNF-α activates the NF-κB, PI3K, and MAPK signaling pathways that lead to Bcl-2 expression, which in turn provokes IRES-dependent HIF-1α mRNA translation and HIF-1α protein synthesis in LLC-PK$_1$ cells (69). In this study, we demonstrated that the NF-κB inhibitors BAY11-7082 and resveratrol do not affect HIF-1α accumulation induced by LPS in differentiated THP-1 cells and that a degradation-resistant form of IκBα does not inhibit HRE-dependent gene expression, as shown in Fig. 5. Our data indicate that the NF-κB pathway does not significantly contribute to HIF-1α protein expression induced by LPS in differentiated THP-1 cells. Taken together, these studies indicate that induction of HIF-1α expression is stimulus or cell-type specific or both.

LPS induces oxidative stress and produces NO and ROS in macrophages (13, 14). NO from macrophages is produced mainly by inducible nitric oxide synthase (NOS2), expression of which is stimulated by LPS and regulated in a NF-κB–dependent manner (37). Under hypoxic conditions, HIF-1 also mediates activation of NOS2 in macrophages (42). We and others have reported that certain NO donors such as NOC18 and GSNO induce HIF-1 activation under nonhypoxic conditions (25, 27, 28, 52–54). We demonstrate that the NOS inhibitor L-NAME does not inhibit HIF-1α accumulation (Fig. 6A), indicating that an NO-dependent pathway is not involved in LPS-induced HIF-1 activation. In contrast, we demonstrated that the antioxidant NAC suppresses LPS-induced HIF-1 activation (Fig. 6). Treatment with the redox-acting protein TRX-1 also suppressed HIF-1α protein accumulation in THP-1 cells (Fig. 7C). The TRX-1 system is composed of NADPH, TRX-1 reductase, TRX-1, and peroxiredoxins. TRX-1 reduces protein disulfide groups in cooperation with TRX reductase and NADPH and scavenges hydrogen peroxide with peroxiredoxins. It is reported that exogenously administrated TRX-1 may interact with membrane-bound TRX reductase on the cell surface or enter the cells (29). HIF-1α protein expression, HRE-dependent gene expression, and lactate accumulation induced by LPS are inhibited by NAC. Moreover, NAC suppresses LPS-induced phosphorylation of Akt and S6 kinase (Fig. 6B). Although the precise intracellular source from which LPS induces ROS generation remains to be clarified, it has been reported that TLR4 directly interacts with NADPH oxidase, which is required for LPS-induced H_2O_2 generation in HEK 293 cells (41, 47). A similar mechanism might be involved in ROS generation by LPS in THP-1 cells. We demonstrated that the NADPH oxidase inhibitor DPI suppressed LPS-induced ROS generation and HIF-1 activation (Fig. 7B and C). We also demonstrated that LPS induced accumulation of HIF-1α protein in a TLR4- and MyD88-dependent manner (Fig. 7A). ROS are important intracellular second messengers generated in response to stimuli with proinflammatory agents including TNF-α, IL-1β, and LPS. ROS induce tyrosine kinase, PKC, and MAPK activity (6, 36, 40). Cytokines such angiotensin II increase HIF-1α translation through the ROS-dependent activation of the PI3K pathway (46). Taken together, we conclude that HIF-1α protein expression induced by LPS is mediated through ROS-mediated signaling pathways that increase the translation of HIF-1α mRNA into protein.

Another important finding of our study is that LPS-induced HIF-1 activation is dependent on the differentiation status of THP-1 cells. As shown in Fig. 1, HIF-1 activation is induced by LPS in differentiated THP-1 cells not but in undifferentiated cells. Moreover, we demonstrate that neither TNF-α nor IL-1β affects HIF-1 activity in THP-1 cells, although they induce NF-κB activation. Thus, the analysis both of differentiation and of pharmacologic inhibitors indicates that HIF-1 and NF-κB are activated by different mechanisms in these cells. The demonstration that HIF-1 is activated in response to LPS after macrophage differentiation complements recent studies demonstrating that HIF-1α is required for inflammatory and bactericidal responses by macrophages (7, 48). The TLR4 and MyD88 loss-of-function studies provide a molecular basis for the increased HIF-1α expression in differentiated THP-1 cells because these proteins are required for generation of the ROS signal in response to LPS (Fig. 8).

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ABBREVIATIONS

CHX, cycloheximide; DFX, desferrioxamine; EPO, erythropoietin; HIF-1, hypoxia-inducible factor 1; HRE, hypoxia response element; mTOR, mammalian target of rapamycin; NAC, *N*-acetylcysteine; PHD, prolyl hydroxylase domain-containing protein; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; S6K, S6 kinase; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor.

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