Activation of hypoxia-inducible factor 1 during macrophage differentiation

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Oda, Tomoyuki, Kiichi Hirota, Kenichiro Nishi, Satoshi Takabuchi, Seiko Oda, Hiroko Yamada, Toshiyuki Arai, Kazuhiko Fukuda, Toru Kita, Takehiko Adachi, Gregg L. Semenza, and Ryuji Nohara. Activation of hypoxia-inducible factor 1 during macrophage differentiation. Am J Physiol Cell Physiol 291: C104–C113, 2006. First published February 15, 2006; doi:10.1152/ajpcell.00614.2005.—Monocytes/macrophages of the myeloid lineage are the main cellular effectors of innate immunity. Hypoxia-inducible factor 1 (HIF-1) is essential for myeloid cell activation in response to inflammatory stimuli. However, it has not been established whether HIF-1 activity is induced during differentiation from monocyte to macrophage. We demonstrate that macrophage differentiation of THP-1 cells or monocytes from peripheral blood induces increased expression of both HIF-1 protein and mRNA levels and increased HIF-1 transcriptional activity leading to increased expression of HIF-1 target genes. The increased HIF-1 activity in differentiated THP-1 cells resulted from the combined effect of increased HIF-1mRNA levels and increased HIF-1 protein synthesis. Differentiation-induced HIF-1 protein and mRNA and HIF-1-dependent gene expression was blocked by treating cells with an inhibitor of the protein kinase C or MAP kinase signaling pathway. THP-1 cell differentiation was also associated with increased phosphorylation of the translational regulatory proteins p70 S6 kinase, S6 ribosomal protein, eukaryotic initiation factor 4E, and 4E binding protein 1, thus providing a possible mechanism for the modulation of HIF-1 protein synthesis. RNA interference studies demonstrated that HIF-1 is dispensable for macrophage differentiation but is required for functional maturation.

translation; RNA interference

MYELOID CELLS ARE RECRUITED to diverse sites of inflammation, such as wounds, arthritic joints, and necrotic tumors, which all share in common a reduction in O2 tension below levels present in normal tissues. Thus effector cells of the innate immune system must maintain their viability and physiological functions in a hypoxic microenvironment (35). Hypoxic conditions affect 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 (32, 33, 36, 42). It is well known that neutrophils and macrophages are dependent on anaerobic glycolysis for production of ATP (26, 40). The dependence of myeloid cells on this metabolic pathway suggests that they are highly adapted to a hypoxic environment.

In response to hypoxia, dramatic changes in gene expression occur, leading to increased synthesis of proteins, such as erythropoietin, glucose transporters, glycolytic enzymes, vascular endothelial growth factor (VEGF), and matrix metalloproteinases, which mediate cellular and tissue adaptation (16). The changes in gene expression are controlled by the transcriptional activator hypoxia-inducible factor 1 (HIF-1) (46). HIF-1 is a heterodimer composed of a constitutively expressed HIF-1β subunit and an inducibly expressed HIF-1α subunit (55). The regulation of HIF-1 activity occurs at multiple levels in vivo. Among these, the mechanisms regulating HIF-1α protein expression and transcriptional activity have been most extensively analyzed. O2-dependent hydroxylation of proline residues 402 and 564 in HIF-1α is required for binding of the von Hippel-Lindau tumor-suppressor protein (VHL), which is the recognition component of a ubiquitin-protein ligase that targets HIF-1α for rapid proteasomal degradation in nonhypoxic cells (19, 20). Under hypoxic conditions, hydroxylation of HIF-1α is inhibited due to substrate (O2) limitation, resulting in HIF-1α protein stabilization (8, 19, 20). Transcriptional activity of HIF-1α is also O2 regulated (22, 39). Asparagine residue 803 of HIF-1α is hydroxylated by factor-inhibiting HIF-1 under normoxic conditions, and the hydroxylated form of HIF-1α cannot bind to the coactivators p300 and CREB-binding protein (CBP) (2, 30, 34). The iron chelator desferrioxamine (DFX) induces HIF-1α stabilization and transcription under normoxic conditions (22), presumably by inhibiting the prolyl and asparaginyl hydroxylases, which contain Fe2+ at their catalytic sites (43). In contrast, HIF-1β is constitutively expressed in most cell types (18, 57).

Several reports (5, 48) have described expression of HIF-1α and VEGF in macrophages. Conditional gene targeting in the myeloid cell lineage has demonstrated that HIF-1α plays essential roles in antibacterial (38) and inflammatory (7) responses. The proinflammatory effect was attributed primarily to regulation of glycolytic metabolism by HIF-1, suggesting a housekeeping role rather than participation in a dynamic response. Proinflammatory cytokines and other stimuli, including TNF-α, IL-1, and LPS, activate HIF-1 (4, 12, 23). However, to the best of our knowledge, changes in HIF-1 activity associated with monocytic differentiation have not been pre-
viously reported. In this study, we used the human monocytic leukemia cell line THP-1 (52), which is a multifaceted model for the study of monocyte/macrophage differentiation (3), and primary monocyte-derived macrophages from healthy volunteers. We demonstrate that HIF-1 is activated during the macrophage-like differentiation of the monocytic THP-1 cell line that is induced by exposure to phorbol-12-myristate 13-acetate (PMA), all-trans-retinoic acid (ATRA), or 1α,25-dihydroxy vitamin D3 (VitD) and primary monocyte-derived macrophages. We also demonstrate that not only HIF-1α but also HIF-1β protein expression is induced during the differentiation process. Finally, we show that in addition to activating HIF-1 under nonhypoxic conditions, differentiated THP-1 cells manifest an augmented induction of HIF-1 activity in response to hypoxia.

EXPERIMENTAL PROCEDURES

Cell culture and reagents. THP-1 human myeloid leukemia cells (a gift from Dr. N. Kume, Kyoto University) were maintained in RPMI supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (49). DFX was obtained from Sigma (St. Louis, MO). PMA, ATRA, and VitD were from Biomol (Plymouth Meeting, PA). Cycloheximide (CHX), genistein, LY-294002, wortmannin, PD-98059, and GF109203X were obtained from Calbiochem (San Diego, CA).

Preparation of macrophages from human peripheral blood. Human peripheral blood monocyte-derived macrophages were prepared as previously described (37). Briefly, mononuclear cells were isolated from heparinized peripheral blood from healthy volunteers by Percoll-gradient centrifugation. All subjects gave written informed consent before enrolling in the study. The monocytes were plated onto plates and were washed twice to eliminate nonadherent cells after 2 h. Complete medium was added to the plates, and the cells were incubated. On day 5 of culture, >98% of adherent cells were macrophages, as determined by staining of nonspecific esterase and fluorescence analysis cell sorting.

Plasmid constructs. Reporter plasmid p2.1 contains a 68-bp hypoxia response element (HRE) from the enolase 1 gene, a simian virus-40 (SV-40) promoter, and firefly luciferase coding sequences (47). Plasmid pVEGF-Kpro contains nucleotides -2274 to +379 of the VEGF gene inserted into luciferase reporter pGL2-Basic (Promega) (9). The Renilla luciferase expression plasmid pRL-SV40 was from Promega (Madison, WI). pCMV-3xFLAG-HIF-1αDN, which encodes a FLAG-tagged dominant negative form of HIF-1α, was generated by subcloning from pCEP4-HIF-1α-NB·AB (21, 22).

Hypoxic treatment. Tissue culture dishes were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA), which was flushed with a 1% O2-5% CO2-94% N2 gas mixture, sealed, and stored at 37°C (13, 24).

Immuno blot assays. Whole cell lysates were prepared by incubating cells for 30 min in cold RIPA buffer containing 2 mM dithiothreitol, 1 mM NaVO3, and Complete protease inhibitor (Roche Applied Science, Tokyo, Japan) (13, 14). Samples were centrifuged at 10,000 × g at 4°C (13, 24). Samples were washed twice to eliminate nonadherent cells after 2 h. Complete medium was added to the plates, and the cells were incubated. On day 5 of culture, >98% of adherent cells were macrophages, as determined by staining of nonspecific esterase and fluorescence analysis cell sorting.

RT-PCR. The RT-PCR protocol was described previously (1, 50, 51). After treatment, cells were harvested and RNA was isolated with TRIzol (Invitrogen). One microgram of total RNA was subjected to first-strand cDNA synthesis using random hexamers (SuperScript II RT kit, Invitrogen) (1, 50, 51). cDNAs were amplified with TaqGold polymerase (Roche, Mannheim, Germany) in a thermal cycler with specific primers. A primer pair for detection of the truncated form of HIF-1α lacking exon 11 is described elsewhere (6). 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 s at 94°C, 60 s at 57°C, and 30 s at 72°C for 30 cycles (CD14, CD36), 28 (glucose transporter 1 (GLUT1)), 25 [carboxypeptidase M, VEGF, lactate dehydrogenase A (LDHA), HIF-1α, and HIF-1β] 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 light.

Reporter gene assays. Reporter assays were performed in THP-1 cells as described previously (13, 14). In each transfection, test plasmid, reporter gene plasmid p2.1, and the control plasmid pRL-SV40, containing a SV40 promoter upstream of Renilla reniformis (sea pansy) luciferase coding sequences (Promega), containing a thymidine kinase promoter upstream of R. reniformis luciferase coding sequences, were premixed with Lipofectamine 2000 transfection reagent (Invitrogen). After treatment, the cells were harvested and the luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). The ratio of firefly to Renilla luciferase activity was determined.

In vitro HIF-1α-VHL interaction assay. Plasmids used in assays were described previously (34). Glutathione S-transferase-HIF-1α(429–608) fusion protein was expressed in Escherichia coli (34). Biotinylated methionine-labeled proteins were generated in reticulo- cyte lysates with the TNT T7-coupled transcription/translation system using Transcend Biotinylated tRNA (Promega). Aliquots (25 μg) of THP-1 cell lysate were preincubated with or without DFX for 30 min at 30°C. A 5-μl aliquot of in vitro-translated biotinylated VHL protein was mixed with 4 μg of glutathione S-transferase fusion protein in a final volume of 200 μl of binding buffer (Dulbecco’s PBS, pH 7.4, and 0.1% Tween 20) and incubated for 2 h at 4°C with rotation, followed by addition of 10 μl of glutathione-Septarose 4B beads (Pharmacia) and incubation at 4°C for 1 h. The beads were pelleted, washed three times in binding buffer, pelleted, resuspended in Laemmli sample buffer, and analyzed with SDS-PAGE. Proteins were transferred to PVDF membrane and visualized using streptavidin-labeled horseradish peroxidase and ECL reagent (Amersham Biosciences).

RNA interference. To generate siRNAHIF-1α, two oligonucleotides consisting of ribonucleosides, except for the presence of 2′-deoxyri- bonucleosides (dTdT) at the 3′ end, 5′-AGAGGGUGGUAGUGGUGGGdTdT-3′ and 5′-GCCACACAUUCACUCCUCUdTdT-3′, were synthesized and annealed (Dharmacon Research, Lafayette, CO) (29). siRNAHIF-1α, was introduced into THP-1 cells by electroporation using the Nucleofector (Amaxa Biosystems) following a protocol provided by the manufacturer.

Measurement of lactate. 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.

RESULTS

Increased HIF-1 in THP-1 cells after macrophage differentiation. Human THP-1 leukemia cells differentiate along the monocytic lineage after 72-h exposure to 100 nM PMA, 1 μM...
ATRA, or 100 μM VitD. PMA, ATRA, or VitD treatment resulted in differentiated phenotype characterized by adherence and loss of proliferation (Fig. 1A). Each treatment induced the expression within 72 h of CD14 and CD36 (Fig. 1B), which are molecular markers of macrophage differentiation. Moreover, carboxypeptidase M mRNA expression, which is another molecular marker of differentiation, was also induced (Fig. 1B, left).

THP-1 cells were treated with 100 nM PMA or vehicle for 78 h and then exposed to 20% or 1% O2 for 4 h and harvested for immunoblot analysis (Fig. 1C). Under nonhypoxic conditions (20% O2), HIF-1α protein expression (Fig. 1C, top) was undetectable in undifferentiated THP-1 cells (lane 1) but was induced in differentiated THP-1 cells (lane 2). Hypoxia induced HIF-1α expression in undifferentiated THP-1 cells (lane 3). Remarkably, the combination of differentiation and hypoxia had a synergistic effect on HIF-1α expression (lane 4). On the other hand, HIF-2α expression was not induced significantly by differentiation. HIF-1β protein expression (Fig. 1C, middle) was observed in THP-1 cells under nonhypoxic conditions and was not induced by hypoxia (compare lanes 1 and 3). However, HIF-1β expression was induced in response to differentiation (lanes 2 and 4). Expression of β-actin protein (Fig. 1C, bottom) was not affected by hypoxia or differentiation.

To determine whether the induction of HIF-1α and HIF-1β expression was specific to PMA-elicited signaling or common to differentiation induced by other agents, we examined the effect of two other inducers of monocytic differentiation, VitD and ATRA (15, 58). VitD (100 μM) induced both HIF-1α and HIF-1β protein expression under nonhypoxic conditions (Fig. 1D, lane 2). Exposure of THP-1 cells to 1 μM ATRA also induced both HIF-1α and HIF-1β protein expression at 20% O2 conditions (lane 3). Finally, we examined the induction of HIF-1 proteins using monocytes from human peripheral blood.

Fig. 1. Effect of macrophage differentiation on hypoxia-inducible factor 1α (HIF-1α) protein levels. A: THP-1 cells were exposed to 100 nM phorbol-12-myristate 13-acetate (PMA), 1 μM all-trans-retinoic acid (ATRA) or 100 μM 1α,25-dihydroxy vitamin D3 (VitD) for 72 h, and analyzed by light microscopy. B: total RNA isolated from THP-1 cells after differentiation (diff.) was subjected to RT-PCR using primers specific for CD14, CD36, or carboxypeptidase M (CPM) mRNA. THP-1 cells were exposed to 100 nM PMA for 72 h (C) under 20% or 1% O2 conditions or 1 μM ATRA or 100 μM VitD for 72 h under 20% O2 conditions (D), and harvested. Whole cell lysates were subjected to immunoblot (IB) assay for HIF-1α (top), HIF-1β (middle), or β-actin (bottom) protein expression. E: monocytes (mono)/macrophages (macro) from human peripheral blood were exposed to 100 nM PMA for the indicated time and harvested. Whole cell lysates were subjected to immunoblot assay for HIF-1α (top), HIF-1β (middle), or β-actin (bottom) protein expression.
Monocytes isolated from peripheral blood were allowed to differentiate to macrophages. The cells were harvested and subjected to Western blot analysis (Fig. 1E). HIF-1α and HIF-1β protein expression was induced in response to the differentiation of peripheral blood monocytes into macrophages, similar to THP-1 cells. In contrast, HIF-2α expression was constant during differentiation.

Next, we investigated the dose dependency and time course of HIF-1 protein expression during the differentiation process induced by PMA or ATRA. PMA induced HIF-1α protein accumulation in a dose-dependent manner with a peak response at 100 nM (Fig. 2A). Exposure of cells to 100 nM PMA resulted in a time-dependent induction of HIF-1α that peaked at 4–6 h and decreased gradually but did not return to the basal level (Fig. 2B) even 7 days after PMA exposure (data not shown). Notably, after 48 h, a form of HIF-1α protein with reduced electrophoretic mobility predominated (lanes 9 and 10). In contrast, HIF-1β expression did not significantly change until 12 h (Fig. 2B) and then increased gradually over 7 days (data not shown). Expression of β-actin was constant. A similar pattern of HIF-1 protein expression was observed in cells exposed to ATRA (Fig. 2C).

We examined the effect of PMA on the Jurkat T lymphocyte line. PMA induced the expression of HIF-1α but not that of HIF-1β (Fig. 2D). The induction of HIF-1α was weak and transient and decreased to the basal level after 12 h, suggesting that the acute effect of PMA-induced signaling on HIF-1 is different from the effect of PMA as a differentiation factor.

Differentiation of THP-1 cells induces HIF-1-mediated transcriptional activation. We investigated by RT-PCR whether differentiation induced gene expression downstream of HIF-1. VEGF, GLUT1, and LDHA mRNA expression was induced under nonhypoxic conditions in cells exposed to PMA (Fig. 3A, lane 2) or hypoxic conditions (lane 3). Consistent with the effect on HIF-1 protein levels, differentiation and hypoxia synergistically induced GLUT1 and VEGF mRNA expression (lane 4). Macrophages typically produce most of their ATP through glycolysis (33), and HIF-1 is a key regulator of glycolysis (44, 45, 47). We performed an experiment to determine the role of HIF-1 in THP-1 cells after macrophage differentiation. As shown in Fig. 3B, lactate levels in culture media increased with differentiation and this was suppressed by treatment with 25 μM ascorbate, a potent HIF-1 inhibitor (27). Lipopolysaccharide induced accumulation of lactate, and the accumulation is also suppressed by ascorbate.

To determine whether the increase in VEGF mRNA levels reflected an increase in HIF-1 transcriptional activity, THP-1 cells were transfected with reporter plasmid p2.1, containing a HIF-1-dependent HRE, or p2.4, containing a mutated HRE (47). PMA or hypoxia induced HRE-dependent gene expression and combined exposure induced the greatest response (Fig. 3C). The mutated reporter p2.4 was not activated by PMA, providing evidence that the PMA-induced gene expression was HRE dependent (data not shown). PMA also induced transcription of pVEGF-KpnI, a reporter plasmid containing nucleotides −2274 to +379 relative to the transcription start site of the VEGF gene (Fig. 3D). Thus PMA-induced transcription, driven either by an isolated HRE upstream of a minimal promoter (p2.1) or by a native promoter sequence, encompasses an HRE (pVEGF-KpnI).
Differentiation does not prolong HIF-1α or HIF-1β protein half-life and does not inhibit interaction between HIF-1-α and VHL. To determine whether the differentiation process affected HIF-1α protein half-life (13, 24), THP-1 cells were treated with PMA for 48 h or with DFX for 4 h to induce HIF-1α expression, and then CHX was added to block ongoing protein synthesis. In the presence of CHX, the half-life of HIF-1α was >30 min in DFX-treated cells, due to inhibition of prolyl hydroxylation, but <15 min in PMA-differentiated cells (Fig. 4A). Neither DFX nor differentiation affected the half-life of HIF-1β protein.

Oxygen-dependent hydroxylation of two proline residues in HIF-1α is required for binding of VHL, the recognition component of a ubiquitin-protein ligase that targets HIF-1α for rapid proteasomal degradation in nonhypoxic cells. To study whether differentiation affected HIF-1α hydroxylase activity, the binding between HIF-1α and VHL was investigated. As shown in Fig. 4B, lysate from undifferentiated THP-1 cells promoted the binding. Lysates from PMA- and ATRA-differentiated THP-1 cells also promoted HIF-1α-VHL interaction.

As shown in Fig. 4, A and B, induction of HIF-1α and HIF-1β protein expression is not due to protein stabilization. Therefore, we evaluated whether differentiation could modify the levels of HIF-1 mRNAs. As seen in Fig. 4C, differentiation increased HIF-1α but not HIF-1β mRNA levels in THP-1 cells and in macrophages from peripheral blood. These experiments suggest that differentiation-stimulated HIF-1α protein expression is due at least in part to increased HIF-1α mRNA levels.

HIF-1α is not required for THP-1 cells to undergo macrophage differentiation. To further investigate whether HIF-1 is required for the macrophage differentiation of THP-1 cells in response to PMA, THP-1 cells were mock transfected or transfected with a small interfering RNA (siRNA<sub>HIF-1</sub>) that targets HIF-1α mRNA for degradation. siRNA targeted to GFP mRNA was also introduced into THP-1 cells as a negative control. Cells were treated with PMA or ATRA (data not shown) for 48 h and were then harvested for immunoblot assay with anti-HIF-1α Ab or RT-PCR with oligonucleotide pairs for CD36, a marker of macrophage differentiation. Introduction of siRNA<sub>HIF-1α</sub> effectively silenced the expression of HIF-1α protein even under hypoxic conditions (Fig. 5A). However, PMA induced macrophage differentiation of siRNA<sub>HIF-1α</sub>-transfected THP-1 cells as well as siRNA<sub>GFP</sub>-transfected cells (Fig. 5B). Moreover, compared with siRNA<sub>GFP</sub>, siRNA<sub>HIF-1α</sub> did not alter expression of CD36 (Fig. 5C).

Differentiation of THP-1 cells does not promote HIF-1α mRNA alternative splicing. We investigated whether macrophage differentiation induced by PMA was associated with the expression of an alternatively spliced form of HIF-1α mRNA lacking exon 11, as previously reported (6). With the use of a primer pair spanning exon 14 (Fig. SD, lanes 1 and 2) or exon

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Fig. 3. Effect of differentiation on HIF-1 target gene expression. A: THP-1 cells were treated with 100 nM PMA for 48 h (lanes 2 and 4) or exposed to 1% O<sub>2</sub> for 14 h (lanes 3 and 4) and total RNA was isolated. Expression of VEGF, glucose transporter-1 (GLUT1), lactate dehydrogenase A (LDHA), and 18S rRNA was analyzed by RT-PCR using specific primer pairs. B: lactate concentrations in supernatant of undifferentiated and differentiated THP-1 cells were measured either under control conditions or after treatment with LPS. When indicated, 25 μM ascorbate was added. Values were normalized to total protein content. THP-1 cells were transfected with pRL-simian virus 40 (SV40) encoding Renilla luciferase and one of the following plasmids encoding firefly luciferase: HRE reporter p2.1 (C) or VEGF promoter reporter pVEGF-K<sub>pro</sub> (D). Cells were exposed to 20% or 1% O<sub>2</sub> with or without PMA for 48 h and then harvested for luciferase assays. The ratio of firefly:Renilla luciferase activity was determined and normalized to the value obtained from nonhypoxic cells transfected with empty vector to obtain the relative luciferase activity (RLA). Results shown represent mean ± SD of 3 independent transfections.
11 (lanes 3 and 4), we did not detect any change in the size of the RT-PCR product obtained by amplification of mRNA isolated from cells before and after differentiation. Thus PMA-induced differentiation of THP-1 cells did not have any detectable qualitative effect on HIF-1α/hIF-1β mRNA expression.

**Impact of kinase inhibitors on HIF-1 activation.** To examine the signaling pathways leading to the induction of HIF-1α protein expression in differentiated THP-1 cells, we focused on various kinases, including phosphatidylinositol 3-kinase (PI3-kinase), tyrosine kinases, mitogen-activated protein kinase (MAPK), and mammalian target of rapamycin (mTOR). We performed assays under two different protocols. One examined the short-term (6 h) effect of PMA on THP-1 cells and the other investigated the long-term (48 h) effect on THP-1 cells that had differentiated in response to PMA treatment.

THP-1 cells were pretreated with LY-294002, wortmannin, genistein, PD-98059, rapamycin, or GF109203X, which are selective pharmacological inhibitors of PI3-kinase, tyrosine kinases, MEK, mTOR, and protein kinase C (PKC), respectively, for 30 min, treated with 100 nM PMA for 6 h, and then harvested for immunoblot assay (Fig. 6A). PMA treatment increased HIF-1α protein expression (Fig. 6A, top, lane 2). All of the agents (lanes 4-8) except genistein (lane 3) inhibited the induction of HIF-1α protein expression in THP-1 cells. HIF-1β protein expression was not induced and not affected by any of the kinase inhibitors (Fig. 6A, middle). β-Actin expression was also constant (bottom).

Next, THP-1 cells were treated with PMA and allowed to undergo macrophage differentiation for 48 h, and then kinase inhibitors were administered for an additional 24 h. The cell lysates were subjected to immunoblot assay (Fig. 6B). Only PD-98059 (lane 6) and GF109203X (lane 8) partially suppressed the expression of HIF-1α protein (Fig. 6B, top). In contrast to the 6-h protocol, HIF-1β expression was induced after the 48-h incubation with PMA, an effect that was not blocked by treatment with kinase inhibitors (Fig. 6B, middle). β-Actin expression was constant (Fig. 6B, bottom). These results are consistent with data presented in Fig. 2, which indicated that the short-term effect of PMA on HIF-1 differs mechanistically from the long-term effect of PMA as a differentiation factor. Next, the effects of the kinase inhibitors on HIF-1 mRNAs were investigated. As shown in Fig. 6C, HIF-1α mRNA induction in response to PMA-induced differentiation was suppressed by the PKC inhibitor GF109203X.
contrast, HIF-1β mRNA expression was not affected by any of the kinase inhibitors.

**Differentiation-induced activation of MAPK, PI3-kinase, and translational regulatory proteins.** HIF-1 activity induced by the stimulation of receptor tyrosine kinases or G protein-coupled receptors requires MAPK and/or PI3-kinase signaling (10, 11, 13, 31). To determine whether the MAPK- and PI3K-mediated pathways were activated in PMA-treated THP-1 cells, the phosphorylation of p42ERK2/p44ERK1 and AKT were analyzed in THP-1 cells and peripheral blood monocyte-derived macrophages (Fig. 7). Phosphorylation of p42ERK2/p44ERK1 was induced by PMA treatment in THP-1 cells in a time-dependent manner. The phosphorylation peaked at 15 min after PMA stimulation and declined thereafter (Fig. 7A). However, increased levels of ERK phosphorylation were sustained for at least 72 h (Fig. 7B). In contrast, AKT phosphorylation (Ser-473) was not prominent until 6 h, and then gradually increased (Fig. 7A). The phosphorylation peaked at 48 h and remained elevated at 72 h (Fig. 7B). The total protein levels of ERK and AKT were constant during the process (Fig. 7, A and B). As in THP-1 cells, phosphorylation of Erk and Akt was induced after differentiation of monocye-derived macrophages (Fig. 3C).

The signal transduction pathway involving PI3-kinase, AKT, and mTOR has been shown to regulate protein translation via phosphorylation of p70S6K (Thr-389), the S6 ribosomal protein (Ser-235/236), eukaryotic initiation factor-4E (eIF-4E) (Ser-209) and eIF-4E binding protein 1 (4E-BP1) (Ser-65). In THP-1 cells, the phosphorylation of p70S6K, S6 ribosomal protein, and 4E-BP1 was induced by PMA stimulation in a
time-dependent manner (Fig. 7D). Ser-209 of the mRNA cap-binding protein eIF-4E was also phosphorylated by PMA treatment of THP-1 cells. This result is consistent with studies (54, 56) indicating that ERK activates the MAPK signal integrating kinases, MNK1 and MNK2, which in turn phosphorylate eIF-4E.

**DISCUSSION**

Monocytes and macrophages are important cellular components of the innate immune system (17). In wounds and abscesses, myeloid cells must function in microenvironments where O2 and nutrient levels are extremely low (33, 36). In this study, we have demonstrated for the first time that during differentiation from monocyte to macrophage, HIF-1 activity increases markedly both in THP-1 cells and monocytes from human peripheral blood. Both HIF-1α and HIF-1β protein levels increase and cells acquire the ability to respond to hypoxia more robustly. Interestingly, HIF-2α expression is not altered by differentiation, suggesting an exclusive role of HIF-1α:HIF-1β heterodimers in activated macrophages. Thus HIF-1 activity in monocytes/macrophages is modulated by both O2-dependent and O2-independent signals.

Since the discovery of THP-1 cells in 1980 by Tsuchiya et al. (53), THP-1 cells have been used for study of monocyte-macrophage differentiation (3, 52). After treatment with phorbol esters, VitD, or ATRA, THP-1 cells differentiate into macrophage-like cells, which mimic native monocyte-derived macrophages in several respects. After differentiation, THP-1 cells acquire phagocytic activity and expression of scavenger receptors are observed (28, 41). Compared with other human myeloid cell lines, such as HL-60, U937, KG-1, or HEL cell lines, differentiated THP-1 cells behave more like native monocye-derived macrophages. Because of these characteristics, the THP-1 cell line provides a valuable model for studying the mechanisms involved in macrophage differentiation, and for exploring the regulation of macrophage-specific genes as they relate to physiological functions displayed by these cells. In this study, human monocyctic THP-1 cells were differentiated into adherent macrophages by treatment with PMA, ATRA, or VitD, as evidenced by the induction of macrophage differentiation markers such as CD14, CD36, and carboxypeptidase M.

Although the precise molecular mechanisms by which PMA, ATRA, or VitD induces THP-1 cells to differentiate into macrophages have not been delineated, the primary cellular targets of these agents are different. Yet they each induced the accumulation of HIF-1α and HIF-1β protein, and expression of the HIF-1-regulated VEGF gene. Differentiation induced by each of these agents was associated with increased levels of HIF-1α, which peaked at 6 h and then gradually decreased but did not return to basal levels. Thus, ATRA, PMA, and VitD each induce both macrophage differentiation and a sustained increase in the expression of HIF-1α and HIF-1β protein. The effect of PMA on Jurkat T cells was different from its effect on THP-1 cells with respect to HIF-1 protein accumulation. PMA treatment induced HIF-1α accumulation only transiently and did not induce HIF-1β expression in Jurkat T cells. In contrast, PMA induced the differentiation of THP-1 cells and sustained accumulation of both HIF-1α and HIF-1β protein. Recently, Chun et al. (6) reported that PMA treatment of Hep3B and human embryonic kidney-293 cells induced the expression of a HIF-1α mRNA splice variant lacking exon 11, which encodes an isoform of HIF-1α that is stable under nonhypoxic conditions. However, the results presented in Fig. 5D demon-
strate that this does not occur during the differentiation of THP-1 cells, providing further evidence that differentiation-induced HIF-1 activation is different from activation induced by acute PMA exposure.

Another important and novel finding of this study is the induction of HIF-1β protein without any increase in the cognate mRNA levels during differentiation of THP-1 cells treated with PMA, ATRA, or VitD. HIF-1β induction was not observed in THP-1 cells after hypoxia treatment, indicating that the induction was specifically associated with differentiation. This co-induction of both subunits of HIF-1 during the differentiation process confers robust and sustained activation of HIF-1 in macrophages. The kinetics of induction were different for HIF-1α and HIF-1β. Kinase inhibitors also had different effects on the expression of HIF-1α and HIF-1β, suggesting that discrete molecular mechanisms underlie the induction of each subunit.

Different molecular mechanisms also control differentiation- and hypoxia-induced HIF-1α expression. Unlike hypoxia, differentiation did not increase the half-life of HIF-1α protein in THP-1 cells. Moreover, because the levels of mRNAs encoding the HIF-1α hydroxylases PHD-2, PHD-3, and the factor inhibiting HIF-1 increased during the differentiation process (T. Oda and K. Hirota, unpublished data), reduced expression of these enzymes is unlikely to be the cause of increased HIF-1α accumulation and HIF-1 activity. The induction of HIF-1α protein during differentiation is largely dependent on activation of the PKC and MAPK pathways. Kinase inhibitors suppress PMA-induced HIF-1 activation in a time-dependent manner. All of the kinase inhibitors other than genistein acutely blocked acute PMA-induced HIF-1α protein accumulation. In contrast, HIF-1α protein expression after 48-h treatment, i.e., after differentiation, was only sensitive to the MEK-ERK inhibitor PD-98059 and the PKC inhibitor GF109203X. The MEK-ERK pathway stimulates the phosphorylation of eIF-4E, which is required for its mRNA cap binding activity. ERK has also been shown to phosphorylate 4E-BP1, which blocks its ability to inhibit eIF-4E. Thus macrophage differentiation both de-represses (via phosphorylation of 4E-BP1) and activates (via phosphorylation of eIF-4E and p70S6K) protein synthesis. On the other hand, HIF-1α mRNA induction is sensitive to only GF109203X. Thus, HIF-1α protein induction is dependent on both increased mRNA levels and an increased rate of protein translation from mRNA, which are regulated by different mechanisms.

Combined with its well-established roles in regulating angiogenesis and metabolic adaptation, these results add yet another dimension to the multifaceted involvement of HIF-1 in macrophage activity. Macrophages must produce sufficient ATP via glycolysis to generate an O2 burst under hypoxic conditions (25, 44). In mice with myeloid-specific deficiency of HIF-1α, monocytes differentiate into macrophages but the metabolic defect results in profound impairment of myeloid cell aggregation, motility, invasiveness, and bacterial killing (7). The dramatic effect of HIF-1α loss of function is consistent with our demonstration that HIF-1α, but not HIF-2α, is induced during monocyte-to-macrophage differentiation. We also show that differentiation induced the expression of mRNAs encoding GLUT1 and LDHA, which are required for intracellular glucose transport and the conversion of pyruvate to lactate, respectively, the first and last steps of glycolysis. Further studies are in progress to delineate the role of HIF-1 in macrophage function and to determine whether HIF-1 inhibitors may be useful as anti-inflammatory agents. In summary, our results suggest that increased expression of HIF-1α and HIF-1β promotes the adaptation of macrophages to the low levels of O2 and glucose that are commonly observed at sites of inflammation.

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REFERENCES

15. Hnana Z, Nandan D, Sly L, Knutson KL, Herrera-Velit P, and Reiner NE. 1α,25-Dihydroxyvitamin D3-induced myeloid cell differenti-


