Normoxic Stabilization of Hypoxia-Inducible Factor-1α by Modulation of the Labile Iron Pool in Differentiating U937 Macrophages: Effect of Natural Resistance–Associated Macrophage Protein 1

Helen J. Knowles,¹ David R. Mole,² Peter J. Ratcliffe,³ and Adrian L. Harris¹

¹Cancer Research UK Molecular Oncology Laboratory, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital and ²Henry Wellcome Building for Molecular Physiology, University of Oxford, Oxford, United Kingdom

Abstract

Hypoxia-inducible factor (HIF) is a transcription factor with major roles in many cellular and systemic responses to hypoxia. Activation of HIF pathways under hypoxia is mediated by suppression of the Fe²⁺- and O₂-dependent HIF hydroxylase enzymes that normally inactivate HIFα subunits. Mechanisms underlying induction of HIF in normoxic conditions are less clearly understood. In human cancers, infiltrating macrophages show up-regulation of HIF and it has recently been shown that normoxic expression of HIF-1α is essential for macrophage function. Here, we report studies of HIF-1α induction following phorbol-12-myristate 13-acetate (PMA)–induced differentiation of monocytic U937 and THP1 cells. HIF-1α was markedly up-regulated under normoxia in this setting and this involved failure of HIF-1α prolyl hydroxylation despite the presence of O₂. Fluorescence measurements showed that differentiation was associated with marked reduction of the labile iron pool. Both the reduction in labile iron pool and the up-regulation of HIF-1α were suppressed by RNA interference–mediated down-regulation of the iron transporter natural resistance–associated macrophage protein 1. Up-regulation of HIF-1α following PMA-induced differentiation was also abolished by addition of Fe²⁺ or ascorbate. These results indicate that physiologic changes in macrophage iron metabolism have an important effect on HIF hydroxylase pathways and suggest means by which the system could be manipulated for therapeutic benefit. (Cancer Res 2006; 66(5): 2600-7)

Introduction

Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor composed of a hypoxia-inducible α-subunit and a constitutively expressed β-subunit. Under hypoxic conditions, stabilization of HIFα results in formation of an active complex that regulates expression of genes, including angiogenic factors, glycolytic enzymes, and survival factors (1). Under normoxic conditions, HIFα is posttranslationally hydroxylated by the prolyl hydroxylase domain enzymes (PHD1-3), targeting it for interaction with the von-Hippel Lindau (VHL) E3 ubiquitin ligase complex and rapid proteasomal degradation (2, 3). These enzymes have an absolute requirement for O₂ and are therefore inactive under hypoxic conditions, allowing HIFα protein to accumulate (2, 3). The PHD enzymes also require 2-oxoglutarate, Fe²⁺, and ascorbate as cofactors. Accumulating evidence indicates that limiting availability of these cofactors may also activate the HIF response by impairing hydroxylation. We recently reported that ascorbate or iron supplementation can significantly blunt the HIF transcriptional response and ablate normoxic induction of HIF-1α by enhancing hydroxylase activity under conditions of growth factor or oncogenic activation of HIF (4).

Hypoxia is a fundamental microenvironmental component of solid tumor tissue and hypoxic induction of HIF-1α is evident in a range of cancers (5, 6). Many solid tumors are also characterized by the presence of an inflammatory infiltrate. High levels of macrophage infiltration have been associated with poor prognosis in cancers of the breast, cervix, and bladder (7). In breast cancer, high macrophage indices have been correlated with poor relapse-free and overall survival (8) and have been positively associated with angiogenic variables, such as increased blood vessel density (8) and high tumor vascular endothelial growth factor expression. In other cancer types, including prostate, non–small cell lung, glioma, and ovarian cancers, the association between macrophage infiltration and prognosis is less clear (7). HIF-positive tumor-associated macrophages have been described in cancers of the breast, lung, ovary, prostate, and pancreas (6, 9). Currently, only two studies have investigated macrophage HIF expression and prognosis. High macrophage expression of HIF-2α in breast cancer correlated strongly with high levels of angiogenesis and moderately with overall survival (10). In bladder cancer, a positive association was observed with both histologic grade and pathologic stage (11).

Recent experiments in vivo have shown HIF-1α expression to be essential for normal macrophage function. Conditional ablation of HIF-1α in murine myeloid cells greatly inhibited homotypic adhesion, motility, and invasion of ex vivo peritoneal macrophages (12). In vivo macrophage infiltration, edema formation, and tissue destruction were significantly reduced inmurine models of acute skin and chronic joint inflammation (12). The authors showed that HIF-1α is essential for the maintenance of intracellular energy homeostasis in macrophages. Even under normoxic tissue culture conditions, HIF-1α null macrophages had ATP levels reduced by >80% (12). This suggests a substantial role for HIF in macrophage physiology in a range of microenvironmental conditions and raises questions as to the mechanism of activation and functional importance of the response.

There is now substantial evidence for HIF induction by nonhypoxic stimuli, especially oncogenic mutation (ras, src, and PTEN) and growth factor stimulation (insulin, insulin-like growth...
factor-I, and angiotensin; ref 1). Other stimuli to induce normoxic HIF in macrophages include oxidized low-density lipoprotein (13), lipopolysaccharide (14), and the macrophage-derived peptide PR39 (15). It is therefore likely that within the complex growth factor-rich microenvironment of solid tumor tissue HIFα expression is regulated by local levels of stimuli as well as classic hypoxia-mediated induction. Supporting evidence for this hypothesis has been observed in a panel of lung carcinoma cases demonstrating neither HIF-1α nor HIF-2α tumor positivity, but strong HIF-2α expression within tumor-associated macrophages (6). The same study reported little HIFα protein expression within normal tissues, with the exception of some Kupffer cells and bone marrow macrophages, suggesting that macrophage HIFα expression may also be a feature of some stages of differentiation (6).

We have investigated normoxic expression of HIF-1α protein in a tissue culture model of macrophage differentiation. We show that HIF-1α accumulates in phorbol-12-myristate 13-acetate (PMA)- differentiated THP1 and U937 cells and that this is associated with a marked reduction in the intracellular labile iron pool, at least in part dependent on expression of the iron transporter natural resistance–associated macrophage protein 1 (Nramp1/Slc11a1). Supplementation with ascorbate to enhance PHD enzyme activity ablated PMA-mediated induction of HIF-1α and increased the labile iron pool. These data show a close interrelationship between the HIF pathway and cellular iron metabolism that might be manipulated with the potential to inhibit a range of protumorigenic macrophage functions.

Materials and Methods

Cell culture. Human monocyctic THP1 (acute monocytic leukemia) and U937 (myelomonocytic) cell lines and the human breast cancer cell line MDA468 were obtained from the Cancer Research UK cell service and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1-glutamine (2 mmol/L), penicillin (50 IU/mL), and streptomycin sulfate (50 μg/mL). Monocytic cells were induced to differentiate by treatment with 20 nmol/L PMA for 48 hours. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Lymphoprep (Axis-Shield UK Ltd., Huntingdon, United Kingdom) as described. Monocytes were selected by adherence and differentiated into macrophages by adhesion over 7 days. Hypoxic exposures (0.1% O2, 5% CO2, balance N2) were done in a Heto-Omega-170 incubator (RS Biotech, Irvine, Scotland). Following digestion of unhybridized species, samples were analyzed on a 6% agarose gel.

RNase protection assay. Total RNA was extracted in TRI Reagent (Sigma, Poole, United Kingdom) and dissolved in hybridization buffer [80% formamide, 40 mmol/L PIPES, 400 mmol/L NaCl and 1 mmol/L EDTA (pH 8)]. Probes for HIF-1α, PHD1, PHD2, PHD3 (2), and a U6 small nRNA internal control were labeled with 32P-CTP (Amersham Biosciences, Little Chalfont, United Kingdom) and hybridized to 20 μg RNA overnight at 55°C. Following digestion of unhybridized species, samples were analyzed on a 6% polyacrylamide sequencing gel.

Western blot. Cells were homogenized in lysis buffer (62 mmol/L urea, 10% glycerol, 5 mmol/L DTT, and 1% SDS plus protease inhibitors). Whole cell extract was separated by 8% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Primary antibodies were monoclonal mouse anti-HIF-1α (BD Transduction Laboratories, Lexington, KY) and anti-β-tubulin (Sigma, polyclonal rabbit anti-Nramp1 (AutoGenBio, Cambridge, United Kingdom), and sheep anticeruloplasmin (Abcam, Cambridge, United Kingdom). Antibody-treated HIF-1α was a rabbit antiserum raised against a synthetic peptide corresponding to residues 558 to 569 of human HIF-1α with a hydroxylated Pro396. Antihuman COOH-terminal ferroporin was provided by Prof. A. Townsend (Weatherall Institute of Molecular Medicine, Oxford, United Kingdom). Immunoreactivity was visualized with horseradish peroxidase–linked serum and chemiluminescence.

Measurement of PHD enzyme activity. Cytoplasmic extract from suspension and PMA-treated THP1 and U937 cells was assayed by measuring the interaction between a bead immunopurified biotinylated synthetic HIFα peptide and [35S]methionine-labeled VHL-α as described (16). Uncomplemented assays were incubated in the presence of extract and peptide alone. Fully complemented assays were additionally supplemented with 50 μmol/L FeCl3, 2 mmol/L 2-oxoglutarate, 2 mmol/L ascorbate, 2 mg/mL bovine serum albumin (BSA), and 0.3 mg/mL catalase. Captured VHL was quantified by SDS-PAGE and autoradiography using a phosphorimagener.

Measurement of the labile iron pool. Monocytic cells were seeded at 5 × 105 per well and either treated with 20 nmol/L PMA or induced to adhere directly to poly-l-lysine-coated six-well plates. The labile iron pool was assayed using the fluorescent iron chelator calcein-AM (Molecular Probes, Leiden, the Netherlands) as described (17). Briefly, cells were incubated in RPMI 1640 with 20 mmol/L HEPES and 1 mg/mL BSA and loaded with 250 nmol/L calcein-AM at 37°C for 10 minutes. Cells were washed with loading medium and transferred into 2 mL HBS [20 mmol/L HEPES, 150 mmol/L NaCl (pH 7.3)] + 10 mM L-glucose. Baseline calcein fluorescence was measured in a fluorescence plate reader (excitation 488 nm, emission 517 nm). The amount of intracellular iron bound to calcein was assessed by addition of the cell permeant Fe3+ chelator isonicotinyl salicylaldehyde hydrazone (100 μmol/L SIH, a gift from Prof. P. Ponka, Lady Davis Institute for Medical Research, Quebec, Canada) ref. 18) with continuous monitoring of the fluorescence signal.

Luciferase assay. U937 cells were transfected with either pGAL-α 775 to 826 (encoding the indicated amino acids of HIF-1α fused to the GAL4 DNA-binding domain; ref 19) or the mutated pGAL-α 775 to 826 (N803A), the GAL4-responsive luciferase reporter pUAS-tk-Luc and the pHRG-TK renilla luciferase reporter (Promega, Southampton, United Kingdom). Cells were then either treated with PMA for 48 hours or maintained in suspension culture. To assay for FII activity, cells were lysed in 200 μL cell culture lysis reagent (Promega). Luminescence in relative light units was read in a FLUOstar OPTIMA luminometer (BMG Labtech Ltd., Aylesbury, United Kingdom) and firefly luciferase values were normalized to renilla.

Nramp1 PCR. Total RNA was amplified using SuperScript One-Step reverse transcription-PCR with Platinum Taq (Invitrogen, Paisley, United Kingdom) and Nramp1-specific primers 5′-GGAACATCTAGGTCAGTACGTCTTC-3′ and 5′-CGAGTGCAAGTAGTGATTGTCG-3′ (20).

Nramp1 RNA interference. Small interfering RNA sequences were designed against NM_000578 (SLC11A1, Nramp1; Eurogentec, Southamp-ton, United Kingdom) and designated R1: sense 5′-GGUCUCGGCAUCUCUA- CUAChdT-3′ and antisense 5′-GUAGAAGAGUGGACACgCdtT-3′ (nucleotides 544-562), R2: sense 5′-GUAGAAGAGUGGACACcGdtT-3′ and antisense 5′-ACAGUGCUAGCGCAUCUGdT-3′ (nucleotides 594- 612) and R3: sense 5′-CCUCUCUUCCUCCUCUCUUdTdT-3′ and antisense 5′-AGGAAAGAGGAAGAGGATGdT-3′ (nucleotides 717-735). Small interfer-RNA sequences against HIF-1α and HIF-1α scrambled control were as described (21). Repeat 200 nmol/L transfections of double-stranded oligonucleotides were done with OligofectAMINE (Invitrogen) at 0 and 24 hours. Cells were harvested for analysis at 48 hours.

Iron uptake and release assays. Iron assays were done according to a modification of Draksmeth et al. (22). To make 5Fe-transferrin (16Fe-TF), apo-TF was loaded with 5FeCl3 (Perkin-Elmer, Boston, MA) to 66% saturation. To make 5Fe-nitroacetic acid (NTA), 17.5 nmol/L in RPMI 1640/0.2% BSA/L-glutamine. For uptake assays, cells were pretreated with ascorbate from 16 hours before iron loading or with 30 μmol/L holotransferrin at 0 hours. At given time points, cells were washed with cold PBS/0.1% NaN3 lysed in dH2O, and incorporated cellular radioactivity measured with a Packard Cobra γ-counter. For release assays, cells were iron-loaded for 48 hours, washed with PBS, and incubated in serum-free RPMI 1640/0.1% glucose. 5Fe-TF-loaded cells were pretreated with ascorbate from 16 hours before washing. At given time points, medium was removed and assayed for released radioactivity.

www.aacrjournals.org 2601 Cancer Res 2006; 66: (5). March 1, 2006
Results

PMA induces HIF-1α protein expression in THP1 and U937. To determine whether differentiation affects expression of HIF-1α, we treated THP1 and U937 cells with PMA for 48 hours and assayed expression of HIF-1α mRNA and protein (Fig. 1A). HIF-1α mRNA was unaffected by hypoxia or PMA treatment. HIF-1α protein accumulated following treatment with either condition alone, whereas treatment with hypoxia + PMA produced an additive or greater than additive effect (Fig. 1A).

We therefore assayed for possible effects of PMA on expression of the PHD enzymes (PHD1-3) that regulate HIF-1α protein stability. PHD1 mRNA was not regulated under any condition, with variable effects of PMA treatment on protein level expression (Fig. 1B). PHD2 mRNA and protein were up-regulated under hypoxic conditions, as reported (24). PHD2 mRNA was down-regulated by PMA, whereas protein level expression was either unaffected or increased (Fig. 1B). PHD3 was not detected in either cell line (data not shown). These data suggest that stabilization of HIF-1α protein under normoxia in PMA-treated cells was not likely because of deficient PHD enzyme expression.

PMA-induced HIF-1α is abrogated by Fe2+ or ascorbate. Under normoxic conditions, HIF-1α is posttranslationally hydroxylated by the PHD enzymes and targeted for proteasomal degradation (2, 3). We monitored the hydroxylation status of PMA-induced HIF-1α to determine whether it is stabilized due to lack of hydroxylation. As hydroxylation controls, cells were treated with either the proteasome inhibitor MG132 or the 2-oxoglutarate analogue MMOG. MG132 prevents degradation of HIF, resulting in accumulation of the hydroxylated species under normoxia (Fig. 2A). MMOG inhibits PHD enzyme activity, thus preventing hydroxylation of HIF-1α protein, which therefore accumulated as a nonhydroxylated species (Fig. 2A). THP1 and U937 cells were exposed to 0.1% O2 for 16 hours and transiently reoxygenated for sufficient time to allow PHD-mediated hydroxylation of HIF-1α but insufficient time for HIF-1α degradation to occur. Duplicate lysates were electrophoresed and blotted to allow comparison of the ratio of hydroxylated HIF-1α to total HIF-1α protein. Hydroxylated HIF-1α was evident following reoxygenation of both monocytic and PMA-stimulated THP1 and U937 cells (Fig. 2A). However, normoxic PMA-treated cells had undetectable levels of the hydroxylated species (Fig. 2A, compare lanes 2 and 3 for each cell line). Lack of hydroxylation of PMA-induced HIF-1α under normoxic conditions could be indicative of suboptimal activity of the hydroxylating PHD enzymes. Consideration of the PMA-treated samples in the MG132 lanes reveals suboptimal hydroxylation of the HIF-1α that accumulates during proteasomal inhibition when compared with monocytic (no PMA) controls, supporting our hypothesis that PMA leads to impaired hydroxylation of HIF-1α.

Ascorbate and Fe2+ are essential cofactors for the PHD enzymes, supplementation with which can enhance enzyme activity (4). Both 25 μmol/L ascorbate and 40 μmol/L FeCl3 completely abrogated PMA-mediated accumulation of HIF-1α in normoxic THP1 and U937 (Fig. 2B). Adhesion-induced differentiation of PBMC-derived monocytes/macrophages also induced normoxic expression of HIF-1α that was abrogated by treatment with 25 μmol/L ascorbate (Fig. 2C). We next assayed PHD activity directly by analysis of the amount of VHL captured by a bead-immunopurified HIFα construct (16). In uncomplemented assays lacking supplementation with Fe2+ or ascorbate, monocytic PHD activity was high compared with PMA-treated cells (Fig. 2D, shaded columns). This difference in activity was not reduced by Fe2+ and ascorbate supplementation (Fig. 2D, open columns). Thus, there may be differences in the ability of supplementary iron to access the enzyme active site in extracts compared with whole cells. Additionally, a decrease in enzyme expression could contribute to this result.

Direct addition of PMA to the assay of monocytic lysates did not inhibit PHD activity, showing that PMA does not directly inhibit the enzyme.

These data show that PMA treatment inhibits PHD enzyme activity and induces expression of a nonhydroxylated HIF-1α species. Although we do not rule out additional mechanisms of HIF accumulation (e.g., enhanced translation), it is evident that supplementation with essential PHD enzyme cofactors in the whole cell situation is sufficient to enhance enzyme activity and abrogate HIF-1α induced by differentiation.

PMA reduces the intracellular labile iron pool. We hypothesized that PMA treatment of monocytic cells might alter intracellular iron concentrations, either directly or via changes in ascorbate levels, resulting in limiting availability of free Fe2+ for use by cellular enzymes. To determine whether changes in the labile iron pool might underlie impairment of PHD enzyme activity following PMA-induced differentiation, we assayed the relative size of the labile iron pool in monocytic and PMA-treated U937 and THP1 cells using the fluorescent iron chelator calcein-AM. Control experiments achieved >60% reduction in the labile iron pool following 8-hour treatment of monocytic cells with the iron chelator desferrioxamine. PMA reduced the labile iron pool in THP1 cells from 419 ± 177 to 30 ± 18 arbitrary units (a.u., P < 0.001, unpaired t test) and in U937 from 80 ± 29 to 13 ± 8 a.u. (P < 0.01, unpaired t test; Fig. 3).

Figure 1. PMA stabilizes HIF-1α protein under normoxic conditions. Suspension and PMA-treated cells were incubated under normoxia (N) or hypoxia (H, 0.1% O2) for 16 hours. Total RNA and protein was assayed by ribonuclease protection assay or Western blot, respectively. Representative results for each experiment. A, top, HIF-1α mRNA with snRNA control. Bottom, HIF-1α protein with β-tubulin control. B, top, PHD1 and PHD2 mRNA with snRNA control. Bottom, PHD1 and PHD2 protein with β-tubulin control.
If the mechanism of PMA-induced HIF activation is through labile iron pool depletion, it might be predicted that other iron-containing enzymes would also be affected. FIH is an asparaginyl hydroxylase that regulates HIF transcriptional activity by hydroxylation of a residue within the COOH-terminal transactivation domain (CAD; ref. 25). FIH also requires Fe^{2+} as a cofactor and is inhibited by treatment with desferrioxamine, resulting in suboptimal availability of iron for use as a cofactor by the PHD enzymes.

**Inhibition of Nramp1 expression prevents accumulation of HIF-1α and enhances the labile iron pool.** Many cellular proteins regulate uptake and release of iron from the cell. Nramp1 is induced during terminal differentiation of HL-60 cells with either PMA or vitamin D (26) and has effects on intracellular iron availability (27, 28). We observed PMA-induced Nramp1 expression in THP1 and U937 cells at both the mRNA and protein level (Fig. 4B). We therefore hypothesized that in PMA-treated cells, Nramp1 might function to alter cellular iron availability and hence potentially contribute to the regulation of HIF-1α. Consequently, we designed RNA interference (RNAi) duplexes (R1-R3) specific for Nramp1 to enable direct analysis of its effect on HIF-1α expression. Initial experiments showed RNAi-mediated reduction in Nramp1 protein to be effective in the order R3 (65-70% knockdown) > R2 (55-60% knockdown; data not shown). R1 had no effect and effectively acted as an Nramp1 RNAi control. Experiments were done in U937 cells due to the greater transfection efficiency achieved with this cell line (data not shown). Both RNAi R3 and R2 caused knockdown of Nramp1 protein paralleled by almost complete ablation of PMA-induced HIF-1α expression (Fig. 4B, left and middle). This was not a nonspecific effect of these duplexes as in MDA468 cells that do not express Nramp1, transfection with Nramp1 RNAi did not affect levels of HIF-1α protein induced by hypoxia (Fig. 4B, right, results only shown for RNAi R3).

To determine whether this effect on HIF might indeed be mediated through effects on the labile iron pool, we measured SIH-chelatable iron levels in U937 + PMA treated with the most effective Nramp1 RNAi compared with those supplemented with 40 μmol/L FeCl₂. Nramp1 RNAi R3 significantly increased the labile iron pool (P < 0.005 versus all other conditions, unpaired t test; Fig. 4C) to 87% of the level of increase achieved with 40 μmol/L FeCl₂. Neither
HIF-1α RNAi or treatment with control RNAi species resulted in a labile iron pool significantly different from mock control.

These results show that, at least in U937 cells, inhibition of Nramp1 expression is sufficient to increase the labile iron pool with a concomitant dramatic decrease in HIF-1α protein expression. It is therefore of interest that PMA increased expression of the iron export proteins ferroportin (29) and ceruloplasmin (Fig. 4D; ref. 30) and that differentiation of macrophage-derived macrophages produced a similar expression profile with respect to Nramp1 and ceruloplasmin (Fig. 4E), providing further potential avenues for manipulating the labile iron pool.

Modest effects of ascorbate on cellular iron disposition. As both Nramp1 RNAi and ascorbate supplementation ablate PMA-induced HIF-1α, we considered whether ascorbate could also mediate this effect via changes in the cellular labile iron pool. We assayed the effect of 25 μmol/L ascorbate on the relative size of the labile iron pool in monocytic and PMA-treated THP1 and U937 cells using calcine-AM. Ascorbate treatment significantly increased the labile iron pool in monocytic (undifferentiated) THP1 and U937 cells 2-fold (P < 0.005) and 3-fold (P < 0.05), respectively (data not shown). However, although ascorbate also consistently increased the labile iron pool in PMA-treated cells, the effect was modest and not statistically significant [THP1 (29 ± 18 versus 57 ± 47 a.u.) and U937 (13 ± 7 versus 20 ± 8 a.u.)].

To pursue possible effects of ascorbate on cellular iron disposition, we assayed rates of cellular iron uptake and efflux in U937 cells treated with PMA.

Physiologic (25 μmol/L) ascorbate concentrations had no effect on the rate of Tf-dependent (59Fe-Tf) iron uptake (Fig. 5A) or on iron efflux following Tf-dependent uptake (Fig. 5B). A small but significant increase in iron uptake was observed via Tf-independent pathways with 59Fe-NTA (Fig. 5C, P < 0.001 versus control cells, two-way ANOVA), with no significant effect on the subsequent rate of iron efflux (Fig. 5D). In agreement with published reports (31, 32), nonphysiologic concentrations of ascorbate had strong effects on Tf-independent (Fig. 5C, P < 0.001 versus control, two-way ANOVA), but not Tf-dependent pathways of iron uptake. This shows a modest overall effect of physiologic (25 μmol/L) ascorbate to increase cellular iron uptake via Tf-independent pathways. Considering that the effect of ascorbate on the labile iron pool in PMA-treated cells was also not significant, these results suggest that direct modulation of cellular iron levels is likely not the primary mechanism whereby ascorbate mediates its effect on HIF-1α and PHD enzyme activity.

Discussion

In addition to mediating cellular responses to hypoxia, the HIF system has major functions in oxygenated cells (12) and can be activated by a range of nonhypoxic stimuli (1, 13–15). To better understand these processes, we analyzed induction of HIF in a cell line model of monocytic/macrophage differentiation. PMA treatment of monocytic cell lines causes them to adhere, cease proliferation, and acquire characteristics of cells of the macrophage lineage (6, 33, 34). We show clear induction of HIF-1α in these cells, despite apparently normoxic culture conditions, and show that this is associated with lack of hydroxylation of at least one of the prolyl residues that normally target normoxic HIF-α for destruction.

PMA did not act by reducing expression of the PHD enzymes that catalyze HIF prolyl hydroxylation. Indeed, PHD2 is thought to be the predominant enzyme regulating HIF-1α levels in normoxia (24), especially when relatively more abundant than the other PHD enzymes (35), and PHD2 levels were either maintained or increased. However, the PHD enzymes require Fe2+ as a cofactor (2, 3) and are readily inhibited by iron chelation (36). Iron and ascorbate supplementation of extracts from PMA-treated cells did not increase enzyme activity. This may be differences in the ability of supplementary iron to access the enzyme active site in extracts compared with whole cells.

However, PMA-induced HIF-1α was abrogated by iron supplementation in the whole cell situation, suggesting that limiting iron availability might be responsible for escape from hydroxylation and prompting us to measure changes in cellular iron.

The labile iron pool is a transitory intracellular pool of chelatable Fe2+ and Fe3+ bridging extracellular iron and that associated with cellular proteins or stored bound to ferritin (37). In quiescent cells, it is homeostatically maintained to meet metabolic demand while minimizing the potential for formation of reactive oxygen species. Measurement of the labile iron pool, by observing dequenching of calcine fluorescence following application of a cell-permeant iron chelator, showed large reductions in the labile iron pool on PMA-induced differentiation of U937 and THP-1.
Differentiation was associated with enhanced expression of the iron transport proteins ferroportin (29), ceruloplasmin (30), and Nramp1 (38, 39); the latter being of interest due to its phagocyte-restricted expression and critical function in macrophage mechanisms of innate immunity. Experimental suppression of Nramp1 expression raised labile iron pool levels and abrogated the induction of HIF-1α. This supports a direct link between changes in labile iron pool and regulation of HIF-1α and suggests that Nramp1 activity itself contributes to both the reduction of labile iron pool and HIF induction in macrophages. This additionally suggests that manipulation of the expression and/or activity of iron-regulatory proteins has the potential to affect PHD enzyme activity via modulation of cofactor availability, implying that the iron at the catalytic center can in some way exchange with the labile iron pool.

Nramp1 is an integral membrane glycoprotein expressed exclusively on endosomal, lysosomal, and phagosomal membranes of phagocytic cells (40). Although its function as a pH-dependent divalent cation transporter mediating iron translocation across vesicular membranes is clear, the direction of transport and manner in which it confers resistance to bacterial infection remain controversial (38, 39). Under one model, it is proposed that Nramp1 has Fe²⁺/H⁺ symport activity, functioning to pump iron out of the phagosome down a proton gradient to create an iron-poor intraphagosomal environment that inhibits bacterial growth (38, 39, 41). The alternative model proposes an Nramp1 antiport function, pumping iron into the phagosome where it serves as a catalyst for the Fenton/Haber-Weiss reaction and formation of highly toxic bacteriocidal hydroxyl radicals (38, 39, 42). The current controversy is resolved by the reported subcellular localization of hydrophilic calcine, the product of cleavage of lipophilic calcine-AM by cytosolic esterases, as being primarily cytosolic or nuclear (37) and being specifically excluded from the endosomal/lysosomal compartments of lymphocytic cells (43). Notably, the observed reduction in labile iron pool is consistent with effects of Nramp1 to reduce both total cellular iron (44) and the chelatable fraction (27), and with increased activity of iron-sensing protein IRP2 observed following transfection of active Nramp1 into RAW264.7 macrophages (45). Whatever the precise process, our findings suggest a further mechanism for Nramp1-associated pathogen resistance, namely a critical function for Nramp1 in normoxic activation of HIF in macrophages. In this respect, the recent demonstration of striking effects of genetic inactivation of HIF-1α on normoxic patterns of macrophage gene expression and on phenotypic properties, such as invasion and bacteriocidal action (12), are consistent with a major role for the HIF system in mediating normoxic macrophage functions relevant to innate pathogen resistance.

Abrogation of PMA-induced HIF-1α by iron supplementation suggests that reduction in the labile iron pool is directly responsible for activation of HIF. Nevertheless, there are other possibilities, including inactivation of the PHD enzymes by Nramp1-dependent free radical species (38, 39, 42), in an analogous manner to that proposed for inactivation of PHD2 by oxygen radical species generated in Ras-transformed junD-deficient fibroblasts (46).

Interestingly, PMA-induced HIF-1α was also abrogated by supplementation with ascorbate. We have previously shown that ascorbate can inhibit normoxic induction of HIF-1α under conditions of oncogenic or growth factor activation of cancer cell lines (4). We additionally showed that this was due to enhanced PHD enzyme activity resulting in enhanced HIF-1α ubiquitination and subsequent degradation, an effect lost under conditions of

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**Figure 4.** PMA-induced Nramp1 modulates HIF-1α and the labile iron pool. A. THP1 and U937 suspension and PMA-treated cells were incubated under normoxia or hypoxia (0.1% O₂) for 16 hours. Total RNA and protein were extracted and assayed for Nramp1 expression by PCR and Western blot. Top, Nramp1 mRNA with β-actin control. Bottom, Nramp1 protein with β-tubulin control. B. MDA468 and PMA-treated U937 cells were exposed to a double transfection of 200 nmol/L RNAi directed against Nramp1 (R1, R2, and R3), HIF-1α (HIF), and HIF-1α scrambled control (scr) or a mock transfection (m, OligofectAMINE alone). Total protein was extracted 48 hours after the initial transfection (including 16 hours hypoxic exposure for MDA468 cells) and assayed for Nramp1, HIF-1α, and β-tubulin expression by Western blot. Representative result of lysates probed from the same blot. C. PMA-treated U937 cells transfected with RNAi against Nramp1 (R3, R1), HIF-1α or HIF-1α scrambled control, mock transfectants (mock), untreated (−) cells, and cells treated with 40 μmol/L FeCl₂ for 4 hours (Fe⁺⁺) were loaded with 250 nmol/L calcine-AM and the labile iron pool assayed using SIH. Columns, mean from at least three independent experiments; bars, SD. D, suspension and PMA-treated THP1 and U937 cells assayed for ferroportin (FPN), ceruloplasmin (CPN), and β-tubulin expression by Western blot. E, PSMB-derived monocytes and macrophages were assayed for Nramp1, ceruloplasmin, and β-tubulin expression by Western blot.
Because ascorbate has been reported to increase the labile iron pool in K562 erythroleukemia cells (31) and monocytic U937 (32), we tested for effects of ascorbate on the labile iron pool and on cellular iron uptake/release. In agreement with these reports, a significant increase in the labile iron pool was observed in undifferentiated cells; however, increases in differentiated cells were modest and did not reach statistical significance. Furthermore, although high concentrations of ascorbate promoted substantial changes in cellular uptake (and subsequent efflux) of non-Tf-bound iron, effects of 25 μmol/L ascorbate (a concentration sufficient to ablate induction of HIF-1α) were modest. Calcein fluorescence measurement of the labile iron pool assays both forms of iron (Fe2+ and Fe3+) that exchange with the chelated species (17). It is thus possible that ascorbate acts to increase the availability of the Fe2+ PHD enzyme cofactor in a manner not detected by changes in the labile iron pool, or acts more specifically at the active site to reduce oxidized forms of the iron-enzyme complex that arise following uncoupled cycles as has been proposed for its action on the collagen prolyl hydroxylases.

Despite uncertainties regarding the mechanism, major effects to suppress HIF-1α were observed with physiologic concentrations of ascorbate (normal plasma range 20-60 μmol/L; ref. 47). Tissue culture systems that lack a source of ascorbate may be particularly sensitive to ascorbate deficiency. However, monocytic cell lines and monocyte-derived macrophages that are ascorbate-deficient in culture (32) can be used to model clinical conditions associated with reduced circulating levels of ascorbate. Plasma ascorbate levels decrease with increasing tumor stage in breast and cervical cancer (48) and to levels indicative of incipient clinical scurvy in lung cancer patients. Reduced WBC ascorbate levels are also evident in myeloid and chronic lymphatic leukemia. Interestingly, both breast and lung cancers show HIFα-positive macrophages (6, 9, 10). It is also evident that ascorbate can affect clinical iron status. Ascorbate supplementation increased serum ascorbate from 0.47 to 0.75 mg/dL in a deficient population, with concomitant improvement in iron status as measured by serum iron and ferritin levels and Fe-Tf saturation. Ascorbate also improved iron status in patients with erythropoietin hyporesponsiveness and functional iron deficiency (49).

In summary, we have shown that HIF-1α is induced during PMA-mediated differentiation of monocytic cells in a manner that is independent of oxygen, dependent on Nramp1, and associated with a reduction in the labile iron pool. The data suggest that physiologic alterations in cellular iron disposition have the potential to manipulate the HIF response through regulation of PHD activity and that external provision of iron/ascorbate should have important biological effects on the pathophysiologic operation of the system. Our results additionally have potential therapeutic implications, especially considering the substantial evidence for a detrimental role of macrophages in some human cancers and that these cells additionally both up-regulate HIFα and require it for normal function.

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References

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Helen J. Knowles, David R. Mole, Peter J. Ratcliffe, et al.


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