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Advances in Brief

Effect of Ascorbate on the Activity of Hypoxia-inducible Factor in Cancer Cells[1]

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Abstract

Hypoxia-inducible factor (HIF) plays an important role in determining patterns of gene expression in cancer. HIF is down-regulated in oxygenated cells by a series of Fe (II) and 2-oxoglutarate dependent dioxygenases that hydroxylate specific residues in the regulatory HIF-α subunits. Because these enzymes require ascorbate for activity *in vitro* we analyzed the effects of ascorbate on HIF in human cancer cell lines. Ascorbate at physiological concentrations (25 μM) strikingly suppressed HIF-1α protein levels and HIF transcriptional targets, particularly when the system was oncogenically activated in normoxic cells. Similar results were obtained with iron supplementation. These results indicate that both ascorbate and iron availability have major effects on HIF, and imply that the system is commonly regulated by limiting hydroxylase activity under normoxic tissue culture conditions.

Introduction

HIF[3] is an $\alpha\beta$ heterodimeric transcription factor that directs a broad range of responses to hypoxia. The HIF transcriptional cascade is activated in cancer and plays a central role in the malignant phenotype, contributing to increased angiogenesis, enhanced glycolysis, and other properties that promote tumor growth (1). HIF-β subunits are constitutive proteins, and regulation of the active complex is achieved through a multistep process affecting the abundance and activity of HIF-α subunits. HIF-α subunits are post-translationally modified by a series of oxygen-dependent enzymatic hydroxylations at specific amino acid residues (2–5). Prolyl-4-hydroxylation at two sites within a central degradation domain by a set of closely related Fe^{2+} and 2-OG-dependent dioxygenases (PHD 1–3) mediates interactions with the VHL E3 ubiquitin ligase complex that targets HIF-α for proteasomal degradation (2, 3). In a second hydroxylation-dependent control, β-hydroxylation of an asparaginyl residue in the COOH-terminal activation domain by another Fe^{2+} and 2-OG-dependent dioxygenase, FIH, inhibits transcriptional activity by preventing interaction with the p300/CBP coactivator (4, 5). The HIF hydroxylases are absolutely dependent on dioxygen, and limitation of activity in hypoxia allow HIF-α to escape proteolysis and become transcriptionally active. Similarly, hydroxylase inhibition by iron chelators, transition metals, and 2-OG analogues account for activation of the HIF system by these compounds (2–5). Enzymes of this type are also known to be variably dependent on ascorbate for full catalytic activity (6), and ascorbate promotes HIF hydroxylase activity *in vitro*, raising important questions as to whether and in what way ascorbate availability affects the HIF system in the intact cell. Here we report major effects of ascorbate and iron supplementation on HIF-1α levels and the HIF transcriptional response, particularly under conditions of oncogenic activation of HIF in normoxic cells.

Materials and Methods

Cell Culture. PC3 (human prostate adenocarcinoma), OVCAR3 (ovarian carcinoma), HS578T, MDA 468, and MCF7 (breast carcinoma) cell lines were obtained from the Cancer Research United Kingdom cell service and maintained in RPMI 1640 or DMEM supplemented with 10% fetal bovine serum, L-glutamine (2 μM), penicillin (50 IU/ml), and streptomycin sulfate (50 μg/ml). The VHL-deficient RCC4 renal carcinoma cell line and its counterpart containing a stably transfected VHL gene were cultured in DMEM and maintained in selection with 500 μg/ml G418. The Chinese hamster ovary cell-derived transfectant E48.4.51, containing a stably integrated HRE-linked firefly luciferase reporter gene, was cultured in Ham's F12 medium supplemented as above, and maintained in selection with puromycin (5 μg/ml) and hygromycin (0.75 mg/ml). Total iron levels in batches of these media (mean $\pm$ ISD) were 23.0 $\pm$ 2.7 μM (RPMI 1640), 25.7 $\pm$ 2.3 μM (DMEM), and 24.5 $\pm$ 7.5 μM (Ham's F12) as measured by ferrozine colorimetric assay. L-Ascorbic acid sodium salt, bovine insulin, and iron-poor (apo) and iron-saturated (holo) transferrin were obtained from Sigma (Poole, United Kingdom), and recombinant human IGF-I was obtained from R&D Systems (Abingdon, United Kingdom). Hypoxic exposures (0.1–3.0% O_2, 5% CO_2, and balance N_2) were performed in a Heto-Holten CellHouse 170 incubator (RS Biotech, Irvine, Scotland).

Western Blotting. Cells were homogenized in lysis buffer (6.2 M urea, 10% glycerol, 5 mM 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 mouse anti-HIF-1α monoclonals reactive to human (BD Transduction Laboratories, Lexington, KY) or mouse (Novus Biologicals, Littleton, CO) HIF-1α and a mouse anti-β-tubulin monoclonal antibody (Sigma). Immunoreactivity was visualized with horseradish peroxidase-linked goat antimouse serum and chemiluminescence.

***In Vitro* Ubiquitination Assay.** HIF-1α polyubiquitylation was assayed as described previously (7). Briefly, [^{35}S]methionine-labeled HIF-1α was synthesized in the presence and absence of 2 mM ascorbate (pH 7.5) by coupled transcription and translation in rabbit reticulocyte lysate (Promega, Southampton, United Kingdom).Ubiquitylation assays were performed by mixing 4 μl of 5 mg/ml ubiquitin (Sigma), 4 μl of 10$\times$ ATP regenerating system, 0.66 μl of 150 μM ubiquitin aldehyde (Affiniti Research Products, Exeter, United Kingdom), 30.5 μl of RCC4/VHL cell extract, and 2 μl of HIF-1α *in vitro* transcription and translation in the presence and absence of 2 mM ascorbate. The reaction mix was incubated at 30°C, and aliquots were removed into SDS sample buffer at the indicated times and analyzed by SDS-PAGE and autoradiography.

Luciferase Assay. E48.4.51 cells were seeded at 1×10^5 cells/well in six-well plates 24 h before experimental treatments. Cells were then lysed in 400 μl of cell culture lysis reagent (Promega) before addition of luciferase assay reagent. Luminescence in relative light units was read in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) and normalized relative to total protein content.

RNase Protection Assay. Total RNA was extracted in TRI Reagent (Sigma) and dissolved in hybridization buffer [80% formamide, 40 mM PIPES, 400 mM sodium chloride, and 1 mM EDTA (pH 8)]. Fifteen μg of RNA was

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[3] The abbreviations used are: HIF, hypoxia-inducible factor; 2-OG, 2-oxoglutarate; VHL, von Hippel-Lindau; IGF, insulin-like growth factor; VEGF, vascular endothelial growth factor; DFO, desferrioxamine; MMOG, dimethyloxalylglycine; PHD, prolyl hydroxylase domain; FIH, factor inhibiting HIF; CBP, CREB binding protein; HRE, hypoxia response element; sn, small nuclear; TOR, target of rapamycin.

analyzed for Glut-1 and VEGF using U6 small nuclear RNA as an internal control.

VEGF ELISA. VEGF secretion into the culture medium was measured using a Quantikine Human VEGF Immunoassay (R&D Systems, Minneapolis, MN).

Results

Effect of Ascorbate on HIF-1α Protein Induced by Hypoxia, Cobalt, and DFO. Initial experiments focused on defining the effects of ascorbate on induced levels of HIF-1α in a cell line that manifests high-level induction of the protein from a low baseline level in normoxic culture. Treatment of MDA468 breast carcinoma cells with 100 μM $CoCl_2$, 100 μM DFO, or exposure to hypoxia (0.4% O_2) induced high-level expression of HIF-1α protein. In the presence of 400 μM ascorbate, levels of HIF-1α induced by DFO were reduced 3-fold, and HIF-1α induction in response to $CoCl_2$ was inhibited completely (Fig. 1*A*). In contrast, ascorbate had little or no effect on the level of HIF-1α induced by severe hypoxia.

Ascorbate Reduces Levels of HIF-1α in Normoxic Cells and Prevents Induction by Growth Factors. A number of transformed cell lines have been reported to manifest increased levels of HIF-1α in normoxic culture. We next wished to determine whether ascorbate had effects on HIF-1α expression in this situation. PC3 prostate carcinoma cells, which are functionally defective for PTEN (8) and p53 (9), and demonstrate high basal levels of HIF-1α protein (10), were therefore exposed to ascorbate under otherwise standard conditions of normoxic tissue culture. Concentrations of ascorbate ranging from 25 to 400 μM reduced HIF-1α to undetectable levels under these conditions, indicating that concentrations well within the physiological range (approximately 25–50 μM) had striking effects on the HIF system. In contrast, hypoxia-induced HIF-1α was again unaffected by even the highest dose of ascorbate (Fig. 1*B*). Time course experiments indicated that 25 μM ascorbate had a striking inhibitory effect under normoxia that lasted for at least 24 h (Fig. 1*C*). Additional experiments on ovarian (OVCAR3) and breast carcinoma (HS578T) cells demonstrated that this phenomenon extends to other cell lines that express high steady state levels of HIF protein (Fig. 1*D*).

High levels of HIF-1α can also be induced under normoxia by stimulation with specific growth factors. Both IGF-I (25 nM) and insulin (100 nM) induced HIF-1α in serum-deprived MCF7 cells. Again this stimulation of HIF-1α expression was strikingly inhibited by 25 μM ascorbate (Fig. 1*E*).

Effects of Ascorbate Are Mediated by Actions on HIF Hydroxylases. Previously, biological requirements for ascorbate have generally been assigned to effects on the procollagen PHDs, which are required for the hydroxylation and stabilization of collagen. The current experiments suggest that, under commonly used tissue culture conditions, there are also major effects of ascorbate on the HIF hydroxylases. However, ascorbate, at least in higher concentrations, has the potential for other biochemical activities. These include generalized redox activities encompassing both reducing and oxidant effects, such as the generation of oxygen radical species by Fenton chemistry (11). Previous work has established that both procollagen and HIF PHDs can be powerfully inhibited by certain oxoglutarate analogues (2, 3, 6). Therefore, we hypothesized that if the action of ascorbate was via promotion of HIF PHD activity, the effects should be lost under conditions of enzyme blockade by an oxoglutarate analogue, whereas potential actions through other redox mechanisms would be unaffected. To test this we exposed PC3 cells to the oxoglutarate analogue MMOG in the presence or absence of ascorbate. As expected, 1 mM MMOG elevated HIF-1α protein levels in normoxic PC3 cells. Strikingly, ascorbate had no effect at all on HIF-1α levels under these conditions, strongly supporting an action on HIF hydroxylase activity (Fig. 2*A*). In cells that are functionally defective for VHL HIF-1α is stabilized irrespective of hydroxylation, leading to constitutively high levels of the protein. Consistent with the action of ascorbate being via hydroxylase activity, no effect was observed in VHL-defective renal carcinoma cells (Fig. 2*B*). Because hydroxylation of HIF-α promotes VHL-dependent ubiquitylation it would be predicted that promotion of hydroxylase activity by ascorbate would be manifest in *in vitro* assays of VHL-dependent HIF-α ubiquitylation. Data shown in Fig. 2*C* indicates that this is indeed the case. Taken together these results demonstrate that the action of ascorbate is on HIF hydroxylase activity, and indicate that in pVHL-competent cells, in the absence of ascorbate supplements, HIF hydroxylase activity is limiting for HIF proteolysis under normoxic tissue culture conditions.

Because ascorbate may act to increase Fe(II) availability at the hydroxylase active site, we hypothesized that it might also be possible to promote hydroxylase activity, and, hence, HIF-1α proteolysis, by iron supplementation. Addition of exogenous iron either as $FeCl_2$ or iron-loaded transferrin, but not addition of an equimolar amount of iron-poor transferrin, strikingly reduced HIF-1α levels in normoxic

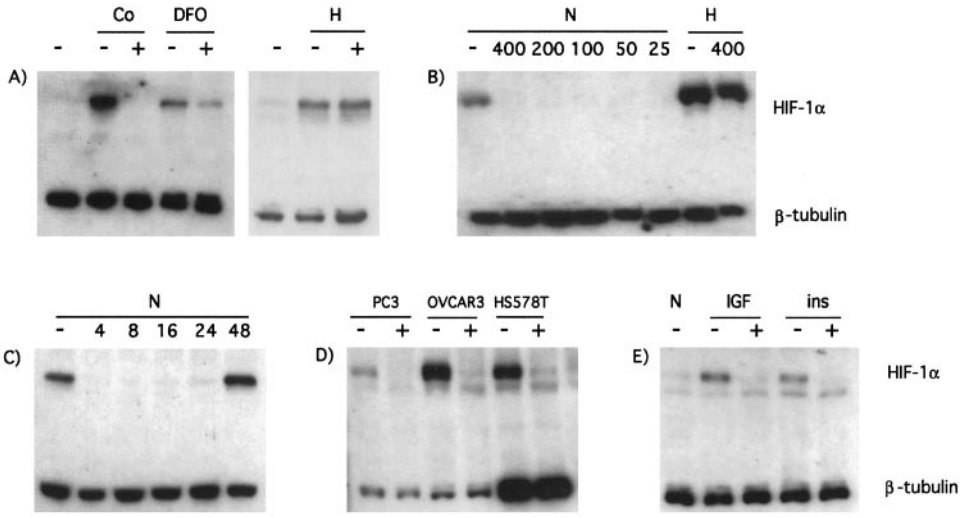

Fig. 1. *A*, Western blot analysis of HIF-1α protein in MDA468 cells treated for 8 h with $CoCl_2$ (*Co*), DFO, or hypoxia (*H*) in the presence (+) or absence (−) of ascorbate in comparison with an untreated control. *B*, Western blot analysis of HIF-1α protein in PC3 cells treated with 0–400 μM ascorbate for 8 h under either normoxic (*N*) or hypoxic conditions (*H*; 0.4% O_2). *C*, Western blot showing the effect of 25 μM ascorbate on HIF-1α in normoxic (*N*) PC3 cells over a 0–48 h time course. *D*, HIF-1α Western blot of total protein extract from normoxic PC3, OVCAR3, and HS578T cells after 24-h treatment with 25 μM ascorbate (+) in comparison with untreated controls (−). *E*, Western blot for HIF-1α in total protein extract from MCF7 cells after 36-h serum deprivation (0.1% fetal bovine serum in DMEM) and 4-h treatment with IGF-I (*IGF*) or insulin (*ins*) in the presence (+) or absence (−) of 25 μM ascorbate. *N*, unstimulated control.

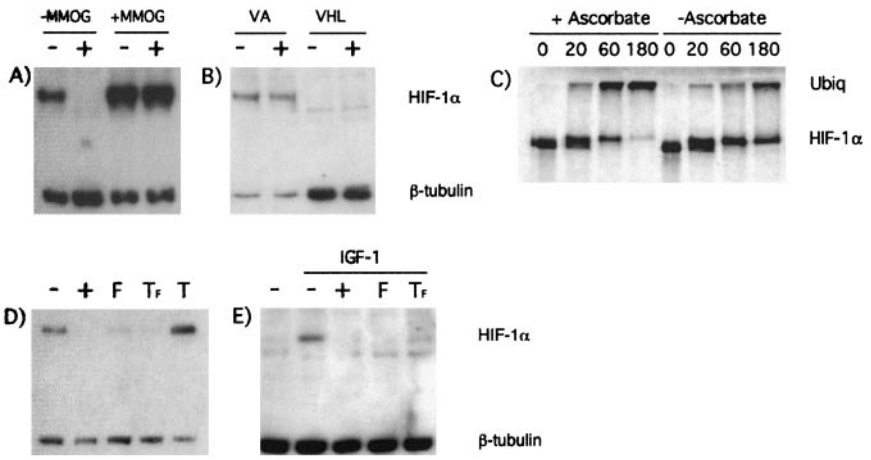

Fig. 2. *A*, Western blot for HIF-1α in PC3 cells after treatment with 1 mM MMOG for 6 h in the presence (+) or absence (−) of 25 μM ascorbate. *B*, Western blot for HIF-1α protein in VHL-defective RCC4 cells (*VA*) and RCC4 cells transfected with a functional VHL gene (*VHL*) treated with 25 μM ascorbate (+) for 4 h. *C*, assay of polyubiquitination of HIF substrate by RCC4 VHL cell extract over a 20–180-min time course in the presence or absence of 2 mM ascorbate. *D*, Western blot for HIF-1α in PC3 cells treated for 4 h with 25 μM ascorbate (+), 40 μM $FeCl_2$ (*F*), 40 μM iron-loaded transferrin (T_F), or 40 μM iron-poor transferrin (*T*). *E*, Western blot demonstrating inhibition of IGF-I (25 nM) induced HIF-1α with 25 μM ascorbate (+), 40 μM $FeCl_2$ (*F*), or 40 μM iron-loaded transferrin (T_F).

PC3 cells (Fig. 2*D*) and prevented the induction of HIF-1α by growth factors in MCF7 cells (Fig. 2*E*).

Effects of Ascorbate on the HIF Transcriptional Response. To determine whether the effects of ascorbate on HIF-1α protein are reflected in the activity of the HIF transcriptional response, we analyzed effects on HRE-mediated reporter gene expression using a stably transfected Chinese hamster ovary cell line (E48.4.51) that expresses an HRE-regulated luciferase gene. Because this cell line has very low basal levels of HIF-1α and manifests minimal luciferase activity in normoxia (data not shown), transcriptional effects of ascorbate under normoxic conditions could not be studied. However, HRE-mediated luciferase reporter activity was strikingly induced by exposure of cells to $CoCl_2$ and DFO, and this activity was reduced by 40–60% by concurrent exposure of cells to ascorbate (Fig. 3*A*).

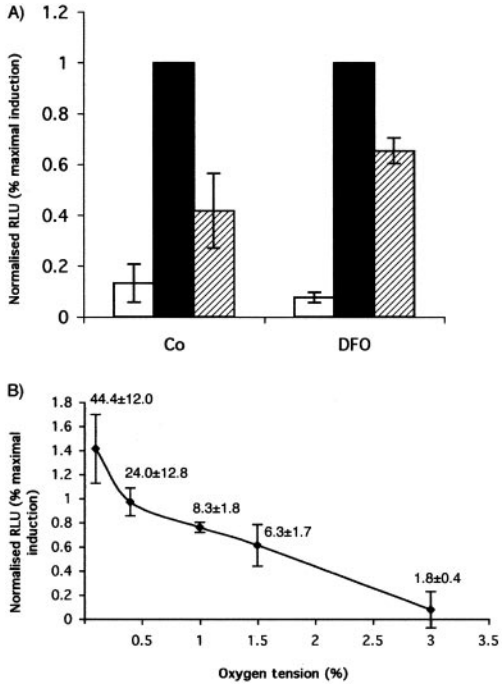

Fig. 3. Effect of ascorbate on HRE reporter gene activity. Values for luciferase assays are expressed as a fraction of the level of induction achieved after 16-h stimulation in the absence of ascorbate. Each point represents the mean of at least three independent experiments; *bars*, ± SD. *A*, stimulation with 100 μM $CoCl_2$ or 100 μM DFO (■) in comparison with the untreated control (□). The level of inhibition achieved with 25 μM ascorbate (▨) is significant at the $P < 0.001$ level in both cases. *B*, hypoxic stimulation with 0.1–3.0% O_2. Each *point* is annotated with the actual level of induction observed at that O_2 tension.

Responses were assessed to graded hypoxia over the range 0.1–3.0% O_2. In concordance with the lack of effect on induction of HIF-1α protein under hypoxia, ascorbate did not inhibit HRE-mediated transcription under stringent hypoxia (0.1–0.4% O_2; Fig. 3*B*). However, the transcriptional response induced by more moderate hypoxia (1.0–3.0% O_2) was partially inhibited by 25 μM ascorbate, with strongest effects being observed at the higher O_2 tensions. Indeed, the 1.8-fold induction of luciferase seen under 3.0% O_2 was blocked completely in the presence of ascorbate (Fig. 3*B*).

To additionally pursue the biological importance of ascorbate in regulation of the HIF transcriptional cascade we examined effects on the expression of HIF target genes. Normoxic mRNA levels of the glucose transporter Glut-1 were measured by RNase protection assay. Glut-1 mRNA was strikingly reduced by ascorbate in PC3 cells, which express relatively high levels of HIF-1α (Fig. 4*A*), and reduced to a lesser extent in MDA468 cells, which express much lower levels of HIF-1α under normoxia (Fig. 4*B*). In neither cell line was the hypoxia-inducible level of Glut-1 mRNA affected by ascorbate. Similar results were obtained in assays of VEGF mRNA levels, although the magnitude of the effect was variable between cell lines and somewhat less than for Glut-1. Results in normoxic unstimulated MDA468 cells are shown in Fig. 4*C*. Consistent with the effects observed on HIF-1α protein levels, ascorbate also had significant effects on the induction of VEGF by growth factors, substantially reducing the level of VEGF mRNA induced by both IGF-I and insulin (Fig. 4*D*).

Finally we tested for effects on VEGF secretion in normoxic cells. In cells grown for 24 h in normoxic culture with and without supplementation, addition of ascorbate (25 μM) inhibited VEGF secretion into the medium by both MDA 468 (56.1 ± 5.4 pg VEGF/10^4 cells *versus* 71.86 ± 4.9, $P < 0.005$) and PC3 cell lines (12.4 ± 1.1 pg VEGF/10^4 cells *versus* 18.9 ± 0.6, $P < 0.01$).

Discussion

In this study we have demonstrated large effects of physiological concentrations of ascorbate on expression and activity of the HIF system. Effects on HIF-1α were specific to particular conditions, strongly suggesting that they are mediated by promotion of PHD activity. Thus, consistent with the absolute requirement for dioxygen as cosubstrate for this class of enzyme, no effect was observed under stringent hypoxia. Equally, ascorbate had no action in the presence of enzyme blockade by the 2-OG analogue MMOG or in the absence of a functional VHL. In contrast, marked effects were seen on cells exposed to Co(II), DFO, or moderate hypoxia, and on oncogenically activated normoxic cells. Effects on HIF transcriptional target genes

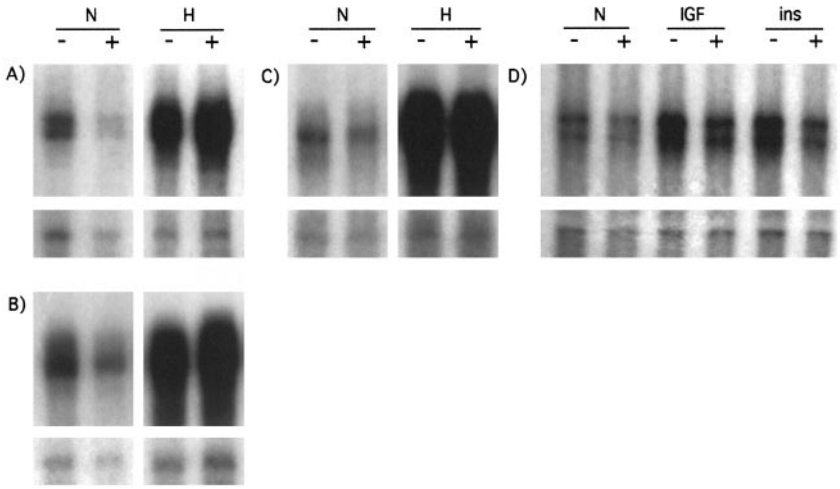

Fig. 4. RNase protection assays demonstrating the effect of 25 μM ascorbate (+) under normoxia (*N*) and after 16 -hypoxic induction (*H;* 0.4% O_2) on the level of Glut-1 mRNA in (*A*) PC3 cells and (*B*) the MDA468 cell line, and also (*C*) on VEGF mRNA levels in MDA468s. *D,* VEGF RPA on serum-deprived MCF7 cells under normoxia (*N*) and after 8-h stimulation with 25 nM IGF-I (*IGF*) or 100 nM insulin (*ins*) in the presence (+) or absence (−) of 25 μM ascorbate. The *bottom panel* in each case represents the U6 snRNA internal loading control.

demonstrate the biological importance of the effect and suggest that ascorbate may also promote FIH activity, although the current study cannot distinguish separate effects on the different HIF hydroxylases.

Previous studies of the biological action of ascorbate have focused on the collagen prolyl hydroxylase a functionally related set of Fe(II) and 2-OG dependent dioxygenases. The mechanism of action remains incompletely understood. Under normal turnover conditions ascorbate is not consumed stoichiometrically (6). However, ascorbate consumption can be promoted using artificial substrates that result in uncoupled decarboxylation of 2-OG. It is postulated that uncoupled cycles result in conversion of the enzyme-bound Fe(II) to Fe(III), that this self-oxidation occurs occasionally even under normal turnover conditions, and that the action of ascorbate is to reduce this to the active Fe(II) form within the enzyme active site (6). An alternative or additional possibility is that ascorbate might support the provision of Fe(II) within an intracellular pool that can replenish the active site with Fe(II). That such a mechanism may account, at least in part, for the effects we describe is suggested by several observations. First, we found that iron supplementation itself was able to reduce steady-state HIF-1α levels in normoxic cells in a manner similar to ascorbate. In previous work it has been shown that iron supplements antagonize the HIF-regulated induction of erythropoietin gene expression by cobaltous ions (12), most probably by increasing the availability of Fe(II) so as to compete out the incorporation of Co(II) at the hydroxylase active site. In the current work we found that ascorbate was also able to antagonize the effect of exposure to cobaltous ions and DFO. Taken together these observations are most compatible with an action of ascorbate to promote the availability of Fe(II) to the active site of the enzyme, although it is also possible that ascorbate itself chelates Co(II).

Whatever the precise mechanism, our findings indicate that ascorbate has large effects on the HIF system through promotion of HIF hydroxylase activity. This implies that without ascorbate or iron supplementation, enzyme activity is submaximal, and that under commonly used normoxic tissue culture conditions this enzyme activity is limiting for HIF proteolysis. Many previous studies have documented positive effects of oncogenic pathways on HIF in tissue culture cells (13–15). In keeping with this we found elevated levels of HIF-1α in normoxic OVCAR3 and PC3 cells, which carry loss-of-function mutations for p53 (16), and both p53 (9) and PTEN (8), respectively, and in HS578T cells that express a constitutively activated H-ras (17). Effects of ascorbate and iron supplementation were particularly striking in these cells and under conditions of growth factor stimulation, amounting to complete abrogation of immunodetectable HIF-1α. These findings fit well with recent reports that oncogenically stimulated HIF-1α may accumulate in a nonhydroxylated form in the presence of oxygen (18) and indicate that in the future it will be important to consider the availability of ascorbate when analyzing the effects of oncogenic stimulation of the HIF system.

The reasons why escape from hydroxylation is particularly evident under these conditions are unclear. Enhanced HIF-1α translation has been demonstrated after activation of the phosphatidylinositol 3′-kinase/TOR pathway by either activated pp60c-src (14) or insulin (15), and it seems likely that in different cells other oncogenic and mitogenic signal pathways enhance HIF translation. Increased HIF translation might present an increased substrate load that exceeds the capacity of the HIF hydroxylases. Alternatively it is possible that during conditions of enhanced cellular growth the supply of Fe(II), ascorbate or both is impaired, effectively reducing hydroxylase activity. In support of this, it is well established that proliferating cells have a greater requirement for iron and manifest greater susceptibility to the toxicity of iron chelators (19).

In addition to the implications for the study of oncogenic activation of HIF in tissue culture cells, the findings could also be relevant to the regulation of the HIF system *in vivo*. At the systemic level, ascorbate is synthesized in the liver and kidney of most mammals (except primates, which lack the necessary gulonolactone oxidase activity) or obtained from dietary sources. At the cellular level ascorbate is transported into the cells by the Slc23a1 anion transporter (20) or replenished by the reduction of dehydroascorbate (21). Whether ascorbate and/or iron deficiency affects activity of the HIF system in cancer *in vivo* will require additional investigation. Database predictions suggest that many additional members of this class of enzyme with as yet unknown functions, and ascorbate dependence, exist. Thus, effects on overall tumor growth could be determined by the summation of several biological effects, and may be difficult to predict, perhaps contributing to the uncertainties and controversies surrounding the effects of ascorbate on cancer progression and treatment (22). Nevertheless, these new insights into the potential effects of ascorbate deficiency should enable a better understanding of its biological role in malignant and nonmalignant tissues.

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