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Cobalt-induced Oxidant Stress in Cultured Endothelial Cells: Prevention by Ascorbate in Relation to HIF-1 α

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Abstract

Endothelial cells respond to hypoxia by decreased degradation of hypoxia-inducible factor 1 α (HIF-1 α), accumulation of which leads to increased transcription of numerous proteins involved in cell growth and survival. Ascorbic acid prevents HIF-1 α stabilization in many cell types, but the physiologic relevance of such effects is uncertain. Given their relevance for angiogenesis, endothelial cells in culture were used to evaluate the effects of ascorbate on HIF-1 α expression induced by hypoxia and the hypoxia mimic cobalt. Although Ea.hy926 cells in culture under oxygenated conditions did not contain ascorbate, HIF-1 α expression was very low, showing that the vitamin is not necessary to suppress HIF-1 α . On the other hand, hypoxia- or cobalt-induced HIF-1 α expression/stabilization was almost completely suppressed by what are likely physiologic intracellular ascorbate concentrations. Increased HIF-1 α expression was not associated with significant changes in expression of the SVCT2, the major transporter for ascorbate in these cells. Cobalt at concentrations sufficient to stabilize HIF-1 α both oxidized intracellular ascorbate and induced an oxidant stress in the cells that was prevented by ascorbate. Whereas the interaction of ascorbate and cobalt is complex, the presence of physiologic low millimolar concentrations of ascorbate in endothelial cells effectively decreases HIF-1 α expression and protects against cobalt-induced oxidant stress.

Keywords

ascorbate transport; dehydroascorbate uptake; HIF-1 α ; hypoxia; cobalt; SVCT2; endothelial cells

1. Introduction

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that regulates many crucial cellular functions based on availability of molecular oxygen in the cell. These functions include activation of the genes for proteins that determine glucose transport and metabolism, iron transport, angiogenesis, and cell survival versus apoptosis [1–3]. HIF-1 functions as a heterodimer composed of α and β subunits; its levels are regulated in part by post-translational modification of the α subunit [3,4]. The latter involves hydroxylation on selected proline and asparagine residues by specific 2-oxoglutarate dioxygenases, which are related to enzymes that hydroxylate proline and lysine to stabilize pro-collagen and allow its folding into mature collagen [5–7]. Proline hydroxylation in HIF-1 α involves reaction of molecular oxygen and 2-oxoglutarate at an active site containing non-heme ferrous iron [2,5,7]. This hydroxylation in turn targets HIF-1 α for proteosomal degradation through its binding to von Hippel-Lindau protein. When oxygen levels are high, HIF-1 α is hydroxylated and degraded, lowering its intracellular levels and downstream effects. During hypoxia,

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HIF-1 α is not modified and thus can accumulate and migrate to the nucleus to generate its actions [4,6,7]. Vitamin C, or ascorbic acid, facilitates hydroxylation of HIF-1 α , probably by maintaining intracellular or active site iron in its reduced and active ferrous form [2,6,8]. The cellular content of ascorbate modifies HIF-1 α levels and its downstream gene transcriptional activities in a variety of cancer [8] and other cell types [9], including endothelial cells. Regarding the latter, Vissers, et al. [10] showed in both immortalized and primary culture endothelial cells that ascorbate was required to suppress HIF-1 α levels that had increased due to both hypoxia and to cobalt.

Non-redox cycling metal ions such as cobalt and nickel inhibit the HIF-1 α hydroxylase and thus prevent proteosomal degradation of HIF-1 α [2,11]. The mechanism by which such metal ions deactivate the HIF-1 α hydroxylase is a matter of controversy. On the one hand, they could replace the active site iron in HIF-1 α hydroxylase [2,12]. On the other hand, it has been suggested the metal ions form complexes with facilitating proteins that in turn oxidize ascorbate and thereby prevent it from lowering HIF-1 α levels [11,13,14]. Thus, treatment of lung cancer cells with nickel oxidized both extra- and intracellular ascorbate, depletion of which was associated with increased stabilization of HIF-1 α [9,13,15]. Conversely, addition of ascorbate to cobalt- or nickel-treated cells prevented both HIF-1 α expression and subsequent downstream HIF effects [13,15]. Yet to be resolved is whether these effects occurred at intracellular ascorbate concentrations that might be expected in cells *in vivo*.

Further, if metal ions such as cobalt can oxidize intracellular ascorbate, they may cause cellular oxidant stress either directly or indirectly through ascorbate depletion. Such an effect might underlie their well known ability to cause cellular toxicity [11]. In this work we used an endothelial cell line, EA.hy926, to evaluate the interaction of cobalt and ascorbate with regard to HIF-1 α expression. EA.hy926 cells have several features expected of endothelial cells, such as cobblestone appearance with formation of capillary-like tubes in culture [16], expression of factor VIII antigen [17], ability to oxidatively modify of human low density lipoprotein [18], and calcium-dependent endothelial nitric oxide synthase activation [18,19]. The results of this study confirm previous findings in different types of endothelial cells that ascorbate suppresses HIF-1 α protein expression and support the notion that cobalt induces and oxidant stress in the cells that consumes ascorbate, but which also is prevented by what are likely physiologic intracellular levels of ascorbate.

2. Materials and methods

2.1. Materials

Sigma/Aldrich Chemical Co. (St. Louis, MO) supplied the ascorbic acid, dehydroascorbic acid (DHA), CoCl₂, and reagent chemicals. Perkin-Elmer Life and Analytical Sciences, Inc. (Boston, MA) supplied the L-[1-¹⁴C]ascorbic acid, which was dissolved in deionized water containing 0.1 mM acetic acid and stored in multiple aliquots at -20 °C until use.

2.2. Cell Culture

EA.hy926 cells were a generous gift from Dr. Cora Edgell (University of North Carolina, Chapel Hill, NC). They were cultured in Dulbecco's minimal essential medium and 10% (v/v) heat-inactivated fetal bovine serum, to which were added D-glucose to 20 mM and HAT media supplement (Sigma/Aldrich Chemical Co., St. Louis, MO). Unless otherwise noted, cells were cultured to near confluence at 37 °C in humidified air containing 5% CO₂. Just before an experiment, cells were rinsed 3 times in 2 ml of Krebs-Ringer Hepes (KRH) buffer at 37 °C. KRH buffer consisted of 20 mM Hepes, 128 mM NaCl, 5.2 mM KCl, 1 mM NaH₂PO₄, 1.4 mM MgSO₄, and 1.4 mM CaCl₂, pH 7.4.

2.3 Assay of ascorbate transport

Following any treatments as noted, confluent EA.hy926 cells in 12-well plates were incubated at 23 °C in KRH that contained 5 mM *D*-glucose, 0.5 mM GSH, and 0.05 µCi of *L*-[1-¹⁴C]ascorbic acid (0.3 µM). Following 30 min of incubation, the supernatant was aspirated, and the cells were rinsed twice in 2 ml of ice-cold KRH, treated with 1 ml of 0.05 N NaOH, and scraped from the plate. The resulting extract was added to 5 ml of Ecolume liquid scintillation fluid (ICN, Costa Mesa, CA) with mixing. The radioactivity of duplicate samples was measured in a Packard CA-2200 liquid scintillation counter, after allowing at least 1 h for decay of chemiluminescence.

2.4 Assay of ascorbate and GSH

After incubations as indicated with confluent cells in 6-well plates, the medium was aspirated, and the adherent cells were gently rinsed twice with 2 ml of ice-cold KRH. The last rinse was removed and the cell monolayer was treated with 0.1 ml of 25% metaphosphoric acid (w/v) for several minutes, followed by 0.35 ml of a buffer containing 0.1M sodium phosphate and 0.05 mM EDTA, pH 8.0. Adherent material was scraped from the plate, and the lysate was removed and centrifuged at 3 °C for 1 min at 13,000 × *g*. Assay of ascorbic acid was performed in duplicate by high performance liquid chromatography as previously described [20]. Intracellular GSH was assayed in duplicate by the method of Hissin and Hilf [21]. Intracellular concentrations of ascorbate and GSH were calculated based on the intracellular distribution space of 3-*O*-methylglucose in EA.hy926 cells. This was measured as described previously for endothelial cells in culture [19] and was 3.5 ± 1.5 µl/mg protein (N=24, ± SD). In some experiments, ascorbate was also measured in 0.1 ml of the incubation medium by adding 0.1 ml of 25% metaphosphoric acid (w/v), mixing, neutralizing with 0.35 ml of the above phosphate/EDTA buffer, and centrifuging to remove any precipitated solids before assay of ascorbate.

2.5. Polyacrylamide gel electrophoresis and immunoblotting

Cell monolayers were washed in phosphate-buffered saline (PBS, deionized water containing 140 mM NaCl and 12.5 mM NaHPO₄, pH 7.4), and solubilized in a lysis buffer consisting of 150 mM NaCl, 1% Nonidet P40 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% sodium lauryl sulfate (w/v), 0.1 mg/ml phenylmethylsulfonyl fluoride, and leupeptin, pepstatin, and aprotinin, each at 0.01 mg/ml. The lysate was mixed and stored on ice for 30 min. To this was added an equal volume of sample buffer, which consisted of 125 mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) sodium lauryl sulfate, 10% (v/v) mercaptoethanol, and 0.0025% bromphenol blue (w/v), pH 6.8. Samples were boiled for 5 min, centrifuged for 10 s at 13,000 × *g*, and the solubilized material was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the method of Laemmli [22]. Electrophoresis and transfer to poly(vinylidene difluoride) membrane, was carried out as previously described [23]. Blots were probed with affinity purified rabbit polyclonal antibodies that are known to detect the human or rodent SVCT2 transporter (SC-9926) or HIF-1α (SC-10790), both obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (#A0545, Sigma-Aldrich, Inc., St. Louis, MO) and used at a 1:5000 dilution. Bands were stained using ECL Plus Western blotting reagents (RPN 2132, Amersham Biosciences, Piscataway, NJ). Locations of the bands were determined using pre-stained molecular weight markers.

2.6. RNA isolation and RT-PCR

Total RNA was isolated using Trizol reagent (GIBCO, Grand Island, NY), and 2 µg was primed with random hexamers and reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Carlsbad, CA) in a final volume of 20 µl. One microliter of this

mixture was amplified in a 25- μ l reaction using Advantage 2 PCR kit (Clontech, Mountain View, CA). The following primers were used to analyze the expression of SVCT2 and G3PDH: human SVCT2, forward primer 5-GAT GCC ATG TGT GTG GGG TA-3 and reverse primer 5-TAT TGT CAG CAT GGC AAT GC-3; G3PDH, forward primer 5-TCT TTT GCG TCG CCA GCC GAG C-3 and reverse primer 5-CCT GCA AAT GAG CCC CAG CCT TC-3. The PCR products were separated by electrophoresis on 1% agarose gels and visualized with ethidium bromide.

2.7. Data Analysis

Results are shown as mean + standard error, except where noted. Statistical comparisons were made using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). Differences between treatments were assessed by two-way analysis of variance with post-hoc testing using Dunnett's test.

3. Results

3.1. HIF-1 α expression and ascorbate in EA.hy926 cells

HIF-1 α protein was usually not detected in immunoblots of EA.hy926 cells that were cultured under ambient oxygen concentrations (Fig. 1A, Lane 1). However, when the cells were cultured for 18 h in the presence of 0.25 mM CoCl₂ (Fig. 1A, Lane 2), or under hypoxic conditions at 1% oxygen (Fig. 1B, Lane 1), HIF-1 α was strongly expressed. It should be emphasized that EA.hy926 cells do not contain measurable ascorbate under the culture conditions used in these experiments [24]. Culture of the cells for 18 h after a single addition of ascorbate alone to 200 μ M had no effect on the low HIF-1 α expression (Fig. 1A, Lane 3). However, HIF-1 α protein expression induced by hypoxia was completely blocked by a single treatment of the cells with ascorbate concentrations as low as 50 μ M when ascorbate was added 18 h before cell sampling (Fig. 1B, lanes 2–4). Cobalt-induced HIF-1 α protein was less sensitive to ascorbate than when HIF-1 α induced by hypoxia, but was nonetheless progressively decreased by increasing concentrations of ascorbate (Fig. 1A, Lanes 4–6). These results fit with the notion that ascorbate inhibits both hypoxia- and cobalt-induced stabilization of HIF-1 α in EA.hy926 cells.

3.2. Cobalt and ascorbate transport

If ascorbate lowers HIF-1 α expression in EA.hy926 cells, then it is possible that HIF-1 α might in turn affect some aspect of ascorbate uptake or maintenance in the cell. Given its marked ability to induce HIF-1 α expression after 18 h in culture (Fig. 1A), cobalt treatment was used to investigate ascorbate homeostasis in EA.hy926 cells. Ascorbate is transported into EA.hy926 cells on a specific ascorbate transporter, termed the SVCT2, which generates a sharp gradient of the vitamin across the endothelial cell membrane [24]. However, as shown in Fig. 2, we failed to see an effect of 18 h of treatment in culture with 200 μ M concentrations of either cobalt or ascorbate itself on SVCT2 message or protein, measured by RT-PCR and immunoblotting, respectively.

3.3. Cobalt effects on cellular ascorbate

Although treatment of EA.hy926 cells in culture with cobalt did not affect SVCT2 message or protein, it did decrease intra- and extracellular ascorbate when it was present. Culture of the cells for 18 h following a single addition of 200 μ M ascorbate markedly increased intracellular ascorbate from undetectable levels to about 1.7 mM (Fig. 3, squares, left Y-axis). This was associated with a decrease in extracellular ascorbate from the added concentration of 200 μ M to about 50 μ M, or 25% of the added amount (Fig. 3, circles, right Y-axis). Addition of increasing amounts of cobalt for 18 h markedly decreased intracellular

ascorbate. This decrease became significant at a cobalt concentration of 100 μM and was nearly complete at 250 μM . Extracellular ascorbate decreased at even lower cobalt concentrations.

Since cobalt depleted ascorbate in the culture medium (Fig. 3), at least part of its effect to decrease intracellular ascorbate could be due to direct oxidation of ascorbate in the medium. Indeed, in results not shown, 250 μM CoCl_2 enhanced the oxidation of 100 μM ascorbate in the absence of cells by about 30% in KRH over the course of one hour of incubation, indicating a direct oxidative effect. To determine the extent to which cobalt depleted or oxidized intracellular ascorbate, we carried out two short-term experiments in which only intracellular ascorbate was exposed to cobalt. In the first experiment, EA.hy926 cells were loaded with ascorbate by incubation for 15 min with 250 μM DHA. The latter is rapidly taken up by the cells on glucose transporters and reduced to ascorbate, which is trapped in the cells. Under such conditions the extracellular ascorbate concentration is less than 1 μM (results not shown), compared to about 1 mM inside the cells (Fig. 4A, at time zero). As shown by the circles in Fig. 4A, subsequent addition of cobalt caused a time-dependent decrease in intracellular ascorbate that was significant by 1 h of incubation, with a maximal 40% decrease at 2–3 h. This loss is comparable to that observed in buffer alone, as noted above. Cells not treated with cobalt had no loss of intracellular ascorbate over this time period (Fig. 4A, squares). Since extracellular ascorbate concentrations were very low following DHA loading, these results suggest that cobalt depletes or oxidizes intracellular ascorbate and does so over a relatively short time course. In the second experiment, cells were pre-incubated for 2 h in KRH with increasing concentrations of cobalt, rinsed to remove extracellular cobalt, and incubated for 30 min with 250 μM ascorbate followed by assay of intracellular ascorbate. As can be seen in Fig. 4B, intracellular ascorbate was decreased by pre-treatment with cobalt by about 30%. Since the cells will still be taking up ascorbate over 30 min, it is possible that these results reflect an effect on ascorbate transport. However, radiolabeled ascorbate transport over 30 min was not affected by a 2 h pre-treatment with 250 μM cobalt (results not shown), indicating that the effect of cobalt was due to loss of intracellular ascorbate and not to prevention of its entry into the cells.

3.4. Ascorbate blunts cobalt-induced oxidant stress in EA.hy926

To determine if cobalt treatment induces an oxidant stress in EA.hy926 cells that might account for the observed decreases in intracellular ascorbate, effects of cobalt on intracellular GSH concentrations and malondialdehyde formation were determined. As shown in Fig. 5, GSH was modestly decreased after 18 h of culture of the cells with increasing concentrations of cobalt, with a significant effect noted at 250 μM and a decrease of 25% at 500 μM . On the other hand, culture of the cells for 18 h with cobalt concentrations as low as 100 μM increased the malondialdehyde content of the cells by 50% (Fig. 6A). The effect of cobalt to induce malondialdehyde formation was partially blocked by a single addition of ascorbate to a concentration of 50 μM in the culture medium and almost completely blocked by 0.5 mM ascorbate (Fig. 6B). In results not shown, ascorbate concentrations up to 0.5 mM alone under these conditions had no effect on cell malondialdehyde content. Inhibition of cobalt-induced malondialdehyde generation by ascorbate shows that ascorbate prevents the oxidant stress induced by cobalt, during which ascorbate itself is oxidized.

4. Discussion

Endothelial cells resemble other cell types in that they express little or no HIF-1 α in culture under oxygenated conditions [8–10,25]. However, as previously demonstrated [10] and confirmed in this work, endothelial cell HIF-1 α expression is markedly increased by culture under hypoxic conditions, presumably because of lack of molecular oxygen as a substrate

for the dioxygenase enzymes required for HIF-1 α hydroxylation. Several divalent metal ions have also been shown to stabilize HIF-1 α , including nickel, Cr(VI), and cobalt [13,15]. In addition to their use as tools to increase HIF-1 α expression, the toxicity and carcinogenicity of several of these metals have been linked to their effects on HIF-1 α expression [11]. Also of interest is their potential interaction with ascorbate, which has been shown to activate the HIF-1 α prolyl hydroxylase by maintaining iron in or near the catalytic site of the enzyme in its active or ferrous state [26,27]. Whereas ascorbate is sufficient to fulfill this function, it is not absolutely necessary. Thus, the endothelial cells used in this work were cultured in the absence of added ascorbate and had undetectable intracellular levels of the vitamin. Yet, HIF-1 α protein levels were very low in these oxygenated cultures. Cells must therefore have other mechanisms for maintaining the iron in or near the catalytic site of the hydroxylase in the ferrous form.

Although cobalt and nickel ions are not as redox-active as iron or copper, they nonetheless can cause oxidative loss of ascorbate in solution and within cells ([13,15], present work). Depletion of intracellular ascorbate has thus been proposed as a mechanism, in addition to directly supplanting iron in the enzyme active site, by which nickel or cobalt can deactivate the HIF-1 α prolyl hydroxylase [11]. The present studies show that in endothelial cells devoid of ascorbate, an overnight treatment with cobalt markedly increased HIF-1 α protein levels. HIF-1 α stabilization by cobalt in the absence of ascorbate shows that cobalt alone is able to inhibit the HIF-1 α prolyl hydroxylase. The effect of ascorbate to prevent cobalt-induced HIF-1 α protein expression suggests a competition between the two agents, especially since ascorbate has been proposed to chelate transition metal ions [11], and cobalt can in turn oxidize ascorbate. Endothelial cells are able to maintain intracellular ascorbate at concentrations of 1–3 mM in culture ([10], Fig. 3 in present work). This occurs even though the extracellular supply of the vitamin has stabilized to about 50 μ M, which is in the normal range for plasma ascorbate concentration [28]. Under these conditions, ascorbate completely prevents the stabilization of HIF-1 α by cobalt (last lane, Fig. 1A). Of note is that the intracellular ascorbate concentration at the end of such an incubation is also very low after 18 h or treatment with 0.25 mM cobalt (0.16 ± 0.04 mM, Fig. 3B, squares). Thus, although pharmacologic concentrations of cobalt do oxidize and deplete ascorbate, what are likely normal physiologic concentrations of ascorbate in endothelial cells overwhelm the effect of cobalt on HIF-1 α . Given that hypoxia-induced HIF-1 α expression was even more sensitive to ascorbate than that induced by cobalt (Fig. 1B), HIF-1 α expression in endothelial cells *in vivo* should also be suppressed by physiologic ascorbate levels. In another context, pharmacologic levels of ascorbate have been shown to suppress the growth of xenograft tumors in mice [29]. The mechanism of this suppression involves pro-oxidant effects of extracellular ascorbate to selectively kill cancer cells by increasing extracellular H₂O₂ [30,31]. Such high ascorbate concentrations might also be expected to suppress HIF-1 α and thus growth in tumor cells, although this might be counteracted by the pro-oxidant effect of ascorbate. The role of HIF-1 α in ascorbate-induced tumor suppression needs further study.

As demonstrated in other cell types [13], treatment in culture with cobalt depleted ascorbate in EA.hy926 cells. This effect could be due in part to oxidation of extracellular ascorbate that had not yet entered the cells. However, since cobalt-induced intracellular ascorbate loss also occurred under situations in which either extracellular ascorbate or cobalt was absent, it seems most likely due to oxidative loss or chelation of intracellular ascorbate, as previously proposed [11]. In support of a role for oxidative modification of intracellular ascorbate in this phenomenon, cobalt treatment at levels sufficient to deplete ascorbate and to stabilize HIF-1 α protein also generated an oxidant stress in the cells. This was evident first as a modest decrease in intracellular GSH concentrations. As in other cells challenged with oxidant stress, intracellular ascorbate was more sensitive to oxidative loss than was GSH [32–34]. Indeed, since ascorbate can be recycled from DHA by GSH itself [35] as well as by

GSH-dependent thiol transferases [36], GSH could have been depleted due to rapid ascorbate recycling. The second effect of cobalt indicating an oxidative stress was to cause a 50% increase in the cell content of malondialdehyde, a marker of lipid peroxidation. The cobalt-induced increase in malondialdehyde was largely prevented by culturing the cells in the presence of ascorbate. These results show that just as ascorbate can counteract the effect of cobalt on HIF-1 α protein levels, it can also decrease the oxidant stress induced by the metal ion.

Since ascorbate reverses HIF-1 α protein stabilization induced by both hypoxia and cobalt ions, one wonders whether HIF might in turn affect ascorbate homeostasis in the cell. The major route for accumulation of ascorbate in EA.hy926 endothelial cells involves its uptake on the SVCT2 [37]. If HIF increased SVCT2 gene expression as part of its transcriptional regulation, this would increase ascorbate accumulation by the cells and thus would provide negative feedback on HIF-1 α protein levels. However, our finding that treatment of EA.hy926 cells with cobalt failed to significantly increase either SVCT2 message or protein argues that regulation of SVCT2 is not part of the many functions of HIF. A previous report showed that a 20 h exposure of a human lung cell line to 0.5 mM nickel decreased SVCT2 mRNA in a Northern blot [9]. This effect was partially reversed by 25 μ M ascorbate. Our failure to see such effects could be due to different cell types or to the use of different transition metals. Nonetheless, both results suggest that the SVCT2 is not among the many proteins induced by HIF.

In summary, ascorbate, at what are likely physiologic intracellular concentrations, is sufficient to completely suppress cobalt- or hypoxia-induced increases in HIF-1 α expression in endothelial cells in culture. At concentrations adequate to induce HIF-1 α expression, cobalt oxidizes intracellular ascorbate and induces an oxidant stress in endothelial cells that is mitigated by ascorbate. Although ascorbate may be involved in the physiologic regulation of HIF-1 α stabilization, the SVCT2 expression is not among the many pathways stimulated by HIF-1 α .

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Abbreviations used

DHA	dehydroascorbic acid
Hepes	N-2-hydroxyethylpiperazine-NN-2-ethanesulfonic acid
HIF	hypoxia-inducible factor
KRH	Krebs-Ringer Hepes
SVCT2	Sodium-dependent Vitamin C Transporter, type 2

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Figure 1. HIF-1 α immunoblotting

Panel A: Confluent EA.hy926 cells were treated with ascorbate or cobalt at the indicated concentrations and then cultured for 18 h under ambient atmospheric oxygen concentrations before rinsing the cells 3 times with KRH, followed by removal of the cells for immunoblotting as described under Experimental Procedures. Blots of HIF-1 α are shown in the top row, with blots of β -actin shown in the second row as a control for gel protein loading. Results are representative of 4 such experiments performed. Panel B: EA.hy926 were cultured to confluence at ambient oxygen concentrations and then cultured with additions as noted for 18 h under a mixture of 1% oxygen, 5% carbon dioxide, and 94% nitrogen. Cells were rinsed 3 times with KRH and taken for immunoblotting of HIF-1 α (top row) and β -actin (bottom row). Results are representative of 2 such experiments performed.

Figure 2. SVCT2 message and protein

Panel A: Confluent EA.hy926 cells in culture were treated for 18 h with ascorbate or cobalt as indicated, rinsed 3 times in buffer, and taken for determination of SVCT2 mRNA (Panel A, top row) or protein by immunoblotting (Panel B, top row), using glyceraldehyde 3-phosphate dehydrogenase (G3PDH) or β -actin as a control for loading of the gel lanes, respectively.

Figure 3. Cobalt-induced loss of ascorbate in overnight culture

Confluent EA.hy926 cells were treated for 15 min with 200 μ M ascorbate, followed by addition of the indicated concentration of cobalt and culture for 18 h before sampling of medium aliquots for ascorbate (circles, right Y-axis) and removal of the cells for assay of intracellular ascorbate (squares, left Y-axis). Results are shown from 4 experiments, with an “*” indicating $p < 0.05$ compared to the sample not treated with cobalt.

Figure 4. Cobalt-induced decreases of intracellular ascorbate

Panel A: EA.hy926 cells were rinsed free of culture medium and incubated in KRH that contained 5 mM D-glucose and 250 μ M DHA for 15 min before addition of 250 μ M cobalt (circles) or no cobalt (squares), followed by culture for the times indicated before 3 cell rinses with KRH and removal of the cells for assay of intracellular ascorbate. Results are from 4 experiments, with an “*” indicating $p < 0.05$ compared to the sample at time zero. Panel B: EA.hy926 cells were incubated for 2 h in KRH that contained 5 mM D-glucose with the indicated concentration of cobalt, followed by 3 rinses and addition of 250 μ M ascorbate. After an additional 30 min, the cells were rinsed 3 times in KRH and taken for assay of intracellular ascorbate. Results are from 4 experiments, with an “*” indicating $p < 0.05$ compared to the sample not treated with cobalt.

Figure 5. Cobalt-induced decreases in GSH

EA.hy926 cells were cultured for 18 h with the indicated concentration of cobalt, rinsed 3 times, and taken for assay of their GSH content. Results are shown from 7 experiments, with an “*” indicating $p < 0.05$ compared to the same not treated with cobalt.

Figure 6. Malondialdehyde formation in response to cobalt and ascorbate

Panel A: EA.hy926 cells were cultured for 18 h with the indicated concentration of cobalt, rinsed 3 times in KRH, and taken for assay of malondialdehyde. Results are shown from 5 experiments, with an “*” indicating $p < 0.05$ compared to cells not treated with cobalt. Panel B: EA.hy926 cells were treated with the indicated concentration of ascorbate for 15 min, and then cultured for 18 h without (square) or with (circles) 250 μ M cobalt. Results are shown from 5 experiments, with an “*” indicating $p < 0.05$ compared to the sample treated with cobalt alone.