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Ascorbate Depletion Mediates Up-Regulation of Hypoxia-Associated Proteins by Cell Density and Nickel

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Abstract Exposure of human lung cells to carcinogenic nickel compounds in the presence of oxygen up-regulated carbonic anhydrase IX (CA IX) and NDRG1/Cap43, both known as intrinsic hypoxia markers and cancer-associated genes. This suggests that factors other than a shortage of oxygen may be involved in this induction. Both proteins can also be induced in the presence of oxygen by culturing these cells to a high density without medium change. The intracellular ascorbate measurements revealed its rapid depletion in both metal- and density-exposed cells. Nickel exposure caused strong activation of HIF-1α and HIF-2α proteins, underscoring activation of HIF-1-dependent transcription. In contrast, cell density-dependent transcription was characterized by minor induction of HIF-1α or HIF-2α. Moreover, the up-regulation of NDRG1/Cap43 in HIF-1α deficient fibroblasts suggested the involvement of different transcription factor(s). The repletion of intracellular ascorbate reversed the induction of CA IX and NDRG1/Cap43 caused by cell density or nickel exposure. Thus, the loss of intracellular ascorbate triggered the induction of both tumor markers by two different conditions in the presence of oxygen. Ascorbate is delivered to lung cells via the SVCT2 ascorbate transporter, which was found to be sensitive to nickel or cell density. Collectively these findings establish the importance of intracellular ascorbate levels for the regulation of expression of CA IX and NDRG1/Cap43. We suggest, that, in addition to low oxygenation, insufficient supply of ascorbate or its excessive oxidation in tumors, can contribute to the induction of hypoxia-associated proteins via both HIF-dependent and independent mechanisms. J. Cell. Biochem. 97: 1025–1035, 2006.

Key words: ascorbate; cell density; nickel; SVCT1; SVCT2; hypoxia-inducible transcription factor; carbonic anhydrase IX; NDRG1/Cap43

Increased expression of hypoxia-inducible genes is a hallmark feature of many solid tumors. It is generally accepted that a shortage of oxygen (hypoxia) in the tumor is the primary cause of the hypoxia-inducible factor 1 (HIF-1) activation, followed by the induction of hypoxia-inducible genes. The existence of hypoxic regions in solid tumors is well documented [Hockel and Vaupel, 2001]. They arise as the result of low vascular density and disorderly vascular architecture. In response to hypoxia, tumor cells modulate the expression of specific genes, allowing for adaptation and survival in the hypoxic environment. The major regulator of hypoxic response is HIF-1, which activates transcription of genes involved in crucial aspects of cancer biology, including a shift in energy metabolism, cell survival, angiogenesis, and invasiveness [Semenza, 2003]. Since hypoxia is widely spread in tumors, hypoxia-inducible genes like carbonic anhydrase IX (CA IX) and NDRG1/Cap43 are used as tumor markers. CA IX first was identified as a marker protein expressed in several types of human carcinomas, but not in the corresponding

Abbreviations used: CA IX, carbonic anhydrase IX; DFX, deferoxamine mesylate; FCS, fetal calf serum; MEF, mouse embryo fibroblasts; HIF, hypoxia-inducible factor; HRE, HIF response element; Luc, luciferase; PBS, phosphate-buffered saline; SVCT1 and SVCT2, sodium-dependent vitamin C transporters.

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non-cancerous tissues [Pastorek et al., 1994]. Additionally, its overexpression has been identified in a number of solid tumors, including renal carcinoma, particularly clear cell adenocarcinoma [McKiernan et al., 1997; Ivanov et al., 2001], and non-small cell lung carcinoma [Vermylen et al., 1999; Ivanov et al., 2001], head and neck squamous cell carcinoma [Beasley et al., 2001; Ivanov et al., 2001], and cervix carcinoma [Loncaster et al., 2001]. NDRG1/Cap43, originally cloned by us as a nickel-inducible gene [Zhou et al., 1998], was found to be induced by hypoxia in an HIF-dependent manner [Salnikow et al., 2000]. NDRG1/Cap43 protein is expressed at low levels in normal tissues; however, it is overexpressed in a variety of cancers, including lung, brain, melanoma, liver, prostate, breast, and renal cancers [Cangul et al., 2002].

Enzymatic hydroxylation of specific amino acids in HIFα proteins has been recently recognized as a mechanism of oxygen sensing [Ivan et al., 2001; Jaakkola et al., 2001; Maxwell and Salnikow, 2004]. It links levels of HIF-1 transcription factor to a shortage of molecular oxygen and explains why hypoxia-inducible genes are up-regulated in poorly oxygenated tumors. However, the role of deficiency of other components of the hydroxylation reaction, including iron and ascorbate that are also delivered with blood, is usually under-rated. In this respect, investigation of the mechanisms involved in the induction of hypoxia-inducible genes in the presence of oxygen is of particular interest since it may clarify the role of iron and ascorbate. Thus, it is likely that iron chelator deferoxamine induces hypoxic genes by facilitating oxidation of intracellular iron, and, in particular, hydroxylase-bound iron. Hypoxia-mimicking metals such as nickel and cobalt represent another interesting research tool for studying hypoxic response. It is known that expression of both CA IX and NDRG1/Cap43 can be induced by nickel or cobalt in the cell culture [Salnikow et al., 2000; Chrastina et al., 2003]. Recently we have shown that the underlying mechanism of the induction of hypoxic genes by metals involves depletion of ascorbate [Salnikow et al., 2004].

It was reported previously that CA IX expression could be induced by the increase in cell density [Pastorek et al., 1994; Ivanov et al., 1998, 2001]. Several mechanisms were proposed to explain this phenomenon, including the suggestion that high cell density lowered pericellular pO_2, which then activated HIF, a crucial factor for the induction of CA IX expression [Chrastina, 2003]. The involvement of other than HIF-1 transcription factors like SP1 was also proposed [Kaluz et al., 2003]. Here we show that similarly to nickel exposure, growing cells in the culture to a high-density results in the depletion of intracellular ascorbate, which in turn activates NDRG1/Cap43 and CA IX. We also show that expression of the SVCT2 ascorbate transporter can be modulated by both nickel and cell density. Thus, the results of this study provide a link between intracellular ascorbate level and expression of tumor markers in the presence of oxygen.

MATERIALS AND METHODS

Reagents

$NiSO_4 \cdot 6H_2O$ was obtained from Alfa Aesar (Ward Hill, MA). $FeSO_4 \cdot 7H_2O$, and L-ascorbic and L-lactic acids were obtained from Sigma (St. Louis, MO), and deferoxamine mesylate (DFX) was obtained from Calbiochem (La Jolla, CA). Protease inhibitor cocktail was purchased from Roche (Indianapolis, IN).

Cell Lines and Culture Conditions

The 1HAEo- cell line was obtained from Dr. D.C. Gruenert [Gruenert et al., 1995]. Cells were grown on plastic coated with the mixture of BSA (Invitrogen Corporation, Carlsbad, CA) and collagen (Cohesion, Palo Alto, CA) in Minimum Essential Medium with Earle's modified salts (Invitrogen) containing 10% FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. Mouse embryo fibroblasts (MEF) and cells with HIF-1α knockout (MEF HIF-$1^{-/-}$) were described previously [Salnikow et al., 2000]. Mouse hepatoma Hepa1 cells were grown in α-Minimal Essential Medium (Invitrogen) supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 U/ml penicillin.

Northern Blot Analysis

Northern blot analysis was performed using a cDNA probe encoding for the open reading frame of SVCT2. The probe was provided by Dr. C. Corpe (NIH). Total RNAs were extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions and the RNA was electrophoresed (15 μg total RNA/lane) in 1.0% agarose/formaldehyde gels. Probes were

labeled with [^{32}P]-α-dCTP using a MegaPrime DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN). Hybridizations were carried out in the presence of ExpressHyb solution (BD Biosciences, Palo Alto, CA), as recommended by the supplier.

RT-PCR Analyis of Ascorbate Transporters Expression

Total RNA was isolated from 1HAEo- and HL-60 cells using TRIzol reagent (Invitrogen). All samples were treated with DNase. RT-PCR was performed by using SuperScript III One-Step RT-PCR (Invitrogen) under standard conditions using 30 cycles and annealing temperature 56°C. For SVCT1 (1018 bp) the sequence was forward, 5′-CACTACCTGACATGCTTC-AG-3′ and reverse, 5′-CCAGGACGCCAATGT-TGG-3′. The sequence was for SVCT2 (647 bp): forward, 5′-GGGGCTACAGCACTACCTG-3′ and reverse, 5′-GGATGGCCAGGATGATAG-3′. For USP11 (1019 bp) primer sequence was forward, 5′-CTTCGACCCCTTCTGCTAC-3′ and reverse, 5′-GGTCCAGCTTCTTGGTTGC-3′.

Western Blot Analysis

Total cell protein extracts were obtained after lysing cells in the lysis buffer (Cell Signaling, Beverly, MA) for 15 min at 4°C. Equal loading of protein was assured by prior quantitation using the Bradford assay. Forty micrograms of protein extracts were separated by gel electrophoresis in 4%–12% NuPage bis–tris minigels (Invitrogen) in MOPS buffer, pH 7.7 and transferred onto a PVDF membrane (Roche, Indianapolis, IN).

Nuclei for nuclear extracts preparation were obtained as described earlier [Salnikow et al., 2004]. Nuclei were resuspended in 50–150 μl of extraction buffer (0.42 M NaCl, 25% glycerol, 2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF and antipain 10 μg/ml, aprotinin 1.5 μg/ml, leupeptin 3 μg/ml, pepstatin 3 μg/ml, and phosphatase inhibitors, 20 mM HEPES, pH 7.9) and incubated on ice for 15 min, followed by maximum speed centrifugation in the Eppendorf centrifuge for 15 min at 4°C. Supernatants were aliquoted and stored at −70°C. Twenty micrograms of nuclear extracts were used for Western blots. Western blot analysis for the hypoxia-inducible NDRG1/Cap43 protein levels was performed using rabbit polyclonal antibody, as described previously [Salnikow et al., 2002]. CA IX antibodies were kindly provided by Dr. E. Stanbridge. HIF-1α antibodies were purchased from BD Biosciences. HIF-2α antibodies were purchased from Novus Biologicals (Littleton, CO). Antibodies against β-actin were purchased from Abcam (Cambridgeshire, UK). Immunoreactive bands were detected using horseradish peroxidase-linked secondary antibody and enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston, MA).

HRE-Luc Reporter Assay

The transient transfection experiments were carried out as previously described [Salnikow et al., 2004]. The transfection experiments were repeated twice and each condition has been tested in quadruplicate. The results shown are the mean ± SD.

Measurement of Ascorbate Levels

HPLC measurements of total intracellular ascorbate levels were performed by HPLC as previously described [Quievryn et al., 2002]. The ascorbate measurements were repeated three times. The results shown are the mean ± SD of one representative experiment.

RESULTS

Induction of NDRG1/Cap43 and CA IX by Nickel Can be Prevented by Ascorbate

Human exposure to nickel occurs primarily via inhalation and can lead to asthma, inflammation, lung fibrosis, and lung cancer. Therefore, the response to nickel was tested on minimally transformed 1HAEo- human lung epithelial cells. In these cells nickel strongly induced NDRG1/Cap43 and CA IX under normal oxygen conditions. The increased expression of CA IX in human lung cancers and preneoplastic lesions has been described [Vermylen et al., 1999; Ivanov et al., 2001]; however, the induction of CA IX by nickel in human lung cells has not been shown before. The time course of induction is shown in Figure 1A,B. The delay in response by 16 h for both proteins suggests the involvement of some precursory metabolic changes. Recently, we have shown that 16 h exposure to nickel can significantly deplete intracellular ascorbate [Salnikow et al., 2004]. Here we tested whether the addition of ascorbate could reverse the induction of two hypoxia-inducible proteins by nickel. The exposure of 1HAEo- cells to as low as 50 μM of soluble $NiSO_4$ for 20 h induced expression of both proteins

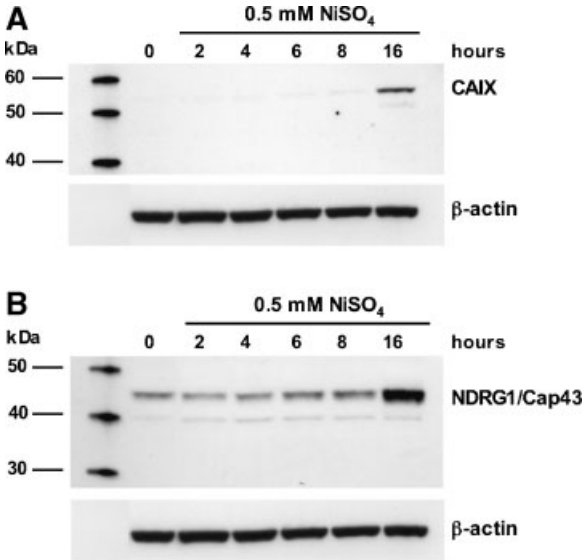

Fig. 1. Time course of CA IX and NDRG1/Cap43 induction by nickel sulfate. 1HAEo- cells were exposed to 0.5 mM $NiSO_4$ for different periods of time. **A**: Western blot analysis of CA IX expression in total cell extracts. **B**: Western blot analysis of NDRG1/Cap43 expression in total cell extracts. Western blot for β-actin was used as a loading control.

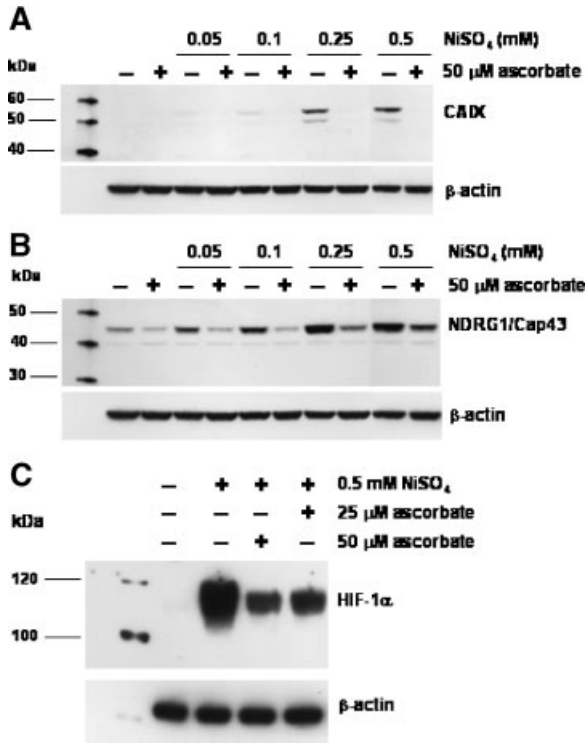

Fig. 2. Effect of ascorbate on CA IX and NDRG1/Cap43 induction by nickel sulfate. Dose-dependent induction of CA IX and NDRG1/Cap43 in 1HAEo- cells exposed to nickel in concentrations shown in the figure alone or in the presence of 50 μM of ascorbate for 20 h. **A**: Western blot analysis of CA IX expression in total cell extracts. **B**: Western blot analysis of NDRG1/Cap43 expression in total cell extracts. **C**: Western blot analysis of HIF-1α expression in nuclear extracts of 1HAEo-cells. Western blot for β-actin was used as a loading control.

(Fig. 2A,B). The effect was much stronger at higher concentrations of nickel ranging from 0.25 to 0.5 mM. Nickel-induced NDRG1/Cap43 and CA IX expression was abrogated by the addition of ascorbate in the culture medium. However, at higher concentrations of nickel the reversion was incomplete.

Both hypoxia-inducible genes are regulated by the HIF-1 transcription factor, which is a heterodimer of HIF-1α or HIF-2α proteins and HIF-1β/ARNT. In concordance with that, the exposure to nickel led to a significant accumulation of HIF-1α and HIF-2α proteins; the addition of ascorbate reversed it (Figs. 2C and 3B). However, the repealing effect of ascorbate was much stronger toward HIF-2α protein.

Induction of NDRG1/Cap43 and CA IX by Cell Density Can be Prevented by Ascorbate

The CA IX and NDRG1/Cap43 proteins can be up-regulated in 1HAEo- cells by cell density (Fig. 3A). Since nickel induced these genes via stabilization of the HIF-1 transcription factor, we next investigated whether the induction of HIF-1 also takes place during culturing cells to a high density. Figure 3B shows that, unlike nickel exposure, no significant induction of HIF-1α or HIF-2α was observed at days 2–4. Thus, the up-regulation of CA IX and NDRG1/Cap43 proteins did not coincide with HIF-1α or HIF-2α accumulation. These data suggested that HIF-1 may not be involved, or only partially involved, in density-dependent up-regulation of CA IX and NDRG1/Cap43 proteins. In order to confirm that HIF-1 was not involved in density-dependent up-regulation of hypoxic genes, we investigated whether NDRG1/Cap43 protein could be up-regulated by cell density in MEF and HIF-$1\alpha^{-/-}$ fibroblasts. Figure 3C shows a similar induction of NDRG1/Cap43 protein at high density, which was independent of HIF-1α. These data suggested the involvement of another transcription factor in density-dependent induction of hypoxia-inducible genes.

Since ascorbate reversed nickel-induced expression of CA IX and NDRG1/Cap43, we decided to test the effect of ascorbate on density-induced expression of these proteins. The addition of ascorbate without medium change reversed the effect of density on the expression

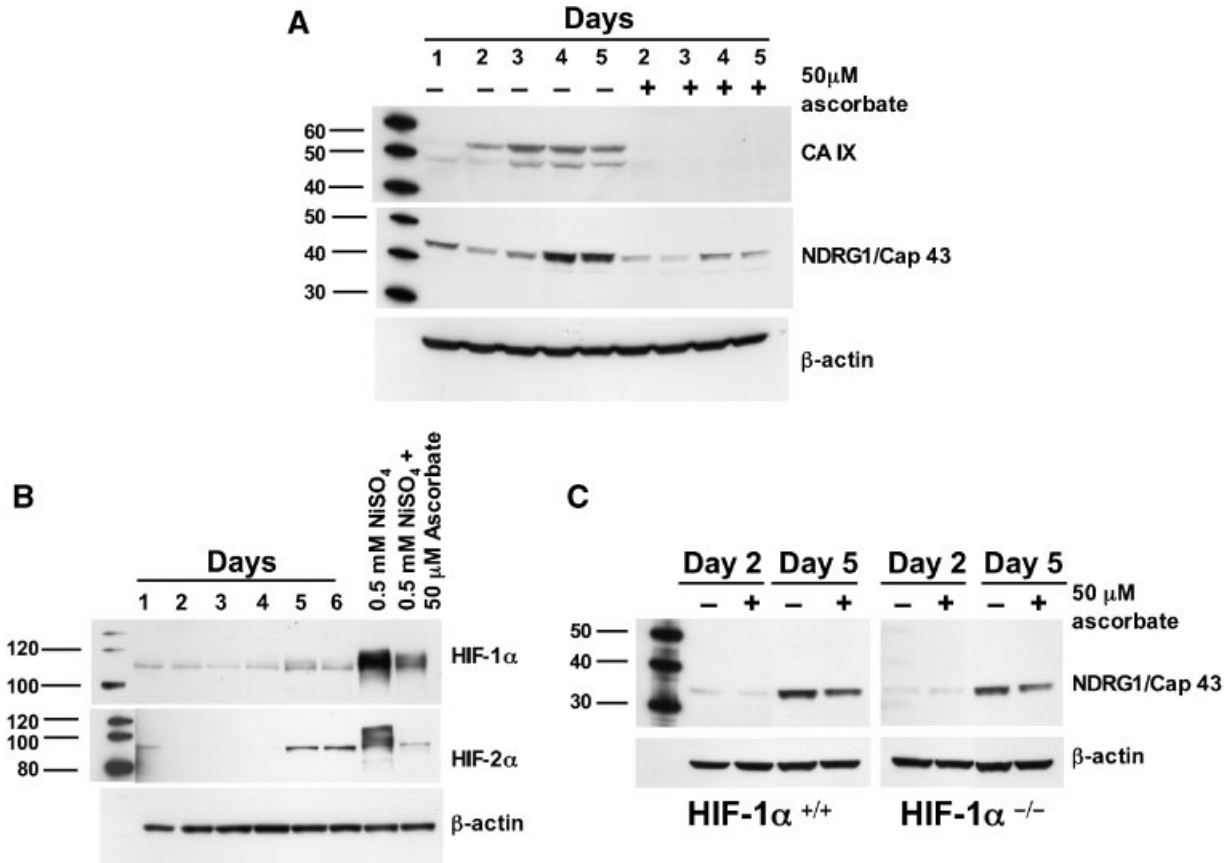

Fig. 3. CA IX and NDRG1/Cap43 induction by cell density is HIF-independent. 1HAEo- cells were plated at a density of 10^6 cells per 100 mm dish. Cells were incubated either without medium change, or at day 2, 50 μM of ascorbate was added daily. **A**: Western blot analysis of CA IX and NDRG1/Cap43 expression in 40 μg of total cell extracts. **B**: HIF-1α and HIF-2α expression in 15 μg of nuclear extracts. The induction of HIF-1α and HIF-2α by nickel is shown for comparison. **C**: Western blot analysis of NDRG1/Cap43 expression in 40 μg of total cell extracts of MEF (**left panel**) and HIF-1α$^{-/-}$ fibroblasts (**right panel**). Western blot for β-actin was used as a loading control.

of both genes (Fig. 3A). Unlike cells in vivo, cultured cells in vitro are highly oxygenated. It was conceivable, therefore, that ascorbate could be oxidized and destroyed more quickly in the cell culture with time. In order to test this possibility, cells were plated at a density of 1.28×10^4 cells/cm^2 (10^6 cells per 100 mm dish) and allowed to grow up to 7 days with or without ascorbate added to a final concentration of 50 μM every day (Fig. 4). During 7 days the cell number was increased from 10^6 cells per 100 mm dish to almost 9×10^6 cells per 100 mm dish. At day 3 cells reached confluency. No differences in the growth rate were observed in ascorbate supplemented versus non-supplemented 1HAEo- cells. At the same time, both CA IX and NDRG1/Cap43 expression was up-regulated after day 2 in cells which were not supplemented with ascorbate (Fig. 3A). We further evaluated changes in the intracellular levels of ascorbate. By day 2, intracellular levels of ascorbate in non-supplemented culture dropped from 60 to 1–2 μM, and after day 3 became undetectable, whereas in the supplemented culture it was slightly decreased by day 3 but remained between 60 and 100 μM thereafter (Fig. 4). Noteworthy is that when cells were placed under hypoxic conditions (1% O_2), the level of ascorbate without supplementation was three to four times higher by day 2 as compared to normoxic conditions (data not shown).

Effect of Nickel and Cell Density on Expression of Ascorbate Transporter SVCT2

Ascorbate is delivered to cells via the sodium-dependent ascorbate transporters SVCT1 and SVCT2, which are expressed in many cells. First, we investigated the level of expression of both SVCT1 and SVCT2 in human lung 1HAEo-

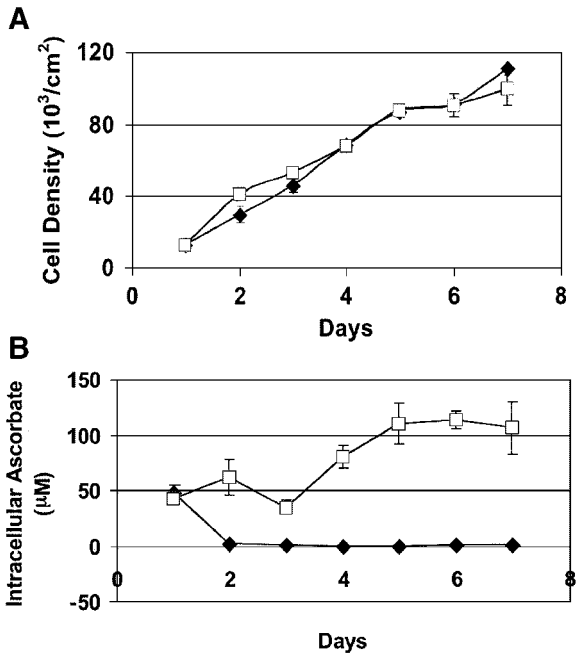

Fig. 4. Time-dependent changes of cell density and ascorbate levels in 1HAEo- cells. **A**: Growth rate of 1HAEo- cells in the presence of ascorbate. 1HAEo- cells were plated at a density of 12×10^3 cells/cm^2 (10^6 cells per 100 mm dish). Cell number was determined by counting cells in three independent dishes. Values shown represent the mean ± SD from one out of three representative experiments. (◆) Cells were incubated either without medium change, or (□) starting at day 1 50 μM of ascorbate was added daily. **B**: Cell density-dependent changes in intracellular ascorbate levels. Intracellular ascorbate level was determined by HPLC method as described in Materials and Methods. (◆) Changes in intracellular ascorbate levels without medium change, (□) or starting at day 1 50 μM of ascorbate was added daily. Mean ± SD are shown.

cells. Using RT-PCR, we found that SVCT2 is predominantly expressed in 1HAEo- and HL-60 cells (Fig. 5A). These data suggested that ascorbate is delivered mostly via SVCT2 transporter in 1HAEo- cells. Previously we found that ascorbate uptake declined with time in nickel-exposed 1HAEo-cells [Salnikow et al., 2004], although the mechanism was not understood. RT-PCR data indicated that nickel exposure partially inhibited the expression of both ascorbate transporters. Northern blot analysis confirmed down-regulation of SVCT2 expression following nickel exposure (Fig. 5B). Addition of ascorbate partially reversed this effect.

We also found density-dependent changes in SVCT2 expression. In cells growing at low density and high levels of ascorbate, the expression of SVCT2 was low. However, SVCT2 expression was augmented in dense cultures (Fig. 5C). The increase in SVCT2 expression concurred with the decline of ascorbate level in the medium. The addition of ascorbate without medium change abolished the increase in SVCT2 expression in dense cultures, but at day 5, the addition of ascorbate did not have any effect on the SVCT2 mRNA expression.

Thus, ascorbate supplementation significantly decreased expression of both the hypoxia-inducible genes and ascorbate transporter. These data emphasized the importance of ascorbate in the regulation of expression of both the hypoxia-inducible genes and ascorbate transporter. However, it was conceivable that other factor(s) in the cell medium might be involved in up-regulation of these genes by high density. One possibility was that the acidification of media by lactic acid could be involved in up-regulation of gene expression by high density.

Effect of Lactic Acid on the HIF-Dependent Gene Expression

Lactic acid is the final product of glycolysis under anerobic conditions. Under aerobic conditions the final product is pyruvate; however, lactate also can be produced when NADH production exceeds oxidative capacity of the respiratory chain. Previously we have shown that nickel induces expression of all glycolytic enzymes, including the enzyme involved in lactate production, lactate dehydrogenase [Salnikow et al., 2003]. This could cause excessive production of lactic acid. To test whether density-dependent expression of CA IX and NDRG1/Cap43 was due to media acidification or/and increased production of lactic acid, cells were incubated with lactic acid at concentrations ranging from 0.5 to 10 mM. No induction of the NDRG1/Cap43 (Fig. 6A,B) or CA IX (not shown) proteins has been observed in 1HAEo-cells or Hepa1 cells. Further, using the HRE-Luc reporter, we investigated whether the acidification of media had any effect on HIF-dependent transcription. In this experiment, 1 mM lactic acid only slightly stimulated HIF-dependent transcription (Fig. 6C), suggesting that lactate was not an important factor in the up-regulation of HIF-dependent genes.

Effect of Iron Supplementation on the Induction of Hypoxia-Inducible Genes

Since the acidification of the medium had no effect on the induction of hypoxia-inducible genes, we investigated the possibility that

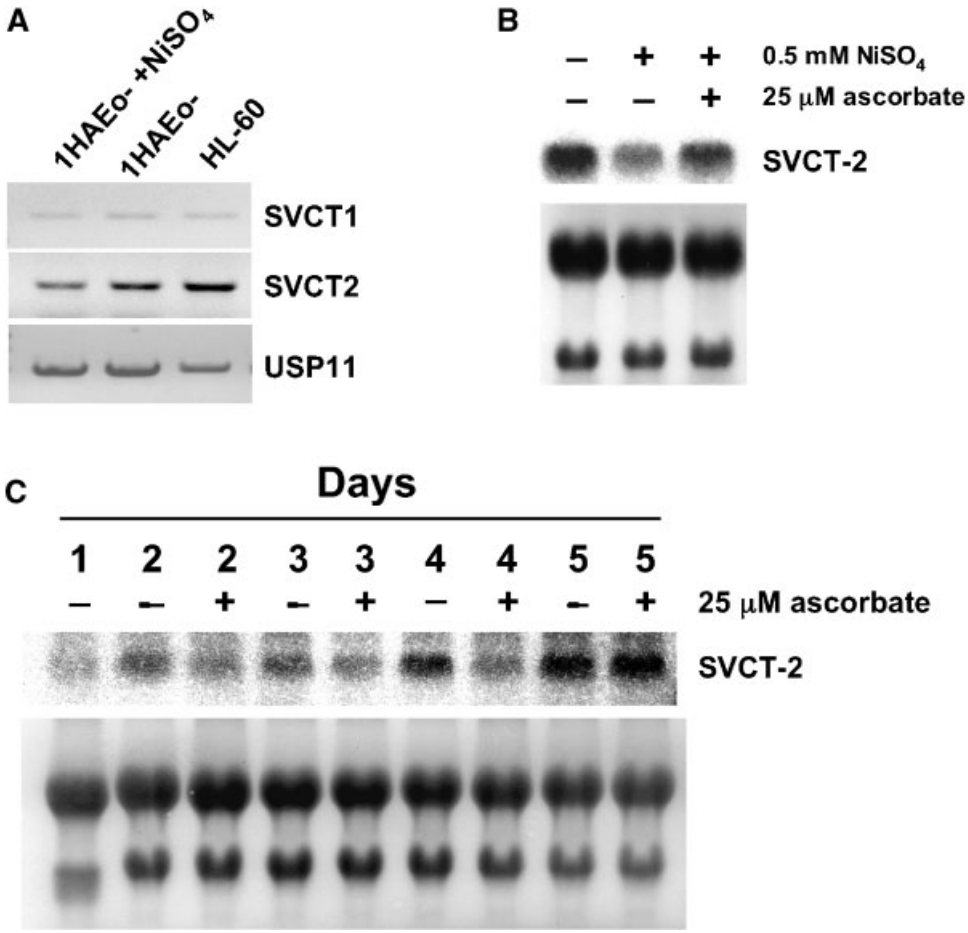

Fig. 5. Effect of nickel and cell density on ascorbate transporter expression. **A**: RT-PCR analysis of SVCT1 and SVCT2 expression. Total RNA was isolated and converted into cDNA as described in Materials and Methods Section. Standard PCR was performed using 30 cycles. USP11 was used as loading control. **B**: Effect of nickel exposure on SVCT2 expression. 1HAEo- cells were exposed to nickel alone or in combination with ascorbate for 20 h. Fifteen micrograms of total RNA were subjected to Northern blot analysis using SVCT2-specific probe. **C**: 1HAEo- cells were plated at a density of 10^6 cells per 100 mm dish and were grown with or without ascorbate for 5 days. Total RNA was isolated each day with 24 h intervals. Fifteen micrograms of total RNA were analyzed by Northern blot analysis using SVCT2-specific probe. Staining of nylon membranes below the Northern blots is shown to assure RNA loading.

enhanced iron consumption by growing cells could lead to iron shortage in dense culture and this might cause the induction of hypoxia-inducible genes. It has to be taken into consideration that EMEM medium does not contain iron and the only source of iron in the culture is serum. The daily addition of ferrous sulfate to maintain 150 μM iron in the cell culture did not prevent up-regulation of NDRG1/Cap43 expression (Fig. 6D), suggesting that the effect was not due to iron deficiency.

DISCUSSION

Since hypoxia is widespread in tumors, a number of hypoxia-inducible genes are considered tumor markers [Loncaster et al., 2001; Potter and Harris, 2004]. The main reason for tumor hypoxia is poorly organized blood vessels and limited blood delivery into a tumor body [Hockel and Vaupel, 2001]. But blood is delivering to cells not only oxygen, but also ascorbate and iron. In our experiments we failed to reverse up-regulation of NDRG1/Cap43 by iron supplementation in a dense culture; therefore, we focused our attention on changes in ascorbate levels in dense cultures. The important role of ascorbate in HIF-α metabolism has been elucidated recently because of studies on mechanisms of metal-induced hypoxic stress [Knowles et al., 2003; Salnikow et al., 2004]. Both nickel and cobalt can up-regulate HIF-1α and hypoxia-inducible genes in vitro under atmospheric

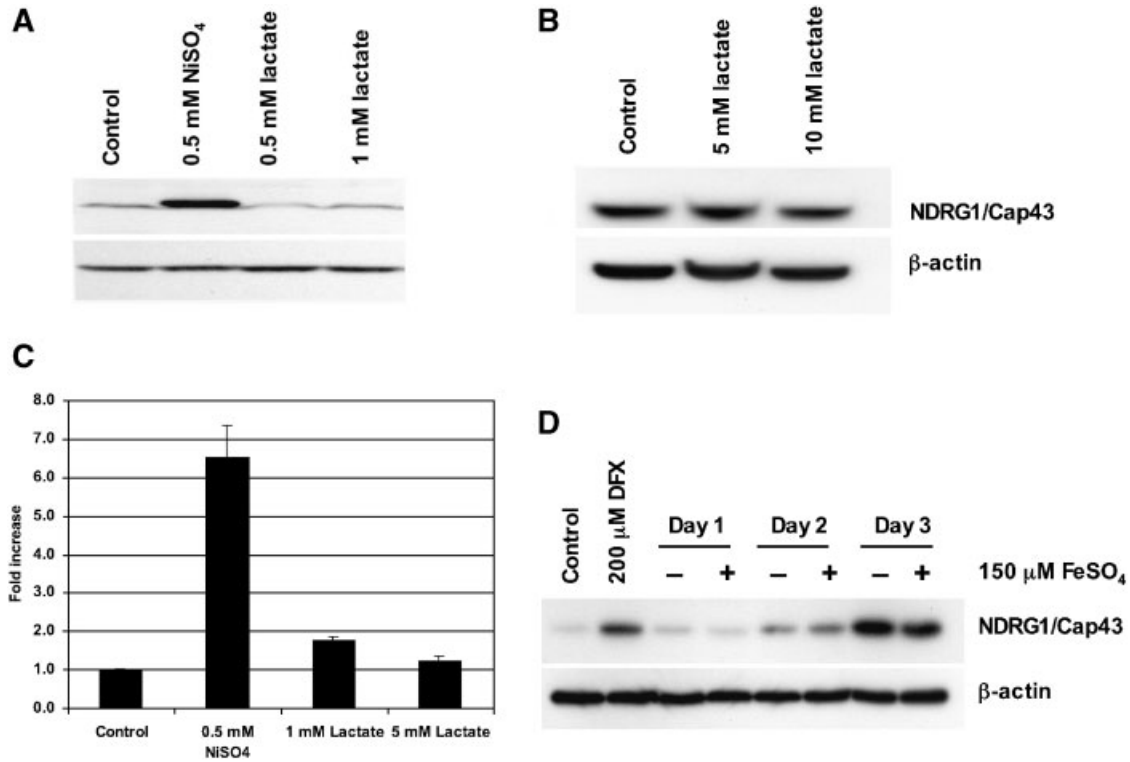

Fig. 6. Medium acidification or iron supplementation does not change HIF-dependent expression. **A**: Effect of lactic acid on HIF-dependent expression. 1HAEo- cells were transiently transfected with HRE-Luc plasmid. Sixteen hours after transfection, lactic acid was added at concentrations indicated in Figure. Relative luciferase activity was detected 20 h after exposure and expressed as fold increase over control. Induction by nickel is shown for comparison. The data are presented as mean ± SD. Effect of lactic acid on NDRG1/Cap43 expression in Hepa1 (**B**) and 1HAEo- cells (**C**). Cells were exposed to lactic acid in concentrations shown in the Figure for 20 h. Forty micrograms of total cell extract was resolved over SDS–PAGE subjected to Western blot analysis using anti-NDRG1/Cap43 antibodies. Exposure to 0.5 mM $NiSO_4$ was used as a positive control. **D**: Effect of iron supplementation on NDRG1/Cap43 expression. 1HAEo- cells were plated at a density of 10^6 cells per 100 mm dish and were grown with or without supplementation of 150 μM $FeSO_4$ for 3 days. Forty micrograms of total cell extract was resolved over SDS–PAGE and subjected to Western blot analysis using anti NDRG1/Cap43 antibodies. Exposure to 0.2 mM DFX (iron chelator) was used as a positive control. In all cases Western blot for β-actin was used as a loading control.

oxygen pressure by lowering intracellular ascorbate [Salnikow et al., 2004]. Since both oxygen and ascorbate are important components of the prolyl hydroxylase reaction, the shortage of either one could result in enzyme inactivation and hypoxia-like stress. In this study, we show that both nickel exposure and continuous growth of cells to high density could up-regulate two well-known tumor and hypoxia-associated proteins, CA IX and NDRG1/Cap43, under normoxic conditions.

The up-regulation of both tumor markers by nickel was clearly due to stabilization of HIF-1α and HIF-2α. This stabilization was likely a result of inhibition of prolyl hydroxylases due to depletion of intracellular ascorbate. The role of the depletion of intracellular ascorbate in nickel-induced HIFα activation is clearly seen in Figure 2B,C. At low nickel concentrations 50 μM of ascorbate can completely reverse NDRG1/Cap43 up-regulation. At higher nickel concentrations 50 μM of ascorbate is insufficient to completely reverse HIF-1α or NDRG1/Cap43 up-regulation.

In contrast, cell density did not stabilize HIFα's at days 2–4 when up-regulation of CA IX and NDRG1/Cap43 was observed. Even at later time points when HIFα's were increased their levels did not reach the levels observed in nickel-exposed cells, suggesting the involvement of another transcription factor(s) in density-dependent up-regulation of CA IX and NDRG1/Cap43. The involvement of another transcription factor, not HIF-1, in density-dependent NDRG1/Cap43 up-regulation was further supported by the observation that the NDRG1/Cap43 was up-regulated by high density regardless of HIF-1, that is, in mouse

fibroblasts originated from HIF-1α knock-out mice. In these cells HIF-1α is not expressed and HIF-2α is transcriptionally inactive [Park et al., 2003]. Our data are in agreement with recently published by Kaluz et al. [2002] observation that CA IX can be induced by cell density without HIF-1α up-regulation. These authors also showed moderate activation of CA IX promoter by cell density in HIF-1α deficient cells.

In this study, we show for the first time that hypothetical density-induced transcription factor is also sensitive to intracellular ascorbate levels, since the addition of ascorbate reversed the CA IX and NDRG1/Cap43 up-regulation by cell density. Although two different pathways appeared to be involved in cell density- and nickel-induced up-regulation of the CA IX and NDRG1/Cap43, in both cases the deficiency of intracellular ascorbate caused the induction of these tumor markers.

Ascorbate is a well-known antioxidant required by all mammalian cells [Rose, 1988; Padayatty et al., 2003]. In addition to its general antioxidant activity, ascorbate plays an important specific role in iron reduction in numerous iron-containing dioxygenases [de Jong et al., 1982; Myllyla et al., 1984]. While most mammals produce ascorbate in the liver due to the presence of a specific enzyme, γ-gulonolactone oxidase, humans have a mutation in the enzyme and are unable to synthesize ascorbate. Therefore humans, in order to maintain necessary intracellular ascorbate levels, must receive it with the diet and transport it into cells. Thus, the level of ascorbate strongly depends on the ascorbate levels in the blood or in the serum used for cell culture. Our measurements showed that the level of ascorbate in freshly prepared culture medium varies between 1 and 5 μM, depending on the source of serum [Salnikow et al., 2004]. Both the oxidized and reduced forms of ascorbate can be transported into cells with the help of glucose transporters or sodium-dependent transporters SVCT1 and SVCT2, respectively. Most ascorbate, however, is delivered into the cell in a reduced form via sodium-dependent transporters. Inside the cell, ascorbate can be accumulated up to 40–60 μM intracellularly [Welch et al., 1993; Goldenberg and Schweinzer, 1994; Daruwala et al., 1999; Takanaga et al., 2004]. Although SVCT1 and SVCT2 have similar functional properties, they are discretely distributed in different tissues. Thus, SVCT1 is confined to the intestine, kidney and liver and, to much extent, to lung, whereas SVCT2 is expressed in brain, eye, lung, endothelial, and neuroendocrine cells [Fischer et al., 2004; Takanaga et al., 2004]. The vital importance of SVCT2 for lung functions was shown in SVCT2 knockout mice [Sotiriou et al., 2002]. Prevailing expression of SVCT2 ascorbate transporter in human lung epithelial cells have been reported previously [Fischer et al., 2004]. Our findings using different human lung epithelial cell line are in agreement with the reported data.

In this study, we found that even 2 days of culturing cells in vitro resulted in a significant loss of intracellular ascorbate in 1HAEo- cells, which caused significant up-regulation of hypoxia-associated proteins CA IX and NDRG1/Cap43. Such loss probably results from the exposure of cultured cells to high oxygen levels, which is a common situation in cell culture. The in vitro "normoxia" is defined as 21% oxygen (pO_2 of 160 mm Hg), which is at least four times higher than the physiological in vivo normoxia [Hockel and Vaupel, 2001]. Oxygen easily diffuses in the medium and it seems unlikely that local, mild hypoxia near the cell surface of dense, but still monolayer cultures could cause up-regulation of hypoxia-associated proteins, as was suggested recently [Kaluz et al., 2002]. Rather, this increased oxygenation can produce rapid oxidation of ascorbate to dehydroascorbate, which is very unstable. Indeed, we observed higher intracellular ascorbate levels under hypoxic conditions as compared with normoxic conditions.

The possibility that a soluble factor is responsible for the differential regulation of VEGF expression in sparse and confluent cultures was suggested previously [Koura et al., 1996]. In this study, we directly showed the loss of intracellular ascorbate by 1HAEo- cells below a detectable level after culturing cells for 2 days. This loss of intracellular ascorbate by 1HAEo- cells due to metabolism and oxidation must be compensated by active uptake via the SVCT2 system. Indeed, at day 1, when concentration of ascorbate inside and outside of the cell was high, Northern blot analysis showed a low level of SVCT2 mRNA expression. Consistent with our hypothesis, by day 5, with a low level of ascorbate inside and outside of the cell, the expression of SVCT2 was increased over 10-fold. A daily addition of ascorbate to the medium

resulted in a decrease in SVCT2 expression, suggesting the existence of a feedback mechanism. Such a marked increase of SCVT2 expression in confluent culture has not been described before; however, similar density-dependent changes in another ascorbate transporter SVCT1 expression were found in the human colon carcinoma cell line CaCo-2 [Maulen et al., 2003]. Thus, we propose that density-dependent changes in ascorbate transporter expression most likely represent normal mechanism of cellular regulation of ascorbate levels.

The exposure to carcinogenic nickel, as shown on Figure 5A,B inhibits expression of SVCT2 and of SVCT1 in human lung cells. Such an inhibition of SVCT1 or SVCT2 expression by a carcinogen, to the best of our knowledge, has not been reported. It is noteworthy that the exposure to phorbol ester, a tumor promoter, significantly inhibits activity of both transporters [Daruwala et al., 1999]. This may cause the induction of HIF-1α demonstrated in phorbol ester-exposed cells [Zhong et al., 2001]. It is not known, however, whether the exposure to phorbol ester suppresses SVCT2 expression similar to that observed for nickel. The modulation of ascorbate transporter's expression by a carcinogen or during spontaneous cell transformation could be translated into the low intracellular ascorbate levels. It is conceivable, therefore, that in some tumor cells expressing hypoxia-associated proteins ascorbate transporters expression could be down-regulated.

The role of hydroxylation in proteasomal degradation of HIF-1α and HIF-2α proteins is well established and the participation of ascorbate in this process has been acknowledged [Knowles et al., 2003; Maxwell and Salnikow, 2004; Salnikow et al., 2004]. The fact that the up-regulation of CA IX and NDRG1/Cap43 by cell density is also ascorbate-sensitive, but does not involve HIFα's, suggests that, in addition to HIF-1, there may be other, differently regulated, but also ascorbate-sensitive transcription factors. We are currently investigating this possibility.

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