Gastric Epithelial Reactive Oxygen Species Prevent Normoxic Degradation of Hypoxia-inducible Factor-1α in Gastric Cancer Cells

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ABSTRACT

The expression of hypoxia-inducible factor (HIF)-1α protein is tightly regulated by cellular oxygen status. Namely, HIF-1α protein is degraded rapidly in normoxic cells, whereas hypoxia stabilizes HIF-1α protein to transactivate hypoxia-responsive genes. Here we show that HIF-1α protein is expressed aberrantly in gastric cancer cells under normoxia in a reactive oxygen species (ROS)-dependent manner. The normoxic expression of HIF-1α in accordance with its DNA binding activity enhances the transcription of target genes such as vascular endothelial growth factor. The aberrant normoxic expression of HIF-1α is not associated with genetic abnormalities such as the loss of von Hippel-Lindau tumor suppressor, but is well correlated with endogenous ROS (hydrogen peroxide) generation. HIF-1α expression is blocked by nonmitochondrial ROS inhibitors, but not by inhibitors of mitochondrial electron transfer, which indicates that nonmitochondrial ROS stabilize HIF-1α protein in these cells. Gastric epithelial ROS have been linked to Helicobacter pylori-induced gastric carcinogenesis. This study demonstrates for the first time that ROS from H. pylori-infected gastric epithelial cells induce HIF-1α expression and subsequently activate HIF-1α-mediated transcription. Taken together, these results provide a novel mechanism of HIF-1α stabilization in gastric cancer, and demonstrate that gastric epithelial ROS, endogenously generated or H. pylori-stimulated, lead to the constant expression of HIF-1α protein under normoxia.

INTRODUCTION

HIF-1α is a transcription factor, composed of HIF-1α and β (1), which transactivates crucial target genes involved in angiogenesis, energy metabolism, and cell proliferation (2–5). Whereas HIF-1β is constitutively expressed, HIF-1α is tightly regulated in an oxygen-dependent manner (6–9). Under normoxia, HIF-1α undergoes rapid degradation, which is dependent on post-translational modification of HIF-1α. If HIF-1α is hydroxylated at its proline residues of its oxygen-dependent degradation domain under normoxic condition, it binds with the pVHL-containing complex, which targets HIF-1α for ubiquitin-proteasome degradation (10–14). When cells are exposed to hypoxia, proline is not hydroxylated and, thus, HIF-1α escapes degradation, allowing it to accumulate and transactivate hypoxia-responsive genes.

The concept that hypoxia plays a major role in HIF-1α regulation has been evolving. In addition to hypoxia, it has been demonstrated that the stabilization of HIF-1α protein is induced by genetic abnormalities, such as the loss of pVHL in renal cancer cells like RCC9 (10) or by an activation of PI3K and Akt in prostate cancer cells such as PC3 (15). In addition, the cellular redox status also affects the expression of HIF-1α protein. It is known that hypoxic ROS are associated with the induction of HIF-1α expression in Hep3B hepatoma cells (16, 17). However, nonglycemic ROS tend to degrade HIF-1α protein in alveolar epithelial cells (18). Thus, the effect of ROS on HIF-1α protein might be determined by oxygen status or cell type. Compared with normal cells, cancer cells have increased metabolism and generate ROS, which has diverse effects on cell survival or cell death (19–24). In gastric epithelial cells, Helicobacter pylori is considered to be a risk factor for gastric cancer. H. pylori-infected gastric epithelial cells generate ROS, which plays an important role in gastric carcinogenesis (25–29). However, the molecular mechanisms by which epithelial ROS is involved in gastric carcinogenesis are unclear. Furthermore, the physiological function of gastric epithelial ROS in gastric cancer is poorly understood.

In the present study, we investigated the expression of HIF-1α protein and the underlying mechanism of its stabilization in gastric cancer under normoxic conditions. Our data provide a novel mechanism of HIF-1α stabilization under normoxia, namely that gastric epithelial ROS protect HIF-1α from normoxic degradation in gastric cancer.
MATERIALS AND METHODS

Cells and Chemicals. Human gastric adenocarcinoma cells (SNU-484, 601, 638, 668, and 719) were obtained from the Korean Cell Line Bank (30) and grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS and gentamicin (10 μg/ml). Human hepatoma cells (HepG2) and prostate cancer cells (PC-3) were obtained from the American Type Culture Collection and grown in RPMI 1640 or DMEM supplemented with 10% FBS and gentamicin (10 μg/ml). All of the cells were incubated under standard culture condition (20% O₂ and 5% CO₂, at 37°C). MG132 (Sigma) was used at a final concentration of 10 μM. ROS inhibitors were purchased from Sigma. DPI and PDTC were both treated at concentrations of 10, 50, and 100 μM, respectively.

Plasmids, Transfection, and Luciferase Reporter Gene Assay. Cells were seeded onto six-well plates, and the medium was changed to serum-free medium 3 h before transfection. Cells were transiently transfected using lipofectin (Life Technologies, Inc.) in duplicate with 1.5 μg of luciferase reporter plasmid containing VEGF promoter or the trimer of a HIF-1 binding site consisting of a denaturation step at 94°C for 1 min, and an extension step at 72°C for 1 min. To estimate the efficiency of cDNA synthesis, β-actin was used as a control. The PCR products were resolved on SeaKem agarose gels.

Antibodies, Immunoblot Analysis, and Immunohistochemistry. Monoclonal antibodies to HIF-1α (NB100–123CS) and Akt (9272) were from Cell Signaling Technology, monoclonal antibody to PTEN (sc-7974) was from Santa Cruz Biotechnology, and monoclonal antibody to α-tubulin was from Sigma. For immunoblot analysis, the cells were washed with ice-cold PBS and suspended in an extraction buffer (20 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin A, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 10 μM aprotinin, 0.5 mg/ml soybean trypsin inhibitor, and 1 mM benzamidine) on ice for 15 min. Samples containing 150 μg of total protein were resolved by SDS-polyacrylamide denaturing gel, transferred onto nitrocellulose membranes (Schleicher & Schuell), and probed with antibodies, as recommended by the manufacturer. Detection was performed using the enhanced chemiluminescence system (Amersham). For immunohistochemical staining, formalin-fixed, paraffin-embedded gastric adenocarcinoma and normal gastric mucosal tissue specimens were obtained, and each tissue section was reacted with anti-HIF-1α monoclonal antibody (NB100–123CS) at a dilution of 1:100 overnight at 4°C. The secondary reagents were contained in a Vector Elite ABC kit (Vector Laboratories), and 3,3′-diaminobenzidine tetrahydrochloride was used as the color reagent. Slides were visualized and photographed under a light microscope (Olympus).

EMSA. Cells were quickly cooled by placing the plates on ice and were then washed twice with ice-cold PBS. Scraped cells were centrifuged and resuspended in five packed cell volumes of buffer A [20 mM Tris (pH 7.6), 10 mM KCl, 0.2 mM EDTA, 20% glycerol, 1.5 mM MgCl₂, 2 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, and 2 μg/ml each of leupeptin, pepstatin, and aprotinin]. Nuclei were pelleted (2,500 × g, 10 min) and resuspended in two packed cell volumes of buffer B (identical to buffer A except that the KCl level was increased to 0.42 M). Nuclear debris was removed by centrifugation (15,000 × g, 20 min). EMSA was performed by incubating 5 μg of the nuclear extract with a 32P-labeled, double-stranded oligonucleotide probe. Sequences containing HIF-1 binding sites were derived from EpoWT. Sequences were as follows: EpoWT (sense), 5′-GCC CTA CGT GCT GCC TCG CAT GCC-3′; and EpoWT (antisense), 5′-GCC ATG CGA GGC AGC ACg TAG GCC-3′. Oligonucleotides were annealed at a concentration of 250 μM in 250 mM Tris (pH 7.8) at 95°C for 15 min, and slowly cooled to room temperature over-night and stored at −20°C. The annealed oligonucleotides were diluted to 5 μM in the same buffer. Probes were labeled using [γ-32P]ATP (NEN) and T4 polynucleotide kinase (Promega). End-labeled probes were purified from unincorporated [γ-32P]ATP by using a ProbeQuanta G-50 microcolumn (Amersham). Binding reactions were set up in a volume of 20 μl, and nuclear extracts (5 μg protein) were incubated in a buffer at a final concentration of 50 mM KCl, 10 mM Tris (pH 7.7), 5 mM DTT, 1 mM MgCl₂, 5% glycerol, 0.03% NP40, and 0.1 μg/μl poly(dIdC). Samples were incubated on ice for 30 min and resolved by electrophoresis in 4% polyacrylamide gels at 4°C. The gels were then dried and analyzed by autoradiography. In competition experiments, 100-fold molar excesses of unlabeled annealed oligonucleotides were added before the addition of the labeled probes.
Measurement of ROS Generation in Gastric Epithelial Cells. Cellular uptake of DCFH-DA (Molecular Probes) and the oxidation of its deacetylated form (DCFH) to yield the fluorescent product, DCF, was used as an intracellular indicator for ROS generation (31). Gastric cancer cells were harvested by trypsinization, washed with DMEM, and resuspended in 2 ml of buffer A (150 mM NaCl, 6 mM KCl, 10 mM HEPES, 1 mM MgCl2, 5.5 mM glucose, and 0.2% BSA). Cells were incubated with 10 μg/ml DCFH-DA for 15 min at 37°C. After incubation, the cells were washed with 5 ml of buffer B (150 mM NaCl, 6 mM KCl, 10 mM HEPES, 1 mM MgCl2, and 5.5 mM glucose) twice and resuspended in 1 ml of buffer B. Intracellular DCF fluorescence was analyzed by a FACScan flow cytometry (Becton Dickinson).

H. Pylori Culture. A H. pylori strain (HP99) was isolated from gastric antral mucosa obtained from a duodenal ulcer patient at Seoul National University Hospital. The isolate was identified as H. pylori based on its morphology and by biochemical testing. HP99 contains cagA+/vaculinating cytotoxin+ (32–34). The bacteria were inoculated onto chocolate agar plates (Becton Dickinson Microbiology Systems) at 37°C under microaerophilic conditions (5% O2, 10% CO2, and 85% N2). Experiments were performed by suspending whole bacteria in RPMI 1640 to a final concentration of 3 × 107 bacteria/ml, and SNU-668 cells were incubated in the bacteria containing medium for 6 h before harvest.

RESULTS

HIF-1α Protein Is Aberrantly Expressed in Human Gastric Cancer Cells under Normoxia. We first examined the expression of HIF-1α mRNA by RT-PCR. HIF-1α mRNA was expressed to a similar extent in all of the gastric cancer cells tested under standard culture condition (20% O2 and 5% CO2; 37°C; Fig. 1A). The expression of HIF-1α protein is tightly regulated by cellular oxygen tension, and, thus, is normally undetectable under normoxia. However, immunoblot analysis showed that gastric cancer cells, such as SNU-484, 601, 638, and 719 express HIF-1α protein despite normoxia at levels comparable with that in PC-3 cells, a positive control, whereas HIF-1α was not detected in SNU-668 gastric cancer cells as was the case in HepG2 cells, a negative control (Fig. 1B). The expression of HIF-1α protein might be affected by some growth factors contained in serum. However, we found that the levels of HIF-1α protein in gastric cancer cells were similar in the presence and in the absence of serum (data not shown). HIF-1α immunostaining showed that HIF-1α-positive cells were clustered within gastric adenocarcinoma cells, but HIF-1α was not detected in nonmalignant epithelial and stromal cells (Fig. 1C). Therefore, we conclude that gastric cancer cells aberrantly express HIF-1α protein under normoxic conditions.

Constantly Expressed HIF-1α Protein Transactivates Hypoxia-responsive Genes. Under hypoxic conditions, stabilized HIF-1α protein binds specifically to promoter sites containing the HRE and transactivates target genes, such as VEGF. To examine the DNA binding activity of HIF-1α protein expressed in normoxic cells, we performed EMSA on two gastric cancer cell lines (SNU-638 and 668), on PC-3 cells (a positive control), and on HepG2 (a negative control; Fig. 2A). The binding of HIF-1α protein to the 24-bp HRE of the EPO gene was detected both in SNU-638 and PC-3 cells, which expressed HIF-1α protein under normoxia, whereas no binding was observed in the SNU-668 or HepG2 cells. We then transfected gastric cancer cells with a luciferase reporter plasmid containing VEGF promoter to determine whether nonhypoxic HIF-1α protein functions as transcriptional activator in these cells. As expected, luciferase activity correlated well with the level of the normoxic expression of HIF-1α protein (Fig. 2B). In addition, luciferase activity correlated well with the expression of VEGF.
mRNA in these cells (Fig. 2C). Taken together, these results suggest that HIF-1α protein, which is expressed in normoxic gastric cancer cells, functions normally to transactivate HIF-1α-mediated transcription.

Aberrant Expression of HIF-1α in Gastric Cancer Cells Is Not Because of the Loss of pVHL or the Activation of the PI3K Pathway. To determine whether the expression of HIF-1α protein in gastric cancer cells depends on the proteasomal degradation pathway, we treated these cells with MG-132, a proteasomal inhibitor. In the presence of MG-132, HIF-1α protein was stabilized in SNU-668 cells under normoxia because MG-132 blocked the degradation of HIF-1α. On the other hand, MG-132 induced only a slight increase in HIF-1α expression in SNU-638 cells (Fig. 3A). These results suggest that the normoxic expression of HIF-1α protein in gastric cancer cells is because of the protection of HIF-1α protein from the normoxic degradation. HIF-1α could be expressed under normoxia by the loss of pVHL (10) or by an activated PI3K/Akt pathway through the loss of PTEN (15) in cancer cells. The gastric cancer cells used in the present experiment contained pVHL and wild-type VHL genes (confirmed by sequencing analysis; data not shown), and the normal expression of PTEN, the later of which results in suppressed Akt activity. These results suggest that the aberrant normoxic expression of HIF-1α protein in gastric cancer cells is not associated with either the loss of pVHL or the activation of the PI3K/Akt pathway (Fig. 3B).

Gastric Epithelial ROS Are Associated with HIF-1α Expression in Gastric Cancer under Normoxia. Recent observations have suggested that cellular redox status affects the stabilization of HIF-1α. To test this suggestion, we examined levels of hydrogen peroxide in gastric cancer cells using DCFH-DA. DCFH-DA enters the cells and can be oxidized to generate the fluorescent DCF in the presence of hydrogen peroxide (31). The level of ROS production tended to be well correlated with the expression of HIF-1α protein in each cell (Fig. 4A). To determine whether ROS activity directly regulates the expression of HIF-1α, we investigated the effect of pharmacologic ROS inhibitors on the expression of HIF-1α. The respiratory chain of the mitochondrial complex or the flavoprotein-containing NADPH oxidase have been proposed as the likely sources of ROS. Mitochondrial ROS are blocked by rotenone, amobarbital, antimycin A, or KCN, which inhibit the mitochondrial electron-transfer system. Likewise, nonmitochondrial ROS are blocked by PDTC or NAC, which enhances the scavenging of hydrogen peroxide. DPI, a specific flavoprotein inhibitor that blocks ROS generation by NADPH oxidase, can also abrogate mitochondrial ROS generation by inhibiting electron transport at the flavin site in mitochondrial complex I (35). In SNU-638 cells, which

Fig. 2 Constantly expressed HIF-1α protein transactivates hypoxia-responsive genes in normoxic gastric cancer cells. A, HIF-1 DNA binding was detected in nuclear extracts by EMSA using an oligonucleotide probe that contained the HIF-1 DNA binding site. The HIF-1 DNA binding complex was detectable in PC-3 and SNU-638 cells under normoxic conditions. C, constitutive protein-DNA complexes. B, gastric cancer cells were transfected with a reporter plasmid containing VEGF promoter-luciferase (pGL2/VEGF-Luc) or luciferase only (pGL2/Luc) and a plasmid encoding β-galactosidase. Forty-eight h later, luciferase activity, normalized for β-galactosidase, was determined. Relative luciferase activity was defined as the values of the others divided by the value of the empty vector control in SNU-484. The results shown are the means of three independent experiments; bars, ±SD. The corresponding expressions of HIF-1α protein are indicated at the bottom. □ empty vector control; ■ pGL2/VEGF-Luc. C, total RNA was isolated from cancer cells incubated under normoxic condition. The expression of VEGF mRNA was analyzed by RT-PCR analysis and detected in PC-3 and SNU-484, 601, 638, and 719 cells under normoxic conditions. To estimate the efficiency of cDNA synthesis, β-actin was used as a control. Control, no DNA.
that normoxic, nonmitochondrial ROS activity is associated with HIF-1α expression in gastric cancer cells. Next, we analyzed the effect of ROS inhibitors on the transcriptional activation activity of HIF-1α in SNU-638 cells under normoxic condition. As shown in Fig. 5, ROS inhibitors decreased both ROS production and HIF-1α expression in a dose-dependent manner. Similarly, VEGF promoter-luciferase activity was decreased by ROS inhibitors. These results suggest that ROS might be a novel target for blocking the normoxic expression of HIF-1α protein and its transcriptional activity in gastric cancer cells.

**H. Pylori-stimulated ROS Induce HIF-1α Expression and Its Transcriptional Activity.** Increased ROS activity has been suggested to play an important role in gastric carcinogenesis. In particular, *H. pylori*-infected gastric epithelial cells produce ROS, and this increased ROS activity might contribute to the initiation and progression of gastric cancer (25–29). Therefore, we hypothesized that *H. pylori*-induced ROS may stabilize HIF-1α protein in gastric epithelial cells. To test theory, we infected gastric cancer cells with *H. pylori*. Infected SNU-638 cells with *H. pylori* induced the expression of HIF-1α in parallel with the increased ROS activity in these cells (Figs. 6, A and B). In addition, the addition of ROS inhibitors to *H. pylori*-infected SNU-668 cells repressed *H. pylori*-induced ROS production, the expression of HIF-1α protein, and transcriptional activation of HRE-luciferase reporter (Fig. 6C). These findings suggest that *H. pylori*-induced ROS generation stabilizes HIF-1α protein under normoxia and subsequently activates HIF-1α-mediated transcription.

**DISCUSSION**

This study demonstrates for the first time that gastric epithelial ROS, endogenously produced or stimulated by *H. pylori*, stabilize HIF-1α protein in human gastric cancer cells under normoxic conditions. In view of the fact that HIF-1α protein transactivates various target genes involved in tumor growth and progression, the constant expression of functional HIF-1α protein may play a crucial role in determining the biological aggressiveness of gastric cancer.

HIF-1α protein is overexpressed in cancer cells not only by hypoxia, but also by genetic abnormalities under normoxic condition. In this regard, our results are unexpected because SNU gastric cancer cells, which were used in the present experiment, do not have associated genetic defects, which might express HIF-1α under normoxia, the addition of DPI, PDTC, or NAC down-regulated HIF-1α expression, whereas the addition of specific inhibitors of mitochondrial electron transfer did not affect the expression of HIF-1α (Fig. 4B). These findings imply that normoxic, nonmitochondrial ROS activity is associated with normoxic expression of HIF-1α. A, intracellular DCF fluorescence was measured to quantify hydrogen peroxide levels. After trypsinization, cells were incubated with DCF-DA (10 μg/ml) for 15 min, and DCF fluorescence was measured by FACS analysis. Relative DCF fluorescence was defined as the DCF fluorescence value of the cell under interest divided by the DCF fluorescence value of SNU-668. The results shown are the means of three independent experiments; bars, ±SD. The corresponding expressions of HIF-1α protein are indicated at bottom. B, SNU-638 cells were treated with DPI (100 μM), PDTC (100 μM), NAC (20 mM), rotenone (10 μg/ml), amobarbital (10 mM), antimycin A (10 μg/ml), or KCN (10 mM) for 6 h under normoxic conditions, and then probed with anti-HIF-1α and anti-α-tubulin antibody.
Fig. 5 Antioxidants effectively block the normoxic expression of HIF-1α protein and HIF-mediated transcriptional activation in gastric cancer cells. SNU-638 cells were grown under normoxic conditions, and treated with ROS inhibitors for 6 h before harvesting. DPI and PDTC were treated at 10, 50, and 100 μM, and NAC was treated at 1, 10, and 20 mM. Immunoblot analysis against HIF-1α and α-tubulin (A), DCF fluorescence analysis by FACS (B), and luciferase assay using VEGF promoter-luciferase reporter (C) were carried out in the absence or in the presence of increasing amounts of antioxidant under normoxia in gastric cancer cells. The relative DCF fluorescence is provided at each given concentration (■) divided by the value of the untreated control (□). The relative luciferase activity is the corrected luciferase value at each concentration (■) divided by the corrected luciferase value of the untreated control (□); bars, ±SD.

induce the normoxic stabilization of HIF-1α. pVHL, a master regulator of HIF-1α expression, is a component of an E3 ubiquitin ligase complex that targets HIF-1α for ubiquitination under normoxia (36, 37). In cells lacking pVHL, HIF-1α is constitutively stabilized under normoxia, and the reintroduction of pVHL degrades HIF-1α (10). In the case of gastric cancer, genetic abnormalities of VHL are very rare; we confirmed that all of the gastric cancer cell lines used in this study contained wild-type VHL. Another factor concerning the normoxic stabilization of HIF-1α involves the activation of the PI3K/Akt pathway (15). To address this possibility, we tested both the expression of PTEN and Akt activity in gastric cancer cells, and observed normal levels of PTEN and the suppressed Akt activity in gastric cancer cells under normoxic condition.

Recent studies have indicated that HIF-1α protein is stabilized by mitochondrial ROS under hypoxic conditions in Hep3B cells (17), and according to this study, mitochondrial generation of superoxide and subsequent hydrogen peroxide induces PI3K
activation leading to HIF-1α stabilization. However, the opposite effect also exists as antioxidants such as NAC or PDTC induce HIF-1α protein on normoxia in alveolar epithelial cells (18). Therefore, the role of ROS as a regulator of HIF-1α protein needs additional investigations. In the present study, we found that gastric cancer cell-derived nonmitochondrial ROS induce HIF-1α expression under normoxia, and our experiments with inhibitors of the mitochondrial electron-transfer chain system provide evidence that nonmitochondrial ROS are associated with HIF-1α stabilization. These results strongly suggest that the ROS-dependent pathway directly mediates the normoxic stabilization of HIF-1α in gastric cancer cells. Moreover, recent studies have suggested that the normoxic degradation of HIF-1α protein is mediated through the hydroxylation of the proline residue in the oxygen-dependent degradation domain (13, 14, 38–42), and that nitric oxide induces nitrosylation at cysteine residues to stabilize the HIF-1α protein (43). We speculate that the stability of HIF-1α protein might be regulated through a ROS-directed post-translational modification, and an experiment to test this hypothesis is now underway.

ROS have been implicated in the pathogenesis of H. pylori-induced gastric cancer, and H. pylori infection is considered to be a risk factor of gastric cancer (25–29). However, the underlying molecular mechanisms of H. pylori-induced gastric carcinogenesis are not completely understood. There is growing evidence that H. pylori induces the generation of ROS in gastric epithelial cells in vitro and that this is important in gastric carcinogenesis (25–29). In this study, we found that H. pylori-infected gastric epithelial cells generate ROS and induce the normoxic expression of HIF-1α protein. Moreover, ROS generated by H. pylori infection stimulate HIF-1α protein, and may act as a driving force to promote tumor initiation and progression. The fact that increased ROS production leads to the normoxic stabilization of HIF-1α implies that H. pylori-induced ROS may be involved in gastric carcinogenesis through the regulation of HIF-mediated gene expression.

In conclusion, gastric cancer cells aberrantly express HIF-1α protein under normoxia, and this normoxic expression of HIF-1α protein is associated with epithelial ROS generation in gastric cancer cells. In addition to endogenous ROS, H. pylori-induced ROS also lead to the stabilization of HIF-1α protein in gastric cancer cells, which activates HIF-mediated transcription. Therefore, the inhibition of ROS generation might provide a novel strategy to block HIF-1α activity in gastric cancer.

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