Mechanism of hypoxia-inducible factor 1α-mediated McI1 regulation in Helicobacter pylori-infected human gastric epithelium

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Bhattacharyya A, Chattopadhyay R, Hall EH, Mebrahtu ST, Ernst PB, Crowe SE. Mechanism of hypoxia-inducible factor 1α-mediated McI1 expression in Helicobacter pylori-infected human gastric epithelium. Am J Physiol Gastrointest Liver Physiol 299: G1177–G1186, 2010. First published September 9, 2010; doi:10.1152/ajpgi.00372.2010.—Hypoxia-inducible factor 1 (HIF1) consists of a hypoxia-inducible α subunit and a constitutively expressed β subunit. Reactive oxygen species (ROS) induced by Helicobacter pylori stabilize HIF1α in the human gastric epithelium in normoxia. HIF1α plays crucial role in carcinogenesis and has been associated with malignant progression of gastric cancer. Several genes contain functional hypoxia-response elements (HREs) in their promoters including Bcl2 family member, McI1. Cellular ratios of anti-apoptotic oncogenic protein, McI1, and tumor suppressor proapoptotic protein, Noxa, determine cell fate by regulating normal cellular growth, cell death and oncogenic processes. The aim of the present study was to examine the mechanism of HIF1α induction in the H. pylori-infected gastric epithelium to better understand disease pathogenesis by H. pylori relevant to gastric carcinogenesis. Our data showed that the dose-dependent increase in HIF1α in H. pylori-infected gastric epithelia is mediated by induction of a ROS-inducible protein, apurinic/apyrimidinic endonuclease 1 (APE1), and an enhanced interaction of APE1 with the transcriptional coactivator p300. Surprisingly, with accumulation of HIF1α, further transcriptional activation of mcI1 was not observed. We identified a HIF-binding site (HBS) in the hif1α promoter and showed that increased HIF1α expression, whether H. pylori-induced or hypoxia-mimetic agent, CoCl2-induced, resulted in enhanced HIF1α binding to its own promoter. This resulted in a transcriptionally inactive hif1α promoter since hif1α HBS lacks HIF ancillary sequence (HAS) required for HIF1 transcriptional activity. We conclude that enhanced binding of “nonfunctional” HIF1α to hif1α promoter and limiting availability of p300 in the cell serves as checkpoints for uncontrolled HIF1α activity. APE1; HIF1α; McI1; H. pylori; gastric cancer.

HYPOXIA-INDUCIBLE FACTOR 1 (HIF1) plays an essential role in the survival of higher organisms during hypoxia by transactivating homoeostasis-maintaining genes (42). HIF1 is involved in apoptosis, cell proliferation, tumor angiogenesis (7), aging (26), central nervous system disorders (20), innate immune responses (35), ischemic and inflammatory diseases (18), and infection (51). The active transcription factor is a heterodimer, with the HIF1β subunit constitutively expressed whereas HIF1α is tightly regulated by cellular O2 concentration (24, 46). HIF1α is constitutively transcribed but degraded rapidly in normoxia. In addition to hypoxia, several other factors induce HIF1α by regulating reactive oxygen species (ROS) or various kinases (47). An increase in HIF1α protein translation results in normoxic accumulation of HIF1α (11). Gastric pathogens, Helicobacter pylori, induce ROS (3, 14, 44), which are implicated in peptic ulcer disease and gastric cancer (32, 37). Gastric epithelial ROS, either endogenous or induced by H. pylori, enhance normoxic HIF1α expression in the gastric mucosa (36).

ROS also activate a ubiquitous multifunctional protein, apurinic/apyrimidinic endonuclease 1 (APE1) (22, 29). We have shown that H. pylori infection and ROS augment APE1 expression in human gastric epithelial cells (GEC) (15). APE1, best known as a base excision repair protein (12), reductively activates various transcription factors (16, 45), such as AP-1, HIF1α, and p53 to name a few (4) and hence is also called redox factor 1 (Ref1). However, despite these recent advances, the underlying molecular mechanism regulating ROS-mediated HIF1α expression is unclear.

In the present study we investigated the mechanism of HIF1α induction in H. pylori-infected GEC with the long-term goal of better understanding mechanisms of H. pylori pathogenesis including gastric carcinogenesis. The transcriptional coactivators p300/CBP with intrinsic protein acetyltransferase activity act as protein scaffolds on which transcriptional complexes can be formed (9) and APE1 interacts with p300 and HIF1α to form such complexes (21, 49). We observed that APE1 increased HIF1α expression, and in conjunction with p300, induced transcriptional activity of hif1α but availability of additional HIF1α did not result in further enhancement of the expression or transcriptional activation of HIF1α-target gene mcI1. Interestingly, we found that increased HIF1α expression, induced by either H. pylori or the hypoxia mimetic cobalt chloride (CoCl2), led to binding of HIF1α protein to a HIF-binding site (HBS) in the hif1α promoter. This study provides the first evidence that HIF1α expression and transcriptional activation in the H. pylori-infected gastric epithelium is critically regulated by the multiplicity of infection and therefore provides new knowledge regarding the role of HIF1α in H. pylori pathogenesis.

MATERIAL AND METHODS

Plasmids and transient transfections. pcDNA3.1-APE1-Flag and pCMV-p300 were used. pcDNA3.1-APE1NΔ41-Flag was kindly provided by Prof. Sankar Mitra (Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX). pcMV6-XL5-HIF1α was purchased from Origene Technologies. Human hif1α promoter (−499 to 0) with either wild-type (WT; CGAGCACGTG) or mutated HBS (CGAACACATA) was cloned in KpnI/HindIII site of pGL3 basic (Promega) by GenScript, Piscataway, NJ. Human mcI1 promoter (−983 to −828) with the HRE was cloned in pGL2 basic vector (Primers are shown in Supplementary Fig. S2; the online version of this article contains
supplemental data). For transient transfections, 1 × 10^6 WT, pSIREN, short hairpin RNA (shRNA) AGS cells were seeded in six-well plates 18–24 h before transfection. For overexpression studies, cells were transfected with 2 μg of plasmid DNA and 10 μl of Lipofectamine 2000 reagent (Invitrogen). Cells were treated after 24 h of transfections. For dual luciferase assays, cells were cotransfected with Renilla luciferase (phRLTK) control plasmid (Promega) along with mcll HRE-containing luciferase construct or HIF1α-hBS containing luciferase construct at a ratio of 1:50 while maintaining the total amount of plasmid DNA per transfection constant at 2 μg. At 24 h after transfection, cells were treated. Firefly and Renilla luciferase gene activities in cell lysates were measured sequentially with a Monolight 2010 Luminometer (Analytical Luminescence Laboratory) by using a commercially available enzymatic assay kit (Dual Luciferase Reporter Assay System; Promega).

GEC cultures and bacterial strains. AGS, MKN 45, NCI-N87, and KATO III cells and H. pylori 26695, a cytotoxin-associated gene (cag) pathogenicity island (PAI) (+) strain (ATCC), and its isogenic mutant, cag PAI (−) strain 8-1, were maintained as previously described (5, 15).

Human GEC isolation from mucosal biopsies. Antral gastric mucosa biopsy specimens were collected from H. pylori-uninfected individuals during diagnostic esophagogastroduodenoscopy following a University of Virginia (UVA) Institutional Review Board-approved protocol. Isolated epithelial cells were resuspended in RPMI 1640 containing 10% FBS (15, 17, 48), and 5 × 10^5 cells were plated in 12-well plates. After 5 h to adhere, cells were infected with multiplicity of infection (MOI) 300 or 600 of H. pylori 26695 or 8-1.

Stable APE1 suppression in AGS. APE1 protein expression was stably knocked down by 50–80% as previously reported (5) by using shRNA in AGS cells referred herein as shRNA cells. Empty vector, pSIREN Retro-Q-expressed cells (pSIREN cells) were used as control cells.

Treatment of cells. Normal (WT), pSIREN, and shRNA cells, MKN 45, NCI-N87, KATO III, and freshly isolated GEC were infected with H. pylori. As determined earlier, MOI 300 for 3 h maximally induced APE1 protein with no further induction by MOI 600. Cells were treated with CoCl₂ (150 and 250 μM for 3 h) to mimic hypoxic conditions.

Real-time RT-PCR. pSIREN and shRNA cells were infected with H. pylori MOI 300 for varying times (1.5–6 h). Total RNA was extracted from pSIREN or shRNA cells with an RNEasy kit (Qiagen). Following reverse transcription using the SuperScript First Strand synthesis system (Invitrogen), real-time dual-labeled probe PCR for human Mcl1 was performed in a SmartCycler (Cepheid) by using primers and TET-labeled probe sets designed in our laboratory (Integrated DNA Technologies). mRNA expression levels were assessed semiquantitatively as previously described (5).

Western blotting and immunoprecipitation. Western blotting with antibodies to APE1 (Novus Biologicals), p300 (Santa Cruz Biotechnology), HIF1α (BD Pharmingen) and Mcl1 (Santa Cruz Biotechnology) were performed. Protein loading was normalized to α-tubulin (Abcam) or HDAC1 (Cell Signaling Technology). HIF1α-immunoprecipitation experiments were performed to analyze the interaction of HIF1α (BD Transduction Laboratories) with p300 and APE1. Protein was detected with enhanced chemiluminescence and visualized by X-ray film.

Affinity purification of HIF1α promoter-bound proteins. Nuclear extracts were prepared as previously described (25). Streptavidin-coated superparamagnetic beads (Invitrogen) coated with biotinylated oligos (Integrated DNA Technologies) containing mcll-promoter specific HRE sequence or WT or mutated HBS in the hif1α promoter (HIF1α oligo is shown in Supplementary Fig. S1B; mcll oligos are shown in Supplementary Fig. S2) were incubated with nuclear extracts prepared from H. pylori 26695-infected or uninfected AGS cells. As the cellular level of p300 and sometimes of HIF1α was difficult to detect by Western blotting, we predicted that, owing to the large size of the HIF1α WT or mutated HBS-containing oligos, steric hindrance could be a problem in p300 and HIF1α detection. We concentrated the nuclear extracts 10 X using Amicon ultracentrifugal filter units (MilliQ) to intensify the protein signals, and 400 μg derivatized beads were incubated with 2,400 μg of nuclear extract in the binding experiments involving the HIF1α WT or mutated HBS-containing oligos.

Metabolic pulse labeling. pSIREN cells were infected with cag PAI (+) strain 26695 and cag PAI (−) strain 8-1. shRNA cells were infected with cag PAI (+) strain for 3 h or left untreated; 150 μM CoCl₂ treated pSIREN cells (3 h) served as a positive control for HIF1α synthesis and 2 μg/ml cycloheximide treated cells as a negative control. After treatment, cells were depleted of Met by incubation in pulse media [MEM (−)]-Glut (−) -Met supplemented with 10% heat-inactivated dialyzed FBS (Thermo Fisher Scientific) and 292 mg/l L-glutamine and metabolically labeled with [35S]Met (Easy tag l-Met, 1 μCi/98 μl, NEN)-containing pulse media (final concentration = 100 μCi/ml) for 30 min and lysed with RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich). Lysates were centrifuged at 16,000 g for 5 min and 10 μg of HIF1α antibody (Novus Biologicals) was used to pull down HIF1α protein. Immunoprecipitated samples were run on 7.5% SDS-PAGE and X-ray films were exposed to the dried gel for 6 wk.

HIF1α-activity assay. APE-1 shRNA cells were transfected with the respective expression plasmids, with a combination of them, or with empty vector. At 24 h after transfection, cells were infected with MOI 300 of H. pylori for 3 h. HIF1 activity of the nuclear lysate was measured by use of the HIF1 activity assay kit (Active Motif). Nuclear lysate was incubated with immobilized oligo from the epo gene. For competition, 20 pmol of WT or mutant probe from the 26-bp epo gene promoter HRE oligo was used.

Immunohistochemistry. Immunohistochemical staining was performed for APE1, HIF1α, and Mcl1 proteins in paraffin embedded H. pylori-infected or uninfected biopsy samples were obtained according to a UVA Institutional Review Board-approved protocol. Surgical sections of gastric adenocarcinomas, for which information regarding H. pylori infection status was unavailable, were obtained from the UVA Biorepository and Tissue Research Facility and stained as above. Sections were deparaffinized and rehydrated prior to antigen retrieval by using the Pascal Pressure Chamber with TRS-9 buffer (DAKO) for 30 s at 125°C and 22 psi. Slides were stained using the Dakoautostainer Universal Staining system. Sections were incubated for 10 min with DAKO Dual Endogenous Enzyme Block prior to incubation with the primary antibody for 30 min
(APE1 = Novus Biologicals, 1:4,000; HIF1α = Novus Biologicals, 1:1,600; and Mcl1 = BD Pharmingen, 1:1,000). Sections were subsequently incubated for 30 min with DAKO Envision Dual Link (anti-mouse, rabbit antibody) prior to incubation with the chromagen substrate diaminobenzidine tetrahydrochloride for 10 min. Slides were counterstained with hematoxylin for 5 min and mounted. Negative controls were created by omitting the primary antibody. Aperio ScanScope XT Slide Scanner (Aperio Technologies) was used to capture digital images.

ChIP analysis. Chromatin immunoprecipitation (ChIP) assay was performed as described (5) using a commercial kit from Imgenex. To assess HIF1α binding to mcl1 and hif1α promoters, immunoprecipitation assay was conducted with HIF1α antibody (Novus Biologicals). For quantitative analysis real-time PCR was performed. After reversal of the DNA-protein cross-links in the immunocomplexes, mcl1 and hif1α promoter sequences in the oligonucleotides containing HRE region (91 BP) were quantitated by real-time PCR using Opticon continuous fluorescence detector (MJ Research). Primer-probes were designed from Applied Biosystems.

EMSA. Nuclear extracts were prepared from \textit{H. pylori}-infected AGS cells and EMSA was performed to analyze mcl1 HRE-bound components following standard protocols as described earlier (5). Oligonucleotides used are shown in Supplementary Fig. S2.

Statistical analysis. Values are given as means ± SE. Statistical differences were determined by Friedman repeated-measures analysis of variance on ranks for multiple groups. Student’s t-test was performed for comparisons of two groups. Statistical significance was determined at \( P < 0.05 \).

RESULTS

\textit{H. pylori} enhance HIF1α expression in cultured and native GEC. Initially, we sought to determine the optimal dose of \textit{H. pylori} to induce HIF1α expression in gastric epithelial AGS cells. Western blot analysis of whole cell lysates prepared from AGS cells infected with \textit{H. pylori} cag PAI (+) strain 26695 for 3 h at different MOI (\( n = 4; P < 0.05 \)) showed a dose-dependent induction of HIF1α protein (Fig. 1A). MOI 300 significantly induced HIF1α protein expression but lower MOIs barely induced HIF1α to a detectable level. CoCl2, a hypoxia mimetic agent at 150 μM dose, was used as a positive control for HIF1α expression. At higher MOI of \textit{H. pylori} and CoCl2 we observed some cell death with associated reduced levels of α-tubulin. We next determined whether MOI 300 was effective in inducing HIF1α protein in other GEC lines. Western blot data confirmed that MOI 300 also induced HIF1α in MKN45 and KATO III cells and, to a lesser extent, NCI-N87 cells (Fig. 1B).

We also assessed APE1 expression in these cell lines with regard to the potential role of APE1 in regulating HIF1α and the HIF1α-target protein, Mcl1. \textit{H. pylori} MOI 300 at 3 h induced APE1 and Mcl1. Previously, we determined that \textit{H. pylori} strain 26695 at MOI 300 induced maximal levels of APE1 in GEC (15). Mcl1 induction by \textit{H. pylori} was greatest 5–8 h after infection whereas maximal APE1 and HIF1α expression was observed at 3–5 h of infection (data not shown). Therefore, for all subsequent experiments, when examining expression of all of these proteins, cells were infected for 5 h. To detect only APE1 and HIF1α in an experiment, 3-h infection was used. To determine whether \textit{H. pylori} induced HIF1α and Mcl1 in native GEC, epithelial cells were isolated from three sets of uninfected human gastric biopsy samples and separately infected with either MOI 300 \textit{H. pylori} cag PAI (+) or (-) \textit{H. pylori} for 5 h. These sets of infected cells were pooled for Western blot analysis, demonstrating that \textit{H. pylori} induced HIF1α and Mcl1 expression in freshly isolated human GEC.

Fig. 1. Helicobacter pylori induce hypoxia-inducible factor (HIF)-1α and HIF1α-regulated Mcl1 expression in native and cultured gastric epithelial cells (GEC). A: Western blot analysis of whole cell lysates prepared from AGS cells infected with varying multiplicities of infection (MOI) of \textit{H. pylori} or 150 μM CoCl2 for 3 h shows a dose-dependent expression of HIF1α. The α-tubulin immunoblot acts as a loading control. Bars depict HIF1α expression normalized to α-tubulin (means ± SE, \( n = 4 \)); \(* P < 0.05\) compared with uninfected cells. B: a representative Western blot (\( n = 3 \)) shows induction of apurinic/apyrimidinic endonuclease 1 (APE1), HIF1α, and the HIF1α-regulated protein Mcl1 in MKN-45, NCI-N87, KATO III and AGS cells after 3 h MOI 300 \textit{H. pylori} infection. C: epithelial cells were isolated from 3 sets of uninfected human gastric biopsy samples and separately infected with either MOI 300 \textit{H. pylori} cag PAI (+) or (-) \textit{H. pylori} for 5 h. These sets of infected cells were pooled for Western blot analysis, demonstrating that \textit{H. pylori} induced HIF1α and Mcl1 expression in freshly isolated human GEC.

\textit{H. pylori} and CoCl2 we observed some cell death with associated reduced levels of α-tubulin. We next determined whether MOI 300 was effective in inducing HIF1α protein in other GEC lines. Western blot data confirmed that MOI 300 also induced HIF1α in MKN45 and KATO III cells and, to a lesser extent, NCI-N87 cells (Fig. 1B).
APE1 expression is related to HIF1α and HIF1α-regulated protein expression in H. pylori-infected and adenocarcinomatous gastric tissue. Expression of APE1, HIF1α, and Mcl1 assessed by immunohistochemistry was compared in gastric biopsy specimens from uninfected (n = 3) and H. pylori-infected (n = 3) subjects. Our data showed that, after H. pylori infection, nuclear expression of APE1 and HIF1α, as well as cytosolic and nuclear Mcl1, was increased (Fig. 2A).

Fig. 2. H. pylori infection upregulates APE1, HIF1α, and Mcl1 in the human gastric epithelium. A: representative immunohistochemistry showing greater expression of APE1, HIF1α, and Mcl1 in H. pylori-infected (n = 2) gastric epithelium and lamina propria than in uninfected (n = 2) samples. All images within a column were obtained from the same biopsy specimen. Omission of the primary antibody served as a negative control (data not shown). Scale shown on Fig. 2A = 100.4 μm. Insets: 3× further magnified images to show nuclear and cytosolic expression. B: immunohistochemical staining of a surgical section of adenocarcinoma showing gastric mucosa with localized nuclear and cytoplasmic expression of HIF1α and Mcl1 in the tumor cells (indicated with arrows) where APE1 expression is also high. Negative (−ve) control (Cont) contains no primary antibody. H&E, hematoxylin and eosin. Original magnification: ×50.
Since H. pylori infection and increased ROS and HIF1α expression are associated with gastric adenocarcinoma, immunohistochemical detection of APE1, HIF1α, and Mc11 was performed in tissues from a surgically resected specimen of gastric adenocarcinoma (Fig. 2B). In general, nuclear and cytoplasmic APE1 expression was greater in adenocarcinoma cells compared with normal or noncancerous GEC (data not shown). Expression of HIF1α and Mc11 was increased in cancer cells that also exhibited increased APE1 expression, implicating an association of APE1 and HIF1α in gastric cancer.

APE1 in association with p300 regulates expression and activation of HIF1α in GEC. APE1 expression is normally high in many cell types, presumably because it is essential for cell survival. We previously established a stable APE1-suppressed cell line. These APE1 shRNA-expressing (shRNA) cells as well as empty vector (pSIREN) and nontransfected AGS cells (WT) were utilized to examine the effect of APE1 on HIF1α expression. Whole cell lysates prepared from pSIREN and shRNA cells were infected with different MOIs for 3 h and analyzed by Western blotting. Our results showed that MOI 300 induced HIF1α in pSIREN cells but to a much lesser extent in shRNA cells suggesting that APE1 is involved in H. pylori-mediated HIF1α expression (Fig. 3A).

To analyze the role of APE1 in HIF1α synthesis during infection, pSIREN and shRNA cells were infected with H. pylori strains 26695 and 8–1 for 3 h or left untreated. Metabolic pulse labeling of cells with [35S]Met followed by analysis of the HIF1α-immunoprecipitate confirmed that APE1 is required for HIF1α synthesis followed by H. pylori infection (Fig. 3B).

We next assessed the role of p300 and APE1 in H. pylori-mediated HIF1α activation. The mean HIF1α activity data from the binding and activity assay demonstrated that both p300 and APE1 overexpression induced H. pylori-mediated HIF1α activity (Fig. 3C). Ectopic APE1 alone induced as much activity as the combined expression of APE1 and p300, suggesting that APE1 is a major regulator of HIF1 activity. H. pylori infection is known to induce an interaction of HIF1α with APE1 and p300 (34). Our data show that the postinfection p300-HIF1α interaction was enhanced in presence of APE1 but no such interaction was detected in CoCl2-treated cells (not shown).

APE1 is required for induction of both HIF1α and HIF1β target proteins. Like other transcription factors, APE1 needs to be localized in the nucleus to function as a transcriptional coactivator of HIF1α (45). We previously showed that APE1 is translocated to the nucleus following H. pylori infection (15). To evaluate the transcriptional regulatory role of APE1 in H. pylori-induced HIF1α expression and activation, we transfected shRNA cells with WT APE1 plasmid and nuclear localization signal-deficient APE1 plasmid (NΔ41) (10) prior to 5-h infection. Western blot analysis of whole cell lysates confirmed that induction of HIF1α and its downstream target, Mc11, after H. pylori infection is significantly potentiated (P ≤ 0.05) by the presence of nuclear APE1 (Fig. 4A).

Initially, we determined by Western blot that basal Mc11 expression was similar in WT, pSIREN, and shRNA cells (data not shown). To assess the role of APE1 in H. pylori infection-induced Mc11 transcription, pSIREN and shRNA cells were infected with MOI 300 of H. pylori. The real-time RT-PCR data (n = 3) showed that APE1 significantly (P < 0.05) enhanced H. pylori-mediated upregulation of Mc11 mRNA expression after 1.5-h infection compared with the uninfected control (Fig. 4B).
MOI 300 and 600 of H. pylori for 3 h. The representative Western blot shows the importance of nuclear APE1 for inducing HIF1α and McI1 expression after H. pylori infection. Corresponding graphs of Western blot analysis (n = 3) show the significant (**P < 0.05) decrease in HIF1α and McI1 protein expression without nuclear localization of APE1. B: real-time RT PCR showing McI1 (at 1.5 h) mRNA expression in MOI 300 H. pylori-infected pSIREN and shRNA cells (means ± SEM, n = 3). McI1 expression was significantly reduced (**P < 0.05) in the H. pylori-infected shRNA cells compared with the H. pylori-infected pSIREN cells. C: dual luciferase assays (means ± SEM, n = 3) show that in the presence of APE1 (pSIREN cells), 3-h infection with MOI 300 of H. pylori induces significant (**P < 0.05) mcl1 HRE-dependent luciferase activity compared with shRNA cells, which have suppressed APE1 expression.

Next, pSIREN and shRNA cells were cotransfected with a Renilla luciferase construct along with a mcl1 luciferase construct containing an HRE (Fig. 4C). Cells were infected with MOI 300 H. pylori for 3 h or left uninfected. The results of the dual luciferase assays (means ± SEM, n = 3) showed that in the presence of APE1, H. pylori infection significantly (P < 0.05) induced mcl1 HRE-dependent luciferase activity compared with the uninfected control and that this induction was also significantly (P < 0.05) potentiated in the absence of APE1.

APE1, Hif1α, and p300 are components of the multiprotein transcriptional complex formed at the mcl1 promoter HRE after H. pylori infection. EMSA was used to identify any multiprotein complex that may have formed at mcl1 promoter HREs in H. pylori-infected GEC. AGS cell nuclear extracts showed substantial increases in the complex formation after H. pylori infection (Fig. 5A, lane 3). As shown, two major HRE-bound oligo-protein complexes were formed. Competition with unlabeled mcl1 HRE oligo diminished only the upper band, indicating that the upper band represented an HRE-specific complex (lane 5). The specificity of binding was further confirmed by using labeled (lane 4) or unlabeled (lane 6) mcl1 promoter-specific HRE-mutant oligos.

We next examined whether APE1, HIF1α, and transcriptional coactivator p300 were also bound to the mcl1 promoter HRE sequence. Streptavidin-coated magnetic beads were coated with mcl1 HRE-containing oligo, and nuclear lysates from H. pylori-infected and uninfected cells were incubated with the beads. Western blotting of the extracted proteins revealed that p300; HIF1α and APE1 were bound to mcl1 HRE after H. pylori infection (Fig. 5B).

HIF1α binds to its own promoter and limits activity of HIF1α. Because more ROS are induced with greater MOI of H. pylori (14) and ROS have also been implicated with both destabilization and accumulation of HIF1α (28), we next examined the effect of higher MOI on expression of HIF1α and HIF1α target, McI1, in GEC. AGS cells were infected with MOI 300 and 600 of H. pylori strain 26695 for 5 h and both nuclear and cytosolic extracts were analyzed by Western blotting. H. pylori infection resulted in predominantly nuclear accumulation of HIF1α and higher H. pylori MOI potentiated that effect (Fig. 5C). Transcriptional coactivator p300 was also induced in the nucleus by higher MOI of H. pylori. APE1 was induced and translocated to the nucleus similarly by both MOI 300 and 600 with a corresponding decrease in cytosolic levels after infection. Surprisingly, we found that, at higher MOI, both cytosolic and nuclear McI1 expression was decreased.

We performed Western blotting on whole cell lysates prepared from AGS cells infected with MOI 300 and 600 or treated with 150 or 250 µM CoCl2 for 3 h (Fig. 5D). Data showed H. pylori dose-dependent increase in p300, HIF1α, and APE1 expression but McI1 expression decreased at higher MOI. Higher dose of CoCl2 had no effect on p300 and APE1, induced HIF1α expression, and suppressed McI1 expression (Fig. 5D).

We next wanted to determine whether the MOI-dependent difference in McI1 expression is due to differences in HIF1α binding to its promoter. To determine in vivo binding of HIF1α to the mcl1 (Fig. 6A) HRE in AGS cells infected with MOI 300 and 600 of H. pylori or treated with 150 and 250 µM CoCl2 for 3 h, sample lysates were subjected to ChIP assay using HIF1α antibody. Our data demonstrated that at higher MOI or with more hypoxia induction, there was a trend to less HIF1α bound to the mcl1 HRE.

Our thorough analysis of the human and mouse hif1α promoter revealed that both species have one HBS in their promoters. The ACGTG motif in the human hif1α promoter is located at −275 to −271 from the transcription start site and a p300 binding sequence is located at −191 to −195 (Supplementary Fig. S1). After overexpressing HIF1α we did not find...
any change in hif1α luciferase activity (data not shown), which indicated that the HBS in the hif1α promoter is nonfunctional.

To assess whether there was any difference in the in vivo binding of HIF1α with hif1α HBS, AGS cells infected with MOI 300 and 600 of H. pylori or treated with 150 and 250 μM CoCl2 treatments for 3 h were assessed by ChIP assay using HIF1α antibody. Our quantitative RT-PCR data confirmed that, at higher MOI or hypoxia induction, significantly (P < 0.05) more HIF1α was bound to the hif1α promoter HBS (Fig. 6B). Taken together, our data suggest that when more HIF1α is bound to the hif1α promoter HBS, there is a less HIF1α bound to the mcl1 promoter HBS.

Streptavidin-coated magnetic beads were used to further assess whether HIF1α and p300 bind to the hif1α promoter after H. pylori infection and whether altered H. pylori-MOI affects binding. We prepared nuclear extracts from uninfected and 5-h H. pylori-infected (MOI 300 and 600) AGS cells. WT or HBS-mutant oligonucleotides were designed based on the human hif1α promoter sequence and the beads were coated with oligos prior to incubation with nuclear lysates. By analyzing the bead-bound proteins, we observed that with higher MOI more HIF1α was bound to the hif1α promoter, which was not seen when the HBS was mutated (Fig. 6C, top). The p300 was bound to the promoter at the lower H. pylori dose but not at the higher MOI (Fig. 6C, top), and mutation at HBS did not change p300 binding to the HIF1α promoter, indicating that p300 was not bound to the HBS but to the p300-binding site. Nuclear lysates analyzed by the binding assay were also run separately in SDS-PAGE and Western blotted. Nuclear expression of p300 was induced equally by H. pylori MOI 300 and MOI 600. HIF1α was predominantly expressed in the nucleus and was further induced with higher MOI. We found that ectopic expression of p300 induced hif1α luciferase activity both at baseline and following H. pylori infection but MOI 300 and 600 showed comparable hif1α promoter activation (data not shown).

Although the mechanism of CoCl2-mediated HIF1α expression and activation is different than the H. pylori-mediated mechanism since the former agent does not induce APE1 (Fig. 3D and 5D), we wanted to know whether our finding of HIF1α binding to the hif1α promoter was a generalized event or only limited to H. pylori infection. To address this question, we treated AGS cells with 150 and 250 μM CoCl2 for 5 h. Nuclear extracts were prepared and incubated with only WT hif1α promoter oligo-precoated magnetic beads. Western
was bound to the quantitative real-time PCR and was expressed as % of input.

not bind to the p300 binds with the HBS-mut oligo to the same extent, indicating that p300 does Ab. The binding of HIF1 region was determined by quantitative real-time PCR and are expressed as % of -infected or CoCl2-treated cells were immunoprecipitated using HIF1 H. pylori /H9251

Fig. 6. HIF1 G1184 HELICOBACTER PYLORI

HIF1/H9251 by use of HIF1 analysis of HIF1 atins from /H9251 in HIF1 /H9251 control for the nuclear lysates. HBS-mut oligo. p300 binding to the putative HBS located in the HIF1, H. pylori- infected or CoCl2-treated cells were subjected to ChIP assay to the HRE was determined by 

Increased expression of p300 and HIF1 is controlled by HIF1 itself, adding further complexity to the current understanding of the regulation of HIF1α.

Nonhypoxic accumulation and activation of HIF1α occurs when HIF1α protein translation surpasses its degradation (13). We show that APE1 enhances HIF1α synthesis in H. pylori-infected gastric epithelia. Since blocking redox activity of APE1 decreases expression of HIF1α target proteins in the infected cells, the DNA binding and transcriptional activity of HIF1α induced by H. pylori are mediated by APE1. Although we did not study the mechanism of HIF1α synthesis by APE1, it is possible that APE1 does so by translocating to the nucleus and reductively activating transcriptional regulators of hif1α such as NF-κB and p300. Additional studies are necessary to examine the role of APE1 in HIF1α synthesis.

APE1 plays important roles in oxygen-regulated gene expression (31). It prepares transcription factors for DNA binding by keeping them in reduced states (2) and recruits p300 to the transcriptional complexes. In vitro studies show that APE1 interacts with both COOH- and NH2-terminal transactivation domains (CTAD and NTAD) of HIF1α (8). APE1 not only interacts with p300 and HIF1α to form transcriptional complexes (21, 49) but is obligatory for the formation of that complex (49). Binding of the transcriptional coactivators p300/ CBP to the CTAD of HIF1α is necessary for HIF1α transcriptional activity. The cysteine-histidine rich 1 (CH1) domain of p300 interacts with HIF1α-CTAD (30, 43, 50). NMR studies have shown that the CTAD domain is mainly unstructured without the CH1 domain of p300 (11, 19). In keeping with previously reported studies, in the present study we have confirmed APE1 as an essential molecule in the formation of the transcriptional complexes (21, 49), including the antiapoptotic protein Mcl1 (38). Similar to a previous study that reported that H. pylori induce the antiapoptotic protein Mcl1 (34), we show that antiapoptotic mechanisms are activated by H. pylori in GEC and HIF1α plays a pivotal role in this. However, unlike Mimuro et al.’s study in Mongolian gerbils (34), our findings in human GECs show this phenomenon is cag PAI independent. Because the balance between the proapoptotic protein Noxa and the antiapoptotic protein Mcl1 determines cell fate, further understanding the role of H. pylori-induced HIF1α upregulation (1, 33) is important in understanding H. pylori-associated disease pathogenesis.

DISCUSSION

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 blot analysis of the bead-bound protein eluates showed more HIF1α binding at higher CoCl2 dose (Fig. 6D, top) in parallel with the amount of HIF1α in nuclear lysates (Fig. 6D, bottom).
Because a dose-dependent increase of ROS was observed with increasing MOI of *H. pylori* infection (14), we expected more HIF1α production and greater expression of McI1 in the infected epithelium with a greater MOI. Instead, we found that, at greater *H. pylori* doses, McI1 expression was reduced despite further induction of HIF1α. While searching for the transcription factors associated with hif1α regulation that could be differentially regulated by varying MOIs, we found a HBS in the hif1α promoter. Functional HREs are complex regulatory elements, comprising a conserved HBS A/GCGTG and an extremely variable flanking sequence (47). The HBS alone cannot function in response to hypoxia and needs an adjacent inverted repeat sequence located immediately downstream of the HBS called HIF ancillary sequence (HAS). Comparison of different hypoxia-sensitive genes demonstrated that these two motifs are usually spaced by eight or nine nucleotides (29). hif1α promoter has no such inverted repeat ancillary sequence. Thus we conclude that, with increased production, either in response to *H. pylori* or CoCl2, HIF1α binds to the hif1α promoter HBS and since the HAS is lacking in the hif1α promoter the bound HIF1α becomes nonfunctional and this sequestering effect limits the intracellular availability of HIF1α to induce HIF1α target gene mcl1.

Adding to the complexity, multiple transcription factors compete with HIF1α to bind with p300 (19) and many of them, such as p53, p73 and STAT2, are induced by ROS. Squelching is described as the interference of transcription factor activity by other factors that bind to different target genes (6). Because p300 is the limiting cofactor within the cell (25), competition for p300 also explains the phenomenon of HIF1α squelching associated with higher *H. pylori* MOI. Further studies are needed to analyze whether this self-regulatory function of HIF1α plays any role in the disease pathogenesis of *H. pylori* and ROS-associated diseases such as gastric ulcers and gastric cancer.

In summary, our results show that, despite enhanced expression of HIF1α with higher doses of *H. pylori*, the intracellular functions of HIF1α can be modulated, in part because of HIF1α binding to its own promoter HBS and becoming unavailable for binding to other genes containing functional HREs. This mechanism may be important in gastrointestinal diseases associated with *H. pylori* infection in which ROS and HIF1α are involved such as gastric cancer and ulceration. Since inappropriate activation of HIF1α generally promotes tumorigenesis, targeting specific components of APE1-mediated HIF1α activation may prove to be useful for designing future therapies for gastric cancer.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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