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Hypoxia-Inducible Factor-2 α Plays a Role in Mediating Oesophagitis in Gastro-Oesophageal Reflux Disease

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Abstract

Objective—In an earlier study wherein we induced acute reflux by interrupting proton pump inhibitor (PPI) therapy in patients with reflux oesophagitis (RO) healed by PPIs, we refuted the traditional concept that RO develops as an acid burn. The present study explored our alternative hypothesis that RO results from reflux-stimulated production of pro-inflammatory molecules mediated by hypoxia inducible factors (HIFs).

Design—Using oesophageal biopsies taken from patients in our earlier study at baseline and at 1 and 2 weeks off PPIs, we immunostained for HIF-1 α , HIF-2 α , and phospho-p65, and measured pro-inflammatory molecule mRNAs. We exposed human oesophageal squamous cell lines to acidic bile salts, and evaluated effects on HIF activation, p65 function, pro-inflammatory molecule production, and immune cell migration.

Results—In patient biopsies, increased immunostaining for HIF-2 α and phospho-p65, and increased pro-inflammatory molecule mRNA levels were seen when RO redeveloped 1 or 2 weeks after stopping PPIs. In oesophageal cells, exposure to acidic bile salts increased intracellular reactive oxygen species, which decreased prolyl hydroxylase function and stabilised HIF-2 α , causing a p65-dependent increase in pro-inflammatory molecules; conditioned media from these cells increased T cell migration rates. HIF-2 α inhibition by shRNA or selective small molecule antagonist blocked the increases in pro-inflammatory molecule expression and T cell migration induced by acidic bile salts.

Conclusions—In patients developing RO, increases in oesophageal HIF-2 α correlate with increased pro-inflammatory molecule expression. In oesophageal epithelial cells, acidic bile salts stabilise HIF-2 α , which mediates expression of pro-inflammatory molecules. HIF-2 α appears to have a role in RO pathogenesis.

Keywords

Oesophageal Disease; Inflammatory Diseases; Cell signaling; Cytokines

Introduction

Reflux oesophagitis has been assumed to develop when cells in the surface layer of oesophageal squamous epithelium succumb to the caustic chemical effects of refluxed gastric acid and pepsin.¹ The caustic death of surface cells is thought to attract granulocytes (neutrophils and eosinophils), and to stimulate basal cell proliferation.^{2,3} However, in earlier studies in a rat model, we found that reflux oesophagitis started with oesophageal infiltration by T lymphocytes and with basal cell hyperplasia preceding the loss of surface cells, findings inconsistent with the traditional concept of reflux oesophagitis as a chemical burn.⁴ Moreover, we showed that acid and bile salts caused human oesophageal epithelial cells in culture to secrete pro-inflammatory and pro-proliferative cytokines.⁴ Thus, we proposed a new concept for reflux oesophagitis pathogenesis in which refluxed gastric juice does not kill oesophageal epithelial cells directly, but rather stimulates them to secrete

cytokines that induce proliferative changes and attract the T lymphocytes and other inflammatory cells that ultimately damage the mucosa.

Recently, we conducted a clinical study designed to test the traditional notion that reflux oesophagitis in humans develops as a chemical burn. In patients with reflux oesophagitis healed by proton pump inhibitors (PPIs), we induced acute oesophagitis by interrupting PPI therapy.⁵ As in our rat model, we found that “acute” reflux oesophagitis in humans began with T lymphocyte-predominant inflammation, and with basal cell and papillary hyperplasia developing before loss of surface cells. These were findings that would be expected if reflux oesophagitis resulted from reflux-stimulated oesophageal production of pro-inflammatory molecules.⁴ This clinical study was the initial part of a project designed to explore the role of pro-inflammatory molecules and hypoxia-inducible factors (HIFs) in the pathogenesis of human reflux oesophagitis.

HIFs, transcription factors that enable cells to respond to hypoxic stress, can mediate inflammatory processes.⁶⁻⁹ HIFs are heterodimeric proteins comprising an oxygen-regulated HIF- α subunit (HIF-1 α or HIF-2 α) and a constitutively-expressed HIF-1 β subunit.⁹ Normally, HIFs are inactive because their HIF- α subunits are rapidly degraded by proteasomes. This degradation begins when prolyl hydroxylases (PHDs) catalyze hydroxylation of proline residues in the oxygen-dependent degradation domain of HIF- α . Hydroxylated HIF- α is bound by von Hippel-Lindau (VHL) protein, which initiates proteosomal degradation via the ubiquitin pathway. Hypoxia decreases PHD activity, preventing proteosomal HIF degradation and enabling HIFs to accumulate. These stabilised HIFs translocate to the nucleus to induce transcription of target genes that contain hypoxia responsive elements (HREs).^{6, 10, 11}

In earlier studies, we showed that human oesophageal squamous cells exposed to acid and bile salts *in vitro* produced reactive oxygen species (ROS).¹² Like hypoxia, ROS can stabilise HIF proteins [Reviewed in¹¹]. In a mouse model of HIF-mediated colitis, furthermore, colonic inflammation developed in a pattern similar to our rat model of reflux oesophagitis, starting with submucosal inflammation and epithelial proliferative effects associated with increased expression of pro-inflammatory cytokines.¹³ These observations led us to hypothesize that refluxed acid and bile salts cause oesophageal squamous epithelium to produce ROS that inactivate PHDs, enabling HIFs to accumulate and to stimulate the secretion of pro-inflammatory molecules. To explore this hypothesis, we studied HIF activation and expression of pro-inflammatory mediators in oesophageal biopsies taken from patients in our earlier study on acute reflux oesophagitis.⁵ We also performed *in vitro* studies in human oesophageal squamous cell lines to explore mechanisms whereby acidic bile salts cause HIF activation and the subsequent events that might contribute to development of reflux oesophagitis.

Materials and Methods

Patient Tissues

These studies were approved by the Institutional Review Board of the Dallas VA Medical Center and registered on clinical trials.gov (NCT01733810). The 12 patients whose

oesophageal biopsies were used in this study are described in our earlier report on acute reflux oesophagitis.⁵ Briefly, the 12 were derived from an original group of 215 patients diagnosed with Los Angeles grade C oesophagitis (Figure 1A). Study patients were treated with PPIs for 1 month, and had baseline endoscopy documenting healing of oesophagitis. PPIs were then stopped, and endoscopy was repeated 1 and 2 weeks later. All patients redeveloped endoscopic evidence of reflux oesophagitis by week 2. At each endoscopy (baseline on PPIs, and 1 and 2 weeks after stopping PPIs), 10 oesophageal biopsy specimens were taken 1-3 cm proximal to the squamo-columnar junction; 4 specimens were fixed in formalin and embedded in paraffin, 6 were snap frozen. Results of routine histologic evaluation of paraffin-embedded specimens are described in our earlier report.⁵

Oesophageal Squamous Cell Lines

We used two non-neoplastic, telomerase-immortalised oesophageal squamous cell lines (NES-G4T and NES-B10T) created from oesophageal biopsies from GORD patients.^{14, 15} Cells were maintained in monolayer culture at 37°C in a 5% CO₂ incubator in growth medium co-cultured with a fibroblast feeder layer as previously described.^{14, 15} For individual experiments, cells were equally seeded into collagen IV coated wells (BD Biosciences, San Jose, CA) and maintained in growth medium.

Exposures to Hypoxia or Acidic Bile Salts

For hypoxia, NES cells were incubated at 1% oxygen by nitrogen in an INVivo2 400 (LAF Technologies, Queensland, Australia) hypoxia work station. For individual experiments, cells were cultured in neutral medium (pH7) alone or with periodic exposures to acidic medium (pH 5.5) containing a mixture of conjugated bile acids (glycocholic acid, glycodeoxycholic acid [both from Calbiochem, San Diego, CA], taurocholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid, and taurodeoxycholic acid [Sigma-Aldrich, St. Louis, MO] in a 20:6:3:15:3:1 molar concentration, total concentration 400 µM) as previously described.^{14, 16-18} Neutral or acidic bile salt medium was added for 10, 30 or 60 minutes, then replaced with growth medium. These exposure durations were chosen to simulate typical episodes of gastrooesophageal reflux.^{19, 20} All assays were performed over a 24-hour time course unless otherwise indicated.

Additional details are provided in the Supplemental Material and Methods section and in Supplemental Tables 1 and 2.

Results

Development of Acute Reflux Oesophagitis in Humans is Associated with Increased HIF-2α Protein Levels in Oesophageal Squamous Epithelium

In oesophageal biopsies from study patients, we observed significant increases in cytoplasmic and nuclear immunostaining for HIF-2α in epithelial cells from baseline to week 1 after stopping PPIs, and HIF-2α staining remained elevated at week 2 (Figure 1B&C and Supplemental Figure 1). In contrast, we found no significant change in staining for HIF-1α protein from baseline through 2 weeks (Figure 1B&C and Supplemental Figure 1).

Thus, the development of acute reflux oesophagitis is associated with increased HIF-2 α protein in oesophageal squamous epithelium.

In Developing Reflux Oesophagitis, Increased Epithelial Immunostaining for HIF-2 α Correlates with Increased Expression of Pro-Inflammatory Mediator mRNAs

We found increases in mRNA for the pro-inflammatory mediators IL-8, IL-1 β , tumor necrosis factor (TNF)- α , cyclooxygenase-2 (COX-2), and intercellular adhesion molecule (ICAM)-1 in oesophageal biopsies taken at 1 and/or 2 weeks after stopping PPIs (Figure 2A-E); elevations in IL-8 and ICAM-1 achieved statistical significance (Figure 2A&E). Statistical analyses revealed large non-linear associations of pro-inflammatory mediator mRNA levels with HIF-2 α immunostaining H scores, and we computed η^2 (η^2) values on quartile data to estimate the extent to which these pairs of variables were correlated (η^2 value $\times$ 100% indicates % of variance in mRNA changes explained by changes in HIF-2 α ; $\eta^2 \times 100\%$ values $\geq 14\%$ indicate a large effect).²¹ We found non-linear correlations between HIF-2 α immunostaining and mRNA levels of all pro-inflammatory mediators, with large associations ($\eta^2 \times 100\% \geq 14\%$) for IL-1 β , TNF- α , and ICAM-1 at week 1, and for IL-8, TNF- α , COX-2 and ICAM-1 at week 2 (Figure 2F). This suggests that increased HIF-2 α in oesophageal squamous epithelium might contribute to increased expression of pro-inflammatory mediators, but even large associations alone do not establish cause and effect.

Oesophageal Squamous Epithelial Cells Have Intact and Functional HIF Machinery

We exposed NES-B10T cells to hypoxia (1% oxygen), and measured nuclear expression of HIF-1 α , HIF-2 α , and hypoxia-mediated genes. Under normoxic control conditions, NES-B10T cells exhibited baseline nuclear expression of HIF-2 α , but not HIF-1 α (Supplemental Figure 2A). Exposure to hypoxia caused a strong, rapid accumulation of nuclear HIF-1 α , peaking within 3 hours and disappearing by 18 hours. In contrast, hypoxic induction of HIF-2 α peaked at 18 hours and remained elevated at 24 hours (Supplemental Figure 2A). Constitutive expression of HIF-1 β and PHD1 was observed in NES-B10T cells both in control and hypoxic conditions, while hypoxia caused a slight reduction in PHD2 expression (Supplemental Figure 2B). PHD3 is not expressed in squamous oesophagus (www.proteinatlas.org).

For HIF-1 α targets, we found that hypoxia caused an expected increase in CA9 and PGK1 mRNAs, and an expected decrease in NBS1 and MSH2 mRNAs (Supplemental Figure 2C).²² For HIF-2 α targets, we found an expected increase in expression of Dcytb1 and COX-2 (Supplemental Figure 2C).^{13, 22} These findings indicate that HIF-1 α and HIF-2 α pathways are intact and functional in oesophageal squamous epithelial cells. Since hypoxia caused a sustained increase in HIF-2 α but not HIF-1 α , and we found increased oesophageal HIF-2 α in our patients with acute reflux oesophagitis, we focused on HIF-2 α for further experiments.

Acidic Bile Salts Cause a Sustained Increase in HIF-2 α Nuclear Protein in Oesophageal Squamous Epithelial Cells

One hour after a 60-minute treatment with acidic bile salts, NES-G4T and NES-B10T cells showed a considerable increase in total HIF-2 α protein, remaining above baseline even at 24 hours (Figure 3A). Shorter exposures to acidic bile salts (10 and 30 minutes) resulted in similar increases in nuclear HIF-2 α at 4 hours, but the 10-minute exposure caused only a small increase at 1 hour (Figure 3B). Therefore, we used either 30- or 60-minute exposures for further experiments.

Acidic Bile Salts Decrease PHD Function by Generating Intracellular ROS via the NADPH Oxidase System in Oesophageal Squamous Epithelial Cells

The rapidity of the observed rise in nuclear HIF-2 α protein in NES cells exposed to acidic bile salts (within 1 hour) suggests an effect on HIF-2 α protein stabilisation, which could be caused either by increased PHD degradation or decreased PHD function. We found that exposure to acidic bile salts for up to 24 hours had no apparent effect on NES cell levels of PHD1 and PHD2 protein (Figure 3C), suggesting that acidic bile salts do not increase PHD degradation. To explore effects on PHD function, we performed an activity assay in which PHD proteins collected from NES cells exposed to acidic bile salts are used to hydroxylate a GST-HIF2 α oxygen-dependent degradation domain (ODD) fusion protein that, when hydroxylated, binds VHL protein. Thus, decreased binding of GST-HIF2 α ODD to VHL reflects decreased PHD function. We found that acidic bile salt exposure resulted in decreased binding of GST-HIF2 α ODD protein to VHL (Figure 3D). For confirmation, we transfected NES cells with an HA-tagged VHL plasmid and, using immunoprecipitation, found decreased binding of HIF-2 α to HA-tagged VHL in NES cells exposed to acidic bile salts (Figure 3E). This demonstrates that acidic bile salts reduce PHD activity in oesophageal squamous cells by decreasing PHD function, not by increasing PHD degradation.

Previously, we showed that acidic bile salts increase intracellular ROS in NES cells via the NADPH oxidase system.^{12, 14} To explore a role for ROS in reducing PHD function, we treated NES cells exposed to acidic bile salts with NAC (an ROS scavenger) or DPI (an NADPH oxidase inhibitor), and repeated the PHD activity assay. Both NAC and DPI (Figure 3F) prevented acidic bile salts from affecting PHD function, suggesting that acidic bile salts decrease PHD function in oesophageal squamous cells by generating intracellular ROS via the NADPH oxidase system.

HIF-2 α Induced by Exposure to Acidic Bile Salts Mediates an Inflammatory Response in Oesophageal Squamous Epithelial Cells

HIF- α proteins induce transcription of target genes containing HREs. To confirm functional activity of HIF-2 α induced by acidic bile salt exposure, we measured HRE reporter activity in NES cells transfected with a specific HIF-1 α siRNA (PCR confirmed siRNA knockdown of HIF-1 α mRNA, Supplemental Figure 3A). A 60-minute treatment with acidic bile salts significantly increased HRE reporter activity in cells transfected with HIF-1 α siRNA or with control siRNA, suggesting that the induced HIF-2 α protein is functionally active (Supplemental Figure 3B). In NES-B10T cells with HIF-1 α knockdown by siRNA,

treatment with acidic bile salts increased IL-8 and COX-2 mRNAs. IL-8 and COX-2 are direct HIF binding targets, supporting a role for HIF-2 α in the pro-inflammatory mediator increase induced by acidic bile salts (Supplemental Figure 3C). Acidic bile salt treatment also increased COX-2, IL-1 β , IL-8, TNF- α , and ICAM-1 mRNAs by one hour after exposure (Figure 4A) in both NES cell lines, a time point corresponding with detection of increased HIF-2 α nuclear protein (Figure 3B). To confirm a role for HIF-2 α , we measured pro-inflammatory mediators in NES-B10T cells stably infected with a HIF-2 α shRNA (PCR and Western blot confirmed knockdown of HIF-2 α mRNA and protein by this shRNA, Figure 5A). In cells infected with control vector, acidic bile salts increased COX-2, IL-1 β , IL-8, TNF- α , and ICAM-1 mRNAs by one hour (Figure 4B). In contrast, HIF-2 α knockdown by shRNA blunted or eliminated increases in COX-2, IL-8, IL-1 β , TNF- α , and ICAM-1 mRNAs at one hour after acidic bile salt exposure (Figure 4B).

We also treated NES-B10T cells with a highly selective small molecule inhibitor of HIF-2 α [active enantiomer (S,R)-37 Peak 2, and inactive enantiomer (R,S)-37 Peak 1] at 10 μ M concentration over 24 hours.²³ Supplemental Figure 4 shows that the active enantiomer decreased mRNA levels of Dmt1 and Dcytb1 (HIF-2 α specific targets), but not PGK1 (a HIF-1 α specific target). In NES-B10 cells treated with inactive enantiomer, exposure to acidic bile salts increased COX-2, IL-1 β , IL-8, TNF- α , and ICAM-1 mRNAs by one hour (Figure 4C); these increases were eliminated by treatment with active enantiomer (Figure 4C). These results demonstrate that HIF-2 α is a transcription factor that regulates an oesophageal epithelial inflammatory response to acidic bile salts.

HIF-2 α Induced by Exposure to Acidic Bile Salts Mediates an NF- κ B/p65-Dependent Inflammatory Response in Oesophageal Squamous Epithelial Cells

The pro-inflammatory mediators in our panel are known to be regulated by NF- κ B and, in earlier studies, we showed that acidic bile salts trigger NF- κ B/p65 signaling that increases IL-8 expression in NES cells.²⁴ In non-oesophageal cell types, interactions (cross-talk) between NF- κ B and HIF-1 α have been described [Reviewed in²⁵]. We explored whether exposure to acidic bile salts might result in cross-talk between HIF-2 α and NF- κ B/p65 in oesophageal cells. First, we studied how knockdown of one gene affected the expression and function of the other. NES-B10T cells were stably infected with a RELA/p65 shRNA or control vector (PCR and Western blot confirmed p65 knockdown by the RELA/p65 shRNA, Figure 5A). In whole cell lysates, HIF-2 α knockdown had no apparent effect on total p65 mRNA and protein, and RELA/p65 knockdown had no apparent effect on HIF-2 α mRNA and protein (Figure 5A). RELA/p65 knockdown also had no apparent effect on nuclear protein levels of HIF-2 α (Figure 5B). In contrast, HIF-2 α knockdown did reduce nuclear protein levels of phospho- and total p65 (Figure 5B). These findings demonstrate crosstalk between HIF-2 α and RELA/p65 in oesophageal cells and suggest that HIF-2 α mediates the functional activity of phospho-p65.

We transfected NES-B10T cells with an NF- κ B reporter construct, and found that HIF-2 α knockdown blocked the increase in NF- κ B/p65 transcriptional activity induced by acidic bile salts (Figure 5C). Knockdown of HIF-2 α also abrogated the increase in phospho-IKK α / β , phospho-I κ B α (serine 32/36), and phospho-p65 (serine 536) induced by acidic bile salt

exposure (Figure 5D). Unlike control vector cells, cells containing RELA/p65 shRNA did not increase their expression of COX-2, IL-8, IL-1 β , TNF- α , and ICAM-1 mRNAs after exposure to acidic bile salts (Figure 5E). These observations suggest that crosstalk between HIF-2 α and members of the NF- κ B cascade play a major role in regulating the oesophageal epithelial inflammatory response to acidic bile salts.

In Oesophageal Squamous Epithelium of Patients Developing Acute Reflux Oesophagitis, Increases in Phospho-p65 Are Associated with Increases in HIF-2 α and in Expression of Pro-Inflammatory Mediators

After our *in vitro* demonstration that HIF-2 α increases pro-inflammatory mediators in oesophageal squamous cells by enhancing NF- κ B/p65 activity, we sought evidence that these effects occur in the oesophagus *in vivo*. In agreement with our *in vitro* data, we found that immunohistochemical staining for phospho-p65 (relative to total p65) in oesophageal biopsies increased significantly from baseline to 1 week after stopping PPIs; at week 2, phospho-p65 staining remained higher than baseline (Figure 6 A&B and Supplemental Figure 5). This increase in phospho-p65/total-p65 resulted from increased phospho-p65 levels; total p65 levels did not change significantly (data not shown). Non-linear correlations between HIF-2 α and phospho-p65/total-p65 demonstrated large associations ($\eta^2 \times 100\%$ = 14%) at both week 1 ($\eta^2 \times 100\%$ = 28%) and week 2 after stopping PPIs ($\eta^2 \times 100\%$ = 46%). Non-linear correlations between phospho-p65/total-p65 and pro-inflammatory mediator mRNA levels demonstrated large associations for IL-8, TNF- α , COX-2 and ICAM-1 at week 1, and for all mediators at week 2 (Figure 6C). These findings demonstrate that development of reflux oesophagitis is associated with increases in HIF-2 α that appear to contribute to increased NF- κ B/p65 activity, which in turn appears to contribute to increased expression of pro-inflammatory mediators in oesophageal squamous epithelium.

HIF-2 α Induced by Acidic Bile Salts in Oesophageal Squamous Epithelial Cells Mediates Their Secretion of T Cell Chemokines

We determined effects of HIF-2 α inhibition on the secretion of T cell chemokines by NES-B10T cells exposed to acidic bile salts for 1 hour (Figure 7 A&B). Conditioned media from control cells treated with acidic bile salts caused a significant increase in T cell migration rates. In contrast, conditioned media from acidic bile salt-treated cells with HIF-2 α inhibition either by shRNA (Figure 7A) or small molecule antagonist (Figure 7B) caused no significant change in T cell migration rates. This shows that exposure to acidic bile salts causes oesophageal squamous epithelial cells to secrete chemokines that attract T lymphocytes through a HIF-2 α -dependent mechanism. A schematic model summarizing mechanisms elucidated by our study is provided in Figure 7C.

Discussion

In patients with reflux oesophagitis healed by PPIs, we have shown that stopping PPIs results in the rapid development of acute reflux oesophagitis associated with increased HIF-2 α in oesophageal squamous epithelium. This increase in epithelial HIF-2 α is associated with increased epithelial NF- κ B/p65 activity and increased mRNA expression of pro-inflammatory molecules (COX-2, IL-8, IL-1 β , TNF- α , and ICAM-1). In oesophageal

squamous epithelial cell lines *in vitro*, we have shown that acidic bile salts decrease PHD function by generating intracellular ROS through the NADPH oxidase system. This causes a sustained increase in HIF-2 α , which mediates an NF- κ B/p65-dependent inflammatory response by squamous cells, increasing their expression of COX-2, IL-1 β , IL-8, TNF- α , and ICAM-1 mRNAs. Finally, we have shown that HIF-2 α induced by exposing oesophageal squamous epithelial cells to acidic bile salts mediates their secretion of T cell chemokines. These observations support our hypothesis that reflux oesophagitis develops, not as a chemical injury, but as a cytokine-mediated process in which refluxed acid and bile salts activate HIF-2 α in oesophageal squamous epithelium, thereby stimulating secretion of pro-inflammatory molecules that attract T lymphocytes and other inflammatory cells that damage the oesophagus.

HIFs are known to mediate inflammation in extra-oesophageal organs.^{6, 8, 26-29} In animal models of colitis, HIF signaling has been shown to regulate genes involved in mucosal inflammation. HIF-1 α appears to have a protective role, enhancing colonic mucosal integrity by regulating genes involved in barrier protection.^{30, 31} In contrast, HIF-2 α signaling augments the colonic epithelial inflammatory response.^{13, 26} Shah *et al.* used cre/loxP technology to delete VHL protein in the mouse intestine, thereby causing an increase in colonic HIF-2 α signaling that mediated the expression of pro-inflammatory molecules.¹³ As in our rat model of reflux oesophagitis,⁴ inflammation in this HIF-mediated colitis began in the submucosa, with epithelial proliferative and regenerative changes that preceded surface ulceration. Moreover, through this HIF-mediated increase in pro-inflammatory molecules, treatment with dextran sodium sulfate caused more severe colitis in VHL-deficient mice than in wild-type mice. Subsequent studies demonstrated that it was HIF-2 α (not HIF-1 α) that mediated the colonic epithelial inflammatory response in VHL-deficient mice.²⁶

We have found evidence of a role for HIF-2 α signaling in the development of reflux oesophagitis. Oesophageal biopsies from our GORD patients demonstrated increases in HIF-2 α , but not HIF-1 α , at 1 and 2 weeks after stopping PPI therapy. In oesophageal squamous cell lines, we found that HIF-2 α inhibition blocked the increased expression of pro-inflammatory mediators induced by exposure to acidic bile salts, suggesting that this epithelial inflammatory response is HIF-2 α specific. In addition, HIF-2 α inhibition blocked the increase in T cell migration caused by conditioned media from cells treated with acidic bile salts. In patients with acute reflux oesophagitis, furthermore, we found large associations between oesophageal immunostaining for HIF-2 α and expression of pro-inflammatory molecules. These findings all suggest that HIF-2 α mediates the oesophageal epithelial inflammatory response in GORD.

HIFs can regulate gene expression directly by inducing transcription of genes containing HREs, or indirectly by influencing the activity of other transcription factors.²⁶ Many pro-inflammatory mediators are known to be regulated by the transcription factor NF- κ B. In earlier studies, we showed that acidic bile salts induce NF- κ B signaling that increases IL-8 expression in oesophageal squamous cells.²⁴ In the present study, knockdown of NF- κ B RELA/p65 in oesophageal squamous cells abolished the increase in pro-inflammatory molecule expression induced by acidic bile salts. Interactions between HIF-1 α and NF- κ B

have been described, [Reviewed in ²⁵] but we are not aware of any report describing links between HIF-2 α and NF- κ B. We found that knockdown of RELA/p65 had no effect on cytoplasmic or nuclear HIF-2 α levels, but knockdown of HIF-2 α reduced nuclear levels of phospho- and total p65, suggesting that HIF-2 α affects NF- κ B transcriptional activity. We confirmed this by demonstrating that HIF-2 α knockdown eliminated induction of an NF- κ B reporter construct by acidic bile salts. It has been proposed that hypoxia enhances NF- κ B activity through effects on PHD function, ¹⁰ and we have demonstrated that ROS induced by acidic bile salts inhibit PHD function in oesophageal squamous cells. If the enhanced NF- κ B activity induced by acidic bile salts was solely the result of PHD inhibition, then we would have found no effect of HIF-2 α knockdown on NF- κ B activity, which was not the case. Thus, our findings demonstrate crosstalk between HIF-2 α signaling and the NF- κ B pathway in oesophageal squamous cells. Studies to further understand the nature of this crosstalk are warranted.

Activation of the canonical NF- κ B pathway generally involves phosphorylation of the IKK kinase complex, which phosphorylates I κ B kinase at serines 32 and 36, thereby enabling it to release RELA/p65 protein. ³² The I κ B kinase also can phosphorylate p65 on serine 536, leading to enhanced p65 transcriptional activity. ³³ We found that HIF-2 α knockdown decreased nuclear RELA/p65, and that HIF-2 α expression was necessary for acidic bile salts to increase phosphorylation of IKK, I κ B (serine 32 and 36), and p65 (serine 536). In patients with acute reflux oesophagitis, furthermore, we found increases in oesophageal levels of phospho-p65, and we found large associations between HIF-2 α and phospho-p65 levels. These findings elucidate a molecular mechanism whereby HIF-2 α enhances NF- κ B/p65 transcriptional activity and target gene expression, and suggest that NF- κ B/p65 is a major effector of the HIF-2 α -mediated oesophageal epithelial inflammatory response to acidic bile salt exposure.

In conclusion, our earlier clinical study in GORD patients refuted the traditional notion that reflux oesophagitis develops as a caustic chemical injury, and our present molecular studies in those patients and in oesophageal squamous cell lines have elucidated molecular mechanisms whereby gastro-oesophageal reflux can cause oesophagitis through cytokine-mediated mechanisms triggered by HIF-2 α . Exposure to acidic bile salts causes oesophageal squamous cells to produce ROS through the NAPH oxidase system, leading to a decrease in PHD function that stabilises HIF-2 α protein by decreasing its binding to VHL. This HIF-2 α increases the production of pro-inflammatory molecules largely by enhancing NF- κ B/p65 transcriptional activity. Inhibition of HIF-2 α in oesophageal squamous cells blunts the increase in pro-inflammatory mediator production and secretion of T cell chemokines induced by acidic bile salts. In support of these mechanisms elucidated by *in vitro* studies, we found large associations between HIF-2 α , phospho-p65, and pro-inflammatory molecule expression in oesophageal biopsies of GORD patients at 1 or 2 weeks after stopping PPIs. Thus, our findings suggest a role for HIF-2 α in the epithelial inflammatory response of oesophageal squamous cells to gastro-oesophageal reflux, and suggest that a HIF-2 α -directed therapy might be a novel approach to the prevention and treatment of reflux oesophagitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

COX-2 cyclooxygenase-2

DPI	diphenylene iodonium
GORD	gastro-oesophageal reflux disease
HIF	hypoxia inducible factor
HREs	hypoxia responsive elements
HIF-2αODD	HIF-2 α oxygen-dependent degradation domain
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
mRNA	messenger RNA
NAC	N-acetylcysteine
NES	normal oesophageal squamous
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PHDs	prolyl hydroxylases
PPI	proton pump inhibitor
RO	reflux oesophagitis
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of the mean
TNF	tumor necrosis factor
VHL	von Hippel-Lindau

Significance of this study

What is already known on this subject?

- A recent study, in which acute reflux oesophagitis was induced by interrupting proton pump inhibitor (PPI) therapy in patients with reflux oesophagitis healed by PPIs, showed that acute reflux oesophagitis starts with T lymphocyte-predominant inflammation of the oesophagus.
- The findings of that study were inconsistent with the traditional concept that reflux oesophagitis develops as a caustic chemical burn, which would first attract neutrophils and eosinophils rather than lymphocytes.
- When human oesophageal epithelial cells in culture are exposed to acid and bile salts, they produce reactive oxygen species (ROS) and secrete pro-inflammatory molecules.
- Within cells, ROS can stabilise hypoxia inducible factors (HIFs), which are important mediators of inflammation.

What are the new findings?

- Oesophageal biopsies from patients with acute reflux oesophagitis show increased immunostaining for HIF-2 α , which correlates with increased expression of pro-inflammatory molecule mRNA.
- Acidic bile salts induce human oesophageal squamous cells to increase their intracellular levels of ROS, thereby stabilising HIF-2 α , which mediates the expression of pro-inflammatory molecules and the secretion of chemokines that increase T cell migration rates in a transwell system.
- These findings support a new hypothesis for reflux oesophagitis pathogenesis in which refluxed gastric juice does not kill oesophageal epithelial cells directly, but rather activates their HIF-2 α , which stimulates their secretion of pro-inflammatory molecules that attract the T lymphocytes and other inflammatory cells that ultimately damage the oesophagus.

How might it impact on clinical practice in the foreseeable future?

- The elucidation of a role for HIF-2 α in the oesophageal inflammatory response to gastro-oesophageal reflux suggests that a HIF-2 α -directed therapy might be a novel approach for preventing and treating reflux oesophagitis.

Figure 1.

Development of acute reflux oesophagitis is associated with increased levels of HIF-2α in patient oesophageal biopsy specimens. (A) Patient flow chart. (B) HIF-2α and HIF-1α immunohistochemistry in biopsies of squamous oesophagus from patients with healed reflux oesophagitis at baseline (on PPIs), and at 1 and 2 weeks after stopping PPIs. Scale bar: 200 μM (C) Quantitation of HIF-1α and HIF-2α staining in patient biopsies using H scores. Bar graphs depict mean ± SEM. NS, not significant. *, p 0.05 compared with baseline

Figure 2.

Induction of acute reflux oesophagitis increases pro-inflammatory mediator mRNA expression that correlates with increased HIF-2 α protein in patient oesophageal biopsies. Representative PCR and semi-quantitative analyses for (A) IL-8, (B) IL-1 β , (C) TNF- α , (D) COX-2 and (E) ICAM-1 expression at baseline (on PPIs), and at 1 and 2 weeks after stopping PPIs. Bar graphs depict mean $\pm$ SEM values for all 12 patients. *, p 0.05; **, p 0.01 compared with baseline (F) Table demonstrating eta scores (n=12) for associations between HIF-2 α protein levels and pro-inflammatory molecule mRNA levels in patients'

oesophageal biopsies. $\text{Eta}^2 \times 100\%$ indicates percent of variance in mRNA changes explained by changes in HIF-2 α ; values $\geq 14\%$ indicate a large effect.

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Figure 4.

Acidic bile salts induce HIF-2 α and increase expression of pro-inflammatory mediators by oesophageal squamous epithelial cells. PCR and semi-quantitative analysis (depicted beneath individual gels) of expression of pro-inflammatory mediators after (A) acidic bile salts, (B) stable infection with a control vector or shHIF-2 α , and (C) treatment with active or inactive enantiomers of a small molecule HIF-2 α antagonist and acidic bile salts. Note that the densitometry scales vary among samples. PCR gels shown are representative of the results from 2 independent experiments.

Figure 5.

Crosstalk between HIF-2α and RELA/p65 regulates expression of pro-inflammatory mediators in oesophageal squamous cells. (A) PCR and Western blot analyses of whole cell lysates demonstrating efficiency of HIF-2α and RELA/p65 knockdown by shRNA in NES-B10T cells. Western blot analysis of (B) nuclear HIF-2α in cells after stable infection with either a control vector (CV) or shRELA/p65 or nuclear phospho-and total p65 in cells after stable infection with either a control vector or shHIF-2α. (C) Luciferase reporter assay for NF-κB activity in response to acidic bile salts in cells stably infected with either a control

vector or shHIF-2 α . (D) Western blot analysis of NF- κ B signaling proteins after exposure to acidic bile salts in cells stably infected with either a control vector or shHIF-2 α . (E) PCR and semi-quantitative analyses of pro-inflammatory mediator expression after exposure to acidic bile salts in cells stably infected with shRELA/p65 or a control vector. Bar graphs depict mean $\pm$ SEM (n=3). *, p < 0.05 compared with control vector. PCR gels and Western blots shown are representative of the results from 2 independent experiments.

Figure 6.

Development of acute reflux oesophagitis is associated with increased expression of phospho-p65 in patient oesophageal biopsies. (A) Immunohistochemical staining for phospho-p65 and total p65 in squamous oesophagus from reflux oesophagitis patients at baseline (on PPIs) and at 1 and 2 weeks off PPIs. Scale bar = 200 μ m (B) Quantitation of phospho-p65/total p65 in patient oesophageal biopsies using an H score. (C) Table demonstrating eta scores for associations between phospho-p65/total p65 protein levels and pro-inflammatory mediator mRNA levels in patients' oesophageal biopsies. Eta² $\times$ 100%

indicates percent of variance in mRNA changes explained by changes in phospho-p65/total p65; values $\geq 14\%$ indicate a large effect. Bar graphs depict mean $\pm$ SEM. *, $p \leq 0.05$ compared with baseline. NS; not significant

Figure 7.

HIF-2 α induced by exposing oesophageal squamous epithelial cells to acidic bile salts mediates their secretion of T cell chemokines. Migration rate of T cells treated with conditioned media (CM) collected from NES-B10T cells exposed to acidic bile salts (A) using cells stably infected with a control vector or shHIF-2 α , or (B) using cells treated with active or inactive enantiomers of a HIF-2 α small molecule antagonist. Bar graphs depict mean $\pm$ SEM (n=3). **, p 0.01, ***, p 0.001 compared to indicated control. (C) Schematic model demonstrating mechanisms of acid and bile salt-induced HIF-2 α stabilisation and

induction of pro-inflammatory mediators that might contribute to development of reflux oesophagitis.

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