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IFN-γ Attenuates Hypoxia-Inducible Factor (HIF) Activity in Intestinal Epithelial Cells through Transcriptional Repression of HIF-1β

Louise E. Glover,* Karina Irizarry,*† Melanie Scully,* Eric L. Campbell,* Brittelle E. Bowers,* Carol M. Aherne,*‡ Douglas J. Kominsky,* Christopher F. MacManus,* and Sean P. Colgan*

Numerous studies have revealed that hypoxia and inflammation occur coincidentally in mucosal disorders, such as inflammatory bowel disease. During inflammation, epithelial-expressed hypoxia-inducible factor (HIF) serves an endogenously protective function. In this study, we sought to explore how mucosal immune responses influence HIF-dependent end points. Guided by a screen of relevant inflammatory mediators, we identified IFN-γ as a potent repressor of HIF-dependent transcription in human intestinal epithelial cells. Analysis of HIF levels revealed that HIF-1β, but not HIF-1α, is selectively repressed by IFN-γ in a JAK-dependent manner. Cloning and functional analysis of the HIF-1β promoter identified a prominent region for IFN-γ-dependent repression. Further studies revealed that colonic IFN-γ and HIF-1β levels were inversely correlated in a murine colitis model. Taken together, these studies demonstrated that intestinal epithelial HIF is attenuated by IFN-γ through transcriptional repression of HIF-1β. These observations are relevant to the pathophysiology of colitis (i.e., that loss of HIF signaling during active inflammation may exacerbate disease pathogenesis). The Journal of Immunology, 2011, 186: 1790–1798.

The inflammatory bowel diseases define a series of chronic disorders with a complex etiology, including Crohn’s disease and ulcerative colitis. Central to their pathogenesis is intestinal barrier disruption, as well as a dysregulated immune response to omnipresent luminal Ags at the mucosal interface (1). The epithelial cell layer that makes up this dynamic barrier is finely regulated by inflammatory mediators (2, 3) and by metabolic shifts that are characteristic of inflammatory lesions (4). Such changes include diminished oxygen availability (hypoxia), resulting, in part, from active inflammatory cell recruitment and inadequate tissue perfusion (5). Adaptive mechanisms that have evolved to facilitate cell survival and functionality under hypoxic conditions include active transcriptional regulation of gene expression by hypoxia-inducible factor (HIF)-1, a member of the Per-ARNT-Sim family of basic helix–loop–helix transcription factors (6). Functional HIF-1 exists as an αβ heterodimer, comprising a constitutive (HIF-1β) and adaptive subunit (HIF-1α); its activation is dependent upon stabilization of an O2-sensitive degradation pathway that is regulated, in part, by a family of prolyl hydroxylase (PHD) enzymes. However, induction of HIF-1α by proinflammatory stimuli, even under normoxic conditions, has been described in phagocytes as a means to regulate priming, differentiation, and functional specialization of these immune cells (7–9).

Although significant progress has been made in delineating the intricate cross-talk that exists between hypoxic and inflammatory regulation of HIF in immune cells (10), a number of studies implicated a protective role for epithelial HIF in mucosal inflammation. Originally guided by microarray analysis of differentially expressed mRNA in cultured epithelial cells subjected to hypoxia, these studies proved to be robust in a number of animal models of inflammation. Further examination of mechanisms related to hypoxia-elicited barrier protection revealed three important features. First, expression of the functional proteins encoded by these mRNAs was localized to the most luminal aspect of polarized epithelia (i.e., apically expressed proteins). Second, molecular dissection of the hypoxia-elicited pathway(s) for this apical gene cluster revealed a high propensity for regulation by HIF. Third, HIF-dependent epithelial barrier-protective pathways driven by hypoxia tend to be more nonclassical regulators of barrier function. Rather than classic junctional proteins, such as occludin or claudin(s), hypoxia-induced enhancement of barrier function occurs through diverse pathways, ranging from increased mucin production (11) and molecules that modify mucus (e.g., intestinal trefoil factor) (12) to xenobiotic clearance (P-glycoprotein) (13) to nucleotide metabolism (ecto-5′-nucleotidase, CD73) (14, 15) and nucleotide signaling (adenosine A2B receptor) (14).

To more fully understand the physiologic implications of intestinal epithelial HIF, Karhausen et al. (16) generated two mouse lines with intestinal epithelial-targeted expression of mutant Hif1a (constitutive repression of HIF-1) or mutant von Hippel-Lindau gene (Vhlh, constitutive overexpression of HIF), which includes HIF-1 and HIF-2. Studies of colitis in these mice revealed that the loss of epithelial HIF-1 correlated with more severe clinical symptoms (mortality, weight loss, colon length, intestinal epithelial permeability), whereas an increase in epithelial HIF was protective for these individual parameters. Further evidence in

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Abbreviations used in this article: DSS, dextran-sodium sulfate; HIF, hypoxia-inducible factor; IEC, intestinal epithelial cell; PHD, prolyl hydroxylase; TSS, transcriptional start site.
support of a protective role for HIF in mucosal inflammation is provided by studies directed at inhibition of the enzymes that degrade HIF (i.e., HIF PDHs) (17). Indeed, studies revealed that administration of these compounds was protective in at least two models of murine colitis (18, 19). More recently, it was revealed that a specific PHD isoform (PHD1) mediates the protective influences of these compounds in murine dextran-sodium sulfate (DSS) colitis (20).

These findings emphasize the role of epithelial HIF during inflammatory diseases in the colon and may provide the basis for a therapeutic use of PHD inhibitors in inflammatory mucosal disease. Less is known about the influence of epithelial mediators on HIF activity in intestinal epithelia. In the current study, we aimed to clarify whether epithelial HIF activation and function are regulated by mediators found in the inflammatory milieu.

Materials and Methods

DSS colitis model

DSS colitis was induced in 10-wk-old C57BL/6 female mice (The Jackson Laboratories) with a modification of the technique of Okayasu et al. (21). Treatment was initiated on day 0 by the addition of 4.5% DSS (m.w. 36,000–50,000; MP Biomedicals) solution to drinking water. Vehicle control animals received water alone. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at University of Colorado.

Cell culture

Human T84 and Caco-2 epithelial cells were cultured as previously described (22). For mRNA and protein analysis, T84 cells were grown to confluence on 5-cm² collagen-coated permeable supports to form polarized monolayers. Where indicated, exposure to hypoxia was performed in a humidified hypoxic cell chamber (Coy Laboratory Products, Grass Lake, MI), as previously described (12), using standard hypoxic conditions of pO₂ 20 torr and pCO₂ 35 torr, with the balance made up by N₂ and water vapor. Normoxic cells were cultured in atmospheric oxygen concentrations (pO₂ 147 torr; pCO₂ 35 torr).

Cytokines, Abs, and reagents

Recombinant human IFN-γ, IFN-β, TNF-α, IL-4 (R&D Systems, Minneapolis, MN), and PGE₂ (BIOMOL, PA) were used at the indicated concentrations. JAK inhibitor 1 and BAY 11-7085 were purchased from Fisher Scientific. Rabbit anti-pSTAT1 (Tyr701) and STAT1 were purchased from Cell Signaling Technologies (Danvers, MA). Monoclonal anti–HIF-1β was purchased from Novus Biologicals (Littleton, CO). Anti–β-tubulin and anti–TATA-binding protein were from Abcam (Cambridge, MA).

Plasmid constructs

The promoter reporter constructs pHRE-Luc (23) and pA2BR-Luc (24) were described previously. A 1.4-kb fragment of the human HIF-1β promoter was cloned by PCR to generate pHIF1-β-Luc in pGL3-basic (Promega, Madison, WI) using primer set For 5'-ACCTTCAGGCT-GAGGCAGGATAACTGC-3' and Rev 5'-GATAAGGC TTCAGAGTG-GCCGGTCTCAGC-3'. Homology to published sequence was confirmed by sequencing at the University of Colorado cancer center DNA sequencing and analysis core facility. Sequential truncations of the cloned HIF-1β promoter sequence were generated by exonuclease III digestion (Erase-a-Base; Promega). Transfection of Caco-2 and HeLa cells was carried out using FuGENE 6 (Roche Diagnostics, Indianapolis, IN), as directed by the manufacturer. T84 cells (2 × 10⁵) were nucleoected with 2 μg plasmid using AMAXA Cell Line Nucleofector kit T, program T005, and the Nucleofector Device (Amaxa Biosystems, Walkersville, MD), as instructed in the manufacturer protocols. Four hours postnucleoect and seeding, cells were treated with the indicated cytokines. To assay promoter activity, transfected cells were harvested, and luciferase activity was measured in extracts using a dual-luciferase reporter-assay system (Promega). All firefly luciferase activity was normalized to a cotransfected Renilla reporter.

Real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol, and quantified by nanodrop spectrophotometer. First-strand cDNA was generated using equal amounts of input RNA and an iSCRIPT cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time quantitative PCR was performed on 100 ng cDNA template, using intron-spanning primers and Power SYBR (Applied Biosystems, Foster City, CA), in a total volume of 20 μl. Primer sets used are outlined in Supplemental Table I. Transcript levels and fold change in mRNA relative to β-actin endogenous control were determined as previously described (25).

Protein analysis

Where indicated, nuclear epithelial cell fractions were prepared using the NE-PER extraction kit, according to the manufacturer’s instructions (ThermoScientific, Rockford, IL). Protein content was quantified using BCA protein assay reagent and 30 μg nuclear extract subjected to SDS-PAGE and Western blot analysis. The antibodies used were: mouse anti–p-STAT1 (Tyr701), goat anti-STAT1, rabbit anti-pM-TOR (Ser2448), rabbit anti-M-TOR, mouse anti-p-p38 (Thr180/Tyr182), rabbit anti-p-p38, rabbit anti-p-PERK, rabbit anti-PERK, rabbit anti-p-eIF2α (Ser51), rabbit anti-eIF2α, rabbit anti-p-ERK1/2 (Thr202/Tyr204), rabbit anti-ERK1/2, rabbit anti-p-EGFR, rabbit anti-EGFR, rabbit anti-MMP-3, rabbit anti-MMP-9, rabbit anti-IL-6, rabbit anti-IL-8, rabbit anti-IFN-γ, rabbit anti-IL-10, rabbit anti-TGF-β, rabbit anti-α-SMA, rabbit anti-β-tubulin, rabbit anti–α-actin, and rabbit anti–β-catenin. Blots were incubated with secondary antibodies and visualized by enhanced chemiluminescence.
PAGE to assay for HIF-1β. For in vivo cytokine screening, whole-colon samples were homogenized in lysis buffer (150 mM NaCl, 20 mM Tris [pH 7.5], 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100) with Halt protease inhibitor mixture (Thermoscientific) and precleared by centrifugation. Nuclear HIF-1α and whole-colon IFN-γ were assayed by chemiluminescence-based sandwich immunoassay, according to the manufacturer’s protocols (MesoScale Diagnostics, Gaithersburg, MD). Plates were analyzed on a Sector 2400 Imager (MesoScale Diagnostics), and samples were normalized to total protein content. For preparation of whole-cell lysates, polarized cultures on permeable supports were washed twice in ice-cold HBSS and scraped directly into whole-cell lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF) supplemented with protease and phosphatase inhibitors (Thermoscientific). Samples were sonicated briefly and centrifuged for 10 min at 4°C to remove insoluble material. Twenty micrograms of total protein was subjected to SDS-PAGE to assay for STAT1, IκB, and NF-κB subunits.

**Statistical analysis**

Data were compared by two-factor ANOVA with the Bonferroni post hoc test or by the Student t test where appropriate. Values are presented as the mean ± SEM from at least three individual experiments.

**Results**

**IFN-γ represses HIF activity in intestinal epithelial cells**

Inflammatory lesions, such as those present in colitis, are associated with prodigious metabolic shifts and focal hypoxia. Adaptive responses to this state of inflammatory hypoxia include pro-survival and proresolution processes, such as angiogenesis, glycolysis, and adenine nucleotide signaling (26). We recently reported that expression of the adenosine receptor AA2BR is inversely correlated with pathogenic severity in a mouse model of colitis and that functional loss of this receptor exacerbated the acute inflammatory colitic phase (27). Furthermore, AA2BR is transcriptionally induced by HIF-1 in hypoxia (24). In initial studies, we elected to examine the impact of inflammatory mediators on HIF regulation in intestinal epithelia, using AA2BR as a model anti-inflammatory molecule. Caco-2 epithelial monolayers were transfected with an AA2BR-promoter luciferase reporter plasmid (24) and exposed to a panel of relevant inflammatory mediators, including TNF-α (10 ng/ml), IL-4 (10 ng/ml), PGE2 (100 nM), or IFN-γ (10 ng/ml; representing 200 reference units/ml) or vehicle control (ethanol) for 24 h. Cells were then exposed to hypoxia or normoxia for an additional 24 h. As shown in Fig. 1 and consistent with previous reports, AA2BR promoter activity was significantly induced by hypoxia. Interestingly, we observed a significant repression of AA2BR and implicating repression of HIF activity by IFN-γ.

**FIGURE 2.** Influence of IFN-γ on HIF expression and activity in human IECs. A, Microarray analysis of T84 monolayers on semipermeable membranes treated on the basolateral aspect with 10 ng/ml of IFN-γ for 0, 6, or 18 h. Data are mean ± SEM from three separate RNA isolations. *p < 0.001. B, T84 monolayers treated basolaterally with 10 ng/ml of IFN-γ for the indicated times were harvested for total RNA, and HIF-1α and HIF-1β mRNA levels were determined by real-time quantitative PCR. Data were calculated relative to β-actin and expressed as fold change relative to control. *p < 0.05. C, Nuclear fractions of T84 monolayers exposed basolaterally to 10 ng/ml of IFN-γ for the indicated times were extracted, normalized for protein content, and resolved by reducing SDS-PAGE to assay for HIF-1β protein levels. Nuclear TATA-binding protein levels were monitored as a loading control. Shown is a representative Western blot of three experiments with densitometric analysis. *p < 0.05. D, T84 monolayers were treated basolaterally with 10 ng/ml of IFN-γ for the indicated times and cultured under normoxic or hypoxic conditions for the last 12 h of treatment. Nuclear fractions were then harvested and subjected to ELISA for analysis of HIF-1α expression. Data are mean ± SEM of three experiments. *p < 0.025.
A recent study by Hiroi et al. (28) revealed that IFN-γ negatively regulates HIF-1-dependent gene induction in glioblastoma cells. Along with our observation that HIF is responsible for the hypoxic induction of AA2BR expression (24), we hypothesized that IFN-γ influences HIF activity in intestinal epithelial cells (IECs). In this study, we examined the direct influence of IFN-γ on HIF activity by reporter assay in T84 intestinal epithelia, given the extensive characterization of IFN-γ responsiveness in this model line (29, 30). T84 epithelial monolayers were nucleofected with an HRE-luciferase reporter plasmid (23) and treated for 24 h with the same panel of inflammatory cytokines as described for Fig. 1A. Treated cells were cultured for an additional 24 h in normoxic or hypoxic conditions, as indicated. As outlined in Fig. 1B, significant attenuation of HIF activity was observed in the presence of IFN-γ (75 ± 7%; p < 0.01 by ANOVA). Exposure of HRE-luciferase–nucleofected T84 epithelial monolayers to 1, 10, or 100 ng/ml of IFN-γ further revealed a dose-dependent decrease in HIF activity in normoxia and hypoxia (Fig. 1C; p < 0.01 by ANOVA).

**IFN-γ selectively represses epithelial HIF-1β expression**

Prompted by the results implicating repression of HIF activity by IFN-γ, we next examined the influence of IFN-γ on the basal expression of HIF in IECs. In this study, we profiled the relative expression of the individual subunits of HIF (i.e., HIF-1α and HIF-1β) by microarray analysis in untreated and IFN-γ–treated T84 epithelial cells. Notably, these experiments demonstrated that HIF-1β was selectively repressed at 6 and 18 h of IFN-γ treatment (Fig. 2A; p < 0.001). As outlined in Fig. 2B, these microarray results were validated by real-time PCR in RNA derived from T84 epithelial cells similarly exposed to a time course of IFN-γ treatment (10 ng/ml) and consistently revealed that the expression of HIF-1β mRNA is prominently decreased by exposure to IFN-γ by 12 h (Fig. 2C; p < 0.01), although repression was not significant at earlier time points tested (e.g., at 6 h, p > 0.05). By contrast, HIF-1α mRNA expression was moderately increased by IFN-γ treatment, strongly suggesting that the observed IFN-γ attenuation of HIF activity results from the selective repression of HIF-1β.

**Extensions of these findings revealed that pre-exposure of T84 cells to 10 ng/ml IFN-γ for the indicated times resulted in a progressive loss of constitutively expressed nuclear HIF-1β protein (Fig. 2C). Because HIF-1α/HIF-1β heterodimerization is required for stable association within the nuclear compartment and precludes formation of the active transcriptional complex (31), we examined whether nuclear HIF-1α was decreased as a consequence of IFN-γ–mediated HIF-1β repression. As shown in Fig. 2D, in response to IFN-γ treatment, nuclear HIF-1α was decreased in a time-dependent manner. Taken together, these findings confirm our results of functional HIF repression in the AA2BR promoter (Fig. 1A) and HRE-luciferase screens (Fig. 1B, 1C) and represent, to our knowledge, the first identification of cytokine-mediated repression of HIF activity that is conferred by selective attenuated expression of the HIF-1β subunit.

**IFN-γ directly represses HIF-1β activity**

In view of the likelihood of a transcription-mediated repression of HIF-1β by IFN-γ, attention was directed to the 5’-region of the HIF-1β gene. Based on available public databases (32) and published annotation of the human HIF-1β gene (33), we cloned 1402 bp of promoter sequence, corresponding to positions −1395 to +8 relative to the major transcriptional start site (TSS) (Fig. 3A), into pGL3-basic luciferase reporter vector and used this construct to assess the impact of IFN-γ on HIF-1β promoter activity. As shown in Fig. 3B, T84 epithelial cells nucleofected with this full-length HIF-1β promoter plasmid and treated with increasing concentrations of IFN-γ (16 h) revealed significant repression (p < 0.001 by ANOVA). Extensions of these findings revealed that pre-exposure of T84 cells to 10 ng/ml IFN-γ for the indicated times resulted in a progressive loss of constitutively expressed nuclear HIF-1β protein (Fig. 2C). Because HIF-1α/HIF-1β heterodimerization is required for stable association within the nuclear compartment and precludes formation of the active transcriptional complex (31), we examined whether nuclear HIF-1α was decreased as a consequence of IFN-γ–mediated HIF-1β repression. As shown in Fig. 2D, in response to IFN-γ treatment, nuclear HIF-1α was decreased in a time-dependent manner. Taken together, these findings confirm our results of functional HIF repression in the AA2BR promoter (Fig. 1A) and HRE-luciferase screens (Fig. 1B, 1C) and represent, to our knowledge, the first identification of cytokine-mediated repression of HIF activity that is conferred by selective attenuated expression of the HIF-1β subunit.

**FIGURE 3. Repression of HIF-1β promoter activity by IFN-γ.**

A. Cloned HIF-1β promoter and series of truncated promoter-luciferase reporter constructs generated using exonuclease III digestion. Relative positions of each clone and major TSSs are annotated. B. T84 intestinal epithelia were transfected with pRenilla reporter plasmid and either pHIF-1β-Luc reporter plasmid or empty vector for 4 h and treated with 0, 0.1, 1, or 10 ng/ml of IFN-γ for 16 h. Luciferase activity was measured normalized to Renilla activity. Data are mean ± SEM from three separate experiments. C. T84 IECs were transfected for 4 h with reporter constructs containing the full-length HIF-1β promoter sequence or indicated 5′ truncations. Cells were then incubated with (white bars) or without (black bars) 10 ng/ml of IFN-γ overnight. Luciferase activity was measured and normalized to Renilla activity. Data are expressed as relative promoter activity; a representative of three experiments is shown. Results are expressed as mean ± SEM. D. HIF-1β promoter sequence spanning positions −189 to +8 relative to the TSS.
by ANOVA) of HIF-1β promoter activity, with 59.6% loss of baseline promoter activity at concentrations as low as 0.1 ng/ml (2 RU/ml). This level of repression correlates with that measured at the mRNA and protein levels. To assess type I versus type II IFN specificity of HIF-1β promoter repression, T84 cells were similarly nucleofected with full-length promoter and subjected to increasing doses of IFN-β. Exposure to IFN-β also repressed promoter activity in a dose-dependent manner, comparable to that observed with IFN-γ, albeit only at the highest concentration of cytokine used (59.07% of control at 100 U/ml; Supplemental Fig. 1A). However, extension of these findings to IFN-β effects on HIF-1β transcript levels revealed only a moderate repression of HIF-1β at 6 and 12 h (18.5 and 17.4%, respectively) that was not sustained at 24 h of IFN-β treatment (Supplemental Fig. 1B).

To further scrutinize the promoter region that mediates transcriptional repression by IFN-γ, we generated a series of sequential 5' truncations of the HIF-1β gene promoter sequence in pGL3 using exonuclease III digestion (Fig. 3A). As outlined in Fig. 3C, baseline activity and repression of HIF-1β promoter activity by IFN-γ is lost upon truncation of the promoter to −39 bp upstream of the TSS. Bioinformatic analysis of the promoter sequence between nucleotides −189 and +8 relative to the TSS (Fig. 3D) revealed the existence of several Sp1 transcription factor-binding sites, as well as a recognition sequence for NF-κB. To define the contribution of the canonical NF-κB pathway to IFN-γ–mediated signaling in intestinal epithelia, levels of IκB family proteins and the NF-κB subunit protein p65 were assayed in cytoplasmic and nuclear extracts of T84 monolayers treated with IFN-γ. As outlined in Fig. 4A, IFN-γ–induced a modest increase in cytoplasmic levels of phosphorylated IκB, with a concomitant decrease in IκB levels by 15 min. Consistent with this, at 15 min of IFN-γ treatment, nuclear levels of active p65 NF-κB subunit were moderately increased. To determine whether IFN-γ–mediated NF-κB signaling is required for repression of HIF-1β levels, T84 epithelial cells were pretreated with increasing concentrations of the NF-κB inhibitor compound BAY 11-7085. Pretreatment with BAY 11-7085 inhibited IFN-γ– and TNF-α–induced nuclear translocation of p65 after 15 min of cytokine treatment (Fig. 4B), but it failed to attenuate IFN-γ–mediated repression of HIF-1β mRNA levels (Fig. 4C). Furthermore, site-directed mutagenesis of the NF-κB–binding site in HIF-1β promoter-luciferase reporter plasmids did not inhibit repression of promoter activity (Fig. 4D).

**FIGURE 4.** Repression of HIF-1β by IFN-γ is not mediated by NF-κB. A. The ability of IFN-γ (10 ng/ml) to activate the canonical NF-κB signaling pathway in T84 epithelial monolayers, treated basolaterally for the indicated times, was assayed by measuring levels of cytoplasmic proteins (IκB, P-IκB, p65) and nuclear IκB (p65) by Western blot. Image is representative of three experiments. B. T84 monolayers were treated with BAY 11-7085 (10 μM) or DMSO control for 30 min before exposure to IFN-γ (10 ng/ml), TNF-α (10 ng/ml), or vehicle at the basolateral surface for 15 min. Inhibition of NF-κB signaling was measured by cytoplasmic IκB levels (upper panel) and nuclear translocation of p65 (lower panel). Actin- and TATA-binding protein levels were monitored as loading controls for cytoplasmic and nuclear fractions, respectively. Images are representative of three experiments. C. Polarized T84 monolayers were treated with BAY 11-7085 (10 μM) or DMSO control for 30 min before exposure to IFN-γ (10 ng/ml) or vehicle at the basolateral surface for 12 h. HIF-1β (left panel) and HIF-1α (right panel) mRNA levels were determined by real-time quantitative PCR. Data were calculated relative to DMSO vehicle control for three experiments. D. The putative NF-κB site located at −49 bp was eliminated in the full-length HIF-1β (−1395) and 5' truncated −189 reporter constructs by site-directed mutagenesis. Resulting constructs were cotransfected with Renilla luciferase into T84 IECs. Cells were incubated with (white bars) or without (black bars) 10 ng/ml of IFN-γ overnight, and luciferase activity was measured and normalized to Renilla activity. Data are expressed as relative promoter activity; a representative of three experiments is shown. Results are expressed as mean ± SEM.
negating a key role for IFN-γ-induced NF-κB signaling in regulating HIF-1β expression. Given the timeline of IFN-γ treatment required for repression of HIF-1β, as well as the lack of STAT- or IRF-binding sites in the proximal 5′ promoter region, we postulate that HIF-1β does not represent an immediate early-response gene but rather is regulated through an undefined IFN-γ-signaling pathway.

Repression of HIF-1β by IFN-γ is mediated through JAK signaling

IFN-γ–dependent cellular responses are mediated predominantly through the JAK–STAT1 signaling pathway (34). JAK-STAT signaling involves sequential IFN-γR recruitment and activation of members of the Janus family of kinases and the Stats to regulate target-gene transcription (35). The inhibitory influence of IFN-γ on HIF-1 activity in vitro was recently described (28); although the precise mechanism of repression was not fully elucidated, a central role for IFN-γ–activated STAT1 as a negative regulator of HIF-1–dependent transcription was defined. To determine the role of JAK/STAT signaling in HIF-1β repression, T84 epithelial cells were pretreated with various doses of JAK inhibitor 1, a pharmacologic inhibitor of JAK enzymes, followed by IFN-γ treatment (10 ng/ml). Inhibitor activity was assessed in vitro by its capacity to inhibit IFN-γ–induced tyrosine (Tyr701) phosphorylation of STAT1 (Fig. 5A, 5B). As outlined in Fig. 5C, HIF-1α mRNA levels were modestly, although not significantly, repressed upon treatment with JAK inhibitor in the presence of IFN-γ. Notably, IFN-γ–mediated repression of HIF-1β transcript levels was attenuated by JAK inhibitor 1 in a dose-dependent manner, thus supporting a role for JAK signaling in HIF-1β transcriptional repression.

Mucosal HIF-1β and IFN-γ levels are inversely correlated in a DSS model of colitis

Given the critical role for HIF in barrier maintenance and resolution of inflammation, we extended our in vitro studies to an in vivo model to characterize colon tissue levels of IFN-γ and HIF-1β in murine colitis. To do this, we profiled IFN-γ and HIF-1β in whole-colon samples derived from mice following exposure to DSS or vehicle for 6 d. Histological examinations of colons from DSS-treated mice confirmed the presence of fulminant disease, as demonstrated by prominent tissue architecture loss and inflammatory cell infiltrates (Fig. 6A). As further disease indicators, weight gain and colon length, determined by measurement of the distance from the most distal aspect of the cecum to the most terminal aspect of the rectum, were assessed. Both parameters were significantly reduced in DSS-treated animals (Supplemental Fig. 2). As shown in Fig. 6B and consistent with previous results (36), colonic IFN-γ transcript levels were highly induced after 6 d of treatment with 4.5% DSS (58 ± 28-fold increase over vehicle control). Notably, analysis of colonic HIF-1 mRNA levels revealed a selective repression of the HIF-1β subunit by 51.2 ± 10% (p < 0.05), thereby serving as a functional correlate of IFN-γ–dependent repression of HIF-1β in vivo. HIF-1α transcript levels were not changed under these circumstances. We next determined whether IFN-γ cytokine was appreciably induced at the protein level and whether this correlated with loss of mucosal HIF-1β protein expression. Analysis of whole-colon protein lysates by ELISA revealed a strong induction of IFN-γ cytokine in DSS-treated animals (256-fold increase over vehicle control; Fig. 6C). In parallel to the observed selective repression of colonic HIF-1β transcript, mucosal levels of HIF-1β protein were also significantly lower than those measured in vehicle-treated controls.

![FIGURE 5](http://www.jimmunol.org/) Transcriptional repression of HIF-1β is dependent on IFN-γ–mediated JAK-STAT1 signaling. The ability of IFN-γ to induce tyrosine (Tyr701) phosphorylation of STAT1 in T84 epithelia (A) is inhibited by JAK inhibitor 1 in a dose-dependent manner (B). Images are representative of two experiments. C, Polarized T84 monolayers were treated with JAK inhibitor 1 or DMSO control for 30 min before exposure to 10 ng/ml IFN-γ or vehicle at the basolateral surface for 12 h. HIF-1β (left panel) and HIF-1α (right panel) mRNA levels were determined by real-time quantitative PCR. Data were calculated relative to DMSO vehicle control for three experiments. *p < 0.01.
We extended these findings to determine whether expression of IFN-γ and HIF-1-regulated gene products were altered in DSS colitis. As outlined in Fig. 6E, the well-characterized IFN-γ-responsive gene products IFN-inducible protein 10 and monokine-induced by IFN-γ were prominently induced. Conversely, expression levels of HIF-inducible gene products of the glycolytic pathway (Eno-1, PGK-1), angiogenesis (VegfA), and oxygen sensing (PHD3) were reduced compared with vehicle animals (*p < 0.05). These findings reveal correlative induction of IFN-γ-responsive genes with repression of a number of HIF-1-regulated genes. To further scrutinize this association, we correlated IFN-γ mRNA fold change and HIF-1β mRNA fold change relative to pooled control (*p < 0.01). F, IFN-γ correlation with HIF-1β. Data are plotted as IFN-γ mRNA fold change and HIF-1β mRNA fold change relative to pooled control (n = 6 animals per condition). $R^2 = +0.80$. p < 0.05 by linear regression.

Discussion

In mucosal inflammatory disorders, a complex, bidirectional cross-talk exists between hypoxia and inflammation, whereby cytokines and hypoxia-inducible genes influence each other in reciprocal manners (10). In this study, we sought to define whether specific inflammatory pathways might influence hypoxia-mediated signaling pathways in IECs. Results from these studies showed that IFN-γ, a well-documented effector cytokine in inflammatory bowel disease, inhibits HIF-1 activity and expression via transcriptional repression of HIF-1β. To our knowledge, this observation represents the first described pathway for HIF attenuation by selective repression of the HIF subunit HIF-1β. These results were surprising to us on a number of levels. First, IFN-γ increment plays a role in balancing intestinal HIF activity through selective repression of the HIF-1β subunit.
is not documented to share the highly regulated, dynamic nature of theα subunit of the HIF heterodimer. Investigations of HIF signaling have largely focused on HIF-α function and regulation. Second, although a role for transcriptional modulation of HIF is becoming more appreciated (37), the stability and activity of HIF-α are largely thought to be predominantly regulated at the protein level. HIF-α is regulated by a number of posttranslational modifications, including hydroxylation, acetylation, phosphorylation, and sumoylation, via interactions with protein complexes that include PHDs, the von Hippel-Lindau tumor suppressor gene product, ARD-1, SUMO, and p300/CBP (38). Transcriptional induction of HIF-1α under normoxic conditions by a type I IFN (IFN-α) has been described in human endothelial cells (39) and is thought to be mediated via IFN-stimulated gene factor 3 binding to IFN-stimulated response elements. Bioinformatic analysis of the HIF-1β 5′ flanking promoter sequence proximal to the TSS did not reveal candidate canonical IFN-stimulated response element or IRF-binding site sequences. Site-directed mutagenesis of NF-κB consensus-binding sites did not reverse the observed IFN-γ-mediated repression of HIF-1β promoter activity. Therefore, further investigation is warranted to define the exact site of IFN-γ-mediated repression of HIF-1β promoter activity. A recent study by Gerber et al. (40) revealed a role for IFN-γ in the induction of the PHD enzyme PHD3 as an early immediate gene in endothelial cells. Analysis of PHD3 transcript levels in IFN-γ–treated T84 IECs revealed an early modest, but insignificant, induction (1.4 ± 0.15-fold over control at 2 h, data not shown). However, this cannot be excluded as a second potential mechanism for repression of HIF-1 activity in vitro and warrants further study.

IFN-γ is secreted by an array of activated immune cells that reside in close proximity to IECs, both within the epithelial compartment and from the underlying lamina propria (41). This population of IFN-γ–producing immune cells has conventionally consisted primarily of T lymphocytes and NK cells; however, more recently it has expanded to include B cells and professional APCs (35). A recent study defined a role for HIF-1α in the production of IFN-γ by macrophages, outlining the contribution of inflammatory hypoxia to APC activation and cytokine production. Furthermore, HIF signaling was shown to enhance phagocytic activity and Ag-presentation capacity in hypoxic macrophages by an IFN-γ–dependent process (42). These findings underscore the importance of hypoxia in immune regulation and highlight a functional cross-talk that exists between HIF and inflammatory mediators, such as IFN-γ.

IFN-γ has established importance in mucosal inflammation and has been shown by a number of groups to significantly contribute to the pathogenesis of murine colitis (3, 37, 43). Proposed mechanisms for disease exacerbation include IFN-γ–mediated chemokine induction (i.e., IFN-γ-inducible protein 10, monokine induced by IFN-γ, and MCP-1) (37, 43), augmented colonic NO activity (43), and dysregulated IEC homeostasis with increased apoptosis via altered β-catenin signaling (3). A protective role for HIF in IEC homeostasis in health and disease was described in several studies. HIF elicits a barrier-protective program in the intestinal mucosa to physically impede penetration of macromolecules and intact microbes at the luminal surface, and it promotes resolution of ongoing inflammation to ameliorate the course of disease (44). Targeted IEC knockout of functional HIF-1α was shown to correlate with augmented disease pathogenesis in a transnitrobenzyl sulfonic acid colitis model (16). Moreover, in vivo delivery of PHD inhibitor compounds induced HIF activity and proved profoundly protective in murine colitis (18, 19). In this study, we determined that mucosal IFN-γ induction in DSS colitis inversely correlated with HIF-1β levels, thereby implicating IFN-γ as a regulator of intestinal HIF activity through selective repression of the HIF-1β subunit.

In summary, we propose that the inhibition of protective intestinal epithelial HIF-1 pathway(s) by IFN-γ represents a novel mechanism for IFN-γ–mediated promotion of the colitic course and may provide a balance between pro- and anti-inflammatory responses in mucosal inflammation.

Disclosures
The authors have no financial conflicts of interest.

References


Table I. Primer sequences used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Targets</th>
<th>Sequence 5'-3'</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H. sapiens HIF1-α</strong></td>
<td><strong>Forward:</strong> TTCCAGTTACGTTCTCTCGATCA</td>
<td>76bp</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse:</strong> GCTGGAATACTGTAACTGTGCTTTG</td>
<td></td>
</tr>
<tr>
<td><strong>H. sapiens HIF1-β</strong></td>
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<td>244bp</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse:</strong> TCCTCACAAGCTACGCTATTC</td>
<td></td>
</tr>
<tr>
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<td><strong>Forward:</strong> GCACTCTTCCAGCTTCTTCC</td>
<td>102bp</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse:</strong> CAGGTCTTTTGCGGTGCTCCAG</td>
<td></td>
</tr>
<tr>
<td><strong>M. musculus HIF1-α</strong></td>
<td><strong>Forward:</strong> CAGCTGCGTGAACTCAACAT</td>
<td>77bp</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse:</strong> TCACACGGATCTCTTACC</td>
<td></td>
</tr>
<tr>
<td><strong>M. musculus HIF1-β</strong></td>
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<tr>
<td></td>
<td><strong>Reverse:</strong> ACACCACCCTACGCTCTCA</td>
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<td><strong>Reverse:</strong> TGGCTCTGCAAGATTTTATG</td>
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<tr>
<td></td>
<td><strong>Reverse:</strong> TCACGCACGATTTCCCTTCAG</td>
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</table>
SUPPLEMENTAL Figure 1: Repression of intestinal HIF-1β levels by IFN-β.
SUPPLEMENTAL FIGURE 2: Disease severity in a mouse model of DSS colitis.