Iron Prevents Hypoxia-Associated Inflammation Through the Regulation of Nuclear Factor-B in the Intestinal Epithelium

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Abstract: Background Aims Hypoxia-associated pathways influence the development of inflammatory bowel disease. Adaptive responses to hypoxia are mediated through hypoxia-inducible factors, which are regulated by iron-dependent hydroxylases. Signals reflecting oxygen tension and iron levels in enterocytes regulate iron metabolism. Conversely, iron availability modulates responses to hypoxia. In the present study we sought to elucidate how iron influences the responses to hypoxia in the intestinal epithelium. Methods Human subjects were exposed to hypoxia, and colonic biopsy specimens and serum samples were collected. HT-29, Caco-2, and T84 cells were subjected to normoxia or hypoxia in the presence of iron or the iron chelator deferoxamine. Changes in inflammatory gene expression and signaling were assessed by quantitative polymerase chain reaction and Western blot. Chromatin immunoprecipitation was performed using antibodies against nuclear factor (NF)-B and primers for the promoter of tumor necrosis factor (TNF) and interleukin (IL)1. Results Human subjects presented reduced levels of ferritin in the intestinal epithelium after hypoxia. Hypoxia reduced iron deprivation–associated TNF and IL1 expression in HT-29 cells through the induction of autophagy. Contrarily, hypoxia triggered TNF and IL1 expression, and NF-B activation in Caco-2 and T84 cells. Iron blocked autophagy in Caco-2 cells, while reducing hypoxia-associated TNF and IL1 expression through the inhibition of NF-B binding to the promoter of TNF and IL1. Conclusions Hypoxia promotes iron mobilization from the intestinal epithelium. Hypoxia-associated autophagy reduces inflammatory processes in HT-29 cells. In Caco-2 cells, iron uptake is essential to counteract hypoxia-induced inflammation. Iron mobilization into enterocytes may be a vital protective mechanism in the hypoxic inflamed mucosa.

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Iron Prevents Hypoxia-Associated Inflammation Through the Regulation of Nuclear Factor-κB in the Intestinal Epithelium

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SUMMARY
Hypoxia induces iron mobilization from the intestinal epithelium in human subjects. In HT-29 cells, hypoxia inhibits iron deprivation–associated inflammation through the induction of autophagy, whereas in Caco-2 cells iron ameliorates hypoxia-associated inflammation through the inhibition of nuclear factor-κB.

BACKGROUND & AIMS: Hypoxia-associated pathways influence the development of inflammatory bowel disease. Adaptive responses to hypoxia are mediated through hypoxia-inducible factors, which are regulated by iron-dependent hydroxylases. Signals reflecting oxygen tension and iron levels in enterocytes regulate iron metabolism. Conversely, iron availability modulates responses to hypoxia. In the present study we sought to elucidate how iron influences the responses to hypoxia in the intestinal epithelium.

METHODS: Human subjects were exposed to hypoxia, and colonic biopsy specimens and serum samples were collected. HT-29, Caco-2, and T84 cells were subjected to normoxia or hypoxia in the presence of iron or the iron chelator deferoxamine. Changes in inflammatory gene expression and signaling were assessed by quantitative polymerase chain reaction and Western blot. Chromatin immunoprecipitation was performed using antibodies against nuclear factor (NF)-κB and primers for the promoter of tumor necrosis factor (TNF) and interleukin (IL)1β.

RESULTS: Human subjects presented reduced levels of ferritin in the intestinal epithelium after hypoxia. Hypoxia reduced iron deprivation–associated TNF and IL1β expression in HT-29 cells through the induction of autophagy. Contrarily, hypoxia triggered TNF and IL1β expression, and NF-κB activation in Caco-2 and T84 cells. Iron blocked autophagy in Caco-2 cells, while reducing hypoxia-associated TNF and IL1β expression through the inhibition of NF-κB binding to the promoter of TNF and IL1β.

CONCLUSIONS: Hypoxia promotes iron mobilization from the intestinal epithelium. Hypoxia-associated autophagy reduces inflammatory processes in HT-29 cells. In Caco-2 cells, iron uptake is essential to counteract hypoxia-induced inflammation. Iron mobilization into enterocytes may be a vital protective mechanism in the hypoxic inflamed mucosa.

Keywords: Inflammatory Bowel Disease; Autophagy; Deferoxamine; Caco-2.

Crohn’s disease and ulcerative colitis, the main clinical phenotypes of inflammatory bowel disease (IBD), are chronic disorders arising from the interplay between genetic and environmental factors.1 During active inflammation, resident macrophages and dendritic cells produce inflammatory cytokines that trigger the recruitment of inflammatory cells into the mucosa. The infiltrated immune cells deplete the local oxygen, rendering the inflamed intestinal mucosa severely hypoxic, particularly within the epithelial cell layer.2 The epithelium plays an integral role in maintaining intestinal homeostasis; therefore, it is not surprising that intestinal epithelial cells (IECs) have evolved many molecular mechanisms that enable them to respond to hypoxia.3 Adaptive transcriptional responses to low oxygen conditions are mediated through the hypoxia-inducible transcription factors (HIFs). HIFs are tightly regulated by prolyl hydroxylases (PHD1, PHD2, and PHD3) and factor-inhibiting HIF, which belong to a family of iron-dependent oxygenases that use oxygen and 2-oxoglutarate

Abbreviations used in this paper: ARE, adenylate and uridylate-rich elements; ChIP, chromatin immunoprecipitation; DFO, deferoxamine; DMT-1, divalent metal transporter 1; FACS, ferric ammonium iron citrate; FP, ferroportin; H2DCF-DA, 2’,7’-dichlorodihydrofluorescein diacetate; HIF, hypoxia-inducible transcription factor; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IL1β, interleukin 1β; IRE, iron-responsive elements; IRF, iron regulatory protein; LC3, light chain 3; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; PCR, polymerase chain reaction; PHD, polyhydroxylase; ROS, reactive oxygen species; Tf, transferrin; TNF, tumor necrosis factor; TTP, tristetraprolin.

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as co-substrates. Under normoxia, prolyl hydroxylation leads to ubiquitination and proteasomal degradation of the HIFα subunits, whereas asparaginyl hydroxylation inhibits HIFα transcriptional activity. Hypoxia blocks these processes, allowing HIFs to accumulate and induce the expression of HIF target genes. The nuclear factor (NF)-κB signaling pathway also is regulated by hydroxylases, and there is considerable interdependence between HIF and NF-κB signaling pathways. Thus, hypoxia-mediated NF-κB activation is dependent on hydroxylation of the NF-κB upstream regulatory factor IkB kinase. Proinflammatory signals, such as cytokines, and lipopolysaccharide promote stabilization of HIF proteins, even under normoxic conditions, indicating the interaction between hypoxic and immune responses to infection and tissue damage. In turn, HIF-1α stabilization promotes the transcription of proinflammatory cytokines, including tumor necrosis factor (TNF) and interleukin 1β (IL1β), which mediates systemic inflammation and triggers important host defense mechanisms. Hypoxia-associated HIF stabilization also activates innate immune protective mechanisms such as autophagy, a self-eating process that serves to maintain energy metabolism during metabolic stress.

Autophagic adaptor proteins, such as p62 (also known as sequestosome 1 (SQSTM1)), deliver target substrates for degradation to the autophagosome via binding to the microtubule-associated protein 1 light chain 3 (LC3). LC3 is linked to phosphatidylethanolamine, yielding a product known as LC3-II, which remains associated with autophagosomes until its destruction. The discovery of a functional polymorphism in the autophagy-related 16-like 1 gene associated with Crohn’s disease brought this pathway into the spotlight in IBD and recent studies have shown impairment of autophagy in IBD patients concomitantly with the stabilization of hypoxia markers when compared with healthy subjects. Environmental stress induces autophagy through the inhibition of mammalian target of rapamycin (mTOR), while energetic favorable states activate mTOR. Hypoxia has been linked to the production of reactive oxygen species (ROS), which cause the hypoxic stabilization of HIF-1α and HIF-2α, and enhance the DNA binding of NF-κB through a redox-dependent mechanism. In the context of innate immunity, hypoxia-induced ROS contribute to intracellular signaling, promoting the release of proinflammatory cytokines.

Iron is an essential functional component of proteins that mediate a broad range of vital biochemical functions, such as oxygen transport, energy production, and host defense. Because there are no physiologically regulated means of iron excretion, regulation of systemic iron balance occurs exclusively at the site of absorption. Dietary iron is absorbed primarily by duodenal enterocytes. After ferric iron (Fe³⁺) is reduced to ferrous iron (Fe²⁺) by apical ferric reductases, such as duodenal cytochrome b, it is taken into the cell through the divalent metal transporter (DMT)-1. If the human body is iron-replete, iron is sequestered into ferritin molecules in a redox-inactive state. However, when the body iron status is low, iron is transported out of the enterocyte by the basolateral iron transporter ferroportin (FPN) and loaded onto the plasma protein transferrin (Tf), which delivers iron to peripheral tissues. Despite the essential role of iron within the body, it also is highly toxic because of its capacity to generate iron-catalyzed ROS through Fenton- and Haber-Weiss-type reactions. Central to systemic iron regulation is the liver-derived hormone hepcidin. When increased, hepcidin reduces the influx of iron into the plasma by causing degradation of FPN, leading to anemia of inflammation. This condition is characterized by decreased release from intracellular iron stores, low plasma iron and Tf concentrations, and mild anemia. The enterocyte iron content regulates iron absorption through its effects on iron regulatory proteins (IRPs), which bind to iron-responsive elements (IREs) that regulate messenger RNA (mRNA) translation of FPN, ferritin, and HIF-2α, or DMT-1 mRNA stability. Recent evidence has suggested that release of ferritin-associated iron involves ferritin degradation via autophagy in response to bacterial infections or iron deficiency. Thus, the iron chelator deferoxamine (DFO) induces autophagy, resulting in ferritin lysosomal degradation.

Many of the processes governed by mTOR are dependent on iron as a cofactor. In turn, the mTOR pathway regulates iron homeostasis by modulating Tf-receptor mRNA stability through the induction of the zinc finger protein tristeproline (TTP). mRNA decay is a critical mechanism to control the expression of many proinflammatory cytokines, including TNF. These transcripts are targeted for rapid degradation through the adenylate and uridylic-rich elements (ARE) present in the mRNA 3’ untranslated region. TTP binds to ARE motifs and destabilizes mRNA of a wide range of proinflammatory cytokines, including IL1β and TNF.

The human IEC lines HT-29, Caco-2, and T84 originate from human colon adenocarcinomas. HT-29 cells essentially are undifferentiated under standard culture conditions, although they can differentiate into mucus-secreting goblet cells. Caco-2 cells differentiate spontaneously in culture into an enterocyte-like phenotype expressing intestinal brush-border enzymes and nutrient transporters. Caco-2 cells also express key proteins involved in iron transport, including duodenal cytochrome b, DMT-1, and ferritin, and generally are regarded as the best available intestinal cell model for studying the mechanisms associated with iron transport. T84 cells also differentiate spontaneously at confluence. Nonetheless, although Caco-2 cells are more reminiscent of small intestinal enterocytes, T84 cells are more similar in structure and function to colonic crypt cells.

In the present study we sought to elucidate how iron availability influences the responses to hypoxia in the intestinal epithelium using HT-29, Caco-2, and T84 cells. Our results suggest that hypoxia-promoted iron absorption might represent an essential regulatory mechanism to counteract hypoxia-associated inflammatory gene expression in the intestinal epithelium.

Results

Hypoxia Promotes Iron Mobilization From the Intestinal Mucosa in Human Subjects

To study the effects of hypoxia on iron metabolism, human subjects were subjected to hypoxic conditions
resembling an altitude of 4000 meters above sea level for 3 hours. The participant characteristics are shown in Table 1. Immunostaining of the iron storage ferritin showed significantly reduced intracellular levels of ferritin right after hypoxia and 1 week after hypoxia (Figure 1A), while immunostaining of the iron transporters DMT-1 (Figure 1B) and FPN (Figure 1C) showed no changes. In accordance with the reduced levels of ferritin observed, iron content in colonic tissue showed a trend toward reduced levels after hypoxia (Figure 1D). Transcriptional analysis showed no changes in the mRNA expression of ferritin (Figure 1E) or DMT-1 (Figure 1F) under hypoxia. Although not significant, transcriptional analysis showed a tendency toward an increase in the mRNA expression of FPN (Figure 1G). Accordingly, levels of the hepcidin inducer IL6 showed a trend toward a decrease in mRNA expression (Figure 1H). Taken together, our results indicate a hypoxia-mediated increase in iron mobilization from the intestinal mucosa. Serum levels of iron (Figure 1I), Tf saturation (Figure 1J), Tf (Figure 1K), and ferritin (Figure 1L) remained unchanged after hypoxia. Laboratory parameters are shown in Table 2.

Hypoxia Promotes Iron Uptake in HT-29 and Caco-2 Cells

To further explore the impact of hypoxia on iron metabolism in the intestinal epithelium, we subjected serum-starved HT-29 and Caco-2 cells to hypoxia (0.2% O2) for 4 and 24 hours in the presence of ferric ammonium iron citrate (FAC) or the classic iron chelator DFO to ensure the absence of available iron. Low oxygen conditions induced the expression of the iron transporters DMT-1 and FPN after 24 hours in both cell lines (Figure 2A-D). Conversely, iron supply abrogated hypoxia-induced DMT-1 and FPN mRNA expression (Figure 2A-D). Of note, hypoxia-mediated FPN expression was reduced under conditions of iron deprivation in HT-29 cells, but not in Caco-2 cells, suggesting reduced iron efflux from iron-deprived HT-29 cells when compared with Caco-2 cells. Iron induced the mRNA expression of the intracellular iron storage protein ferritin under hypoxic conditions in both cell lines, confirming iron uptake and storage (Figure 2E and G). Hypoxic conditions were confirmed through the induction of the hypoxia marker vascular endothelial growth factor (Figure 2F and H). Low oxygen tension as well as DFO-mediated iron deprivation induced HIF-1α protein accumulation after 4 and 24 hours in HT-29 (Figure 2I) and Caco-2 cells (Figure 2J). Iron-treated cells showed accumulation of ferritin under both normoxic and hypoxic conditions in both cell lines (Figure 2I and J). Notably, 4 hours sufficed for the induction of ferritin protein expression under hypoxia, but not under normoxia, in Caco-2 cells, confirming the enhanced effects of hypoxia on iron uptake in these cells.

Iron Reduces Hypoxia-Induced IL1β and TNF Expression in Caco-2 Cells

Next, we sought to evaluate the impact of hypoxia and iron availability on inflammatory gene expression and signaling. DFO-mediated iron deprivation induced the expression of TNF in HT-29 (Figure 3A), Caco-2 (Figure 3C), and T84 cells (Figure 3E). DFO-mediated iron deprivation also induced the expression of IL1β in HT-29 cells (Figure 3B), but not Caco-2 or T84 cells (Figure 3D and E), showing hypoxia-associated mechanisms in addition to HIF/NF-κB that activated the orchestration of the expression of IL1β. Strikingly, hypoxia induced TNF and IL1β expression in Caco-2 cells (Figure 3C and D) and T84 cells (Figure 3E and F), but not in HT-29 cells (Figure 3A and B), suggesting a differential regulation of hypoxia-associated factors involved in inflammatory gene expression in these cells. Importantly, DFO-associated inflammatory processes also were ameliorated under hypoxia in HT-29 cells (Figure 3A and B), but not in Caco-2 (Figure 3C and D) or T84 cells (Figure 3E and F), where only iron supplementation reduced DFO-associated inflammation. These results suggest hypoxia-protective mechanisms in addition to HIF activation in HT-29 cells and highlights the crucial role of iron in counteracting hypoxia-associated inflammatory gene expression in Caco-2 and T84 cells. At the protein level, DFO increased p65 phosphorylation under normoxic conditions in all cell lines analyzed, confirming the proinflammatory effects of iron depletion (Figure 3G–I). In accordance with the transcriptional analysis, hypoxia abrogated constitutive p65 activation in HT-29 cells (Figure 3G) while inducing p65 phosphorylation in Caco-2 cells (Figure 3H) and T84 cells (Figure 3I), further highlighting the different responses to hypoxia and iron deprivation in IECs. Notably, iron supplementation was unable to reduce hypoxia-mediated p65 phosphorylation to basal levels in Caco-2 or T84 cells, suggesting inhibitory effects downstream of the NF-κB signaling cascade (Figure 3G–I). Given the differential responses to hypoxia in HT-29 cells when compared with Caco-2 or T84 cells, we next analyzed the pattern of PHD mRNA expression under hypoxia in these cell lines. Individual PHD isoforms showed similar patterns of regulation by hypoxia in all 3 cell lines. Although PHD1 mRNA levels were not affected by hypoxia (Figure 3J, M, and P), PHD2 (Figure 3K, N, and Q) and PHD3 (Figure 3L, O, and R) mRNA expression was increased significantly under hypoxia, with PHD3 induction being particularly striking in Caco-2 and T84 cells compared with HT-29 cells.

| Table 1. Participant Characteristics |
|-------------------------------------|
| Number of subjects                  | 10 |
| Female sex                          | 3 (30%) |
| Age, y (means ± SD)                 | 28.2 ± 4.9 (20–36) |
| Age in males (means ± SD)           | 29 ± 5.5 (20–36) |
| Age in females (means ± SD)         | 26.3 ± 3.2 (24–30) |
| Weight, kg (means ± SD)             | 69.25 ± 11.0 (48–82) |
| Smokers                             | 2 of 10 (20.0%) |
Iron Reduces Hypoxia-Induced Inflammation Through an Autophagy-Independent Mechanism in Caco-2 Cells

Previous reports have highlighted the importance of hypoxia-associated autophagy to reduce inflammation in several mouse models of IBD. To elucidate the effects of iron availability on hypoxia-associated autophagy, HT-29 and Caco-2 cells were subjected to hypoxia in the presence of DFO or FAC. As expected, hypoxia induced autophagy in both cell lines as evidenced by the degradation of p62 and LC3 (Figure 4A). Conversely, iron supplementation blocked constitutive autophagy under normoxic conditions, as evidenced by an increase in phosphorylated mTOR, in HT-29 and Caco-2 cells (Figure 4A). In normoxic HT-29 cells, iron also reduced the conversion of LC3-I into LC3-II, and blocked autophagosome degradation as evidenced by the accumulation of total LC3. Interestingly, hypoxia reversed iron-mediated blockage of autophagy in HT-29, but not in Caco-2, cells, where iron overload triggered mTOR phosphorylation and blocked hypoxia-associated autophagosomal degradation leading to the accumulation of p62 and LC3 (Figure 4A). HT-29 and Caco-2 cells were subjected to hypoxia in the presence of DFO, FAC, or chloroquine, a late-stage autophagy inhibitor that prevents the fusion of autophagosomes with lysosomes. Autophagy was induced 24 hours after hypoxia as shown by a significant increase in staining for LC3 (green) in the presence of chloroquine (Figure 4B). Likewise, iron blocked autophagosomal degradation in Caco-2, but not HT-29, cells under hypoxia, as shown by an increase in LC3 staining (Figure 4B). To further elucidate the role of autophagy in hypoxia-mediated anti-inflammatory effects, HT-29 cells were subjected to hypoxia in the presence of chloroquine, under iron-deprived and iron-overload conditions. Hypoxia no longer was able to abrogate DFO-mediated induction of TNF (Figure 4C) and IL1β (Figure 4D) in the presence of chloroquine, evidencing the crucial role of autophagy in hypoxia-associated inhibition of proinflammatory gene expression in HT-29 cells. Interestingly, autophagy seems to play a crucial role in suppressing constitutive TNF mRNA expression in HT-29 cells as evidenced by an increase in TNF expression in the presence of chloroquine (Figure 4C). Moreover, in the absence of iron, autophagy becomes essential to inhibit IL1β expression in HT-29 cells (Figure 4D). In line with these results, Western blot analysis showed an increase in phosphorylated p65 in the presence of chloroquine (Figure 4E). To elucidate whether the lack of inhibitory effects of hypoxia in Caco-2 cells could be owing to a deficient autophagy activation we stimulated Caco-2 cells with the mTOR inhibitor and autophagy inducer rapamycin under hypoxic conditions in the presence of DFO or iron. Interestingly, rapamycin was able to inhibit TNF (Figure 4F), but not IL1β (Figure 4G) expression, suggesting a reduced activation of autophagy in Caco-2 cells under hypoxia. Moreover, Western blot analysis showed that rapamycin reduced phosphorylated p65 under iron-depleted conditions, but not in the presence of iron, highlighting the strong anti-autophagy effects of iron in Caco-2 cells even under hypoxia (Figure 4H).

Iron Reduces TNF, but Not IL1β mRNA Stability in Caco-2 Cells

The fact that iron reduces hypoxia-associated inflammation in Caco-2 cells, while blocking autophagy, one of the most important protective mechanisms elicited by hypoxia, prompted us to study the molecular mechanisms underlying iron anti-inflammatory effects in these cells. The expression of proteins involved in iron metabolism is regulated at the post-transcriptional level. To assess mRNA stability, we incubated Caco-2 cells with actinomycin D for 1 and 2 hours after 24-hour hypoxia. Iron promoted mRNA decay of TNF (Figure 5A) but not IL1β (Figure 5B) when compared with control or DFO treatment, leading to reduced amounts of TNF mRNA. Iron induced TTP protein expression, suggesting a mechanism for iron-induced TNF mRNA decay after mTOR activation (Figure 5C). Strikingly, DFO-associated iron depletion also induced TTP expression, evidencing further mechanisms for iron-mediated TNF mRNA degradation (Figure 5C).

We also monitored ROS production upon iron supply and iron deficiency under normoxic and hypoxic conditions in the Caco-2 cell system. For this purpose we used cell-permeable 2',7'-dichlorofluorescein diacetate (H2DCF-DA). Low oxygen and iron-repleted conditions triggered the production of ROS, although ROS generation under hypoxic conditions was significantly higher than iron-induced ROS formation (Figure 5D). Under normoxia, iron-mediated ROS formation was abrogated in the presence of DFO (Figure 5D).

Iron Prevents Binding of p65 to the Promoter of TNF and IL1β Under Hypoxic Conditions

Following our results on iron-mediated reduction of IL1β and TNF expression under hypoxic conditions, we sought to elucidate the molecular mechanisms governing...
| Laboratory Parameters | Screening | CRP, μmol/L | Fe, μmol/L | Hemoglobin in females, g/L | Hemoglobin in males, g/L | Leukocytes, 10^9/L | Thrombocytes, 10^9/L |
|-----------------------|-----------|-------------|------------|-----------------------------|---------------------------|-------------------|---------------------|
|                       |           | 1.8 (0.8-3.0) | 8.2 (10-33) | 12.3 (15-34)                 | 12.3 (15-34)               | 2.2 (2.9-3.9)    | 4.9 (2.3-5.0)       |
| T0                    |           | 2.4 (1.0-3.0) | 7.9 (12-35) | 12.8 (15-35)                 | 15.8 (18-35)               | 3.0 (2.3-3.9)    | 1.9 (1.0-2.3)       |
| T1                    |           | 2.7 (1.2-3.4) | 11.3 (12-33) | 12.7 (15-34)                 | 15.7 (18-34)               | 3.9 (2.3-3.9)    | 2.2 (1.0-2.3)       |
| T2                    |           | 2.5 (1.0-3.0) | 8.2 (10-33) | 12.3 (15-34)                 | 12.3 (15-34)               | 2.2 (2.9-3.9)    | 4.9 (2.3-5.0)       |
| T3                    |           | 3.6 (1.0-3.0) | 8.2 (10-33) | 12.3 (15-34)                 | 12.3 (15-34)               | 2.2 (2.9-3.9)    | 4.9 (2.3-5.0)       |

NOTE: Values are shown as means ± SD.

**Discussion**

Our results show that iron uptake reduces hypoxia-associated IL1β and TNF expression through the inhibition of NF-κB promoter binding activity in Caco-2 cells. We also found important differences in the responses to hypoxia and iron availability when comparing Caco-2 and T84 cells with HT-29 cells, which appear to be attributable, at least in part, to the contrasting influences of both processes in autophagy in these cells (Figure 7). Colonic tissue from subjects exposed to hypoxia showed a significant reduction of ferritin levels as well as a trend toward reduced levels of intracellular iron, suggesting iron mobilization from the intestinal epithelium. Of note, iron mobilization did not require an increase in the protein expression of DMT-1 or the iron export protein FPN. Nonetheless, we observed a tendency toward an increase in the mRNA expression of FPN, which is in line with reports indicating that HIFs induce FPN mRNA expression through their binding to the FPN promoter. Lack of changes in FPN protein expression highlights the important role of iron in the post-transcriptional regulation of FPN. Thus, under iron-depleted conditions, IRPs bind to 5′ IRE mRNA, thereby repressing FPN protein expression. The liver hormone hepcidin induces the internalization and degradation of FPN, thereby reducing iron efflux from the enterocyte. Of note, the hepcidin inducer protein IL6 showed a trend toward reduced mRNA expression under hypoxia, which is in accordance with the mobilization of iron observed in colonic biopsy specimens. Taken together, our results are in agreement with previous reports showing that hypoxia promotes iron absorption and efflux from the intestinal mucosa. Moreover, in a recent study, Goetze et al. have shown that altitude-associated hypoxia increases the duodenal mRNA expression of DMT-1 and FPN, and decreases serum ferritin levels in healthy individuals. In vitro, hypoxia significantly increases the expression of the iron transporters DMT-1 and FPN under iron-depleted conditions, thereby promoting cellular iron uptake and efflux in HT-29 and Caco-2 cells. These results are in accordance with studies showing that hypoxia-mediated HIF stabilization induces the expression of DMT-1 and FPN in mice and is essential for iron absorption into the intestine. Previous reports also have shown that ROS formation and iron deficiency increases DMT-1 and iron uptake in the transcriptional regulation of both genes. p65 consensus binding sites in the TNF (-586 to -579, -211 to -202, and -99 to -89) and IL1β (-300 to -289) promoters have been described previously (Figure 6A). To investigate the binding activity of p65 to the TNF and IL1β promoters, Caco-2 cells were subjected to hypoxia (0.2% O2, 4 and 24 h) and chromatin immunoprecipitation (ChIP) analysis was performed. Constitutive binding of p65 to the promoters of TNF and IL1β was abrogated in the presence of iron (Figure 6B and C). Iron inhibited binding of NF-κB to the IL1β promoter under normoxic and hypoxic conditions (Figure 6C), while binding to the TNF promoter was abrogated only under hypoxic conditions (Figure 6B).
enterocytes. Strikingly, hypoxia-mediated FPN induction was reduced under iron-starving conditions in HT-29 cells, but not in Caco-2 cells, suggesting lower rates of iron efflux from iron-depleted HT-29 cells when compared with Caco-2 cells. Previous studies have shown that Caco-2 monolayers show the same seemingly selfless characteristics as enterocytes do in vivo, absorbing more dietary iron and releasing most of it under iron-deprived conditions. Iron supplementation had no effect on DMT-1 or FPN expression, but significantly increased mRNA and protein expression of the iron storage protein ferritin in HT-29 and Caco-2 cells. Several studies have shown that influx of iron into the cell induces ferritin synthesis, while DFO-associated iron depletion triggers ferritin degradation in Caco-2 cells and rat hepatocytes. Ferritin mRNA expression was significantly induced under hypoxic, but not normoxic, conditions, stressing the important role of hypoxia in the expression of iron uptake and storage proteins. Of note, ferritin protein expression also was induced under normoxic conditions in HT-29 and Caco-2 cells, reflecting post-transcriptional regulation of ferritin protein through the dissociation of IRPs from the ferritin mRNA IREs.

Over the past decade, hypoxia has become increasingly recognized as an important factor in the development of IBD. Higher levels of hypoxia-induced transcription factors have been detected in colonic samples from IBD patients and mouse models of colitis. However, new insights have shown that hypoxia also can elicit anti-inflammatory responses depending on the context and cell type studied. Thus, hypoxic IECs show an altered gene expression pattern to ameliorate the inflammatory burden compared with other epithelia. In IECs, hypoxia triggers protective mechanisms that contribute to the resolution of inflammation in several models of IBD. In transgenic mice, loss of HIF-1α expression in the epithelium results in a more severe colitis, whereas active HIF-1α is protective. Conversely, HIF-1α stabilization promotes the transcription of TNF and IL1β in macrophages. In our hands, hypoxia induced TNF and IL1β expression, as well as p65 phosphorylation in Caco-2 and T84 cells, but not in HT-29 cells. Moreover, hypoxia abrogated constitutive p65 activation in HT-29 cells. The NF-κB signaling pathway interacts with HIF-associated signaling cascades, and also is regulated by hydroxylases. Thus, prolyl hydroxylases inhibit NF-κB
Figure 3. Hypoxia induces inflammatory processes in Caco-2 cells, but not in HT-29 cells. (A–C, E, and F) HT-29, Caco-2, and T84 cells were subjected to normoxia (21% O₂) or hypoxia (0.2% O₂) for 4 and 24 hours in the presence or absence of 150 μmol/L DFO or 200 μmol/L FAC, and transcript analysis for (A and C) TNF and (B and D) IL1β was performed. Results represent means ± SEM of 3 independent experiments performed in triplicate. (G–I) Total protein was isolated and Western blot analysis for phospho-p65, total p65, and β-actin was performed. Representative Western blots of 2 independent experiments. (J–R) Transcript analysis for (J, M, and P) PHD1, (K, N, and Q) PHD2, and (L, O, and R) PHD3 was performed. Results represent means ± SEM of 3 independent experiments performed in triplicate. *P < .05; **P < .01; ***P < .001; ****P < .0001. CTRL, control.

through the inactivation of IκB kinase-β and IκB kinase-γ.54,55 The reduction in p65 activation observed in hypoxic HT-29 cells is also in agreement with reports showing that hypoxia elicits anti-inflammatory processes in HT-29 cells through the activation of autophagy.52 Furthermore, inflammatory gene expression and signaling associated with iron deprivation were ameliorated under hypoxia in HT-29, but not in Caco-2 or T84, cells, where only iron supplementation reduced proinflammatory gene expression. More importantly, to understand the molecular mechanisms of iron anti-inflammatory effects in Caco-2 and T84 cells, iron supplementation did not reduce hypoxia-induced p65 phosphorylation, indicating that iron exerts its inhibitory effects downstream of the NF-κB signaling pathway in these cells. Taken together, our results point to a differential activation of elements orchestrating the balance between hypoxia-associated protective and proinflammatory effects in HT-29 when compared with Caco-2 or T84 cells. The differences observed between these cells are possibly a consequence of their different morphologic, biochemical, and functional characteristics.28,59 HT-29 cells remain undifferentiated under standard conditions, although they can differentiate into mucus-secreting cells,29 while Caco-2 and T84 cells form polarized monolayers and differentiate into cells resembling absorptive enterocytes.34,60,61 Consequently, differentiated Caco-2 and T84 cells are both well-established models of human intestinal epithelium. It is interesting to note that, although all 3 cell lines studied showed a similar pattern of PHD expression, hypoxic induction of PHD3 was remarkably higher in Caco-2 and T84 cells when compared with HT-29 cells. In a study comparing more than 10 human cancer cell lines, Appelhoff et al63 have shown a similar pattern in the expression of PHDs. Thus, although PHD3 expression during normoxia is the lowest of the 3 PHDs, PHD3 was induced by an average of 36.2-fold after 16 hours of hypoxia, compared with a 3-fold induction of PHD2, and no induction of PHD1. PHD2 is the most abundantly expressed PHD under normoxic conditions and previous reports have shown a dominant role of PHD2 in the regulation of HIF-1α.54 The strong induction of PHD3 in Caco-2 and T84 cells would alter the proportion of PHDs, substantially leading to a greater contribution of PHD3 to the regulation of HIF and other PHD3 targets.52 Of note, PHD3 has been shown to play an important role in the control of metabolism,65 immune responses in macrophages,66 and neutrophil survival.67
Iron Reduces Hypoxia-Associated Inflammation

A

HT-29

Caco-2

B

HT-29

Caco-2

C

D

E

F

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The contrasting responses to low oxygen tension and iron availability in HT-29 cells when compared with Caco-2 or T84 cells prompted us to study the impact of these factors in the activation of adaptive mechanisms shared by hypoxia and iron metabolism, such as autophagy. Previous reports have highlighted the importance of hypoxia-associated autophagy to reduce inflammation in several mouse models of IBD.\(^\text{52,68}\) Autophagy also plays a critical role in maintaining physiological iron balance in the cell by autophagocytosis of ferritin and subsequent release of iron into the cytosol under iron-depleted conditions.\(^\text{24}\) Our results show that iron reduces the conversion of LC3-I in LC3-II and triggers accumulation of total LC3 in normoxic HT-29 cells, suggesting both a reduction of upstream autophagy and a blockage of autophagosome degradation, thereby preventing lysosomal degradation of ferritin and release of iron. Interestingly, hypoxia was able to counteract iron-associated blockage of autophagy in HT-29, but not in Caco-2 cells, where iron-replete conditions induced phosphorylation/activation of mTOR, as well as the accumulation of p62 and total LC3 similarly to the late-stage autophagy inhibitor chloroquine. Of note, iron induced the phosphorylation of mTOR, but not accumulation of p62 or LC3, in normoxic Caco-2 cells, pointing to the fact that blockage of autophagosome degradation predominantly occurs under hypoxia. Our results are in accordance with reports showing that DFO-mediated iron deprivation induces autophagy by suppression of the mTOR signaling pathway\(^\text{69}\) via a HIF-1α-dependent pathway.\(^\text{70}\) Several studies have shown that the clinical use of rapamycin, an inhibitor of mTOR, is linked to the development of microcytic anemia.\(^\text{71-73}\) A recent report has evidenced a differential activation of the autophagic p38/Akt/mTOR pathway in HT-29 and Caco-2 cells, as well as a higher conversion of LC3-I into LC3-II in HT-29 compared with Caco-2 cells.\(^\text{74}\) The association between NF-κB activation and ferritin accumulation in Caco-2 cells is in line with recent reports showing that NF-κB activation is required for the induction of ferritin after iron uptake in dendritic cells.\(^\text{75}\) Autophagic activity in HT-29 cells might account for the lower levels of ferritin and phospho-p65 observed under hypoxia when compared with normoxia. Contrarily, iron blockage of hypoxia-associated autophagy in Caco-2 cells could explain the accumulation of phospho-p65 and ferritin in these cells, and is in line with previous studies indicating that iron disturbs normal responses to hypoxia, including autophagy.\(^\text{76}\) Hypoxia was unable to abrogate DFO-mediated induction of TNF and IL1β in the presence of the autophagy inhibitor chloroquine in HT-29 cells, reinforcing the crucial role of hypoxia-mediated autophagy in the inhibition of proinflammatory gene expression in these cells. Conversely, the activation of autophagy in Caco-2 cells with the autophagy inducer rapamycin reversed hypoxia-associated TNF expression, suggesting that responses to hypoxia are shifted toward inflammation owing to a diminished autophagic response in these cells. Rapamycin was unable to reduce hypoxia-mediated p65 phosphorylation in the presence of iron, further showing the strong inhibitory effects of iron on autophagy while confirming that iron elicits anti-inflammatory effects downstream of NF-κB activation in Caco-2 cells.

The fact that in Caco-2 cells iron blocked autophagy, a crucial protective mechanism induced by hypoxia, while reducing proinflammatory gene expression, prompted us to study the molecular mechanisms of iron anti-inflammatory effects in these cells. The activation of mTOR has been shown to cause mRNA decay through the induction of mRNA-associated proteins that bind to ARE motifs present in many cytokines, including TNF and IL1β.\(^\text{77}\) Thus, Bayeva et al.\(^\text{26}\) recently showed that mTOR activation modulates iron homeostasis through the expression of the anti-inflammatory protein TTP, which in turn binds to the ARE motifs in Tf receptor 1 mRNA, causing its degradation. In line with iron-mediated activation of mTOR, iron-replete conditions enhanced the expression of TTP together with the mRNA decay of TNF, but not IL1β, suggesting differential post-transcriptional regulatory mechanisms orchestrating the expression of these cytokines. Our results suggest that the observed changes are at least partially mediated by TTP. Nonetheless, the induction of TTP by both DFO and iron suggests additional mechanisms underlying the decay of TNF mRNA. Further research is needed to unveil the impact of iron on the regulation of TNF mRNA stability under hypoxia. Low oxygen tension and iron

**Figure 4.** (See previous page) Iron reduces hypoxia-induced inflammation independently of autophagy in Caco-2 cells. (A) HT-29 cells and Caco-2 cells were subjected to normoxia (21% O\(_2\)) or hypoxia (0.2% O\(_2\)) for 24 hours in the presence or absence of 150 μmol/L DFO or 200 μmol/L FAC. Total protein was isolated and Western blot analysis for phospho-mTOR, total mTOR, p62, LC3, and β-actin were performed. Autophagy was measured by variations in the ratio of LC3-II/LC3-I and the total amount of LC3 (LC3-I plus LC3-II) relative to β-actin. Representative Western blots of 2 independent experiments. (B) HT-29 and Caco-2 cells were subjected to normoxia (21% O\(_2\)) for 24 hours in the presence or absence of 150 μmol/L DFO, 200 μmol/L FAC, or 20 μmol/L chloroquine. Cells were stained for LC3 (green) or cell nuclei (4′,6-diamidino-2-phenylindole; blue). Examples of cells containing both markers are presented in the magnified insets. Scale bars: 10 μm. LC3 puncta were calculated relative to 4′,6-diamidino-2-phenylindole staining from at least 8 areas of interest pooled from 2 independent experiments. Results represent means = SEM. (C–E) HT-29 cells were subjected to hypoxia for 24 hours in the presence or absence of 150 μmol/L DFO, 200 μmol/L FAC, or 20 μmol/L chloroquine, and transcript analysis for (C) TNF and (D) IL1β was performed. Representative Western blot analysis for (E) phospho-p65, LC3, and β-actin was performed. Representative Western blots of 2 independent experiments. (F–H) Caco-2 cells were subjected to hypoxia for 24 hours in the presence or absence of 150 μmol/L DFO, 200 μmol/L FAC, or 250 μmol/L rapamycin, and transcript analysis for (F) TNF and (G) IL1β was performed. Results represent means = SEM of 2 independent experiments performed in triplicate. (H) Total protein was isolated and Western blot analysis for phospho-p65, LC3, and β-actin was performed. Representative Western blots of 2 independent experiments. \(**P < .01; ***P < .001; ****P < .0001.\) CTRL, control.
challenge induced the generation of ROS in a time-dependent manner. Hypoxia-mediated ROS production was significantly higher and overcame iron-generated ROS, probably owing to ferritin storage of iron in a nonredox active form. These results suggest that iron-mediated effects counteracting hypoxia-mediated inflammatory processes are not linked to a reduction in ROS generation. Under iron-starved conditions, ChIP analysis showed constitutive binding of NF-κB to the promoter of IL1β and TNF. Conversely, iron challenge prevented NF-κB binding to both promoters, thereby reducing the expression of IL1β and TNF, and confirming that iron exerts its inhibitory effects downstream of the NF-κB signaling cascade. Notably, iron inhibited binding of NF-κB to the IL1β promoter under normoxic and hypoxic conditions, while binding to the TNF promoter was abrogated only under hypoxic conditions. These results show differential molecular mechanisms regulated by oxygen and iron availability underlying the expression of both cytokines. There is mounting evidence that iron availability has an impact on inflammatory transcriptional regulation. Recently, Dlaska and Weiss showed that lipopolysaccharide- and interferon-γ-induced binding of the transcription factor nuclear factor-IL6 to the inducible nitric oxide synthase promoter was reduced in the presence

Figure 5. Iron reduces TNF, but not IL1β, mRNA stability under hypoxia. (A and B) Caco-2 cells were subjected to hypoxia (0.2% O₂) for 24 hours in the presence or absence of 150 μmol/L DFO or 200 μmol/L FAC. Cells then were incubated with 5 μg/mL actinomycin D for 1 and 2 hours and the consequent decay of (A) TNF and (B) IL1β mRNA was monitored by quantitative PCR. Results represent means ± SEM of 2 independent experiments performed in triplicate. *P < .05. (C) Total protein was isolated and Western blot analysis for TTP and β-actin was performed. Representative Western blots of 2 independent experiments. (D) Caco-2 cells were incubated with 50 μmol/L H₂DCF-DA for 30 minutes at 37°C. Cells then were subjected to normoxia (21% O₂) or hypoxia (0.2% O₂) for 1 and 4 hours in the presence or absence of 150 μmol/L DFO or 200 μmol/L FAC, and ROS-induced fluorescence was measured. Results represent means ± SEM of 2 independent experiments performed in triplicate. **P < .01; ***P < .001. CTRL, control.
of iron in mice, and was enhanced by DFO. DFO also promotes the expression of the growth arrest and DNA damage 45α gene through the induction of binding activity of NF-Y, the stabilizing factor v-MYB and CCAAT/enhancer-binding protein through mechanisms yet to be identified. In summary, our results suggest that hypoxia-promoted iron uptake in enterocytes may be a vital protective mechanism in the maintenance of mucosal homeostasis. Ensuring adequate dietary iron levels would be particularly important to the restoration of homeostasis in the hypoxic mucosa of patients with IBD, especially those with iron-deficiency anemia, a common complication of IBD.

**Materials and Methods**

**Human Subjects**

Healthy subjects (n = 10) were exposed to hypoxic conditions resembling an altitude of 4000 meters above sea level for 3 hours using a hypobaric pressure chamber at the Swiss aeromedical center in Dubendorf, Switzerland. Distal colon biopsy specimens were taken the day before entering the hypobariuc chamber, immediately after hypoxia, and 1 week after hypoxia. Serum samples were taken before hypoxia, and 1 day, 1 week, and 1 month after hypoxia at the Department of Gastroenterology and Hepatology of the University Hospital Zurich. Routine laboratory assays were performed by University Hospital Zurich. This study was approved by the Ethics Committee of the Canton of Zurich (KEK-ZH 2013-0284) and all participants signed informed consent. We confirm that all methods were performed in accordance with the relevant guidelines and regulations.

**Immunohistochemistry**

Intestinal specimens from the distal third of the colon were embedded in paraffin and cut in 5-μm sections with a microtome. Immunostaining for ferritin was performed on Leica Bond Max instruments using Refine horseradish-peroxidase kits (Leica Biosystems, Newcastle, UK). Paraffin-embedded tissue was pretreated for 20 minutes at 100°C with citrate-based buffer at pH 6.0 (Leica Biosystems) before incubation with rabbit anti-ferritin...
antibody (cat. no. ab76768; Abcam, Cambridge, UK) at a 1:600 dilution, rabbit anti–DMT-1 antibody (cat. no. PA5-33022; Thermo Fisher Scientific, Reinach, Switzerland) at a 15 μg/mL concentration, or rabbit anti-ferroportin (cat. no. PA5-22993) at a 1:500 dilution. Rabbit anti–guinea pig (cat. no. P0141; Dako, Glostrup, Denmark) was used as a secondary antibody at a 1:100 dilution. Quantification was performed using ImageJ Analysis Software (National Institutes of Health, Bethesda, MD) using the Colour Threshold tool, and the resulting value was normalized to quantification of nuclei staining.

**Measurement of Iron Content**

Tissue samples were weighted and afterward dissolved in 1 mL tetramethylammonium hydroxide solution at 60°C–70°C for 1 hour. Iron concentrations were determined by atomic absorption spectrometry on an AAnalyst 200 (Perkin Elmer, Inc, Waltham, MA) by reference to a calibration curve. Results are means of duplicate measurements. Internal quality controls were included in each measurement series to ensure reliability of the test.

**Cell Culture and Hypoxia Treatment**

HT-29, Caco-2, and T84 cells were obtained from the German Collection of Cells and Microorganisms (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany), and cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum (VWR, Dietikon, Switzerland). Serum-starved cells were treated with 150 μmol/L of the iron chelator DFO or 200 μmol/L FAC (Sigma-Aldrich, St. Louis, MO) to induce iron-replete conditions, and exposed to hypoxia (0.2% O₂) in a hypoxia workstation incubator (In vivo 400; Baker Ruskin, Bridgend, UK). Cells maintained in normoxia (21% O₂) for the same time period were used as controls. In some experiments, Caco-2 cells were treated with 250 nmol/L of the iron chelator DFO or 200 μmol/L FAC, rapamycin for 1 hour before hypoxia (Sigma-Aldrich). mRNA synthesis was inhibited using 5 μg/mL actinomycin D (Sigma-Aldrich).

**Western Blot**

Total protein was harvested in M-PER lysis buffer (Thermo Fisher Scientific). Human HIF-1α (cat. no. NB100-479; Novus Biologicals, Littlestown, CO), phospho-p65 (cat. no. 3033), p65 (cat. no. 8242), phospho-mTOR (cat. no. 2971), mTOR (cat. no. 2972), p62 (cat. no. 5114) (all Cell Signaling Technology, ZA Leiden, The Netherlands), ferritin (cat. no. ab75973; Abcam), TTP (cat. no. sc-374305; Santa Cruz Biotechnology, Heidelberg, Germany), LC3 (cat. no. 2019 Iron Reduces Hypoxia-Associated Inflammation 351

**RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction**

Total RNA from IECs was extracted using the RNeasy Plus Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer’s instructions. For reverse transcription, the High-Capacity Complementary DNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), was used following the manufacturer’s instructions. Quantitative polymerase chain reaction (PCR) was performed using TaqMan Gene Expression Assays, and the TaqMan system 7900HT (Applied Biosystems), under the following cycling conditions: 20 seconds at 95°C for 3 seconds and 60°C for 30 seconds with the TaqMan Fast Universal Mastermix. Relative mRNA expression was calculated by the comparative delta-delta Ct (ΔΔCt) method using β-actin as the endogenous control.
L8918; Sigma-Aldrich) and β-actin (cat. no. A5441; Sigma-Aldrich) antibodies were used at 1:1000 dilution for Western blot.

Confocal Microscopy

After 24 hours under hypoxia, HT-29 and Caco-2 cells were fixed with 4% paraformaldehyde for 20 minutes and then permeabilized in 100% methanol. After blocking with 3% bovine serum albumin, cells were incubated with LC3 antibody (cat. no. 2775; Cell Signaling Technology) at 1:500 dilution overnight at 4°C. Cells then were incubated with Alexa 488-conjugated secondary rabbit antibody (Invitrogen, Carlsbad, CA) for 1 hour and 4′,6-diamidino-2-phenylindole for 5 minutes before mounting with anti-fade medium (Dako). Cells were analyzed by a Leica SP5 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). Fluorescence images of the same sample were acquired and processed using Leica confocal software (LAS-AF Lite; Leica Microsystems). Quantification of puncta was performed by 2 independent blinded investigators in at least 8 areas of interest from 2 independent experiments.

Measurement of ROS Production

Caco-2 cells were incubated with medium containing 50 μmol/L H2DCF-DA (Sigma-Aldrich) for 30 minutes at 37°C. Inside the cell, H2DCF-DA is converted to the highly fluorescent DCF upon oxidation. Thus, DCF is a nonradiometric ROS probe that accumulates inside the cell with time.29 ROS-induced fluorescence was detected using a microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

ChIP analysis

ChIP was performed using the ChIP-IT Express Enzymatic kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Immunoprecipitation of 25 μg DNA was performed overnight at 4°C using anti-p65 antibodies (cat. no. 8242; Cell Signaling Technology). DNA isolated from an aliquot of the total nuclear extract was used as a loading control for the PCR (input control). PCR was performed with total DNA and immunoprecipitated DNA using the following promoter-specific primers: IL1β promoter-binding site for NF-κB: 5′-CAGCAAGGGACGTATTGG-3′ (forward), 5′-GGTTCTATGAGGCAAGGA-3′ (reverse) (297 bp), and TNF promoter binding site for NF-κB: 5′-GATGGGAGTGTGAGGGA-3′ (forward), 5′-CAAGGAGAAAAACTCCTTGGTCTG-3′ (reverse) (159 bp). The PCR products (10 μL) were subjected to electrophoresis on a 2% agarose gel.

Statistics

Statistical analysis was performed using 1-way analysis of variance followed by the Tukey post-test. The results are expressed as means + SEM and significance was set at P < .05. All authors had access to the study data and reviewed and approved the final manuscript.

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