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Hydroxylase Inhibition Abrogates TNF-α–Induced Intestinal Epithelial Damage by Hypoxia-Inducible Factor-1–Dependent Repression of FADD

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Hydroxylase inhibitors stabilize hypoxia-inducible factor-1 (HIF-1), which has barrier-protective activity in the gut. Because the inflammatory cytokine TNF-α contributes to inflammatory bowel disease in part by compromising intestinal epithelial barrier integrity, hydroxylase inhibition may have beneficial effects in TNF-α–induced intestinal epithelial damage. The hydroxylase inhibitor dimethyloxalylglycin (DMOG) was tested in a murine model of TNF-α–driven chronic terminal ileitis. DMOG-treated mice experienced clinical benefit and showed clear attenuation of chronic intestinal inflammation compared with that of vehicle-treated littermates. Additional in vivo and in vitro experiments revealed that DMOG rapidly restored terminal ileal barrier function, at least in part through prevention of TNF-α–induced intestinal epithelial cell apoptosis. Subsequent transcriptional studies indicated that DMOG repressed Fas-associated death domain protein (FADD), a critical adaptor molecule in TNFR-1–mediated apoptosis, in an HIF-1α–dependent manner. Loss of this FADD repression by HIF-1α-targeting small interfering RNA significantly diminished the antiapoptotic action of DMOG. Additional molecular studies led to the discovery of a previously unappreciated HIF-1 binding site in the FADD promoter, which controls repression of FADD during hypoxia. As such, the results reported in this study allowed the identification of an innate mechanism that protects intestinal epithelial cells during (inflammatory) hypoxia, by direct modulation of death receptor signaling. Hydroxylase inhibition could represent a promising alternative treatment strategy for hypoxic inflammatory diseases, including inflammatory bowel disease. The Journal of Immunology, 2010, 185: 6306–6316.

Inflammatory bowel disease (IBD) comprises chronic, relapsing inflammatory disorders of the digestive tract (i.e., Crohn’s disease [CD] and ulcerative colitis).

The important contribution of TNF-α to inflammation in IBD is widely recognized. This pluripotent, proinflammatory cytokine can compromise intestinal epithelial barrier integrity, a very early event in the pathogenesis of IBD, in part by enhancing intestinal epithelial apoptosis (1–4). By exposure of the inflammatory cells within the lamina propria (LP) to intraluminal Ags in a nonselective way, an unspecific inflammatory response is elicited, which in turn could promote additional tissue damage or even evolve into sustained inflammatory activity. Not surprisingly, molecules that are able to protect or restore intestinal epithelial barrier integrity could play a pivotal role in the treatment of IBD.

In this view, prolyl hydroxylase domain (PHD) inhibitors are increasingly being explored for therapeutic purposes (5–7). Intestinal epithelial cells (IECs) have a limited baseline oxygen supply, rendering them particularly susceptible to conditions associated with increased hypoxia. Chronically inflamed intestinal tissue is generally deprived of oxygen due to increased metabolic demands and vascular dysfunction. Under such conditions, IECs may themselves become a source of proinflammatory cytokines (including TNF-α) that promote disruption of epithelial barrier function and contribute to the ongoing inflammation (8–11). The intestinal epithelium however also has a built-in protection system against low-oxygen conditions, tightly governed by PHD proteins (12).

PHD proteins are ubiquitously expressed and control the transcriptional activity of hypoxia-inducible factor–1 (HIF-1) by hydroxylating the HIF-1α protein subunit when sufficient amounts of oxygen are present. This hydroxylation makes HIF-1α less stable, which in turn leads to its proteasome-mediated degradation. In hypoxic conditions, PHD proteins are inhibited, leading to a gradual increase in HIF-1α protein levels and formation of the HIF-1α–binding heterodimeric transcription factor HIF-1. After translocation to the nucleus of the enterocyte, HIF-1 not only induces the transcription of numerous genes involved in cell metabolism but
also of genes implicated in the preservation of intestinal epithelial barrier integrity (13, 14).

Increased HIF-1α protein levels have been described in patients with active IBD (15). Data on the functional role of HIF-1α in intestinal inflammation are derived from animal studies. Protective effects of PHD inhibitors and HIF-1α have been reported in chemically induced, acute colonic injury in mice (5, 6, 14). However, these models study acute colonic inflammation, whereas human IBD is characterized by a chronic inflammatory process (16, 17). The impact of PHD inhibition on chronic intestinal inflammation is unknown. The present study addressed this issue by evaluating the application of the PHD inhibitor dimethyloxalylglycine (DMOG) in a murine model of TNF-α-driven chronic terminal ileitis that strikingly resembles human CD. This led to the discovery of a previously unappreciated role of HIF-1 in the modulation of TNF-mediated intestinal epithelial apoptosis through direct regulation of Fas-associated death domain (FADD) transcription during (inflammatory) hypoxia.

Materials and Methods

TNF-ARE/+ mice as a model for ileal CD

C57BL/6 TNF-ARE/+ mice were kindly provided by Dr. George Kollia (Alexander Fleming Biomedical Sciences Research Center, Vari, Greece). TNF-ARE/+ mice have a targeted disruption of the TNF AU-rich element (ARE), which is important for TNF mRNA destabilization and translational repression. As a consequence, these mice chronically overproduce TNF-α, which results in spontaneous development of Crohn’s-like intestinal inflammation by Weeks 5–6 of age, exclusively located at the terminal ileum. Clinically, these mice are characterized by severely reduced weight gain compared with that of healthy littermate controls. Histologically, terminal ileal lesions consist of mucosal abnormalities with intestinal villous blunting, broadening, and shortening as well as mucosal and submucosal infiltration of acute and chronic inflammatory cells (18).

All procedures were in accordance with institutional animal health care guidelines, following study approval by the Institutional Review Board at the Faculty of Medicine and Health Sciences of Ghent University.

Isolation of primary IECs for ELISA/Western blot

For use in ELISA experiments, primary terminal ileal epithelial cells were isolated as described previously (19). Commercially available kits were used for HIF-1α and vascular endothelial growth factor (VEGF) protein assessment (for HIF-1α, Human/Mouse Total HIF-1α DuoSet IC ELISA; for VEGF, Quantikine Mouse VEGF; both from R&D Systems, Minneapolis, MN).

A Western blot for FADD was performed using a 1:500 rabbit anti-FADD polyclonal primary Ab (Novus Biologicals, Littleton, CO) and a secondary alkaline phosphatase-linked anti-rabbit Ab (Cell Signaling Technology, Beverly, MA) at a 1:2,000 dilution. Equal loading was verified by immunodetection of β-tubulin.

Treatment of TNF-ARE/+ mice and assessment of intestinal epithelial permeability

Seventeen-week-old, PCR-confirmed C57BL/6J mice (n = 6) were treated for 16 d with eight injections of DMOG (8 mg in 0.5 ml sterile, endotoxin-free PBS, i.p.) on alternating days, a treatment protocol previously shown to be effective in vivo (6). Corresponding littermate TNF-ARE/+ controls (n = 6) underwent the same treatment regimen but received vehicle alone. Healthy littermate C57BL/6J TNF-ARE/- mice (n = 6) were used as a negative control. Weight evolution was monitored every other day. At Day 16 of treatment, all of the mice were sacrificed for histological evaluation.

In a separate experiment, 10 seven-week-old mice (four DMOG-treated TNF-ARE/+; four vehicle-treated TNF-ARE/+; and two healthy control mice) were used to analyze epithelial permeability of the terminal ileum after 4 d of either DMOG or vehicle treatment by using the previously described Evans blue method (20). In short, mice were anesthetized, the abdomen was opened, and a small polyethylene tube was inserted carefully and secured into the ileum at 5 cm from the ileocecal junction. The terminal ileum was flushed with saline and subsequently infused with Evans’s blue (1% in saline; Sigma-Aldrich, St. Louis, MO) for 5 min. To wash out dye sticking to the mucus, the lumen was perfused with 6 mM acetylcysteine (Sigma-Aldrich) for 5 min. After a final flush with saline, animals were sacrificed by cervical dislocation. Next, the terminal 1.5 cm of the ileum was removed and placed in 5 ml N,N-dimethylformamide (Sigma-Aldrich). After overnight extraction, the dye concentration was measured by spectrophotometry at a wavelength of 570 nm.

Histological assessment of intestinal inflammation

H&E-stained slides of 5 μm terminal ileal sections were blindly scored by two observers. At least 10 fields of view were evaluated per mouse, using the following scoring system: A) villous damage: 0, normal villi; 1, blunted villi (drumstick appearance); 2, shortening of villi; and 3, complete destruction of villi; and B) inflammatory cell infiltration: 0, no increase of inflammatory cells in LP; 1, mild increase of inflammatory cells in LP; 2, moderate increase of inflammatory cells in LP; 3, strong increase of inflammatory cells in LP; 4, mild increase of inflammatory cells in the submucosa; 5, moderate increase of inflammatory cells in the submucosa; and 6, strong increase of inflammatory cells in the submucosa. Total inflammation score was calculated as the sum of A and B.

HIF-1α and FADD small interfering RNA

HT29 cells (human colon carcinoma epithelial cell line, HTB-38; American Type Culture Collection, Manassas, VA) were seeded at 150,000 cells per well in a 24-well plate and transfected the next day either with small interfering RNA (siRNA) duplexes targeting human HIF-1α or FADD or with control siRNA (Biologeo, Nijmegen, The Netherlands), using 5 μl lipofectamine (Invitrogen, Carlsbad, CA) in 500 μl RPMI 1640 medium (21, 22).

In vitro apoptosis assay

HT29 cells were seeded in 6-well plates, each well containing 2.5 × 10⁶ cells. After transfection with control or HIF-1α siRNA, cells were pretreated for 24 h with the HIF-1α activator DMOG (1 mM; Echelon Biosciences, Salt Lake City, UT) or vehicle alone (PBS) in culture medium (DMEM).

After pretreatment, medium was washed away and cells were incubated with human rTNF-α (100 μg/ml; R&D Systems) and human IFN-γ (100 U/ml; Invitrogen) to induce apoptosis. After 24 h, cells were harvested and apoptosis was quantified by measuring caspase-3 activity, using a highly sensitive caspase-3 assay (Genescript, Piscataway, NJ).

In vivo TNF-α–induced acute intestinal epithelial apoptosis

Four 7-wk-old healthy C57BL/6J mice were pretreated for 4 d and received two injections of DMOG (8 mg in 0.5 ml sterile, endotoxin-free PBS, i.p.) on alternating days. Littermate controls (n = 4) received vehicle alone.

After pretreatment, a single i.v. injection of murine rTNF-α (10 μg in 200 μl saline; BioLegend, San Diego, CA) was administered to induce acute enteropathy, characterized by massive apoptosis of the enterocytes, as previously reported (23–25). Control animals (n = 2) received i.v. saline without TNF-α.

Mice were sacrificed 1 h after injection of TNF-α. Terminal ileal apoptosis was visualized by TUNEL, according to the manual instructions of a commercial apoptosis detection kit (Genescript). Computerized semiquantitative analysis of the TUNEL-stained sections was performed using an Optronics Color digital camera (Olympus, Tokyo, Japan) and specialized software (cell D; Olympus Imaging Solutions, Münster, Germany).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from HT29 cells (in vitro experiments), terminal ileal specimens (mouse experiments), and human terminal ileal mucosal samples using the RNasy Mini Kit (Westburg, Leusden, The Netherlands) and converted to cDNA for real-time quantification.

All of the reactions were run in duplicate and normalized to stably expressed reference genes. Primer sequences are given in Table I. All of the human participants in this study gave their written informed consent. Patient characteristics are given in Table II.

HIF-1α and FADD immunohistochemistry

Immunohistochemistry for HIF-1α was performed on murine paraffin-embedded terminal ileal specimens. Immunohistochemistry for FADD was performed on terminal ileal biopsy specimens of the above characterized patients (Table II). All of the sections were pretreated with citrate and blocked for endogenous peroxidase activity with 3% H₂O₂ in PBS. The primary Ab (for HIF-1α, 1:100 mouse HIF-1α clone H16b6; EMD Chemicals, Gibbstown, NJ; for FADD, 1:200 rabbit anti-FADD clone Ser191; Abnova; Taipei City, Taiwan) were applied for 60 min. A peroxidase-based visualization kit was used according to the manufacturer’s protocol (linked streptavidin-biotin immunoperoxidase kit; DakoCytomation, Carpenteria,
CA), followed by the application of 3,3′-diaminobenzidine (DakoCyto- 
mation) for 2 min. Counterstaining was performed with hematoxylin.

**HIF-1α transcription factor binding assay**

Potential HIF-1α binding sites in the promoter region of FADD were identified by consulting the TRANSFAC database (Table III). Specific RNA oligonucleotides for each of the putative binding sites were syn-
thesized (Biologeo) and used in a HIF-1α EZ-TFA Transcription Factor 
Assay (Millipore, Billerica, MA), according to the manufacturer’s manual. 
Input DNA from DMOG-treated HT29 cells was used in the assay.

**Chromatin immunoprecipitation assay**

HT29 cells were placed in a hypoxic chamber (~1% oxygen) for 24 h. After 
isolation, sheared chromatin was incubated with an anti–HIF-1α Ab (1:500 
rabbit polyclonal anti–HIF-1α; Millipore) or with an isotype (IgG) ir-
relevant Ab. Immune complexes were precipitated, and HIF-1α binding 
to the promoter of FADD was quantified by quantitative real-time PCR 
(qRT-PCR) (primers: sense, 5′-GCACACACAAAGTGTCCTCACGGGG-
3′; antisense, 5′-ACGTACGTTGCCTCACACTCT-3′), which amplified a 
111-bp region spanning the putative HIF-1 binding site.

**Luciferase reporter assay**

Genomic PCR fragments of 300 bp of the promoter region of FADD, 
starting at base −1 to −155 upstream from the initiation codon and sur-
rounding the putative binding site −531, were cloned between the SacI 
and XhoI sites of the PGL3Enhancer and PGL3Control reporter vectors 
(Promega, Madison, WI). Deletion of the 17-bp predicted binding site in 
these constructs was obtained by PCR. As a control for response to hyp-
oxia, two tandem repeats of a known HIF-1 enhancer sequence from the 3 
region of the erythropoietin gene were cloned into the PGL3Enhancer 
vector (26, 27). Construct sequences were verified by direct sequencing. 

Gene of FADD, human FADD; hGAPDH, human GAPDH; hHIF-1, human HIF-α; hMHS, mouse hydroxymethylbilane syn-
these; hHPRT, human hypoxanthine-guanine phosphoribosyltransferase; hSDHA, human succinate dehydrogenase complex 
subunit A; hVEGF-A, human VEGF-A; mFADD, mouse FADD; mTNF, mouse TNF-α.

**Statistical analysis**

Statistical analyses were performed using SPSS, version 16.0, for Windows 
(SPSS, Chicago, IL). Continuous data are expressed as mean ± SEM, and categorical data as mean ± range. Groups were compared by using the 
Mann-Whitney U test. Weight evolution data of TNFαAREFia 
mice in different treatment groups were compared by ANOVA 
for repeated measurements. The p values <0.05 were considered significant.

**Results**

DMOG treatment stabilizes functionally active intestinal 
epithelial HIF-1α proteins in vitro and in vivo

Prior to our experiments, we confirmed that DMOG treatment 
resulted in a stable increase of functionally active intestinal epithelial 
HIF-1α proteins in vitro and, at the terminal ileum, in vivo. 
In vitro, low basal HIF-1α protein levels were detected in HT29 
cells (ELISA). A treatment for 24 h with DMOG resulted in a 7.5-
fold increase of HIF-1α protein levels (PBS versus 1 mM DMOG, 
p < 0.001). Similar results were obtained with cells exposed 
to hypoxia (1% oxygen) for the same time period (PBS versus 1% 
oxxygen, p < 0.001; DMOG versus 1% oxygen, NS; Fig. 1A). 
In vivo, 4 d of treatment with DMOG (8 mg in PBS, i.p.) resulted in 
a 3-fold increase of HIF-1α protein levels in freshly isolated 
terminal ileal enterocytes (PBS versus DMOG, p < 0.01; Fig. 
1B). Immunohistochemistry on terminal ileal sections showed 
that DMOG treatment stabilized HIF-1α mainly in the IECs (Fig. 
1E).

To confirm functional activity for the DMOG-induced HIF-1α 
proteins, qRT-PCR (in vitro) and ELISA (in vivo) assays were per-
formed using VEGF as positive control, because its gene is typi-
cally HIF-1α–induced.

In vitro, DMOG treatment resulted in a 6-fold increase of intestinal 
epithelial VEGF transcripts (PBS versus DMOG, p < 0.001). A total of 1% oxygen induced VEGF transcription in a 
similar manner (PBS versus 1% oxygen, p < 0.001; DMOG versus 
1% oxygen, NS; Fig. 1C).

**Table I. Sequence of used qRT-PCR primers**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward Primers (5′−3′)</th>
<th>Reverse Primers (5′−3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSDHA</td>
<td>TGGGACACAAAGGCGCCTTG</td>
<td>CCACACCTGCTCAAAATTCATG</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>TGGCCCAACACCTCTCTAC</td>
<td>GCAGCTTGGCTGTCAGTAGAG</td>
</tr>
<tr>
<td>hHPRT</td>
<td>TGGCCACCTGCAAAACACTACA</td>
<td>GCTCTTTTTCACAGCAGAGCT</td>
</tr>
<tr>
<td>hVEGF-A</td>
<td>TCTCACCACATTGGAACCA</td>
<td>GATCTCCGCTCTCTCCTTG</td>
</tr>
<tr>
<td>hFADD</td>
<td>TATTAAATGGCTTCCGCCGAC</td>
<td>CTTGACACGCTGCCACTCTT</td>
</tr>
<tr>
<td>hHIF-1</td>
<td>TGGCACCTCAAAGAAAGAAA</td>
<td>ACGCAACAGGFCAGGCAGAC</td>
</tr>
<tr>
<td>mMHS</td>
<td>AAGGCGCTTTTCTGAGGACC</td>
<td>AGOTTCCCACTTTCTACATCG</td>
</tr>
<tr>
<td>mTNF</td>
<td>CATCTTCCATCAAAATCTGAGTA GCACAA</td>
<td>TGGAGTAGAACAGGTCACACCC</td>
</tr>
<tr>
<td>mFADD</td>
<td>TGGTCCTCCCACACAGAAA</td>
<td>CTTGAGAAGAGGGTTCTTG</td>
</tr>
</tbody>
</table>

**Table II. Characteristics of patients from whom terminal ileal biopsies were obtained for analysis of FADD transcription**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean Age</th>
<th>Medication at Moment of Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>16</td>
<td>51 (27–69)</td>
<td>None</td>
</tr>
<tr>
<td>CD patients, history of ileal involvement</td>
<td>12</td>
<td>37 (24–57)</td>
<td>6 (50%) no medication</td>
</tr>
<tr>
<td>CD patients, never had ileal involvement</td>
<td>6</td>
<td>44 (15–64)</td>
<td>1 (8%) immunomodulating drugs + corticosteroids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 (8%) corticosteroids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 (17%) aminosalicylates</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 (50%) no medication</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 (33%) immunomodulating drugs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 (17%) aminosalicylates</td>
</tr>
</tbody>
</table>

All of the patients had an endoscopically normal appearing ileum.
In vivo, 4 d of DMOG treatment doubled VEGF protein levels in terminal ileal enterocytes (vehicle versus DMOG, \( p, 0.05 \); Fig. 1D).

**DMOG treatment abrogates chronic intestinal inflammation in**

**TNF\(^{ΔARE/+}\) mice**

Given the highly protective effects of DMOG in chemically induced models of acute colitis, we investigated the application of this molecule in the setting of chronic gut inflammation, using the TNF\(^{ΔARE/+}\) mouse model of ileal CD (18).

We initiated treatment in the TNF\(^{ΔARE/+}\) mice when they were 7 wk of age. At that time point, TNF\(^{ΔARE/+}\) mice had a significantly lower weight as compared with that of their healthy littermates and were expected to have clear histological signs of terminal ileitis (Supplemental Fig. 1). Starting from Day 4 of treatment, TNF\(^{ΔARE/+}\) mice showed a clinical benefit from DMOG treatment, represented by significantly improved weight gain compared with that of littermate, vehicle-treated TNF\(^{ΔARE/+}\) mice (\( p < 0.01 \); data not shown).

After 16 d of treatment, TNF\(^{ΔARE/+}\) mice were sacrificed for evaluation. The treatment efficiency was ensured by finding increased HIF-1\(α\) protein levels in terminal ileal enterocytes. Each condition of in vitro experiments was tested 6-fold. In vivo results are of a single experiment with \( n = 3–5 \) mice per group. Histological analysis showed that DMOG stabilized HIF-1\(α\) protein mainly in the IECs. Transcriptional activity of DMOG-induced HIF-1\(α\) protein was verified by qRT-PCR (in vitro) and ELISA (in vivo) for VEGF, because its gene is typically HIF-1\(α\)–induced. *\( p < 0.05 \); **\( p < 0.01 \); ***\( p < 0.001 \).

FIGURE 1. DMOG treatment stabilizes functionally active intestinal epithelial HIF-1α proteins in vitro and in vivo. Low basal HIF-1α protein levels are detectable by ELISA in IECs under normoxic conditions. A, An exposure for 24 h to DMOG (1 mM in DMEM) or 1% oxygen strongly augmented HIF-1α protein levels in HT29 cells in vitro. B, In vivo, 4 d of DMOG treatment (8 mg in 0.5 ml sterile PBS, i.p., every other day) resulted in stably enhanced HIF-1α protein levels in freshly isolated terminal ileal enterocytes. C, In vitro, DMOG and 1% oxygen strongly induced transcription of VEGF. D, In vivo, DMOG treatment resulted in a 2-fold increase of VEGF protein in terminal ileal enterocytes. Each condition of in vitro experiments was tested 6-fold. In vivo results are of a single experiment with \( n = 3–5 \) mice per group. E, Immunohistochemistry for HIF-1α on terminal ileal sections (original magnification \( ×200 \)) showed that DMOG stabilized HIF-1α protein mainly in the IECs. Transcriptional activity of DMOG-induced HIF-1α protein was verified by qRT-PCR (in vitro) and ELISA (in vivo) for VEGF, because its gene is typically HIF-1α–induced. *\( p < 0.05 \); **\( p < 0.01 \); ***\( p < 0.001 \).
At the cytokine level, terminal ileal TNF-α transcription was decreased significantly in DMOG-treated versus vehicle-treated TNF-DARE/+ mice (p < 0.01; Fig. 2B).

**DMOG treatment rapidly restores intestinal barrier function in TNF-DARE/+ mice**

Because DMOG-treated TNF-DARE/+ mice showed an increase in weight gain starting from Day 4 of DMOG treatment, additional experiments focused on that specific time frame. Taking into account that DMOG has been shown previously to have barrier-protective effects in the gut (6), the terminal ileal epithelial barrier function of TNF-DARE/+ mice was compared with that of healthy controls after 4 d of treatment with either DMOG or vehicle alone.

Intestinal epithelial permeability (Fig. 3) was scored per centimeter of terminal ileum (spectrophotometry after Evans blue staining). DMOG restored epithelial barrier integrity, as detected with Evans blue. Four days of DMOG treatment (8 mg in 0.5 ml sterile PBS, i.p., every other day) restored terminal ileal barrier integrity to almost normal levels. Results are representative of two independent experiments with n = 3–5 mice per group. *p < 0.05; **p < 0.01; ***p < 0.001.
staining). As expected, TNF^{ARE/+} mice showed markedly increased terminal ileal epithelial permeability compared with that of their corresponding healthy littermates (vehicle TNF^{ARE/+} versus TNF^{+/+}, p < 0.05). Only 4 d of DMOG treatment was sufficient to restore this barrier dysfunction to near-normal levels (DMOG TNF^{ARE/+} versus vehicle TNF^{ARE/+}, p < 0.01, and DMOG TNF^{ARE/+} versus TNF^{+/+}, NS).

_Pretreatment with DMOG protects the intestinal epithelium against TNF-α–induced apoptosis in vitro and in vivo_

Enhanced intestinal epithelial apoptosis largely contributes to the impaired intestinal barrier reported in IBD (28). Because DMOG treatment rapidly restored barrier function in TNF^{ARE/+} mice, a subsequent experiment evaluated whether pretreatment with DMOG could protect IECs from TNF-α–induced apoptosis.

In vitro, HT29 cells that were pretreated with DMOG (1 mM for 24 h) showed a 35–40% reduction in TNF-α–induced caspase-3 activation (DMOG versus vehicle, p < 0.01; Fig. 4B). To investigate the role of HIF-1α in this antiapoptotic action of DMOG, we transfected HT29 cells with siRNA against HIF-1α, which repressed HIF-1α transcripts 4-fold (p < 0.001; data not shown). After 24 h of DMOG treatment, HIF-1α protein levels were three times lower in HIF-1α siRNA-transfected cells compared with those of control siRNA-transfected cells (p < 0.001; Fig. 4A). This knockdown of HIF-1α was associated with increased susceptibility to TNF-α–induced apoptosis (Fig. 4B), confirming a significant role for HIF-1α in the antiapoptotic properties of DMOG.

To verify the in vivo relevance of these findings, wild-type C57BL/6J littermates were injected (i.v.) with 10 µg rTNF-α after 4 d of treatment with either DMOG or vehicle alone. One hour after injection, massive detachment of TUNEL-positive apoptotic enterocytes was seen in vehicle-treated mice, accompanied by villous deformation as described previously (23–25). In contrast, DMOG-treated mice were strongly protected from TNF-α–induced enterocyte apoptosis and showed general preservation of the villi (Fig. 4C).

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**FIGURE 4.** DMOG protects IECs against TNF-α–induced apoptosis in an HIF-1α–dependent manner. A, B, DMOG pretreatment (1 mM in DMEM for 24 h) prevented intestinal epithelial cells against TNF-α–induced apoptosis in vitro. Transfection of HT29 cells with HIF-1α-siRNA prior to DMOG treatment resulted in highly reduced HIF-1α protein stabilization (A) and significantly diminished protective effects of DMOG on TNF-α–induced IEC apoptosis (B). C, Four days of pretreatment with DMOG (8 mg in 0.5 ml sterile PBS, i.p., every other day) protected C57BL/6J mice against TNF-α–induced apoptosis in vivo. As such, DMOG-pretreated mice did not show the characteristic villous deformation due to the massive shedding of apoptotic enterocytes seen in vehicle-treated mice, accompanied by villous deformation as described previously (23–25). In contrast, DMOG-treated mice were strongly protected from TNF-α–induced enterocyte apoptosis and showed general preservation of the villi (Fig. 4C).
DMOG reduces intestinal epithelial FADD both in vitro and in vivo in a HIF-1α–dependent manner

Transcriptional studies were performed to address the question of how pretreatment with DMOG could prevent subsequent TNF-α–induced damage in IECs. Because TNF-α induces apoptosis via TNFR-1, we focused on critical proteins involved in this pathway.

FADD was investigated as one of the potential targets of DMOG, due to its pivotal role in TNF-mediated apoptosis (29, 30). Interestingly, a 24-h exposure to 1 mM DMOG or 1% oxygen were both found to reduce FADD levels 2.5– to 3-fold in vitro. (compared with PBS, p < 0.001 for DMOG and 1% oxygen; Fig. 5A).

Because the above results suggested a potential role for HIF-1α in transcriptional repression of FADD, subsequent experiments were directed to this hypothesis.

First, the effect of siRNA-induced HIF-1α knockdown on the transcriptional activity of FADD was assessed. DMOG-induced FADD inhibition was diminished significantly (HIF-1α siRNA versus control siRNA; p = 0.01), confirming that DMOG-induced inhibition of FADD is HIF-1α–dependent (Fig. 5B).

As a next step, FADD protein levels were analyzed in vivo by Western blot of terminal ileal mucosal scrapings from healthy C57BL/6J mice after 4 d of DMOG or vehicle treatment. FADD was detectable in all of the vehicle-treated mice (n = 3) but not in DMOG-treated littermates (n = 5; Fig. 5C).

To investigate the functional relevance of this finding, TNF-α–induced apoptosis was compared between cells transfected with either FADD siRNA or control siRNA. FADD siRNA repressed FADD in a way similar to DMOG (Fig. 5D, 5E) and significantly protected IECs against subsequent TNF-α–induced apoptosis (Fig. 5F).

In conclusion, DMOG treatment reduced intestinal epithelial FADD both in vitro and in vivo, rendering the enterocytes less susceptible to TNF-α–induced apoptosis.

The promoter of FADD contains a functional binding site for HIF-1α

It has been appreciated recently that HIF-1 is able to both induce and repress transcription by binding on hypoxia-responsive elements (HREs) in the promoter of the target gene (10, 31, 32).

In a subsequent experiment, we questioned whether HIF-1α was able to regulate FADD transcription in a direct way. A search for transcription factor binding sites using the TRANSFAC database revealed three putative binding sites for HIF-1α, located downstream at −740, −531, and −468 relative to the ATG initiation codon (Table III). All three candidate oligonucleotides were screened for HIF-1α binding activity, using a commercially available transcription factor binding assay. One of the three binding sites (at −531; Fig. 6A) was shown to bind HIF-1α. To ensure that HIF-1α bound the capture probe in a sequence-specific manner, competition experiments were performed by adding increasing amounts of an identical, unlabelled competitor oligonucleotide to the biotin-labeled capture probe. The competitor oligonucleotide dose-dependently diminished the signal intensity of the biotin-labeled capture probe, thereby confirming sequence binding specificity (Fig. 6B).

Effective binding of HIF-1α to the endogenous FADD promoter was confirmed by chromatin immunoprecipitation (Fig. 6C).

To analyze the functional relevance of the −531 HIF-1α–binding site, two plasmids were constructed (PGL3Control−531 and PGL3Enhancer−531) containing part of the FADD promoter sequence that surrounded the confirmed HIF-1α binding site. Whereas luciferase activity was increased significantly during hypoxia in cells transfected with an established erythropoietin–HRE construct (fold increase of 2.82 ± 0.34; p < 0.01 as compared with that of empty vector), a significant decrease of luciferase activity was seen in cells transfected with the FADD promoter constructs (fold increase of

FIGURE 5. DMOG represses intestinal epithelial FADD, which renders IECs less susceptible to TNF-α–mediated apoptosis. A, Transcription of FADD was reduced 2.5– to 3-fold in HT29 IECs by a 24-h exposure to DMOG (1 mM in DMEM) or 1% oxygen. B, Reduction of HIF-1α protein by transfecting HT29 cells with HIF-1α siRNA prior to DMOG treatment resulted in significantly increased FADD transcription, confirming a significant role for HIF-1α in FADD inhibition. C, In vivo, a Western blot revealed strongly reduced FADD protein levels in the terminal ileal mucosa of C57BL/6J mice after 4 d of DMOG (8 mg in 0.5 ml sterile PBS, i.p., every other day). D, FADD siRNA was used to check the functional consequences of FADD down-regulation on TNF-α–mediated apoptosis. E, siRNA repressed FADD 2.5– to 3-fold (similar to DMOG) in HT29 cells, resulting in undetectable protein levels by Western blot. F, FADD repression protected IECs against TNF-α–mediated apoptosis. Each condition of in vitro experiments was tested six times. In vivo results are representative of two independent experiments with n = 3–5 mice per group. *p < 0.05; **p < 0.01; ***p < 0.001.
Evidence for a role for FADD in ileal CD

Because TNFΔARE/+ mice are representative of ileal CD, we studied the expression of FADD in the terminal ileum of patients with CD (n = 18) versus that in healthy controls (n = 16). To

Table III. Putative binding sites for HIF-1α in the promoter region of FADD

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence (5′–3′)</th>
<th>Orientation</th>
<th>Similarity Score</th>
<th>Matrix Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>−740</td>
<td>GGTCCCCGCCGTTGCCC</td>
<td>−</td>
<td>1</td>
<td>0.934</td>
</tr>
<tr>
<td>−531</td>
<td>GGAGAAACCTGGAAPA</td>
<td>−</td>
<td>1</td>
<td>0.964</td>
</tr>
<tr>
<td>−468</td>
<td>AGCCCGAAGCTCAGTG</td>
<td>−</td>
<td>1</td>
<td>0.914</td>
</tr>
</tbody>
</table>

The number of hits for HIF-1α in a 1000-bp region upstream of the initiation codon found by MalInspector (Genomatix, TRANSFAC PRO 8.4, Ann Arbor, MI) using a core matrix match of 100% and a matrix match of 75% relative to the ATG initiation codon.

0.43 ± 0.09; p < 0.001 as compared with that of empty vector) (Fig. 6D). In addition, deletion of the core nucleotides in the HIF-1α binding sequence resulted in the loss of hypoxia-induced luciferase repression (Fig. 6E).
exclude an effect of inflammation on FADD expression, we used biopsy specimens of endoscopically normal appearing terminal ileum. Terminal ileal FADD transcription was enhanced significantly in CD patients with inactive disease compared with that of healthy controls \((p < 0.01; \text{Fig. 7A})\). Interestingly, this finding was only made in patients with a history of ileal CD. CD patients that had never had ileal involvement did not have increased FADD levels compared with those of healthy controls (Fig. 7B). The same findings were made at the protein level using immunohistochemistry for FADD. Expression of FADD was mainly seen in the gut epithelium and, to a lesser extent, inflammatory cells. Increased FADD protein was detected in CD patients with a history of ileal inflammation compared with that of healthy controls, despite the absence of inflammation (representative images are given in Fig. 7).

**Discussion**

In this study, we demonstrate a beneficial effect of the prolyl hydroxylase inhibitor DMOG on chronic intestinal inflammation. We found that DMOG treatment led to very fast abrogation of barrier dysfunction and chronic villous architectural changes in TNF^{ARE/−/−} mice, a model of TNF-α–driven chronic ileitis that strikingly resembles human CD.

The timeframe in which DMOG treatment was able to achieve restoration of the intestinal epithelial barrier suggested an interference with processes that can rapidly influence barrier function, such as IEC apoptosis. IEC apoptosis is known to be a major factor in TNF-α–induced barrier disruption and seems to be the most important structural correlate of barrier dysfunction in human CD (28, 33–35). We could confirm the strong protective effect of DMOG on TNF-α–induced IEC apoptosis both in vitro and in vivo. DMOG-pretreated HT29 cells showed a significant reduction in TNF-α–induced caspase-3 activation, and this effect was largely dependent on HIF-1α. To demonstrate the antiapoptotic action of DMOG on IECs in vivo, we used a murine model of acute enteropathy induced by i.v. administration of a high dose of rTNF-α. DMOG pretreatment highly protected mice against the massive apoptosis and detachment of IECs induced by TNF-α.

![FIGURE 7. CD Patients with a normal appearing terminal ileum have increased FADD mRNA levels compared with those of healthy controls. A, Biopsy specimens of normal appearing terminal ileum of patients with CD and healthy controls were analyzed for mucosal FADD expression. Terminal ileal FADD transcription was enhanced significantly in CD patients versus healthy controls. B, Subanalysis showed that this finding was only true for patients with a history of ileal CD and not for CD patients that had never had ileal involvement. The qRT-PCR results were confirmed at the protein level by immunohistochemistry. Representative images (FADD, original magnification ×100, detail magnification ×200) are given. \(*p < 0.05; **p < 0.01; ***p < 0.001.\)
thereby preventing the associated blunting and shortening of the intestinal villi. Interestingly, these findings correlated with the abolishment of the villous architectural changes by DMOG treatment in TNFαARE/+ mice. In line with our results, Marini et al. (33) found that the prevention of IEC apoptosis by anti–TNF-α Abs resulted in the restoration of the disturbed villous architecture in SAMP1/YitFc mice (another murine model that closely resembles CD).

Strong efforts were made to clarify the mechanism underlying the protective effects of DMOG on TNF-α–induced IEC apoptosis. TNF-α elicits its proapoptotic effect through TNFR-1 (29). Because no changes in mucosal TNFR1 levels were detected after DMOG treatment (data not shown), we focused on critical molecules downstream to the receptor, one of them being FADD. The FADD gene is highly conserved across all species and encodes an essential adaptor molecule of several death receptors, including TNFR-1 (30, 36, 37). After binding of TNF, TNFR-1 is activated and binds to the TNFR-associated death domain, which in turn recruits several proteins to the activated receptor such as TRAF2, RIP1, and IAPs, forming complex I, which is involved in NF-κB and MAPK activation. After internalization of the TNFR1 receptor, a secondary cytosolic complex II is formed involving the recruitment of the FADD adaptor protein and the proximity-induced activation of caspase-8, leading to proteolytic activation of the downstream executioner caspase-3 and caspase-7 (38). We found that DMOG treatment led to a >60% reduction of FADD transcription in IECs. Moreover, we were able to find a functional repressive binding site for HIF-1α in the FADD promoter. As such, our findings revealed an innate mechanism that protects IECs during (inflammatory) hypoxia by direct modulation of death receptor signaling.

Because FADD is a common adaptor of several death receptor systems, DMOG (pre)treatment is expected to reduce intestinal epithelial Fas- and TRAIL-activated IEC apoptosis as well (30, 39). For TRAIL-induced apoptosis, this already has been demonstrated in vitro (6).

Interestingly, the activity of all members of the death receptor family has been correlated positively with disease activity in IBD (40–42). The precise role of FADD in CD is unknown yet, but in our opinion, warrants further research. Our data show that increased FADD transcription is present in endoscopically normal appearing terminal ileum of IBD patients compared with that of healthy controls. Moreover, the human FADD gene is positioned at chromosome 11q13.3, in proximity to a previously established locus for CD (43).

It has been appreciated recently that HIF-1α is able to both induce and repress genes, although the exact mechanism by which HIF-1 leads to transcriptional repression remains to be established. Functional repressive HIF-1α binding sites have been found previously in the promoters of the equilibrative nucleoside transporter 2 gene (10), the cystic fibrosis transmembrane conductance regulator gene (31), and the peroxisome proliferator-activated receptor α gene (32). In line with our results, a downregulation of FADD under hypoxia in human prostate cancer cells was found using microarray analysis, strengthening the concept that FADD is a hypoxia-responsive gene (44).

Even though our data clearly demonstrate the importance of HIF-1α–induced FADD repression in the protective action of DMOG against intestinal epithelial damage, a contribution of other HIF-1α–dependent and/or independent processes cannot be excluded. HIF-1α is a transcription factor of numerous genes, including several genes involved in intestinal epithelial barrier function (e.g., intestinal trefoil peptide and mucin) and genes involved in dampening of inflammation (e.g., netrin-1) (13, 45, 46). In addition, DMOG also has been shown to augment NF-κB activity, which has important interactions with HIF-1α and protects IECs against apoptosis as well (6, 47).

Prolyl hydroxylase inhibitors like DMOG seem to be a promising alternative treatment not only for IBD. Beneficial effects of HIF-stabilizing therapy have been found for a wide variety of ischemic disorders, such as myocardial and cerebral infarction or chronic kidney failure (48–50). An important focus of future research will be to specify the therapeutic targets of nonspecific pan-PHD inhibitors like DMOG to develop more specific, targeted drugs. In this light, Tambuwala et al. (51) have found recently that the protective action of PHD inhibition in experimental colitis is restricted to inhibition of the PHD1 isoform.

Future studies also should address the potential oncogenic consequences for pharmacological HIF-1α stabilization. HIF-1α is overexpressed in many malignancies and has been associated with tumor aggressiveness and a poor prognosis (52). Moreover, because of its suppressive effects on HIF-1α, YC-1 is being developed currently for clinical use as an anticancer drug (21, 53). However, the role of HIF inhibitors as a potential treatment for malignancies has been questioned recently. Mazzone and co-workers (54) found that upregulation of HIF activity in PHD2 haplodeficient mice in fact diminished tumor aggressiveness and inhibited metastasis by improving oxygenation through endothelial normalization.

In conclusion, we showed that DMOG treatment has positive effects in TNF-α–driven Crohn’s–like chronic ileal inflammation and propose downregulation of TNF-α–induced IEC apoptosis by HIF-1α–dependent FADD repression as at least a contributing mechanism. The direct modulation of death receptor signaling by HIF-1 represents a previously unappreciated endogenous adaptive pathway in response to hypoxia that may guide the development of novel therapeutics in hypoxic chronic inflammatory diseases, including IBD.

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Disclosures

The authors have no financial conflicts of interest.

References


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