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Dimethyloxalylglycine preserves the intestinal microvasculature and protects against intestinal injury in a neonatal mouse NEC model: role of VEGF signaling

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Abstract

Background—NEC is a devastating neonatal disease characterized by intestinal necrosis. Hypoxia inducible factor-1α (HIF-1α) plays a critical role in cellular oxygen homeostasis. Here, we hypothesized that prolyl hydroxylase (PHD) inhibition, which stabilizes HIF-1α, protects against NEC by promoting intestinal endothelial cell proliferation and improving intestinal microvascular integrity via VEGF signaling.

Methods—To assess the role of PHD inhibition in a neonatal mouse NEC model, we administered DMOG or vehicle to pups prior to or during the NEC protocol, and determined mortality and incidence of severe intestinal injury. We assessed intestinal VEGF by Western blot and quantified endothelial cell and epithelial cell proliferation following immunofluorescence.

Results—DMOG decreased mortality and incidence of severe NEC, increased intestinal VEGF expression, and increased intestinal villus endothelial and epithelial cell proliferation in experimental NEC. Inhibiting VEGFR2 signaling eliminated DMOG's protective effect on intestinal injury severity, survival, and endothelial cell proliferation while sparing DMOG's protective effect on intestinal epithelial cell proliferation.

Conclusion—DMOG upregulates intestinal VEGF, promotes endothelial cell proliferation and protects against intestinal injury and mortality in experimental NEC in a VEGFR2-dependent

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manner. DMOG's protective effect on the neonatal intestinal mucosa may be mediated via VEGFR-2 dependent improvement of the intestinal microvasculature.

Introduction

Necrotizing enterocolitis (NEC), a devastating disease of the neonatal intestine, affects 7–10% of very low birth weight (VLBW) infants. It is characterized by variable degrees of intestinal tissue necrosis (1). NEC pathogenesis is multifactorial (2, 3). Translocation of bacteria across a weakened intestinal barrier in association with impaired intestinal mucosal immunity leads to an exaggerated inflammatory response, which contributes to a vicious cycle of inflammation and secondary mucosal epithelial injury (2, 3). Mortality is reported as high as 30% (4), and survivors suffer from significant long-term morbidities including short bowel syndrome, and significant neurodevelopmental delays (5). No specific therapies are currently available for NEC, with treatment limited to supportive care and surgery in severely affected infants.

The intestinal microvasculature may play an important role in NEC (6, 7). We recently found that vascular endothelial growth factor (VEGF), a major regulator of angiogenesis, and its main receptor VEGF receptor 2 (VEGFR2) are strongly expressed during late fetal life (7) and decreased in experimental NEC (6). Furthermore, VEGF is decreased in human NEC (6). Inhibition of VEGFR2 kinase activity decreased villous endothelial cell proliferation and increased mortality and severe NEC incidence in neonatal mice (7). VEGF is regulated by the transcription factor hypoxia inducible factor-1 (HIF-1), but HIF-1's role in NEC remains unknown.

HIF-1, the master regulator of the cellular response to hypoxia and ischemia, is a highly conserved transcription factor present in all mammals (8, 9). HIF-1α mediates angiogenesis in response to hypoxic injury by regulating expression of growth factors including VEGF-A, which stimulates endothelial cell proliferation, promotes migration of endothelial cells and vascular smooth muscle cells, and inhibits apoptosis (10, 11). The intestinal mucosa experiences profound fluctuations in blood flow and oxygenation during normal physiologic conditions, and accordingly, intestinal epithelial cells (IECs) are uniquely resistant to hypoxia (12). IECs cope with hypoxia through basal regulation of HIF (12). Oxygen dependent hydroxylation of HIF-1α by prolyl hydroxylase domain enzymes (PHDs) signals the Von Hippel Lindau protein (pVHL) to bind and target HIF-1α subunits for proteasomal degradation, thereby regulating HIF-1α abundance (13). In hypoxia, HIF-1α is stabilized, permitting heterodimerization with HIF-1β. The active HIF-1 heterodimer translocates into the nucleus and binds to HIF response elements present in the promoter of HIF target genes, thereby activating transcription of genes that regulate oxygen homeostasis, including VEGF-A (11). While studies have shown increased HIF-1α expression in the intestine during experimental NEC (14), whether this represents a protective response to intestinal injury or contributes to injury development remains unknown.

Here, we hypothesized that HIF-1α stabilization protects against tissue injury in experimental NEC via upregulation of VEGFR2 signaling thus improving intestinal microvascular integrity. In this study, using dimethyloxalylglycine (DMOG), a PHD

inhibitor that prevents HIF-1α degradation in normoxia (15), we investigated whether PHD inhibition ameliorates intestinal tissue injury in our well-characterized neonatal mouse NEC model (16). We further evaluated whether DMOG's protective effects on mortality and severity of intestinal injury in experimental NEC are dependent on VEGFR2 signaling. To elucidate the mechanism by which PHD inhibition protects against NEC, we examined DMOG's effects on intestinal VEGF expression and intestinal villus endothelial cell and intestinal epithelial cell (IEC) proliferation, and evaluated whether DMOG-mediated preservation of IEC and endothelial cell proliferation in experimental NEC is dependent on VEGFR2 signaling in the neonatal mouse intestine.

Material and Methods

Materials

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animal breeding and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Stanley Manne Children's Research Institute/Northwestern University. Animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85Y23, revised 1996). Anti-CD31 (#ab28364) and anti-BrdU (#ab6326) antibodies were purchased from Abcam (Cambridge, MA), anti-VEGF-A (#sc-7269) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and Horseradish peroxidase (HRP)-conjugated antibodies (#A11037 and #A11007) from Life Technologies (Grand Island, NY). DMOG was purchased from Sigma-Aldrich (St. Louis, MO) and Ki8751 from R&D Systems (Minneapolis, MN).

Animal Experiments

To study the effect of PHD inhibition on NEC incidence, litters of newborn (P0) pups were injected with PHD inhibitor DMOG 40 mg/kg IP (in 30 μl phosphate buffered saline (PBS)/1% carboxymethyl cellulose (CMC)) or vehicle (PBS/1% CMC) and 18h later subjected to our NEC protocol (16). We induced NEC via a 72-hour protocol consisting of inoculation with standardized murine adult commensal bacteria (10^8 CFU) and 5 mg/kg LPS to perturb normal intestinal flora colonization, Esbilac formula feeding every 3h (200 mL/kg/day), and exposure to asphyxia (60 seconds in 100% N_2) followed by cold stress (10 minutes in 4°C) twice daily. This protocol induces within 72h a range of intestinal injury from mild (epithelial) to severe (transmural) resembling human NEC. During this 72h-experimental period, animals were continuously monitored for clinical signs of NEC (severe abdominal distension, lethargy, and apnea). Pups surviving for 72h without signs of distress and pups who developed signs of clinical distress or imminent death were euthanized by decapitation and time of death was recorded. Whole intestinal tissues were collected and H&E stained slides evaluated. The most severely affected area was scored by an investigator unaware of the group assignment, using a pre-established scoring system (16). Severe NEC was defined as histological score ≥ 2, encompassing mid-villous necrosis (score -2-), complete villous necrosis (score -3-), or transmural necrosis (score -4-), corresponding to clinically significant NEC. Mild epithelial sloughing (score -1-) is non-specific and may not represent illness. 6/50 vehicle-treated pups and 3/54 DMOG-treated pups were found dead while not under direct observation and excluded from histological analysis due to concern

that autolysis following a brief delay between death and tissue processing would compromise intestinal injury assessment validity.

To study DMOG's route of delivery and timing of administration, three groups were used: Group 1 (DMOG-gavage) received prophylactic DMOG 40 mg/kg by orogastric gavage and 18h later was subjected to the NEC protocol; Group 2 (IP-DMOG-therapeutic) received 40 mg/kg DMOG IP at 18h of NEC induction; Group 3 received IP vehicle only at 18h of NEC induction.

To study whether DMOG's protective effect persists when VEGFR2 signaling is inhibited, pups received either IP DMOG (40 mg/kg) or vehicle. A subset of pups also received the selective VEGFR2 inhibitor Ki8751 (0.75 mg/kg, IP), 1h after DMOG injection. Ki8751 is over 40-fold more selective for VEGFR2 than c-Kit, PDGFRα and FGFR-2, and has little activity on EGFR, HGFR and insulin receptor (17). 18h after DMOG or vehicle, pups were exposed to a NEC protocol or left with dams for 72h.

To study the effect of DMOG on VEGF in the mouse intestine, P8 littermates were randomized to receive either DMOG (40 mg/kg, IP) or vehicle. 48h later, collected small intestines were analyzed for VEGF protein expression by Western Blot.

For endothelial cell proliferation assessment, P0 pups were divided into 4 groups: DF pups injected with DMOG (40 mg/kg, IP); DF pups injected with vehicle only; pups injected with DMOG and subjected 18h later to our NEC protocol; pups injected with vehicle and subjected 18h later to our NEC protocol. After 24h, pups were euthanized and intestinal tissues were collected for immunofluorescence staining. DF pups were euthanized ~42h after injection with DMOG or vehicle (to correspond to 18h pre-treatment with DMOG and 24h NEC induction in littermates exposed to the NEC protocol). To label proliferating cells, BrdU (0.3 mg IP) was injected 4h prior to euthanasia. To study whether DMOG promotes endothelial cell proliferation via VEGFR2, littermates were injected with DMOG or vehicle only. A subset of pups received Ki8751 (IP 0.75 mg/kg). BrdU was administered and intestinal tissue collected. All time-points described refer to hours after NEC induction in experimental pups and their littermate DF controls unless otherwise specified.

Immunofluorescence Staining

5-μm-thick formalin-fixed paraffin-embedded intestinal tissue sections were stained using standard immunofluorescent methods for CD31 and BrdU. Slides were incubated with primary antibodies [anti-CD31 (1:50), anti-BrdU (1:50)] overnight at 4°C. Washed slides were incubated for 1h with fluorescent dye-conjugated secondary antibody [goat anti-rabbit secondary antibody (1:500), goat anti-rat secondary antibody (1:1000)] for 1h at room temperature. Slides were mounted in Prolong Gold anti-fade reagent with DAPI (Life Technologies, Grand Island, NY) and images were captured using a Leica DMR-HC upright microscope. Proliferating endothelial cells (co-staining for CD31 and BrdU) were assessed in 5–10 20X power fields with 5 mice per group, by a blinded investigator. Negative controls in which the primary antibody had been omitted were run concomitantly and did not show staining. The number of proliferating $BrdU^+$ IECs per crypt was also assessed in 5–7 20X power fields with 5–7 mice per group.

Western Blotting

Snap frozen small intestines were homogenized in lysis buffer (10 mM Tris-Cl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM DTT, 0.25% Nonidet P-40, and Complete Mini Tablet Protease Inhibitor Cocktail from Roche Biochemical Reagents (Sigma-Aldrich, St. Louis, MO)) and lysate protein concentration determined (Bradford method). 50 μg of protein were run on 10% or 15% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes. Membranes were blocked (milk in Tris-buffered saline-Tween 20, 1h), incubated with primary antibodies overnight (4°C) then HRP-conjugated secondary antibodies (1h, room temperature). Membranes were developed using the Pierce ECL method. Relative protein expression was determined by densitometry and normalized to β-actin.

Statistical Analysis

ANOVA analysis was performed when more than two groups were compared. Two-tailed unpaired Student t-test was used for comparison between two groups. Results were expressed as mean ± SEM unless otherwise specified. Differences in severe NEC incidence (score ≥ 2) were evaluated uising χ^2 analysis. Animal survival data were analyzed by Log-rank test. Differences were considered statistically significant when p values were < 0.05.

Results

DMOG decreases mortality and the incidence of severe intestinal injury in a murine neonatal NEC model

To determine whether HIF-1α stabilization plays a role in intestinal injury in NEC, we injected pups intraperitoneally (IP) with the prolyl hydroxylase inhibitor DMOG (40 mg/kg) or vehicle only 18h before NEC induction (Fig 1a). The treatment dose of 40 mg/kg was chosen based on several *in vivo* studies showing that DMOG at this dose increases HIF-1α gene expression and protects against cerebral and myocardial ischemic injury in several rodent models (18, 19). We found that DMOG significantly decreased the mortality of neonatal mice exposed to the NEC protocol by 2.16 fold (DMOG: 12/54 vs. 24/50, $p < 0.005$, Fig. 1b). Furthermore, when pups were injected with DMOG prior to NEC induction, the incidence of severe intestinal injury (histological injury score ≥ 2) was decreased by 1.83-fold compared to those pups injected with vehicle only (DMOG: 18/51 vs. 28/44, $\chi^2 = 7.6$, $p< 0.01$, Fig. 1c).

As expected, mortality was correlated to intestinal injury severity. We found that 83% of pups with histology scores of 0–1 survived the 72h-experimental period vs. 68% of pups with scores of 2 vs. 42% of pups with scores of 3 vs. 12.5% of pups with scores of 4 ($p<0.0001$) (Fig. 1d). There was a 6.6-fold higher mortality in pups with the highest intestinal injury scores (scores of 4) when compared to pups with mild or no intestinal injury (scores of 0–1) ($\chi^2 = 25.3$, $p<0.0001$). However, there were no statistically significant difference in mortality between DMOG-treated pups and vehicle-treated pups at similar histology scores.

To determine whether timing or route of administration affected DMOG's protective effect, littermates were randomly assigned to 3 groups: 1) 40 mg/kg DMOG prophylaxis by orogastric gavage 18h prior to NEC induction; 2) DMOG (40 mg/kg, IP) at 18h of NEC induction; or 3) vehicle only by IP injection (Fig 1A). We found that DMOG's protective effect on survival and intestinal injury in experimental NEC was also present when administered enterally prior to NEC induction. When compared to controls, enterally administered DMOG significantly decreased NEC-associated mortality by 1.87 fold (11/27 vs. 19/25, $p < 0.05$) (Fig. 1e) and decreased incidence of severe NEC (histological injury score ≥ 2) by 2.89 fold (7/27 vs. 18/24, $\chi^2 = 3.5$, $p<0.001$) (Fig. 1f). Administration of DMOG at 18h of NEC induction was still associated with a decreased incidence of severe NEC 3.5 fold compared with vehicle treated littermates (6/28 vs. 18/24, $\chi^2 = 14.92$, $p<0.0001$) (Fig. 1f), while no significant improvement in mortality was noted (18/29 (62%) vs. 19/25 (76%), NS) (Fig. 1e).

DMOG increases intestinal VEGF expression in pups

To determine whether DMOG affects intestinal VEGF expression, dam fed (DF) pups were injected with 40 mg/kg IP DMOG or vehicle only and intestinal tissues were collected 48h later. VEGF expression was assessed by Western blot analysis of whole intestinal tissue lysates. We found that injection with DMOG significantly increased VEGF protein expression in the intestine of DF pups (Fig. 2a–b).

Inhibition of VEGFR2 signaling abrogates DMOG's protective effect on mortality and intestinal injury

To determine whether DMOG's protective effect on mortality and intestinal injury in NEC was dependent on VEGFR2 signaling, newborn pups were injected with DMOG (IP 40 mg/kg) or vehicle only (PBS-CMC) (littermate controls). A subset of pups in each group was also injected with Ki8751 (0.75 mg/kg IP), a selective VEGFR2 tyrosine kinase inhibitor. Pups in each group were subjected to the NEC model, with a small subset of pups in each group remaining with their dams. Survival over the 72h NEC model and histological injury analyses were performed. As expected, DMOG prophylaxis significantly decreased the mortality of neonatal mice exposed to the NEC protocol compared to vehicle-treated littermates (11/16 vs. 15/17, $p<0.05$, Fig. 3a). We previously showed that inhibition of VEGFR2 tyrosine kinase activity by Ki8751 increased the incidence of severe NEC and mortality (7). Ki8751 completely abrogated DMOG's protective effect on 72h-mortality (17/17 vs 11/16, $p<0.05$), to levels similar to Ki8751 treatment alone (17/17 vs. 17/17, NS) (Fig. 3a). Similarly, DMOG's protective effect on severe intestinal injury was eliminated by VEGFR2 inhibition (14/16 vs 3/16, χ^2 15.2, $P<0.0001$) (Fig. 3b) to a level similar to Ki8751 alone (13/15) (Fig. 3B). However, Ki8751 did not affect the 72h survival in DF pups as 90% (9/10) of DF Ki8751-treated pups survived the 72h observation (vs. 9/10 of DF DMOG/ Ki8751-treated pups and 8/8 of DMOG-treated pups, NS)(Fig. 3a). As expected, none of the DMOG-treated DF pups (0/8) had severe intestinal injury on histological examination. Interestingly, 3/9 Ki8751 treated DF pups and 4/8 DMOG-Ki8751 treated DF pups had severe intestinal injury following Ki8751 treatment alone (Fig. 3c), consistent with our previous findings that VEGFR2 inhibition predisposes to intestinal injury in neonatal mice (7).

Endothelial cell and epithelial cell proliferation are decreased in the intestinal villi of neonatal pups during NEC induction, and both effects are mitigated by DMOG administration

To determine whether DMOG preserves intestinal endothelial cell and epithelial cell proliferation during NEC, P0 mouse pups were injected with DMOG (IP 40 mg/kg) or vehicle only (littermate controls). 18h later, half the pups in each group underwent NEC induction and the other half were left with the dams and DF. 24h later, small intestinal tissues were collected and tissue sections immunostained for BrdU and CD31. Intestinal endothelial cell proliferation was decreased in vehicle-treated NEC pups compared to vehicle-treated DF littermate controls (7.8 ± 0.9 cells/field vs. 19.0 ± 1.2 cells/field, $p< 0.0001$), an effect that was mitigated by DMOG administration (DMOG 11.5 cells/field vs. VT-NEC 7.8 ± 0.9 cells/field, $p<0.005$) (Fig. 4b). Also, in DF pups, DMOG increased villous endothelial cell proliferation compared to vehicle-treated littermate controls (26.8 ± 1.8 cells/HPF vs. 19.7 ± 2.0 cells/HPF, $p< 0.05$) (Fig. 4a–c).

We also found that the number of proliferating epithelial cells per intestinal crypt was decreased in vehicle-treated NEC pups compared to vehicle-treated DF littermate controls (3.1 ± 0.24 vs. 6.17 ± 0.35, $p<0.0001$)(Fig. 5). Treatment with DMOG partially rescued intestinal epithelial cell proliferation compared with vehicle-treated littermates (4.9 ± 0.50 vs. 3.1 ± 0.24, $p = 0.001$) (Fig. 5).

Inhibition of VEGFR2 signaling abrogates DMOG's protective effect on villous endothelial cell proliferation but does not affect DMOG's protective effect on intestinal epithelial cell proliferation in a neonatal mouse model of NEC

To determine whether increased intestinal endothelial cell proliferation in DMOG-treated pups is mediated via VEGFR2 signaling, DF mouse pups were injected with DMOG (IP 40 mg/kg) or vehicle only (PBS-CMC)(littermate controls). Half the pups in each group were also injected with Ki8751 (0.75 mg/kg IP), a selective VEGFR2 tyrosine kinase inhibitor. In pups submitted to the NEC protocol, VEGFR2 inhibition abrogated the protective effect of DMOG on villous endothelial cell proliferation (1.6 ± 0.4 cells/field vs VT 11.5 ± 0.88 cells/field, $p< 0.0001$) to a level similar to VEGFR2 inhibition alone (1.6 ± 0.4 cells/field vs. 1.7 ± 0.3 cells/field, NS) (Fig. 4b). Also, in DF DMOG-treated pups, VEGFR2 inhibition decreased endothelial cell proliferation (3.5 ± 0.4 cells/HPF vs. 26.8 ± 1.8 cells/HPF, $p< 0.0001$) to a level similar to VEGFR2 inhibition alone (3.5 ± 0.4 cells/HPF vs. 5.1 ± 0.9 cells/HPF, NS) (Fig. 4a–c). However, VEGFR2 inhibition had no effect on intestinal epithelial cell proliferation (2.98 ± 0.27 vs. 3.1 ± 0.24, NS) or on DMOG-improved intestinal epithelial cell proliferation (5.1 ± 0.32 vs. 4.9 ± 0.50, NS) in pups exposed to experimental NEC (Fig. 5).

Discussion

Despite advances in neonatal intensive care medicine, necrotizing enterocolitis continues to be associated with significant morbidity and high mortality in affected infants (2–5). Currently there are no specific therapies available for NEC, with treatment limited to supportive care, bowel rest, parenteral nutrition, and empiric antibiotic therapy (3–5).

Although protective factors have been identified which decrease the incidence of NEC in VLBW preterm infants, such as human milk (20, 21) and antenatal betamethasone administration to mothers at risk for preterm delivery (22), innovative targeted therapies are needed to protect against intestinal injury in this vulnerable patient population. Here, we found that treatment with DMOG, a PHD inhibitor that stabilizes HIF-1α and upregulates downstream HIF-1 targets including VEGF, significantly decreased mortality and ameliorated intestinal injury in an experimental mouse model of NEC.

Studies have shown increased HIF-1α expression in neonatal mouse intestine during experimental NEC *in vivo* (14). Whether the increase in intestinal HIF-1α expression contributes to intestinal injury in murine NEC or protects against it remains unknown. Our lab recently demonstrated that the number of cells expressing VEGF, a downstream target of HIF-1α, is decreased in the intestine of human neonates with NEC (6). In our murine NEC model, we found that endogenous VEGF protein expression and both VEGFR2 protein expression and phosphorylation are decreased prior to tissue necrosis during NEC development (7). Furthermore, administering SU5416 to mouse pups to block VEGFR2 signaling prior to the NEC protocol led to increased severity of tissue injury and mortality (7). Here, we show that treatment with DMOG, a HIF-1α stabilizing agent, prior to NEC induction protects against intestinal injury and increased endothelial cell proliferation in the lamina propria of the intestinal villi in neonatal mice. The effect of DMOG on survival, incidence of severe intestinal injury, and endothelial cell proliferation was dependent on VEGFR2 signaling, as it was completely abrogated by Ki8751, a selective inhibitor of VEGFR2 kinase activity.

DMOG is a non-specific prolyl hydroxylase domain enzyme inhibitor and accordingly, its effects are not limited to the intestinal microvasculature. Since HIF plays an important role in intestinal epithelial cell physiology (12), we also evaluated whether DMOG treatment affects the intestinal epithelium. We found that DMOG administration prior to NEC induction attenuated the NEC-induced decrease in intestinal epithelial cell proliferation. However, the protective effect of DMOG on intestinal epithelial cell proliferation persisted when pups were pre-treated with VEGFR2 inhibitors. DMOG administration prior to NEC induction no longer conferred a protective benefit against mortality or severity of intestinal injury in pups co-treated with VEGFR2 inhibitor, suggesting that DMOG's protective effect in NEC is mediated via its impact on intestinal endothelial cells rather than epithelial cells.

Risk factors for NEC include conditions that predispose the intestine to ischemia, including congenital heart disease, birth asphyxia, maternal preeclampsia with associated placental vascular insufficiency, severe anemia, and alterations in mesenteric blood flow (23–25). Hypoxia alone has been implicated in NEC development, with clinical studies linking lower target oxygen saturations with increased incidence of NEC in extremely premature infants (26). Our previous studies suggest that the intestinal microvasculature likely plays an important role in NEC pathogenesis (6, 7). Inadequate VEGF-VEGFR2 signaling alters mucosal angiogenesis and contributes to intestinal vascular insufficiency that may predispose premature infants to NEC development when exposed to the stressors of extrauterine life (7). These studies support the notion that stabilizing HIF prevents inadequate intestinal microvasculature development by regulating VEGF-VEGFR2

signaling, thus promoting intestinal perfusion and mesenteric oxygen delivery in premature infants at risk for necrotizing enterocolitis.

The activation of HIFs is part of a wide range of physiologic responses that are necessary at the cellular level for tissue survival at low oxygen tension (8). In addition to promoting angiogenesis and tissue regeneration in the setting of ischemia, HIF-1α plays a critical role in regulating oxygen homeostasis during fetal and postnatal life (27). A physiologic hypoxic environment is required for normal mammalian fetal development, in order to activate HIF-mediated transcriptional regulation of multiple genes with developmentally critical functions (24). When premature infants are exposed to “room air” or ≥21% FiO2, oxygen levels increase to supraphysiologic levels for this developmental stage, which may lead to inactivation of HIF-1α. An untimely decrease in HIF-mediated signaling may contribute to inadequate responses to hypoxic or ischemic insults and can lead to lack of VEGF production during a critical window of intestinal vascular development.

The expression of VEGF and other downstream HIF-1 targets is tightly developmentally regulated in the developing fetus. Nonspecific systemic upregulation of HIF-1 targets such as VEGF at the wrong time could have dangerous unintended effects on several organ systems outside of the gastrointestinal tract (28–29). In particular, systemically inhibiting PHD in premature infants whose microvasculature is still developing may increase the risk of retinopathy of prematurity, a potentially blinding disease of premature infants caused by abnormal vascularization of the developing retina (28). Furthermore, while numerous studies report that HIF-1 is beneficial for cells exposed to hypoxia or undergoing stress (18–19), prolonged HIF-1 activation has been linked to loss of gut barrier function, bacterial translocation and intestinal cell apoptosis, ultimately resulting in villous injury (30). Therefore, before DMOG can be considered as a potential innovative therapy for NEC, additional studies clarifying its mechanism of protection and its effects on other signaling pathways are warranted (15, 31). Careful evaluation of the drug’s effects on other organs and optimal timing for therapeutic intervention will be critical. Treatment strategies that selectively inhibit prolyl hydroxylase enzyme activity in the intestine may be preferable.

Systemic DMOG treatment has proven effective in models of vascular injury. Specifically, in a rat model of stroke, increased regional cerebral blood flow, reduced infarct volumes and improved behavior both 24h and eight days after brain injury occurred in rats that were given DMOG before and after transient middle cerebral artery occlusion (18). These protective effects were associated with increased mRNA and protein levels of the HIF-1 targets VEGF and eNOS (18). PHD inhibition has also been shown to be a promising strategy to protect against ischemic injury in several clinical applications (18, 19, 32). Although the role of hypoxia-inducible factors (HIFs) in neonatal NEC is not well understood, there is accumulating evidence that hypoxia-induced changes in HIF-mediated genes are important in intestinal diseases characterized by ischemia and inflammation (33, 34). Since oxygen-sensing PHDs control transcriptional adaptation to intestinal tissue hypoxia, therapeutic strategies to modulate these hypoxia-signaling pathways for the treatment of inflammatory intestinal diseases have become an important target for study (31). In this study, we demonstrated that PHD inhibition with DMOG drastically improved survival and protected against intestinal injury in an experimental NEC model. Furthermore,

our *in vivo* findings suggest that this may be, at least in part, via improving microvascular integrity as suggested by up-regulation of VEGF and increased intestinal villus endothelial cell proliferation.

Currently there are no targeted therapies for NEC. In this study, to investigate the role of HIFs in NEC pathogenesis, we utilized DMOG, a PHD inhibitor, to stabilize HIF alpha subunits prior to exposure to the NEC protocol. We found that: 1) PHD inhibition by DMOG prior to NEC induction significantly decreased mortality and the incidence of severe intestinal injury in neonatal mice subjected to an experimental NEC model whether administered enterally or intraperiteonally; 2) PHD inhibition by DMOG preserved both villous endothelial cell and epithelial cell proliferation during NEC; 3) PHD inhibition by DMOG increased intestinal VEGF expression in DF pups; 4) VEGFR2 inhibition by Ki8751, a selective inhibitor of VEGFR2 tyrosine kinase activity, abrogated DMOG's protective effect on villous endothelial cell proliferation without affecting DMOG's protective effect on intestinal epithelial cell proliferation; and 5) VEGFR2 inhibition abrogated DMOG protective effect on intestinal injury or mortality. Taken together, these findings provide a basis for testing therapeutic strategies that can selectively inhibit prolyl hydroxylase enzyme activity and preserve VEGF-VEGFR2 signaling in neonates at risk for NEC to prevent the development of the disease. Further investigation is needed to evaluate whether specific inhibition of individual PHDs or activation of specific HIF isoforms are effective therapeutic strategies to protect against intestinal injury in NEC.

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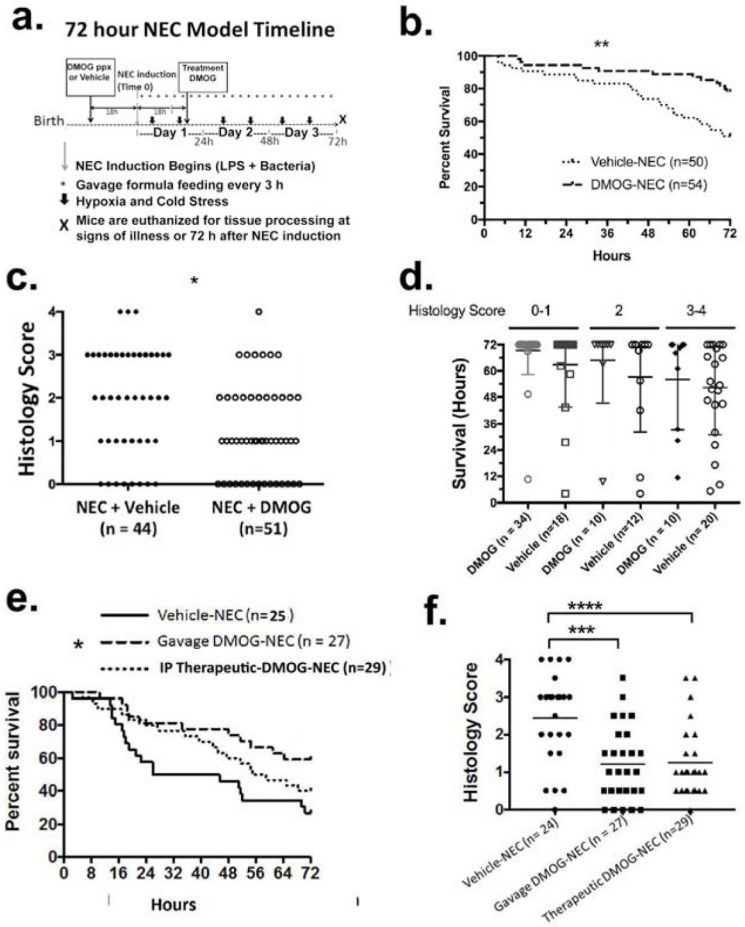

Figure 1. DMOG decreases 72h mortality and the incidence of severe intestinal injury in experimental NEC

(a-b-c) Newborn mice (were injected with DMOG (40 mg/kg, IP) or vehicle 18h prior to a NEC protocol. Mice were euthanized when presenting signs of distress or at 72h of the NEC protocol and whole intestines processed for histology. A timeline of the experiment (a), survival curves (b) and histology scores (c) are presented. Survival was correlated with severity of intestinal injury in both groups studied (d). Data represent 3 independent experiments combined. (e–f) To study the impact of DMOG's delivery route and timing of administration, littermates were divided into 3 groups: Group 1 received prophylactic DMOG (40 mg/kg) by orogastric gavage followed 18h later by NEC induction; Group 2 (DMOG-IP therapeutic) received 40 mg/kg DMOG or vehicle via IP injection at 18h of NEC induction; Group 3 received vehicle (IP) at 18h of NEC induction. All 3 groups were exposed to a 72h NEC protocol. Survival curves (e) and histology scores (f) are presented. *p<0.05, **p<0.005, *** p<0.001, **** p<0.0001. Data represent 2 independent experiments combined.

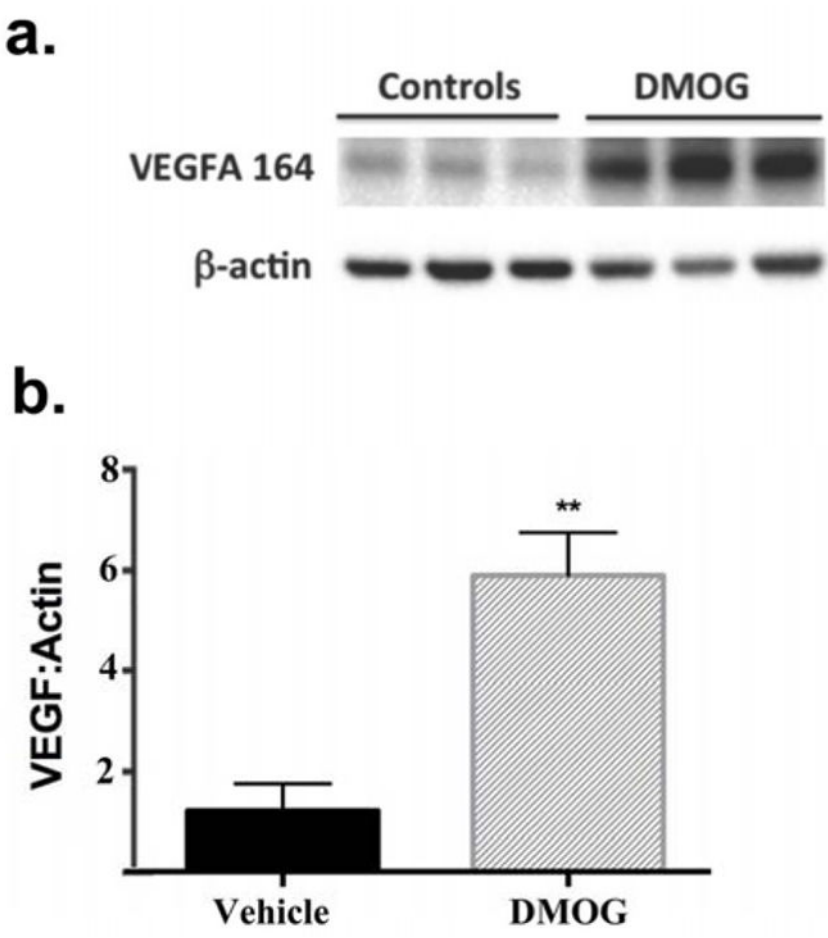

Figure 2. VEGF-A expression is increased in the mouse intestine following injection with DMOG

Intestinal tissues from 8-day-old pups were obtained 48h after DMOG (40 mg/kg, IP) or vehicle only (n=3/group). VEGF-A protein was analyzed by western blot of small intestinal tissue lysates (a). Band densitometry values normalized to β-actin are presented in panel b. **p<0.005.

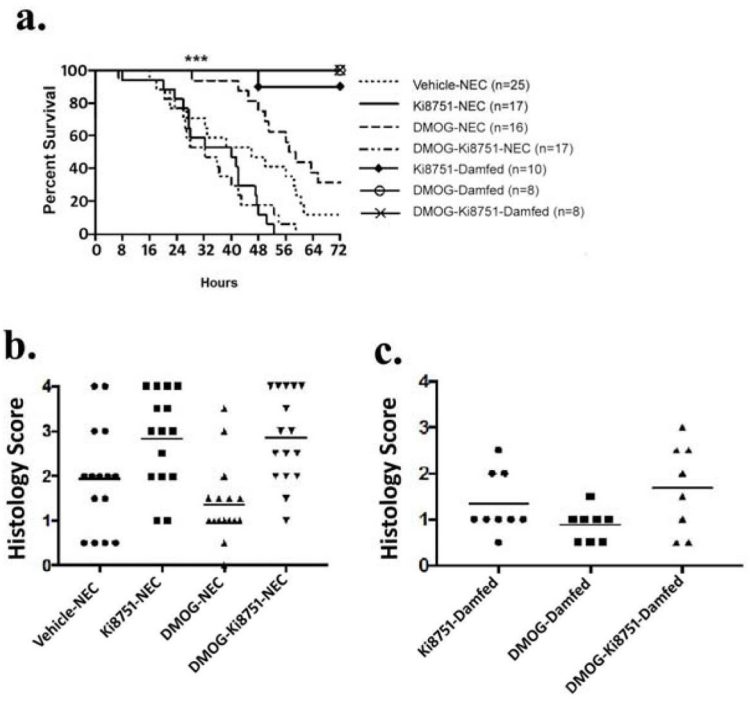

Figure 3. Inhibition of VEGFR2 signaling abrogates DMOG's protective effect on mortality and intestinal injury

Newborn pups received either DMOG (40 mg/kg, IP) or vehicle. A subset of pups in each group also received Ki8751 (0.75 mg/kg, IP) or vehicle. 18h later, pups were exposed to the NEC protocol or left with their dams. Mice were euthanized when presenting signs of distress or at 72h (Fig. 1a) and whole intestines processed for histology. Survival curves (a) and histology scores are presented representing NEC (b) and DF (c) *p<0.05, ** p<0.005, *** p<0.0001.

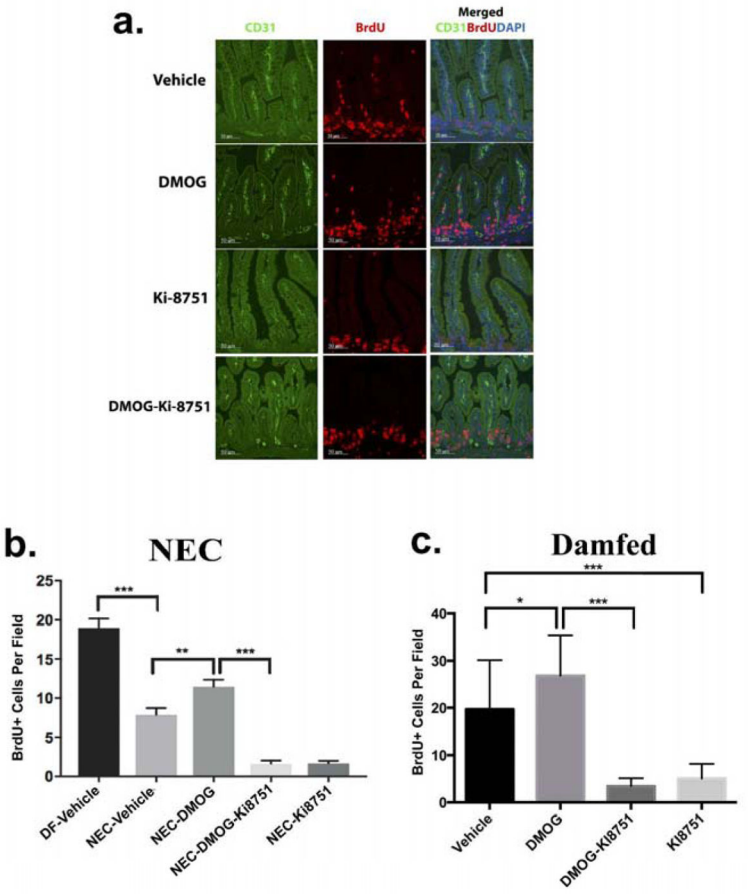

Figure 4. Inhibiting VEGFR-2 signaling abrogates DMOG's protective effect on villous endothelial cell proliferation in DF and NEC-exposed newborn mice

P0–1 littermates received either DMOG (40 mg/kg, IP) or vehicle. A subset in each group also received Ki8751 (0.75 mg/kg IP). 18h later, pups were either exposed to the NEC protocol for 24h or left with their dams and dam fed (DF). Pups were injected with BrdU (0.3 mg IP) 4h prior to euthanasia. IF staining for BrdU (red) and CD31 (green) was performed on small intestinal tissues (a). Proliferating endothelial cells ($CD31^{+}BrdU^{+}$ cells) in the intestinal villi were counted by an investigator unaware of group assignment. 5–7 fields were examined per section with 3–5 mice per group, scale bar = 50 μm. Mean numbers of proliferating endothelial cells per field (± SEM) are presented (b–c). NEC (b) and DF (c). *p<0.05, ** p<0.005 ***p<0.0001.

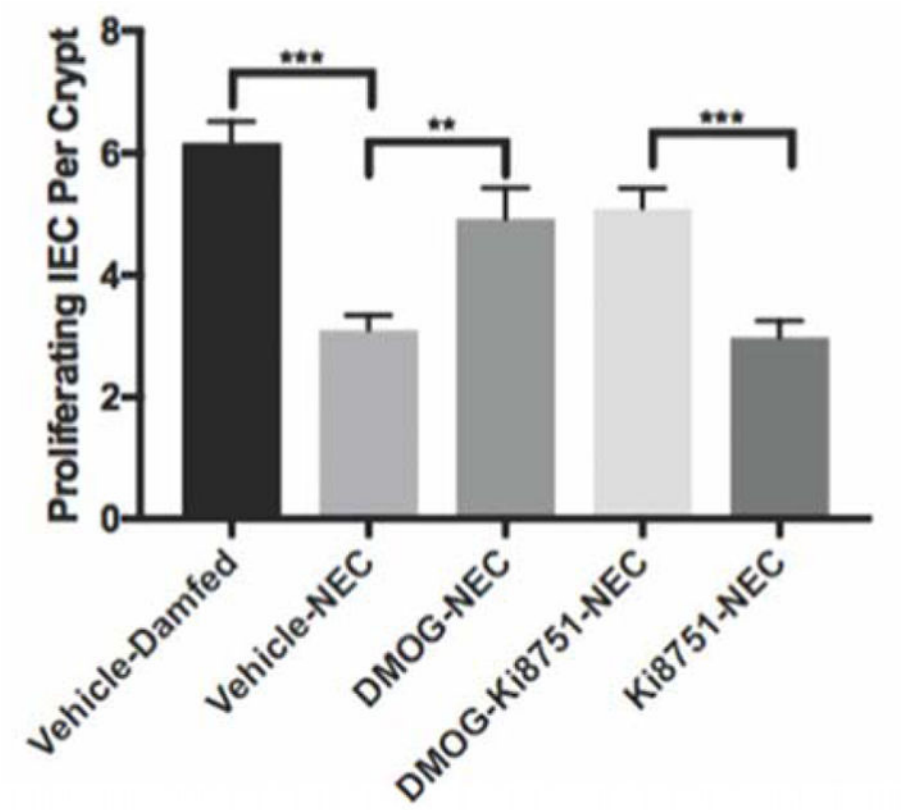

Figure 5. DMOG partially rescues intestinal epithelial cell proliferation in experimental NEC in a VEGFR2-independent manner

Littermates received either DMOG (40 mg/kg, IP) or vehicle. A subset in each group also received Ki8751 (0.75 mg/kg, IP). 18h after DMOG or vehicle injection, pups were submitted to a NEC protocol for 24h. DF vehicle-treated pups were studied as controls. Pups were injected with BrdU (0.3 mg IP) 4h prior to euthanasia. Small intestinal tissues were processed for BrdU IF staining. Proliferating BrdU+ intestinal epithelial cells per crypt were quantified. 5–7 fields were examined per section with 5–7 mice per group. Mean numbers of proliferating epithelial cells per crypt (± SEM) are presented. *$p<0.05$, ** $p<0.005$, *** $p<0.0001$.