Original Paper

Effects of Nitric Oxide and Reactive Oxygen Species on HIF-1 α Stabilization Following *Clostridium Difficile* Toxin Exposure of the Caco-2 Epithelial Cell Line

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Key Words

CDI • Epithelial permeability • Intestinal inflammation • Hypoxia-inducible factor • Nitrosylation • iNOS

Abstract

Background/Aims: Stabilization of the hypoxia-inducible factor (HIF-1 α) is proposed to provide a protective host-response to *C. difficile* intoxication. Here, we aimed to elucidate whether nitric oxide and/or reactive oxygen species produced during *C. difficile* toxin exposure could influence HIF-1 α stability and initiate protection against epithelial cell damage. **Methods/Results:** HIF-1 α and inducible nitric oxide synthase (iNOS) proteins were up-regulated whereas factor-inhibiting HIF-1 (FIH-1) protein was down-regulated in Caco-2 epithelial cell monolayers with *in vitro* toxin exposure. We demonstrate using the biotin-switch assay that the stabilization of HIF-1 α protein occurred via iNOS-dependent nitrosylation. Inhibition of iNOS activity by selective inhibitor (1400W) attenuated HIF-1 α stabilization and exacerbated toxin-dependent disruptions in Caco-2 monolayer morphology and tight junctional integrity *in vitro*. Treatment of Caco-2 cell monolayers with N-actylcysteine (NAC), a scavenger of reactive oxygen species (ROS), attenuated toxin-dependent increases in iNOS and HIF-1 α protein levels but had no effect on FIH-1 responses. In addition, mice that were exposed to *C. difficile* toxin *in vivo* also demonstrated a significant increase in HIF-1 α protein and nitrosylation levels. **Conclusion:** Taken together, these data suggest that important synergistic actions exist between nitric oxide and ROS to stabilize HIF-1 α and its innate, protective actions in the context of *C. difficile* toxin-mediated epithelial injury.

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Introduction

Clostridium difficile is an anaerobic gram-positive bacterium that is the leading cause of hospital-acquired diarrhea and colitis; otherwise known as *C. difficile*-infection (CDI) [1]. CDI is an increasingly common problem with the incidence in the United States almost tripling from 1996 to 2005 [2, 3]. While relatively little is known of the mechanisms by which *C. difficile* colonizes to cause severe colitis and why some patient groups appear relatively protected and others are more susceptible, the emergence of NAP1/ribotype 027 strain that is both hypervirulent and more antibiotic resistant has led to increased disease severity with mortality occurring in 6.9% of cases [2, 4]. Unfortunately the only therapies for CDI include halting exposure to the offending antibiotics, starting *C. difficile* specific antibiotics, initiated fecal microbiota transfer and in severe cases providing a total colectomy.

Upon colonizing the colon, *C. difficile* begins producing potent toxins (Toxin A, TcdA; Toxin B, TcdB) that are the primary effectors of CDI pathogenesis [5]. The toxins are responsible for initiating a severe inflammatory response, with increases in pro-inflammatory cytokines such as interleukin-8 (IL-8), interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) [6]. Furthermore, the *C. difficile* toxins are taken up into the colonic epithelial cells via endocytosis. Once inside the cytosol, the toxins glucosylate and inactivate Rho proteins to elicit dramatic effects on the actin cytoskeleton which in turn can compromise epithelial barrier by destabilizing tight junction contacts [7].

Within intestinal epithelial cells, there are a variety of genes that encode proteins for the direct (or indirect) support of epithelial barrier maintenance and repair. Notably, a number of epithelial barrier-protective pathways are controlled by hypoxia-inducible factor-1 (HIF-1)-mediated transcriptional responses. HIF-1 is a heterodimer made up of α and β subunits that are members of the basic helix-loop-helix/PAS (per-arnt-sim homology) family of transcription factors (reviewed in: [8]). HIF-1 demonstrates protective functions within intestinal inflammatory pathologies such as colitis [9-14]. Its transcriptional activity influences key target genes which are involved in mucosal barrier maintenance and restitution, including vascular endothelial growth factor (VEGF) [15], inducible nitric oxide synthase (iNOS) [16], intestinal trefoil factor (ITF) [11], P-glycoprotein 1 or multidrug resistance protein 1 (MDR-1) [17], adenosine A_{2A/2B} receptors [15, 18] and ecto-5'-nucleotidase (CD73) [19]. The transcriptional activity of HIF-1 is dictated by the bioavailability of the HIF-1 α subunit [8, 10]. Despite the fact that HIF-1 α is expressed at a constant rate, the cellular levels of HIF-1 α tend to be extremely low in normoxic conditions. When oxygen is readily available, the HIF-1 α subunit is rapidly targeted for proteosomal degradation by prolyl hydroxylation. Despite the heavy influence of oxygen on the control of HIF-1 α protein levels, other factors, such as nitric oxide (NO), reactive oxygen species (ROS) and inflammatory mediators have also been found to play an essential role in regulating HIF-1 α activity [10, 20-22].

NO, with its ability to impact upon a great number of essential physiological responses, is known to have important implications with respect to oxygen sensing and HIF-1 signaling. HIF-1 and its associated proteins contain thiol groups on cysteine residues that can act as targets for NO-related post-translational modifications. Recent studies have demonstrated that chemically diverse groups of NO donors (such as S-Nitrosoglutathione for example) or increased endogenous NO (produced via iNOS) can enhance HIF-1 α stabilization, DNA-binding and transcriptional activation of target genes in normoxic and/or inflammatory conditions [21, 23-25]. There have also been a number of investigations of the frequently opposing effects of ROS and NO in the gastrointestinal environment. NO can, through its interactions with proteins in the NOX complex, prevent the generation of ROS [26]; however, elevations in NO can also occur concurrently with increased ROS production. Individually, ROS and NO appear to provide beneficial if not necessary effects in maintaining homeostasis. This delicate balance can easily be altered with the introduction of an infection or the initiation of an inflammatory response. Since both molecules have important homeostatic roles in the gastrointestinal tract, it is important to consider the interactions between the

two molecules in the investigation of *C. difficile* toxin-associated intestinal injury and the role of HIF-1 therein.

An upregulation of HIF-1 α in mucosal biopsies isolated from patients with CDI has been previously reported [27]. In addition, the protective effects of HIF-1 in the context of *C. difficile* toxin-mediated damage have been demonstrated in a murine model of toxin exposure. In this case, mice that were genetically deficient in HIF-1 α (targeted deletion in intestinal epithelium) were more susceptible to *C. difficile* toxin-induced damage when compared to wild type mice [27]. These results implicate an essential role for HIF-1 α in modulating the innate protective functions of the intestinal epithelial mucosa in response to *C. difficile* toxin exposure and intestinal injury. Due to the suggested importance of the HIF-1 transcription factor, we conducted studies to determine the mechanisms of stabilization (i.e., interactions with NO and ROS). NO and ROS produced during *C. difficile* toxin exposure were hypothesized to act as important signals to influence HIF-1 α stability and initiate protection against epithelial cell damage.

Materials and Methods

Inoculation of C. difficile cultures and production of toxin

The bacterial toxin was recovered from the NAP-1/027 strain of *C. difficile* (positive for both TcdA and TcdB) provided by Dr. Tom Louie (Calgary, AB, Canada). The toxin production and extraction was completed as previously described [27]. The protein concentration of the final TcdA/B mixture was determined by Bradford assay, and this mixture was used as the toxin source for all experiments.

Intestinal epithelial cell culture

Caco-2 colorectal adenocarcinoma cell line (ATCC, USA - cell #: HTB-37, designation: Caco-2) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, USA) with 20% fetal bovine serum (FBS; Invitrogen, USA), 1% penicillin-streptomycin solution (PenStrep; Invitrogen, USA), 1% non-essential amino acid solution (NEAA; Invitrogen, USA) and 1% sodium pyruvate solution (Invitrogen, USA). In some experiments, Caco-2 cells with stable expression of siRNA targeting HIF-1 α (HIF-1 α -KD) or the corresponding scrambled control (HIF-1 α -SCR) were used for some experiments. The chosen siRNAs with the sequence 5'-ACC TCG CTG ACC AGT TAT GAT TGT GAT CAA GAG TCA CAA TCA TAA CTG GTC AGC TT-3' and 5'-CAA AAA GCT GAC CAG TTA TGA TTG TGA CTC TTG ATC ACA ATC ATA ACT GGT CAG CG-3' corresponding to position 2666–2685 of the HIF-1 α gene were used as previously described [18]. HIF-1 α -KD and HIF-1 α -SCR cells were cultured in DMEM (Cellgro – Mediatech, USA), with 10% FBS, 1% PenStrep, 1% L-glutamine (Invitrogen, USA) and 3 μ g/mL of puromycin (Invitrogen, USA). Maintenance of the HIF-1 α -KD and HIF-1 α -SCR Caco-2 cells followed the same protocol as described for the wild-type Caco-2 cells. Cell cultures were incubated at 37 °C under 5% CO₂ in 100 cm² Petri dishes and used for experiments at 90% confluence.

Caco-2 cell treatment and extraction of cellular proteins

Monolayers were treated with: *C. difficile* TcdA/B toxin mixture, the selective iNOS inhibitor 1400W dihydrochloride (1400W, dissolved in PBS; SIGMA-Aldrich, USA), or the ROS scavenger, N-acetyl L-cysteine (NAC, dissolved in PBS; SIGMA-Aldrich, USA). For the majority of the experiments, TcdA/B toxin was added at a concentration of 70 μ g/mL. Treatments (i.e., 1400W [28], 100 μ M and NAC, 25 mM) were added 1 hour prior to the addition of TcdA/B. Cells were lysed with HEN/2 buffer (125 mM HEPES, pH 7.7, 0.5 mM EDTA, and 50 mM neocuproine with 0.2% (v/v) NP-40 and Complete Protease Inhibitor). Supernatants, collected via centrifugation at 17,500g (i.e., cytosolic fraction), were collected. Pellets were reconstituted in 150 μ L nuclear extract buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% (v/v) glycerol, and 0.1 mM neocuproine). The reconstituted pellets were then incubated for 2 hours at 4 °C, followed by high-speed centrifugation at 17,500g for 5 minutes at 4 °C. The resulting supernatant represented the nuclear fraction of the cell. Due to the light-sensitive nature of NO and its associated modifications, all samples were prepared in opaque Eppendorf tubes.

Immunoprecipitation of HIF-1 α

Protein G agarose bead slurry (25 μ L; diluted 1:2 with HEN/2 buffer) was combined with nuclear extract sample or colonic tissue homogenate, pre-cleared at 4 °C for 15 min and then centrifuged at 17,500g. The protein concentration of the resulting supernatant was determined by Bradford assay, and samples were normalized based on protein concentration. Each sample was incubated with HIF-1 α antibody (1 μ g; ab1/H1 α 67, Abcam, USA) at 4 °C for 12 hours with gentle rocking. Protein G agarose (25 μ L) was added, and the beads were collected by centrifugation. The supernatant was carefully extracted and discarded, and the beads were washed 4 times with ice-cold PBS. The HIF-1 α -immunoprecipitates were used immediately for immunoblotting or the biotin-switch assay.

Immunoblotting

Western blotting was performed on cell extracts or mouse tissue homogenates. Membranes were incubated with a specific primary antibody diluted 1:1000 in TBST containing 5% milk overnight at 4 °C. The following primary antibodies were used: HIF-1 α (ab1/h1 α 67 – Abcam, USA), iNOS (ab3523 – Abcam, USA), FIH-1 (ab63163/HIF1AN – Abcam, USA), and β -actin (A5316/AC-74 – SIGMA Aldrich, USA). The following secondary antibodies were used: HRP conjugated goat anti-mouse IgG (Bio-Rad, USA) and HRP-conjugated donkey anti-rabbit IgG (GE Healthcare, USA).

Biotin Switch Assay

The biotin switch assay was conducted using the S-nitrosylated protein detection kit (Cayman Chemical, USA). The protocol was performed in very low-light conditions to protect the labile, nitrosylated residues. The manufacturer's suggested protocol was modified slightly to incorporate HIF-1 α immunoprecipitation samples instead of whole cell extracts. In brief, the PBS-suspended HIF-1 α immunoprecipitates were centrifuged (200g, 4 °C) to pellet the G protein agarose beads. The PBS was slowly decanted off the beads, and the samples were placed on ice. Blocking Buffer was added to each sample (250 μ L of Blocking Buffer for 25 μ L of beads) prior to incubation at 4 °C for 30 minutes. The beads were then washed extensively with ice-cold PBS before incubation with Reducing and Labeling Buffer for 1 hour at room temperature. After extensive washing with PBS, the supernatant was decanted from the beads and discarded. An equal volume of 2X Non-reducing Buffer was added to the samples and then heated to boiling for 5 minutes, placed on ice for 5 minutes, and then centrifuged at 17,500g. The supernatant was carefully decanted from the beads and then loaded onto a 10% SDS-PAGE gel. The gel was then subjected to standard immunoblotting protocol. Membranes were blocked overnight in 5% (w/v) bovine serum albumin (BSA) in TBST. This was followed by incubation with Streptavidin-HRP (Millipore, USA) at 1:1000 dilution in 5% (w/v) BSA in TBST.

Nitric oxide detection assay

For the detection of NO in cytosolic cell extracts, the Bioxytech Nitric Oxide Assay kit (OXIS Health Products Inc., USA) was used. The concentration of nitrite in each sample of cell extract was determined from the standard curve and then related to the protein content of each sample as determined previously by the Bradford assay.

In vivo model of C. difficile toxin exposure

C57/BL6 mice (male; 10-12 weeks; Charles River, Sherbrooke, QC, Canada) were used. *C. difficile* toxins were provided by intrarectal instillation as previously described [29]. TcdA/B (50 mg; 100 μ L in PBS) or control saline (PBS alone) was slowly administered over 30 seconds while pressure was applied to the anal area to prevent leakage. All animal experiments were approved by the Animal Care Committee of the University of Calgary and conform to the guidelines set forth by the Canadian Council for Animal Care. The health and welfare of each mouse was assessed in 30 min intervals until the animals were euthanized.

Statistics

All numerical data are reported as the mean $\pm$ standard error of the mean (SEM). For some immunoblotting experiments, representative duplicate samples are shown in figures; however, these were combined and considered to be $n = 1$ experimental value. All graphical data and associated statistical analyses were generated with GraphPad PRISM 4 (GraphPad, USA). Statistics for experiments comparing 2 sets of parametric data were calculated using the Student's t-test. Experiments comparing more than 2

Fig. 1. Exposure of Caco-2 epithelial cell monolayers to *C. difficile* toxins elicits alterations in iNOS, FIH-1 and HIF-1 α expression as well as HIF-1 α nitrosylation. Caco-2 monolayers were exposed to *C. difficile* toxins (TcdA/B, 70 μ g/mL) for 2–12 h with control (vehicle) cells harvested at 12 h. Cell extracts were examined for iNOS (A), HIF-1 α protein (B) and nitrosylation (C), and FIH-1 (D). Expression levels of iNOS and FIH-1 were normalized against β -actin as a loading control. HIF-1 α nitrosylation was measured by the biotin switch assay on immunoprecipitates with the data presented as biotin switch signal relative to HIF-1 α protein levels. Values are reported as mean $\pm$ S.E.M., $n=3$. * - significantly different from control (one-way ANOVA with Neuman-Keuls *post hoc* test, $p < 0.01$).

sets of parametric data were calculated using a 1-way ANOVA followed by the Neuman-Keuls *post-hoc* test. Statistical significance was assigned to values where $p < 0.05$ between groups.

Results

In a previous study, intestinal HIF-1 α expression and signaling was linked to NO generated by iNOS [27]. We further assessed the association among *C. difficile* toxin exposure, iNOS expression and HIF-1 α stabilization in Caco-2 IECs. A 2.4-fold increase in iNOS expression relative to control was observed at 4 hours exposure of Caco-2 IECs with *C. difficile* toxins (Fig. 1A). Elevated levels of NO were also detected in these cells suggesting that the increase in iNOS expression was linked to a direct increase in NO production. With respect to HIF-1 α , an increase in the expression relative to control (no toxin exposure) was also observed at 4 hours (Fig. 1B), indicating a possible correlation with changes in iNOS expression and cellular NO concentrations. As previously identified, nitrosylation of HIF-1 α on specific cysteine residues can effectively stabilize the cellular protein levels [22]. Furthermore, nitrosylation of other modulators of HIF-1 signaling have also been suggested, including von-Hippel Lindau protein (pVHL; [23, 30]), factor inhibiting HIF-1 (FIH-1; [23]

Fig. 2. Inhibition of iNOS suppresses HIF-1 α protein stabilization and nitrosylation as well as changes in FIH-1 expression in Caco-2 IEC monolayers exposed to *C. difficile* toxins. Caco-2 cell monolayers were treated with 1400W (100 μ M, 4 h), TcdA/B (70 μ g/mL, 4 h) or 1400W (100 μ M) with TcdA/B (70 μ g/mL, 4 h). (A), NO production was quantified as the concentration of nitrite in cell extracts using a modified Griess assay. Cell extracts were immunoblotted for HIF-1 α (A) and FIH-1 (B), and expression levels were normalized to β -actin as a loading control. Nitric oxide was quantified as the concentration of nitrite in cell extracts using a modified Griess assay (C), and iNOS protein levels (D) were examined by immunoblotting. In (E), HIF-1 α was immunoprecipitated from nuclear extracts and then subjected to the biotin switch assay

for measurement of protein nitrosylation. The data are presented as biotin switch signal relative to HIF-1 α protein levels. Values are reported as mean $\pm$ S.E.M., n=4. * - significantly different from control; # - significantly different from TcdA/B exposure (one-way ANOVA with Neuman-Keuls *post hoc* test, $p < 0.01$). In (F), Caco-2 cell monolayers were examined by immunocytochemistry of ZO-1 staining for epithelial tight junction integrity. ZO-1 immunostaining was visualized with fluorescence microscopy of Cy3-conjugated secondary antibody. The images are representative of n=3 independent treatments of different Caco-2 monolayers. Scale bar = 100 μ m.

and thioredoxin [31]. So we also examined if *C. difficile* toxin exposure influenced HIF-1 α nitrosylation levels with a Biotin Switch Assay (Fig. 1C). Levels of nitrosylated HIF-1 α reached a peak at 4 hours suggesting that its accumulation occurred coincident with *C. difficile* toxin-dependent increases in iNOS expression and NO production. FIH-1, a known inhibitor of HIF-1 signaling, was also analyzed following exposure of cells to *C. difficile* toxins. Over the course of a 12 hour exposure, Caco-2 IEC monolayers experienced a significant decrease in FIH-1 protein levels when compared to control beginning at the 4 hour mark and continuing through to 12 hours (Fig. 1D).

The results of experiments presented in Figure 1 suggested that the TcdA/B effects on iNOS expression could influence HIF-1 α stabilization via a feed-forward mechanism. The highly selective iNOS inhibitor, 1400W, was applied in order to verify that HIF-1 α nitrosylation, and therefore HIF-1 α protein stabilization, was directly linked to NO derived from iNOS. Similar to previous experiments, we observed significant stabilization of HIF-1 α protein levels following exposure of Caco-2 IEC monolayers to TcdA/B for 4 hours (Fig. 2A). In addition, FIH-1 expression was reduced with TcdA/B exposure (Fig. 2B). However, when cells were pre-incubated with 1400W and then exposed to TcdA/B, HIF-1 α expression

Fig. 3. HIF-1 α protein expression is not induced with CoCl₂ exposure in Caco-2 cells stably expressing HIF-1 α siRNA. Scrambled (HIF-1 α -SCR) or knockdown (HIF-1 α -KD) HIF-1 α siRNA cells were treated with CoCl₂ (0.5 mM, 4 hr). Whole cell extracts were generated and immunoblotted for HIF-1 α . Expression levels of HIF-1 α were normalized against β -actin as a loading control. Values are reported as mean $\pm$ S.E.M., n=3. * - significantly different from vehicle control; (one-way ANOVA with Neuman-Keuls *post hoc* test, p < 0.01).

was suppressed (to control levels; Fig. 2A) and FIH-1 expression was elevated (returned to control levels; Fig. 2B). Coincident with these responses, NO and iNOS expression levels (Fig. 2C and 2D, respectively) returned to basal levels in the presence of 1400W. Finally, the nitrosylation of HIF-1 α , which was elevated 2.5-fold above control with TcdA/B exposure, was also attenuated with iNOS inhibition (Fig. 2E). These results suggest that NO generated by iNOS in response to *C. difficile* toxin exposure plays a key role in modulating the levels of both HIF-1 α and FIH-1 proteins.

C. difficile toxins influence the stability of the cytoskeleton (via glycosylation of the Rho family of small G-proteins [32]) and cause disruption of tight junctions between adjacent intestinal epithelial cells [7]. A key tight junctional protein found along the periphery of IECs is zonula occludens (ZO)-1 [33], and we assessed the integrity of epithelial tight junctional complexes upon exposure to *C. difficile* toxins with immunocytochemistry of ZO-1 (Fig. 2F). Control monolayers displayed the characteristic membrane-distributed staining of ZO-1. Exposure to *C. difficile* toxins resulted in noticeable changes to the continuity of the monolayer, cellular morphology (e.g., the cells were reduced in size with some rounding occurring at the edges) and dramatic reorganization of ZO-1 staining. Application of 1400W had no effect on cellular structure or tight junctional integrity as neither ZO-1 staining nor appearance of the monolayer was altered. However, administration of 1400W in concert with *C. difficile* toxins appeared to exacerbate disruptions in monolayer morphology and tight junctional integrity. ZO-1 staining became increasingly diffuse and cytosolic in nature, and cells were greatly reduced in size with significant rounding with almost complete disruption of the monolayer.

From the data presented, NO produced by iNOS appears to be important for the modulation of HIF-1 α stabilization and signaling during exposure of Caco-2 IECs to *C. difficile* toxin. The regulation of iNOS expression by HIF-1 has been previously reported [16, 34], so we employed Caco-2 IECs with constitutive siRNA-mediated knockdown of HIF-1 α (HIF-KD) [35] to determine whether TcdA/B effects on iNOS were HIF-1 α -dependent. Prior to performing any experiments, we confirmed that the HIF-KD cells, along with scrambled siRNA (HIF-SCR) control cells, demonstrated the appropriate HIF-1 α response. Only the HIF-SCR Caco-2 cells showed the expected increase in HIF-1 α (Fig. 3) when a HIF-1 α stabilizing compound (i.e., CoCl₂) was applied. It should be noted that these cells do not represent a genetic deletion of HIF-1 α ; therefore it is not surprising that some basal level of HIF-1 α protein expression was present in the HIF-KD cells. Upon administration of TcdA/B, HIF-1 α protein levels were increased in the HIF-SCR cells (Fig. 4A, panel *iii*) but not in the HIF-KD cells (Fig. 4B, panel *iii*). Exposure to *C. difficile* toxins caused a significant increase in both iNOS expression and NO availability in the HIF-SCR cells (Fig. 4A, panels *i* and *ii* respectively), but the increases in NO and iNOS expression were not observed in HIF-KD cells (Fig. 4B, panels *i* and *ii*). A lack of measurable increase in HIF-1 α nitrosylation coincided with the absence of changes in NO in the HIF-KD cells (Fig. 4B, panel *iv*). On the surface, these findings suggest a possible

Fig. 4. N-acetylcysteine treatment restricts the stabilization of HIF-1 α protein in Caco-2 cells exposed to *C. difficile* toxins. Monolayers of Caco-2 cells (stably expressing HIF-1 α -SCR (A) or HIF-1 α -KD (B) siRNAs) were treated with a ROS scavenger (N-acetylcysteine, NAC; 25 mM, 4 h), TcdA/B (70 μ g/mL, 4 h) or NAC (25 mM) with TcdA/B (70 μ g/mL, 4 h). NO production was quantified as the concentration of nitrite in cell extracts using a modified Griess assay, (A, i & B, i). Cytosolic extracts were immunoblotted for iNOS, (A, ii & B, ii) and HIF-1 α , (A, iii & B, iii), and expression levels were normalized to β -actin as a loading control. HIF-1 α was immunoprecipitated from nuclear extracts and then subjected to the biotin switch assay for measurement of nitrosylation, (A, iv & B, iv). The data are presented as biotin switch signal relative to HIF-1 α protein levels. Values are reported as mean $\pm$ S.E.M., n=4. * - significantly different from control (one-way ANOVA with Neuman-Keuls *post hoc* test, $p < 0.01$).

feedback loop between *C. difficile* toxin-stimulated iNOS expression and NO production and the nitrosylation and stabilization of HIF-1 α protein in intestinal epithelial cells.

Previous studies have also demonstrated that exposure to *C. difficile* can result in elevated concentrations of ROS *in vivo* [36]. NO signals can be neutralized by compensatory accumulations of ROS [21, 26, 37]. So, it was important to consider the role of ROS in our system. To do this, we examined whether the addition of a ROS-scavenging compound (i.e., N-acetylcysteine; NAC) could increase the bioavailability of NO for the nitrosylation of HIF-1 α . Interestingly, NAC stimulation of HIF-SCR cells alone or in combination with *C. difficile* toxins caused increases in NO levels (Fig. 4A, panel i); however, no alteration in iNOS expression was observed when NAC was provided in the absence of TcdA/B (Fig. 4A, panel ii). The addition of NAC suppressed increases in HIF-1 α expression observed with TcdA/B exposure (Fig. 4A, panel iii), yet no effect on HIF-1 α nitrosylation was observed with administration of NAC during toxin exposure (Fig. 4A, panel iv). The HIF-KD cells generally showed no remarkable effects of NAC treatment. However, HIF-1 α expression was suppressed with exposure to TcdA/B in the HIF-KD cells (Fig. 4B, panel iii), and no change from control was observed when NAC was provided. In summary, NAC was able to increase availability of NO in Caco-2 cells. Notwithstanding that less HIF-1 α protein was present in the cell during exposure to both TcdA/B and NAC, there appeared to be a relative increase in the proportion that was nitrosylated.

The effects of *C. difficile* toxin on FIH-1 responses in Caco-2 HIF-SCR cells were also assessed (Fig. 5A). FIH-1 levels were depressed by approximately 40% relative to control treatments, and NAC treatment in the absence or presence of TcdA/B had no effect on FIH-1

Fig. 5. N-acetylcysteine treatment has no effect on the *C. difficile* toxin-dependent suppression of FIH-1 protein expression in Caco-2 cells. Caco-2 cell monolayers, stably expressing HIF-1 α -SCR (A) or HIF-1 α -KD (B) siRNAs, were treated with a ROS scavenger (N-acetylcysteine, NAC; 25 mM, 4 h), TcdA/B (70 μ g/mL, 4 h) or NAC (25 mM) with TcdA/B (70 μ g/mL, 4 h). Following treatments, cell extracts were immunoblotted for FIH-1, and expression levels were normalized to β -actin as a loading control. Values are reported as mean $\pm$ S.E.M., n=3. * - significantly different from control (one-way ANOVA with Neuman-Keuls *post hoc* test, $p < 0.01$).

expression levels. Caco-2 HIF-KD cells also experienced a 50% decrease in FIH-1 protein with application of either TcdA/B alone or NAC plus TcdA/B (Fig. 5B). As described earlier, we also assessed the membrane-distributed staining of ZO-1 as a marker of monolayer integrity. In this case, Caco-2 HIF-KD cells were exposed to *C. difficile* toxins in the absence and presence of NAC (Fig. 5C). Control Caco-2 HIF-SCR monolayers displayed similar responses to *C. difficile* toxin (data not shown) as shown for the WT Caco-2 cells. Simultaneous exposure of HIF-KD cells to *C. difficile* toxins and NAC appeared to elicit differences in cellular morphology and ZO-1 staining characteristics. Cellular structures were more defined and some retention of ZO-1 pericellular immunostaining was observed.

Given the effects observed *in vitro*, we further explored whether HIF-1 α nitrosylation could be demonstrated in response to *C. difficile* toxin exposure *in vivo*. Mice were provided with TcdA/B or saline control *per rectum* as described previously [29]. With the intra-rectal administration of *C. difficile* toxins, mice demonstrated a significant increase in HIF-1 α protein levels in the colonic tissue (Fig. 6A). The increase in HIF-1 α protein levels was also correlated with a significant increase in nitrosylated HIF-1 α (Fig. 6B), suggesting that the stabilization of HIF-1 α via NO also occurs *in vivo*.

Discussion

These results elaborate on previous studies conducted on HIF-1 α in the context of *C. difficile*-induced intestinal injury. It was proposed that the NO generated as a result of *C. difficile* toxin-induced damage acted as an important signal to stimulate the protective capacity of HIF-1 α signaling in the gastrointestinal tract [27]. In the current investigation, it was demonstrated that HIF-1 α stabilization was dependent upon NO generated by iNOS during *C. difficile* toxin exposure. In Caco-2 intestinal epithelial cells, TcdA/B was capable of inducing iNOS expression, which in turn, was associated with subsequent increases in NO.

Fig. 6. Intrarectal administration of *C. difficile* toxin increases HIF-1α nitrosylation and stabilization. *C. difficile* toxin (TcdA & TcdB; 100 μg in 100 μL) was administered *per rectum* to C57Bl/6 mice. Control mice were provided 100 μL of PBS as a vehicle control. Colonic tissue homogenates were immunoblotted for HIF-1α (A); expression levels were normalized to β-actin as a loading control. In (B), HIF-1α was immunoprecipitated from tissue homogenates (200 μg total protein) and subjected to the biotin switch assay. The data are presented as biotin switch signal relative to HIF-1α protein levels. Values are reported as mean ± S.E.M., n=3. * - significantly different from vehicle control (Student's t-test, p < 0.05).

When cells were pre-treated with the selective iNOS inhibitor, 1400W, the increases observed for both iNOS and HIF-1α were effectively abolished along with the increase in NO itself. Further examination of the HIF-1α subunit demonstrated that the protein was subjected to nitrosylation when Caco-2 IEC monolayers were exposed to TcdA/B. Moreover, isolation of HIF-1α from an *in vivo* model of *C. difficile* intoxication also revealed nitrosylation events that correlated with increases in HIF-1α stabilization. These findings suggest that NO generated via iNOS is necessary for the nitrosylation and stabilization of HIF-1α and are particularly important as they represent the mechanism whereby HIF-1α protein levels are elevated to offer innate protection in the presence of *C. difficile* toxins.

Other studies have identified that *C. difficile* toxins and other actin cytoskeleton-disrupting agents are capable of inducing iNOS expression [38-40]. It was demonstrated that iNOS expression was induced in the presence of cytokines during TcdB exposure [39]. TcdA is also capable of rapidly inducing cytokine and chemokine production, the increases seen as early as 10 minutes with a peak response occurring after 2 hours of toxin exposure [41]. These data correspond with our observed increases in iNOS induction that occur after 4 hours of toxin exposure. A number of studies have identified NF-κB as being a major inducer of cytokine-mediated iNOS expression [41, 42]. However, iNOS can also be induced by disruption of the actin cytoskeleton in an NF-κB-independent fashion [40]. Since the major action of the Clostridial toxins is to facilitate the disruption of the cytoskeleton, it is possible that *C. difficile* toxins induce iNOS expression independent of NF-κB signaling.

In addition to these mechanisms, the data gathered herein from Caco-2 HIF-KD cells reveal a positive feedback nature of the iNOS/HIF-1 regulation in the context of *C. difficile* toxin exposure. When treated with Clostridial toxins, WT Caco-2 and HIF-SCR cells respond with increased HIF-1α stabilization and iNOS induction. In contrast, HIF-KD cells that displayed deficiencies in HIF-1α induction, toxin exposure was accompanied by suppressed HIF-1α responses and iNOS induction. With HIF-1α implicated in a number of cellular processes, it comes as no surprise that the iNOS gene also contains a hypoxia response element for HIF-1 transcriptional regulation within its promoter region [16]. Additional studies investigating the HIF-1α-mediated induction of bactericidal factors (which include NO) identified mice with macrophages deficient in HIF-1α to harbor greater amounts of colonized bacteria compared to WT, suggesting NO production and bactericidal responses to be suppressed by the lack of HIF-1α [14]. Taken together, these findings support a requirement for HIF-1α

signaling in the induction of iNOS expression as an innate protective response to *C. difficile* toxin exposure. Moreover, our data suggests that a positive feedback loop exists in this signaling pathway, with NO derived from the increased iNOS expression providing additional HIF-1 signaling capacity via nitrosylation and stabilization of HIF-1 α protein levels.

It is important to note that elevations in NO are frequently associated with concurrent increases in ROS production. In the context of *C. difficile* intoxication, both TcdA and TcdB are capable of causing significant increases in ROS levels [36, 43]. As a therapeutic, NAC has been shown to possess cytoprotective properties by acting as a potent ROS scavenger in a variety of experimental models [44]. NAC has been demonstrated to impair cellular intoxication during TcdB exposure with the protective effect of NAC hypothesized to result from its ability to alter the redox environment and provide stabilization of cytoskeletal integrity [45]. TcdB exposure of human epithelial cells created an oxidizing cellular environment (represented by an increase in oxidized glutathione), and this oxidative imbalance as well as cytoskeleton-dependent cell retraction and rounding was rectified with NAC treatment. Based on our results, it appears that ROS also interacts with the NO/iNOS system to promote the nitrosylation and stabilization of protective HIF-1 α signaling. Assuming that NAC acts as a ROS scavenger in our system, the findings suggest that ROS may be an essential factor in the stabilization of HIF-1 α in the context of *C. difficile* toxin exposure.

FIH-1, as mentioned previously, is asparagine hydroxylase enzyme that is involved in the suppression of HIF-1 downstream transcriptional activity. Our experiments demonstrate a possible novel connection between NO and FIH-1. Namely, NO may be involved in destabilizing FIH-1 expression to hinder its hydroxylation of HIF-1 α . Studies previously demonstrated that NO donors could inhibit FIH-1 activity, which subsequently promoted interactions of HIF-1 α with its transcriptional co-activators [23]. It was initially suspected that nitrosylation of the Cys-800 site on HIF-1 α protected against the asparagine hydroxylation by FIH-1; however, the data of Park et al. [23] suggest NO could directly inhibit the hydroxylase activity of FIH-1 itself. These data support our findings concerning FIH-1 regulation in the presence of TcdA/B and 1400W. However, in the HIF-KD cells, NO levels were not altered, yet FIH-1 expression was still suppressed in both TcdA/B and NAC + TcdA/B treatments. Since NAC alone does not affect FIH-1 expression, it is also possible that FIH-1 expression was suppressed by TcdA/B in an NO-independent manner.

In another model of intestinal injury, the integrated actions of NO and HIF-1 were investigated in rats that were subjected to intestinal damage with indomethacin [46]. iNOS, HIF-1 and intestinal trefoil factor (ITF; a downstream gene target of HIF) were found to be elevated, and HIF-1 α was also shown to be nitrosylated in the animals that were provided the indomethacin treatment. Moreover, when rats were given 1400W in conjunction with indomethacin, a reduction in the amount of HIF-1 α detected was observed. This attenuation did not significantly modify the damage induced by indomethacin; however, it did serve to impede ITF function with respect to epithelial barrier restitution. This resulted in a delayed healing response in mice treated with both 1400W and IM, compared to those treated with just indomethacin. Taken together the findings of this study suggest that the decrease in HIF-1 α nitrosylation (and subsequent stabilization) was connected to a decrease in barrier restitution via HIF-1-mediated transcriptional activation of ITF. These findings agree with our study in that iNOS is necessary for the induction of HIF-1 α stabilization via nitrosylation. Therefore it is possible that the decrease in stabilized HIF-1 α has a greater consequence with respect to downstream signaling and wound healing following *C. difficile* toxin exposure rather than the immediate damage response. Finally, a recent study demonstrated that NO produced in response to *C. difficile* elicited nitrosylation of *C. difficile* toxins themselves [30]. The trans-nitrosylation of different cysteine residues, including ones located in the catalytic cysteine protease region, were shown to inhibit both autocatalysis and cell entry of the toxin [30]. These studies, along with our current study, demonstrate the increasing importance of NO in mounting a crucial cellular defense response to *C. difficile* toxins.

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Abbreviations

CDI (*C. difficile* infection); HIF (hypoxia-inducible factor); FIH (factor inhibiting HIF); NO (nitric oxide); iNOS (inducible nitric oxide synthase); NAC (N-acetylcysteine); ROS (reactive oxygen species); TcdA (Toxin A); TcdB (Toxin B).

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