**Clostridium difficile** Toxin A Inhibits Erythropoietin Receptor-Mediated Colonocyte Focal Adhesion Through Inactivation of Janus Kinase-2

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Previously, we demonstrated that the erythropoietin receptor (EpoR) is present on fibroblasts, where it regulates focal contact. Here, we assessed whether this action of EpoR is involved in the reduced cell adhesion observed in colonocytes exposed to *Clostridium difficile* toxin A. EpoR was present and functionally active in cells of the human colonic epithelial cell line HT29 and epithelial cells of human colon tissues. Toxin A significantly decreased activating phosphorylations of EpoR and its downstream signaling molecules JAK-2 (Janus kinase 2) and STAT5 (signal transducer and activator of transcription 5). *In vitro* kinase assays confirmed that toxin A inhibited JAK 2 kinase activity. Pharmacological inhibition of JAK2 (with AG490) abrogated activating phosphorylations of EpoR and also decreased focal contacts in association with inactivation of paxillin, an essential focal adhesion molecule. In addition, AG490 treatment significantly decreased expression of occludin (a tight junction molecule) and tight junction levels. Taken together, these data suggest that inhibition of JAK2 by toxin A in colonocytes causes inactivation of EpoR, thereby enhancing the inhibition of focal contact formation and loss of tight junctions known to be associated with the enzymatic activity of toxin A.

**Keywords:** *Clostridium difficile*, toxin, gut inflammation, epithelial cell adhesion, erythropoietin receptor, JAK/STAT pathways

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*Clostridium difficile* is the most common known cause of antibiotic-associated diarrhea and pseudomembranous colitis in humans [8, 13, 14, 16, 19, 24-26, 29]. *C. difficile* produces intestinal damage and diarrhea by releasing two exotoxins, A and B [10]. Toxin A elicits acute enteritis and fluid secretion in several animal species. Toxin A has glucosyltransferase activity, which monoglucosylates the small GTPases Rho, Rac, and Cdc42 at threonine 37, resulting in actin disaggregation [9–11] and microtubule depolymerization [22]. Massive colonic epithelial detachment induced by toxin A is associated with loss of cell-matrix adhesion, considered a cause of acute inflammation [13]. However, the signal transduction pathways by which toxin A induces detachment of epithelial cells and barrier dysfunction are not entirely known.

Erythropoietin (Epo), the principal growth factor responsible for promoting the viability, proliferation, and differentiation of mammalian erythroid precursor cells, exerts its effects by binding to the erythropoietin receptor (EpoR) [6, 18, 23, 31]. Binding of Epo to EpoR induces a conformational change in the receptor complex and triggers a cascade of intracellular events that includes signaling by Janus kinase 2 (JAK2) and signal transducer and activator of transcription 5 (STAT5) [28]. In hematopoietic cells, this cascade stimulates the rapid formation of functional red blood cells [23]. However, EpoR is also expressed in nonhematopoietic cells, including endothelial cells and cells of the central nervous system, where it is mainly involved in regulating cell proliferation [5]. Our recent study also demonstrated that the Epo/EpoR complex increases paxillin phosphorylation and focal contact in rat fibroblasts [12].
Here, we investigated the possible role of EpoR in cell adhesion of colonocytes and changes in EpoR signaling in the loss of cell adhesion of colonocytes following toxin A exposure. We found that EpoR was present and active in human colonic epithelial cells and its activity was inhibited by toxin A. We also found that inactivation of EpoR by toxin A appears to require reactive oxygen species (ROS), which are known to inhibit the kinase activity of JAK2 [4, 7, 17, 20] and are highly produced in colonocytes exposed to toxin A [14]. Consistent with our previous report that EpoR regulates fibroblast cell adhesion [12], the results of the present study also suggest that EpoR, which contributes to focal contacts and tight junctions of colonocytes, is targeted by toxin A, initiating a process that may potentiate the decrease in cell adhesion and tight junctions.

MATERIALS AND METHODS

Toxin A Preparation and Cell Culture
Toxin A was purified from C. difficile strain VPI 10463 (American Type Culture Collection, Manassas, VA, USA) as described previously [14]. The purity of native toxin A was assessed by gel electrophoresis, which confirmed a single protein with the expected molecular mass of 307 kDa. HT29 and CaCo2 cells derived from human colorectal adenocarcinoma were maintained in McCoy’s 5A medium (Invitrogen, Carlsbad, CA, USA) and Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), respectively. Cells were cultured in a 37°C humidified incubator with 5% CO2 [2].

Antibodies and Reagents
Polyclonal antibodies against EpoR, phospho-EpoR, phospho-ERK2, phospho-Smad 2/3, occludin, and tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies against phospho-paxillin (Tyr-118), total JAK2, phospho-JAK2 (Ty-1007/1008), and phospho-STAT5 (Tyr-694) were from Cell Signaling Technology (Beverly, MA, USA). The β-actin antibody, hydrogen peroxide (H2O2), recombinant α-tubulin protein, AG490 (JAK2 inhibitor), erythropoietin (Epo), and uridine diphosphate (UDP)+2'-3'-dialdehyde (monoglucosyltransferase inhibitor) [13] were from Sigma-Aldrich (St. Louis, MO, USA).

Measurement of Transepithelial Resistance (TER)
Colonocytes (CaCo 2 cells) were cultured on polycarbonate filters (Transwells; Costar, Cambridge, MA, USA) and incubated until reaching confluence (5 days). After exposing the cells to toxin A or AG490 for 3 h, TER was measured with a Millicell ERS Volt-Ohm Meter (Millipore, Bedford, MA, USA) [22]. The resistance of the supporting membrane and medium was subtracted from all readings.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)
Total RNA (1 µg) was reverse transcribed using a GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA, USA). Human EpoR was amplified from cDNA using the primer pair 5'-GCT CCC TTT GTC TCC TGC T-3' (sense) and 5'-CTC CCA GAA ACA CAC CAA GTC CT-3' (antisense). β-Actin was amplified as an internal control. PCR was conducted using the thermocycling conditions of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with an empirically optimized number of cycles (i.e., conditions under which generated PCR products did not reach saturation). PCR products were separated on agarose gels and stained with ethidium bromide [3].

Immunoblot Analysis
Colonocytes were washed with cold phosphate-buffered saline (PBS), and then lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% Nonidet P-40). Equal amounts of protein were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels. Antigen–antibody complexes were detected with LumiGlo reagent (New England Biolabs, Ipswich, MA, USA) [16].

Preparation of Human Tissue Samples and Immunohistochemistry
Tissue samples were obtained from three colorectal cancer patients undergoing surgery at the Chungnam National University Medical Center (Daejeon, Korea). Normal tissues were harvested according to an institutional review board-approved protocol. Samples were immediately frozen in liquid nitrogen and stored at −80°C [12]. Tissue sections were heated in a microwave (for antigen retrieval), followed by incubation with 1.5% normal horse serum for 30 min in a humidifier (to block nonspecific protein binding). The sections were then incubated with polyclonal rabbit anti-phospho-EpoR antibody (diluted 1:200) followed by incubation with a biotinylated secondary antibody for 2 h. An avidin–biotinylated enzyme complex (ABC) kit and a 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA, USA) were used to process the sections. The sections were counterstained with hematoxylin, fixed, and then mounted and observed under a microscope.

Immunofluorescence Staining and Laser-Scanning Confocal Microscopy
For visualization of focal adhesions, colonocytes (HT29) grown on glass coverslips were exposed to control medium, toxin A (1 µg/ml), or AG490 (50 µM) [30] for 3 h and then fixed in 4% paraformaldehyde dissolved in PBS. Cells were permeabilized with 0.5% Triton X-100 and then blocked with PBS containing 5% bovine serum albumin (BSA). After washing with PBS, cells were incubated with a polyclonal anti-phospho-paxillin antibody (diluted 1:500) in PBS containing 0.05% Tween 20 for 6 h at room temperature. After three washings, cells were incubated with FITC-labeled anti-mouse IgG (1:500) for 2 h at room temperature, and then mounted and analyzed using a Bio-Rad MRC 1024 laser-scanning confocal microscope equipped with a krypton/argon mixed-gas laser as a light source [12].

In Vitro Kinase Assay
Colonocytes were incubated with medium or toxin A for 1 h and immunoprecipitated with a protein A/G-agarose-conjugated antibody against JAK2 (Santa Cruz Biotechnology). Recombinant α-tubulin protein (Novus Biological, Littleton, CO, USA) was used as a substrate for immunoprecipitated JAK2. Immunoprecipitated JAK2, 3 µg of tubulin protein, and diluted [32P] ATP (specific activity, 3000 Ci/mmol; NEN Life Science Products, Boston, MA, USA) were mixed in kinase assay buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na2VO4, 10 mM MnCl2) and incubated for 30 min at 30°C. The reactions were stopped by the addition of 20 µl of SDS sample buffer, and radioactivity was detected by exposure of gels to X-ray film [13].
Statistical Analysis
The results are presented as mean values ± SEMs. Data were analyzed using the SIGMA-STAT professional statistics software program (Jandel Scientific Software, San Rafael, CA, USA). Analyses of variance with protected t-tests were used for intergroup comparisons.

RESULTS AND DISCUSSION

EpoR is Expressed and Functionally Active in Human Colonocytes
Because our previous study reported that EpoR was present on nonhematopoietic cells fibroblasts, where it regulated focal contact, we assessed whether human colonic epithelial cells also expressed functional EpoR. As shown in Fig. 1A, human colonic tissues from both males and females (age 54 to 70) exhibited marked expression of EpoR and showed activating phosphorylation at Tyr-456, indicative of a functional protein (Fig. 1A). RT-PCR revealed that EpoR mRNA, reflecting transcriptional regulation, was also detectable in human colonic tissues (Fig. 1B). Immunohistochemistry experiments using an anti-phospho EpoR antibody confirmed that active Tyr-456-phosphorylated EpoR was mainly present in epithelial cells of human colon, and not in muscle layers or connective tissues (Fig. 1C). To assess whether colonocyte EpoR was activated by Epo stimulation, HT29 cells were maintained in serum-free medium for 48 h and then incubated with 2 U/ml Epo for 1 h. As shown in Fig. 1D, EpoR was present and markedly phosphorylated by Epo stimulation in HT29 human colonic epithelial cells. Activating phosphorylations of JAK2 and STAT5, known as critical mediators of EpoR signaling, were also highly increased by Epo treatment, and activated forms of these proteins were significantly correlated with the level of activated EpoR. These results suggest that human colonic epithelial cells express functionally active EpoR.

C. difficile Toxin A Decreases Activation of EpoR and its Downstream Signaling Molecules in Human Colonocytes
Because C. difficile toxin A induces a decrease in cell adhesion and promotes subsequent cell rounding, we investigated whether EpoR, which is known to regulate focal contact of nonhematopoietic cells, is inactivated in colonocytes exposed to toxin A. As shown in Fig. 2A, toxin A exposure decreased activating phosphorylation of EpoR in a time-dependent manner. In HT29 cells exposed to toxin A for 6 h, EpoR phosphorylation was completely eliminated and remained for 8 h, whereas total EpoR protein levels were not affected. Toxin A had no effect on the phosphorylation levels of Smad2/3 (an essential regulator of TGF receptor signaling), suggesting that the
dephosphorylation of EpoR induced by toxin A was a specific cellular response. Toxin A also markedly decreased activating phosphorylations of JAK2 (Tyr-1007/1008) and STAT5 (Tyr-694), critical mediators of EpoR-dependent signal transduction; the decreases in phospho-JAK2 and phospho-STAT5 levels were directly correlated with EpoR inactivation following toxin A treatment (Fig. 2B). Similar to C. difficile toxin A, cholera toxin has been shown to catalyze ADP-ribosylation of cytoskeletal proteins, resulting in disorganization of the cytoskeleton [27]. On the basis of the similarity of the cellular toxicity of these toxins, we next assessed whether cholera toxin also induced inactivation of EpoR signaling in human colonocytes. However, cholera toxin did not affect EpoR phosphorylation in HT29 cells (Fig. 2C).

**EpoR Dephosphorylation by Toxin A is Dependent on Toxin A-Induced ROS but Not Toxin A Enzymatic Activity**

Toxin A has glucosyltransferase activity and induces monoglucosylation of Rho family proteins, thereby inactivating the Rho proteins and resulting in actin disaggregation [10]. To explore whether EpoR dephosphorylation by toxin A was associated with toxin A enzymatic activity, we pretreated HT29 cells for 1 h with UDP-2′3′-dialdehyde, which is known to inhibit the enzymatic activity of toxin A without inducing structural changes [13], and measured changes in EpoR phosphorylation following toxin A

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**Fig. 2.** *C. difficile* toxin A inhibits EpoR-dependent signaling pathways in human colonocytes. (A) HT29 cells were incubated with toxin A (TxA; 1 µg/ml) for the indicated times. Cell lysates were resolved on a 10% polyacrylamide gel and then probed with antibodies against phospho-EpoR, EpoR, phospho-Smad2/3, and β-actin. All results are representative of three separate experiments. (B) Blotted membranes were probed with antibodies against phospho-EpoR, JAK2, phospho-JAK2, phospho-STAT5, and β-actin. All results are representative of three separate experiments. (C) Cholera toxin had no effect on EpoR phosphorylation. Cells were incubated with cholera toxin (1 µg/ml) for the indicated times, and then cell lysates were probed with antibodies against phospho-EpoR, total EpoR, and β-actin.

**Fig. 3.** Toxin A-induced ROS generation is involved in EpoR dephosphorylation. (A) HT29 cells were exposed to medium (con), toxin A (TxA; 1 µg/ml) alone, UDP-2′3′-dialdehyde alone (UDP, 1 mM) [13], or UDP-2′3′-dialdehyde plus toxin A for 4 h. Cell lysates were resolved on a 10% polyacrylamide gel and probed with antibodies against EpoR, phospho-EpoR, phospho-JAK2, and β-actin. The results shown are representative of three separate experiments. (B) Colonocytes were treated with H$_2$O$_2$ (500 µM) for the indicated times, and the levels of phosphorylated EpoR were measured. All results are representative of three separate experiments.
exposure. As shown in Fig. 3A, toxin A markedly reduced activating phosphorylations of EpoR and JAK2, but these modifications were unaffected by UDP-2’3’-dialdehyde pretreatment. Because toxin A has been shown to rapidly cause H$_2$O$_2$ production as an early downstream signal [14], we next assessed whether direct exposure of HT29 cells to H$_2$O$_2$ decreased EpoR phosphorylation. Exposure of cells to H$_2$O$_2$ (500 µM) abrogated EpoR phosphorylation in a time-dependent manner and also significantly decreased the levels of activating phosphorylation of JAK2 (Fig. 3B). These results suggest that H$_2$O$_2$ produced by toxin A, but not the enzymatic activity of toxin A, is critical for the decrease in activation/phosphorylation of EpoR and JAK2. This observation is comparable to our previous finding that toxin A-induced colonocyte apoptosis is associated with ROS production but not with enzymatic activity [13]. Numerous reports have shown that oxidative stress attenuates or inhibits JAK activity in several classical cytokine

Fig. 4. Toxin A decreases EpoR phosphorylation through inhibition of JAK2.
(A) JAK2 was immunoprecipitated from colonocytes exposed to medium or toxin A (1 µg/ml) for 1 h using an antibody against JAK2. JAK2-containing immune complexes were incubated with [32P] ATP and recombinant α-tubulin protein (3 µg), and samples were analyzed by SDS-PAGE. Upper panel: autoradiography. Lower panel: densitometric quantitation of autoradiographs. All results are representative of three separate experiments. (B) Colonocytes were treated with AG490 (50 µM) or toxin A (TxA) for the indicated times. Cell lysates were resolved on a 10% polyacrylamide gel and probed with antibodies against phospho-EpoR, phospho-paxillin, phospho-ERK1/2, and β-actin. The results shown are representative of three separate experiments. (C) Colonocytes were incubated with medium (con), DMSO, AG490, or toxin A for 3 h, fixed in 4% paraformaldehyde for 20 min, and then immunostained with an anti-phospho-paxillin antibody. The images shown are representative of three separate experiments. (D) Upper panel: Colonocytes (CaCo2) were grown in Transwell inserts until reaching confluence and then incubated with medium, AG490, or toxin A for the indicated times. TER was measured with a Millicell ERS Volt-Ohm Meter. The results shown represent three independent experiments (*, p < 0.005 vs. medium-treated cells). Lower panel: Colonocytes were treated with AG490 for the indicated times. Cell lysates were resolved on a 10% polyacrylamide gel and probed with antibodies against occludin and β-actin. The results shown are representative of three separate experiments.
Toxin A Decreases EpoR Phosphorylation Through Inhibition of JAK2 and Thereby Influences Cell Adhesion and Tight Junctions in Human Colonocytes

The results presented above show that toxin A inhibits EpoR and its downstream signaling pathways in colonocytes, and suggest that this inhibitory effect of toxin A may be associated with suppression of JAK2 kinase activity (Fig. 2 and 3). To confirm this latter supposition, we next assessed whether toxin A inhibits the kinase activity of JAK2, the essential mediator of EpoR signal transduction. As shown in Fig. 4A, the JAK2 immune complex obtained from control cells (i.e., those exposed to culture medium only) mediated a marked increase in the tyrosine phosphorylation of α-tubulin. However, the kinase activity of JAK2 was significantly attenuated in cells exposed to toxin A. Inhibition of JAK2 activity with 50 µM AG490, a chemical inhibitor of JAK2, also abrogated EpoR phosphorylation in a time-dependent manner (Fig. 4B), decreasing phospho-EpoR to a level similar to that in cells exposed to toxin A. Neither AG490 nor toxin A affected phosphorylation of ERK (extracellular signal-regulated kinase) 1/2. Interestingly, paxillin, which is a focal adhesion molecule known to be inactivated by toxin A, was markedly dephosphorylated by AG490 treatment (Fig. 4B). As shown in Fig. 4C, exposure of colonocytes to toxin A caused a marked decrease in focal contact formation compared with untreated control cells. AG490 treatment also significantly reduced colonocyte focal contact. In vitro tight junction experiments further revealed that AG490 (50 µM) treatment significantly abrogated tight junction formation in human colonocytes (CaCo2 cells), although the degree of the reduction was less than that in cells exposed to toxin A (Fig. 4D, upper panel). Cells treated with AG490 also exhibited a marked decrease in the expression of occludin, a tight junction regulator (Fig. 4D, lower panel). Our previous study reported that toxin A caused a decrease in focal contact in colonocytes through Src inhibition mediated by direct binding to and subsequent inactivation of paxillin [15]. The current findings suggest that JAK2 is another intracellular target for toxin A and indicate that JAK2 inhibition is likely associated with enhancement of various cellular toxicities caused by toxin A. These results collectively suggest that inactivation of JAK2 by toxin A may be a critical step in EpoR signaling-pathway inhibition, and also contributes to the decreases in focal contact formation and tight junctions that are well known to be caused by the enzymatic activity of toxin A.

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