Clostridium difficile Toxin A Regulates Inducible Cyclooxygenase-2 and Prostaglandin E₂ Synthesis in Colonocytes via Reactive Oxygen Species and Activation of p38 MAPK*

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Clostridium difficile toxin A induces acute colitis with neutrophil infiltration and up-regulation of numerous pro-inflammatory mediators, but the contribution of cyclooxygenase-2 (COX-2) induction in this infection is unknown. We report here that toxin A induces expression of COX-2 and secretion of prostaglandin E₂ (PGE₂) in a dose- and time-dependent manner in cultured NCM460 human colonocytes and in human intestinal xenografts. This induction was blocked by SB203580, a p38 MAPK inhibitor, which also decreased the phosphorylation of MSK-1, CREB/ATF-1, and COX-2 promoter activity following toxin A stimulation. Gel shift assays indicated that CREB/ATF-1 was the major proteins binding to the COX-2-CRE. Moreover, colonocytes exposed to toxin A produced reactive oxygen species (ROS), which activated p38 MAPK, MSK-1, and CREB/ATF-1, leading to subsequent COX-2 induction and PGE₂ secretion. In intact mice, blockage of p38 MAPK inhibited toxin A-mediated induction of COX-2 in enterocytes as well as lamina propria cells, and significantly blocked the toxin A-induced ileal fluid of secretion and PGE₂. Furthermore, a selective COX-2 inhibitor also diminished toxin A-associated ileal fluid and PGE₂ secretion. The main signaling pathway for toxin A induction of human COX-2 involves ROS-mediated activation of p38 MAPK, MSK-1, CREB, and ATF-1. Toxin A triggers ileal inflammation and secretion of fluid via COX-2 induction and release of PGE₂.

Clostridium difficile is the major cause of antibiotic-associated colitis, a disease with significant morbidity and mortality (1), and a major economic burden for hospitalized patients (2). C. difficile produces intestinal damage and diarrhea by releasing two exotoxins, A and B, into the intestinal lumen (3). Toxin A, a 308-kDa heat-labile protein, elicits acute enteritis and secretion of fluid from ileum and colon of several animal species (4). The toxin elicits an inflammatory exudate containing lymphocytes, neutrophils, and serum proteins and pro-inflammatory cytokines that mediate a profound and rapid inflammatory response (5–7). The induction of fluid secretion and inflammation by toxin A involves extensive signaling cross-talk between epithelial cells, mast cells, sensory neurons, and inflammatory cells of the intestinal lamina propria (4). The cellular mechanism of toxin A involves glucosylation of a threonine residue at position 37 on Rho, Rac, and cdc42 (8), small GTP-binding proteins that regulate cell shape through modulation of the actin cytoskeleton. Monoglucosylation and inactivation of Rho proteins by the toxin causes severe cytoskeletal abnormalities in cultured and intact human colonocytes (9, 10). However, the signal transduction pathways by which toxin A induces intestinal inflammation are not entirely known.

Toxin A binds to a G protein-coupled receptor (11) on the luminal aspect of the apical intestinal epithelial cell membrane (12) and is then internalized where it activates MAPKs (13), intracellular calcium release (14, 15), release of reactive oxygen species (ROS) (12, 16), and secretion of pro-inflammatory mediators (16, 17). We previously reported that toxin A releases prostaglandin E₂ (PGE₂) into the ileal lumen of intact rats (5) and Alcantara et al. (18) reported that toxin A-induced water and electrolyte secretion in vivo was significantly blocked by a COX-2 inhibitor. COX-2 is induced by pro-inflammatory cytokines, lipopolysaccharide, growth factors, and infectious agents in a variety of cell types (19–23). PGE₂ is a potent stimulator of intestinal chloride and water secretion in mammalian gut (24), and PGE₂ is released during various forms of intestinal inflammation and infection. For example, injection of cultured enterocytes with Salmonella organisms induces COX-2 expression, followed by PGE₂-induced apical chloride secretion (25).

In view of the potential importance of COX-2 in toxin A-induced enteritis, we studied the signaling pathway of toxin A induction of COX-2 in colonocytes, the natural target of toxin A, using the non-transformed human colonic epithelial line, NCM460. Because toxin B also elicits inflammatory and cytoxic responses in human colon in vitro (10) and in vivo (26), we also examined whether it is also able to stimulate COX-2 expression in these cells. We found that toxin A, but not toxin B,
induced COX-2 expression at both mRNA and protein levels through ROS-mediated activation of p38 MAPK, mitogen- and stress-activated protein kinase-1 (MSK-1), ATF-1, and CREB. We also demonstrate that a p38 inhibitor and a selective COX-2 inhibitor reduced toxin A-induced fluid and PGE₂ secretion in vivo.

MATERIALS AND METHODS

C. difficile Toxin A—Toxin A and toxin B were purified from culture supernatants of C. difficile strain VPI 10463 (American Type Culture Collection, Rockville, MD) using anion exchange chromatography and fast protein liquid chromatography as previously described (13, 26). Reagents—The transfection reagent TransIT-LT1 was from Qiagen (Hilden, Germany). The polyclonal antibodies for IxI-B, COX-1, and COX-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-p38, p38, phospho-ERK1/2, ERK1/2, phospho-ATF-2, ATF-2, phospho-CREB, CREB, and phospho-MSK1 were from Cell Signaling Technology (Beverly, MA). The MAPK/ERK kinase specific inhibitor, PD98059, the JAK inhibitor, AG490, the NF-κB inhibitor, PD-98059, the JNK inhibitor, SP600125, the p38 MAPK inhibitor, SB203580 and the selective COX-2 inhibitor, NS-398, were from Calbiochem, N-Acetyl-L-cysteine (NAC), sodium formate, and 2,7-dichlorofluorescein-diacetate (DCFH-DA) were from Sigma. The non-transformed colonocytes NCM460 and the culture medium M3D were obtained from INCELL Corp. (San Antonio, TX) (28). NCM460 cells were cultured in M3D medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% l-glutamine, 10 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C in air supplemented with 5% CO₂. 

PGE₂ and cAMP Measurements in Cultured Colonocytes—Enzyme-linked immunosorbent assay (ELISA) was used to measure the level of human PGE₂, using the appropriate kits from R&D Systems (Minneapolis, MN). Colonocytes were plated 1 day prior to the experiment and then treated with toxin A (3 nm) or toxin B (20 nm). Conditioned medium was collected and aliquots were used to assay PGE₂ and cAMP following the manufacturer’s protocol. To measure intracellular cAMP, cells were initially treated with toxin A and lysed in 500 μl of HCl (0.1 N, 10 min). The suspension was centrifuged (14 min, 3,000 g), and the supernatant was assayed for cAMP by ELISA (R&D Systems, Minneapolis, MN) following the manufacturer’s protocol.

Immunofluorescence Analysis—Cells were harvested and fixed with either ice-cold 1× PBS (pH 7.5), or 4% paraformaldehyde and RT-PCR in microcentrifuge tubes, after which cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium dodecyl sulfate (SDS) and blocked with 2% bovine serum albumin (BSA) in PBS. Cells were incubated in 10% normal goat serum for 20 min at room temperature, followed by incubation in the primary antibody (1:1000 in 0.1% BSA and 2% normal goat serum). After washing with PBS, cells were incubated in the appropriate secondary antibody. Confocal microscopy was used to analyze the localization of the different proteins in the fixed cells. 

Measurement of ROS Generation—DCF-DA is a non-polar compound that enters the cell and is cleaved to form DCFH. Trapped DCFH is oxidized by oxygen free radicals to produce fluorescent DCF. NCM460 cells were preincubated on a 96-well microplate (2 × 10⁴ cells per well) in M3D medium lacking serum for 1 h at 37 °C in the presence of 10 μM DCF-DA. Cells were washed three times in pre-warmed PBS, and then exposed to toxin A (3 nm). Fluorescence intensity was analyzed by fluorospectroscan (Fluorocan Ascent FL, Labsystems) using 485 nm excitation and 538 nm emission filters. DCF fluorescence was also measured by fluorescence microscopy. To avoid photo-oxidation of DCFH, fluorescent images were collected by a rapid scan (total scan time, 3 s). Five groups of 10–20 subconfluent cells were randomly selected for analysis from each sample.

Electrophoretic Mobility Shift Assays—The following sequences derived from the human COX-2 promoter containing the CRE were used: CRE-sense, 5'-AAAAGCATTATTCGTACATGGCGGT-3'; CRE-antisense, 5'-CAAGCCGATGAGCAAGATGACTTGT-3'. Probes were annealed and 5'-overhangs were labeled by incorporation of ²²PdATP (PerkinElmer Life Sciences) with T4 polynucleotide kinase. For gel shift assays, 2 μg of nuclear protein extracts were incubated at room temperature for 5 min with a mixture containing 6 nM HEPES (pH 7.9), 0.4 mM EDTA, 125 mM KCl, 10% glycerol, 0.05 mg/ml poly(dI-dC) 1, 1 mM dithiothreitol, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM NaF, 50 mg/ml aprotinin, 50 μg/ml leupeptin. Approximately 1 ng of labeled probe was added, and the reactions were incubated at room temperature for 5 min. In all supershift experiments, mixtures were preincubated with various amounts of antibodies on ice for 1 h before the addition of probe. DNA-protein complexes were separated on 5% polyacrylamide gel in low ionic strength Tris borate buffer, dried under vacuum, and visualized with a PhosphorImager (Amersham Biosciences).

RNA Isolation and Semi-quantitative RT-PCR—Total cellular RNA was isolated from colonocytes and RT-PCR was performed according to Nakayama et al. (29). Two micrograms of total RNA was reverse transcribed at 42 °C for 1 h in 20 μl of the reaction mixture containing mouse Moloney leukemia virus reverse transcriptase with oligo(dT) primers. Thereafter, the result cDNA was amplified together with Taq polymerase (PerkinElmer Life Sciences) using the specific sets of primers. The primers used were: human COX-2 (300 bp), 5'-ATGAGAGATGGGAAAAATTTGCT (sense) and 5'-GGGCTGGGGTTGOCAATGGAT (antisense), and mouse COX-2 (333 bp), 5'-GCAATACTCTTGTTTCACATC (sense) and 5'-GGGAGAGGCTTCCCAGCTTTG (antisense). β-Actin mRNA was used as the control, respectively. PCR of each molecule was conducted with the optimal numbers of cycles consisting of 94 °C for 1 min, optimal annealing temperature for 1 min, and 72 °C for 1 min, followed by incubation at 72 °C for 3 min. The generated PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and visualized.

Effects of SB203580 and NS-398 on Toxin A-induced Secretion of PGE₂ and Fluid in Mouse Intestine in Vivo—This study was approved by the Beth Israel Deaconess Medical Center Animal Care and Use Committee (Boston, MA). CD1 mice were purchased from Charles River Laboratories (Wilmington, MA) weighting 30–35 g. Mice were acclimatized to these conditions at least 7 days before the experiment. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and ileal loops (3–4 cm) were prepared and injected with buffer alone or with the specific p38 inhibitor SB203580 (10 mg/kg) or with the selective COX-2 inhibitor, NS-398 (1 mg/kg), in a volume of 200 μl. After 30 min, toxin A (10 μg in PBS) or PBS alone was injected intraluminally, and animals were sacrificed 2 h later by CO₂. Ileal loop fluid was collected and centrifuged at 50,000 × g for 15 min. Secretion of PGE₂ was measured in the supernatant with specific ELISA kits according to the manufacturer's instructions. Ileal loops were excised and weighed, and length was measured. Fluid secretion was expressed as the loop weight-to-length ratio (mg/cm). Ileal tissue samples were fresh frozen for immunohistochemical analysis and for protein determination. Frozen intestinal sections were placed on Fisher Superfrost slides, fixed in 80% acetone at room temperature for 60 s, and air-dried. Before labeling, sections were rehydrated in PBS and incubated with 10% normal goat serum for 20 min. Sections were incubated with a fluorescein isothiocyanate conjugated goat anti-COX-2 antibody (Santa Cruz, CA, sc-1745-fluorescein isothiocyanate) (1:100) for 16 h. The slide was then counter-stained for 30 min in propidium iodiode staining solution at a concentration of 5 μg/ml in PBS. After final washing, slides were overslipped with VECTASHIELD (Vector Laboratories) and viewed on a fluorescence microscope (Nikon, Tokyo, Japan) equipped with an attached computer. Images were analyzed using the NIH Image program.

Human Intestinal Xenografts—Human fetal intestine was obtained from Brigham and Woman’s Hospital (Boston, MA) (mean age, 14.2 ± 1.6 weeks) after therapeutic abortion as previously described (30). Procurement and procedures involving xenografting of human fetal tissues into C.B.-17 SCID/SCID mice were performed with full approval from

21238

Toxin A Induces COX-2 in Intestinal Epithelial Cells

Additional Material

Measurement of ROS Generation—DCF-DA is a non-polar compound that enters the cell and is cleaved to form DCFH. Trapped DCFH is oxidized by oxygen free radicals to produce fluorescent DCF. NCM460 cells were preincubated on a 96-well microplate (2 × 10⁴ cells per well) in M3D medium lacking serum for 1 h at 37 °C in the presence of 10 μM DCF-DA. Cells were washed three times in pre-warmed PBS, and then exposed to toxin A (3 nm). Fluorescence intensity was analyzed by fluorospectroscan (Fluoroscan Ascent FL, Labsystems) using 485 nm excitation and 538 nm emission filters. DCF fluorescence was also measured by fluorescence microscopy. To avoid photo-oxidation of DCFH, fluorescent images were collected by a rapid scan (total scan time, 3 s). Five groups of 10–20 subconfluent cells were randomly selected for analysis from each sample.
Colonocytes were exposed to toxin A with or without the selective COX-2 inhibitor NS-398, or toxin B for the indicated times, and PGE2 levels were measured in the conditioned medium by ELISA.

We previously reported (26) that epithelial cells in the grafted intestinal xenografts growing subcutaneously in SCID mice. We found no expression of COX-2 that seen in animal models and in the colon of patients with C. difficile-associated colitis. We found no expression of COX-2 in control xenografts, but robust increases of COX-2 expression after toxin A exposure (Fig. 2), similar to the results observed in cultured colonocytes (Fig. 1, A and B).

**RESULTS**

**Induction of COX-2 by C. difficile Toxin A in Colonocytes**—Because COX-2 has been associated with intestinal inflammation, monocyte infiltration, and prostaglandin production, we examined COX-2 expression levels and PGE2 synthesis in colonocytes exposed to purified toxin A (3 nM). Toxin A induced the expression of COX-2, but had no effect on the expression of COX-1 (Fig. 1A). This response was evident at 1–3 h and peaked at 6 h. Under basal conditions, we observed weak expression of COX-2 mRNA in cultured colonocytes. Toxin A also increased COX-2 mRNA in a time pattern that paralleled the COX-2 protein expression (Fig. 1B). The effect of toxin A on COX-2 protein levels was concentration-dependent between doses of 0.8 and 3 nM (Fig. 1C). Despite the close structural relationship between toxins A and B and their common enzymatic activity against rho proteins, toxin B at 20 nM showed no effect on COX-2 expression (Fig. 1D). Colonocytes were transiently transfected with a construct containing the nucleotide sequence from −1432 to +59 of the human COX-2 promoter and then treated with 1.5 and 3 nM of toxin A or with 10 and 20 nM of toxin B. Toxin A, but not toxin B, increased COX-2 promoter activity in a dose-dependent manner (Fig. 1E). Next, we examined whether toxin A induces prostaglandin E2 secretion in colonocytes and determined the COX-2 dependence of this response using the selective COX-2 inhibitor NS-398. The basal concentration of secreted prostaglandin E2 was ~17 pg/ml/2 × 10⁶ cells (Fig. 1F). Toxin A increased PGE2 secretion by 2- to 3-fold at 6–12 h (Fig. 1F), which was completely blocked by the selective COX-2 inhibitor NS-398. As expected, toxin B did not induce PGE2 secretion up to 12 h (Fig. 1F). These results indicate that toxin A-mediated secretion of PGE2 in colonocytes required COX-2 induction.
Toxin A induces COX-2 in Intestinal Epithelial Cells

Phosphorylation of CREB, ATF-1, ATF-2, MSK-1, and p38 by Toxin A in Colonocytes—We next investigated toxin A-associated phosphorylation of p38 MAPK and its downstream transcription factors ATF-1, ATF-2, and CREB. Toxin A strongly increased the phosphorylation of p38, CREB, ATF-1, and ATF-2 after 1 and 3 h of exposure with return to basal levels at 6 h (Fig. 3A). Mitogen- and stress-activated protein kinase-1 (MSK-1) is directly activated by p38 MAPK and may mediate activation of CREB, as shown in embryonal human kidney cells activated by growth factors and oxidative stress (31). We therefore determined phosphorylation of threonine 581 of MSK-1 following toxin A stimulation. We observed phosphorylation of MSK-1 at 1 and 3 h after toxin A exposure, similar to the time course of CREB/ATF-1 phosphorylation.

Because NF-κB, and ERK1/2 are known regulators of COX-2 expression (32, 33), we examined phosphorylation of IκB, and ERK1/2 in colonocytes exposed to toxin A. As shown in Fig. 3B, we did not observe phosphorylation of IκB and only slight phosphorylation of ERK1/2.

Activation Pathways for COX-2 in Colonocytes Exposed to Toxin A—To define specific induction pathways of COX-2 by toxin A, we treated colonocytes with the p38 MAPK inhibitor SB203580, the JAK1 (Janus tyrosine kinase 1) inhibitor AG490, the NF-κB inhibitor 6-amino-4-quinazoline, the ERK1/2 kinase inhibitor PD98059, and toxin A for 6 h. We found that inhibition of p38 by SB203580 completely prevented the expression of COX-2 by toxin A (Fig. 4A). None of the other inhibitors had any significant effect on toxin A-induced COX-2 expression, apart from the JAK1 inhibitor AG490, which slightly inhibited COX-2 protein expression (Fig. 4A). Similarly, only the p38 MAPK inhibitor, SB203580 reduced the secretion of PGE2 in supernatants (from 49 pg/ml/21240 to 21 pg/ml/2, p < 0.001) (Fig. 4B), whereas, none of the other inhibitors exerted any significant effect at the doses used. Blockade of JAK1 by AG490 weekly inhibited toxin A-induced COX-2 protein (Fig. 4A), but failed to inhibit toxin A-mediated PGE2 secretion (Fig. 4B). In view of the lack of inhibition of COX-2 expression by the ERK1/2 kinase inhibitor PD98059 (Fig. 4A) as well as by the weak phosphorylation of ERK1/2 induced by toxin A (Fig. 3B), we conclude that the ERK1/2 and JAK1 pathways are not significantly involved in the regulation of COX-2 expression by toxin A.

We next examined the involvement of p38 MAPK on toxin A-induction of COX-2 mRNA. The p38 MAPK inhibitor, SB203580, at 10 μM completely blocked the toxin A effect on COX-2 mRNA in colonocytes (Fig. 4C). Because weak expression of COX-2 mRNA was detected in basal condition of cultured colonocytes and previous studies report that p38 activation stabilizes COX-2 messenger RNA (34–36), we investigated the role of toxin A on the stability of COX-2 transcripts after treatment with toxin A and/or actinomycin D for 30, 60, and 90 min. Both the toxin A-induced COX-2 transcripts 6 h following toxin A exposure and the basal expression of COX-2 transcripts disappeared at the same rate in the presence or absence of toxin A exposure (data not shown), indicating that toxin A-mediated p38 MAPK activation does not affect the stability of COX-2 mRNA.

Inhibition of p38 MAPK with SB203580 also blocked the toxin A-induced activation of the human COX-2 promoter in a dose-dependent manner (Fig. 4D), consistent with the results of COX-2 mRNA inhibition by these antagonists (Fig. 4C). COX-2 reporter activity was decreased to basal levels by 10 μM SB203580 (Fig. 4D).

p38 MAPK and Phosphorylation of CREB/ATF-1 and MSK-1—Because our results indicated that toxin A activates CREB/ATF-1 and MSK-1 (Fig. 3A), and p38 is phosphorylated by this toxin (Fig. 3A), we next explored the involvement of the p38 pathway on CREB/ATF-1 and MSK-1 activation in response to toxin A. Incubation of colonocytes with the p38 inhibitor SB203580 (10 μM) diminished toxin A-induced phosphorylation of CREB and ATF-1, whereas PD98059 (20 μM), a selective inhibitor of the ERK1/2 pathway, had no detectable effect (Fig. 5A). In addition, neither an NF-κB inhibitor nor AG490, a JAK inhibitor blocked phosphorylation of CREB and ATF-1. MSK-1, an upstream kinase of ATF-1 and CREB, was also completely blocked by SB203580, whereas toxin A-induced phosphorylation of ATF-2 was not affected by any of the inhibitors used (Fig. 5A). These data suggest that phosphorylation of ATF-2 associated with toxin A is not regulated by p38, but probably by other kinase(s), such as JNK. In addition, these results also reflect that toxin A-induced ATF-2 activation is not involved in COX-2 induction in colonocytes following toxin A exposure. Next, we examined whether a p38 MAPK dominant negative plasmid (p38-DN) prevents induction of COX-2 promoter activity following toxin A exposure. Overexpression of p38 dominant negative at 500 μg and 1000 μg, but not 100 μg, markedly diminished the COX-2 promoter response (Fig. 5B). These re-
results indicated that the p38 MAPK pathway is the major regulator of the COX-2 response to toxin A.

CREB and ATF-1 Bind to the COX-2 CRE—We performed electrophoretic mobility shift assays to identify the transcriptional factors that bind to the COX-2 cAMP-responsive element (CRE) following toxin A treatment. Incubation of colonocytes nuclear extracts with the COX-2 CRE probe produced two major bands and the maximum complex formation was detected 1 h after toxin stimulation (Fig. 6A). The upper complex was more strongly competed than the lower complex by excess cold COX-2 CRE. The upper complex contained a CRE-binding protein (CREB), as shown by supershift experiments using an anti-phospho CREB antibody (Fig. 6B). Because this antibody reacts with both phospho-CREB and phospho-ATF-1, the CREB-ATF-1-DNA complex shown in Fig. 6B may also contain ATF-1. However, we were unable to supershift or clear the complex using antibodies against ATF-2 and c-Jun (Fig. 6B). Taken together, these results suggest that CREB and ATF-1 are important transcription factors in COX-2 gene expression in response to toxin A.

Toxin A Induction of COX-2 Involves ROS—We recently reported that toxin A exposure of Chinese hamster ovary cells generates reactive oxygen species (ROS) reflecting mitochondrial damage (12, 16). It is also known that CREB is regulated by cAMP (37, 38). Based on these considerations, we examined whether colonocytes exposed to toxin A generated ROS or cAMP. In line with our previous results (12), ROS levels measured either by fluorospectroscan (Fig. 7A, upper panel) or fluorescence microscopy (Fig. 7A, lower panel) were increased by ~4- to 5-fold following toxin A exposure (Fig. 7A). In contrast, intracellular cAMP levels were not significantly increased by toxin A (Fig. 7A), indicating that toxin A-induced activation of CREB in colonocytes is not cAMP-dependent.

Next, we tested whether NAC, a hydrogen peroxide scavenger (39), or sodium formate, a hydroxyl radical scavenger (40), could attenuate toxin A-mediated induction of COX-2. Serum-starved colonocytes were preincubated with NAC and sodium formate for 30 min and then exposed to 3 nM toxin A for 6 h. We found that 20 mM NAC preincubation markedly reduced toxin A induction of COX-2, whereas sodium formate had no effect (Fig. 7B). Moreover, colonocytes exposed to 100 and 500 μM H₂O₂ showed increased expression of COX-2 (Fig. 7C, left panel), and activation of p38 in a time-dependent manner (Fig. 7C, right panel). In addition, NAC, but not sodium formate, completely blocked toxin A-induced MSK-1 activation and partially blocked CREB and ATF1 phosphorylation (Fig. 7D, left panel). Phosphorylation of p38 following toxin A exposure was slightly inhibited by NAC (Fig. 7D, right panel). Colonocytes exposed to 100 and 500 μM H₂O₂ also showed increased activation of p38, MSK-1, CREB, and ATF-1 (Fig. 7D, right panel). In addition, toxin A-induced PGE₂ secretion after 36 h was substantially inhibited by 10 μM, and 20 μM of NAC, but not sodium formate (Fig. 7E), whereas hydrogen peroxide exposure for 36 h also increased PGE₂ secretion in a dose-dependent manner (Fig. 7E) but to a lesser degree than toxin A (Fig. 7F). Thus, toxin A rapidly stimulates (within 10 min) release of ROS that mediates activation of p38 MAPK, MSK-1, CREB, and ATF-1, leading to COX-2 induction and PGE₂ secretion.
Toxin A Induces COX-2 in Intestinal Epithelial Cells

DISCUSSION

We report here that C. difficile toxin A strongly induces colonic COX-2 expression and PGE_2 secretion both in vitro and in vivo in human colon and mouse ileum, and this contributes to toxin A-induced enteritis and fluid secretion. The signal transduction cascade following toxin A-receptor binding involves release of hydrogen peroxide, which mediates activation of p38 MAPK and the downstream effectors, MSK-1, CREB, and ATF-1, leading to subsequent COX-2 induction and PGE_2 secretion. In contrast, toxin B did not have an effect on COX-2 induction and PGE_2 secretion. Different expression of receptors for these two distinct toxins in cultured colonocytes probably accounts for these observed responses.

The CRE element in the COX-2 promoter is necessary for the induction of COX-2 transcription mediated by platelet derived growth factor, and lipopolysaccharide (41–43). NF-κB mediates COX-2 induction by lipopolysaccharide and other stimuli (42), but this transcription factor is not involved in the toxin A-mediated COX-2 transcription. We found that toxin A did not
induce NFκB activation in NCM460 colonocytes, in contrast to earlier studies indicating a rapid toxin A-induced NFκB activation in colon adenocarcinoma HT29 cells (16). Non-transformed (NCM460) versus transformed (HT-29) cell phenotypes may account for these different toxin A-associated responses. Our results show that the CRE element in the COX-2 promoter plays a major role in toxin A-mediated induction of intestinal COX-2 transcription. Yamaoka et al. (44) reported that lipopolysaccharide activated STAT5, which induced COX-2 through granulocyte macrophage-colony stimulating factor production in human monocytes. These authors also showed a γ-interferon-activated sequence in the promoter region of human, mouse, and rat COX-2 genes, and reported binding of activated STAT5 to this element. Although our results indicated that toxin A-induced phosphorylation of STAT5 was regulated by JAK1 in colonocytes (data not shown), we found that inhibition of JAK1 by AG490 had no effect on the PGE2 secretion following toxin A exposure (Fig. 4B). Moreover, activation of ERK1/2, a known regulator of COX-2 expression, was not clearly evident in colonocytes following exposure to toxin A. These data suggest that p38 MAPK, but not JAK/STAT5, ERK1/2, and NFκB is the major pathway regulating the COX-2 response to toxin A.

Gel shift assays using a CRE probe from the COX-2 promoter also indicate that CREB and ATF-1, but not c-Jun and ATF-2, bind to the COX-2 CRE. Toxin A induces the phosphorylation of MSK-1, CREB, and ATF-1, which are downstream of p38 MAPK. As expected, we found that the p38 inhibitor SB203580 blocks toxin A-induced COX-2 promoter activity and phosphorylation of MSK-1 and CREB/ATF-1.

We reported earlier that p38 MAPK regulates toxin A-mediated induction of interleukin-8 expression in human monocytes (13). In addition, prostaglandin E2 stimulates interleukin-8 gene expression in human colonocytes by a post-transcriptional mechanism (45). These observations suggest that toxin A activates p38 MAPK, which up-regulates COX-2 expression in intestinal epithelial cells and secretion of PGE2 that in turn up-regulates interleukin-8 gene transcription. Thus, activation of colonocyte p38 MAPK by toxin A is a key modulator of several target genes that ultimately control infiltration of monocytic cells, acute intestinal inflam-
mation, epithelial permeability, and intestinal electrolyte and water secretion.

PGE2 is a potent stimulator of acute fluid secretion by the intestine particularly during bacterial infection. PGE2 and other prostanoids are elevated in luminal secretion from experimental animals and human with infectious diarrhea (46), and administration of PGE2 analogues caused diarrhea in volunteers (47). Epidermal growth factor receptor activation induces basolateral release of PGE2 by COX-2 in colon cancer cells (48). Infection of intestinal epithelial cell lines with Salmonella induces the expression of COX-2 and PGE2 with subsequent apical chloride secretion (25). PGE2 induces cAMP-mediated apical chloride secretion and inhibits electroneutral sodium chloride and water absorption in enterocytes (49, 50). Cholera toxin also induces PGE2 and fluid secretion that can be prevented by selective COX-2 inhibitors (51, 52).

In summary, we demonstrate that toxin A directly induces COX-2 expression in intestinal epithelial cells through activation of p38 MAPK, MSK-1, and CREB/ATF-1 pathways (Fig. 9). Activation of p38, MSK-1, and CREB/ATF-1 pathways appears to be impor-
Toxin A Induces COX-2 in Intestinal Epithelial Cells

The main signaling pathway for toxin A induction of human COX-2 involves ROS-mediated activation of p38 MAPK, MSK-1, CREB, and ATF-1 but not NFκB. Identical to intact intestine. Inducible COX-2 and PGE2 release are tant as determined by chemical inhibitor studies in cell culture secretion in human colonocytes.

COX-2 activation and PGE2 release may represent a potential therapeutic target in toxin A-mediated colitis. Our findings suggest that COX-2 activation and PGE2 release may represent a potential therapeutic target in C. difficile toxin A-mediated colitis.

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