Clostridium difficile Toxin A Induces Reactive Oxygen Species Production and p38 MAPK Activation to Exert Cellular Toxicity in Neuronal Cells

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Clostridium difficile releases two exotoxins, toxin A and toxin B, which disrupt the epithelial cell barrier in the gut to increase mucosal permeability and trigger inflammation with severe diarrhea. Many studies have suggested that enteric nerves are also directly involved in the progression of this toxin-mediated inflammation and diarrhea. C. difficile toxin A is known to enhance neurotransmitter secretion, increase gut motility, and suppress sympathetic neurotransmission in the guinea pig colitis model. Although previous studies have examined the pathophysiological role of enteric nerves in gut inflammation, the direct effect of toxins on neuronal cells and the molecular mechanisms underlying toxin-induced neuronal stress remained to be unveiled. Here, we examined the toxicity of C. difficile toxin A against neuronal cells (SH-SY5Y). We found that toxin A treatment time- and dose-dependently decreased cell viability and triggered apoptosis accompanied by caspase-3 activation in this cell line. These effects were found to depend on the up-regulation of reactive oxygen species (ROS) and the subsequent activation of p38 MAPK and induction of p21Cip1/Waf1. Moreover, the N-acetyl-L-cysteine (NAC)-induced down-regulation of ROS could recover the viability loss and apoptosis of toxin A-treated neuronal cells. These results collectively suggest that C. difficile toxin A is toxic for neuronal cells, and that this is associated with rapid ROS generation and subsequent p38 MAPK activation and p21Cip1/Waf1 up-regulation. Moreover, our data suggest that NAC could inhibit the toxicity of C. difficile toxin A toward enteric neurons.

Keywords: Clostridium difficile, toxin A, gut inflammation, neuronal cells (SH-SY5Y), enteric nerve dysfunction, apoptosis, reactive oxygen species, caspase-3

Introduction

Many lines of evidence indicate that there is a pathophysiological connection between enteric nerves and the gut [1–7]. For example, colonic inflammation activates sensory neurons through an increase in brain-derived neurotrophic factor (BDNF) [6, 8]. Nitric oxide released from neuronal cells inhibits intestinal smooth muscle growth [9]. A component of the gut luminal content, lipopolysaccharide, has been used to establish a neuronal damage model [10]. Many experimental mouse models of colitis exhibit various alterations in enteric neurotransmission [7]. Moreover, gut inflammation is associated with the electrical excitability of colonic dorsal root ganglia neurons [1–4]. Together, these results suggest that enteric nerves are closely connected to mucosal components of the gut, such as epithelial cells, muscle cells, and secretory cells.

Clostridium difficile, which is a pathogenic bacterium responsible for pseudomembranous colitis, releases two exotoxins, toxin A and toxin B; these are the main causative agents for the characteristic inflammatory responses, mucosal damage, and severe diarrhea of this colitis [11–
The glucosyltransferase activities of these toxins inactivate Rho family proteins (Rho, Rac, and Cdc42), leading to cytoskeletal disruption in the mucosal epithelial cells that are essential for forming the physical barrier in the gut [11–13]. The toxin-mediated disruption of the mucosal barrier, which is believed to be an initial process in barrier dysfunction, is followed by increased permeability, diarrhea, and inflammation. However, C. difficile toxin-induced abnormalities in the enteric nerve system are also considered to be critical factors in the progression of gut inflammation [21–23].

Numerous studies have reported that C. difficile toxins excite enteric neurons, and that this effect is associated with inflammatory responses and diarrhea in the gut [21–23]. For example, luminal injection of toxin A increases neurotransmitter secretion and enhances gut motility [21], and toxin A suppresses sympathetic neurotransmission in the guinea pig colitis model [24]. Studies have also shown that chemical agents capable of inhibiting neurotransmission can reduce inflammatory responses and diarrhea in the gut [25, 26]. For example, hexamethonium, which acts as an antagonist for nicotinic receptors on neuronal cells, ameliorates toxin-induced inflammation and diarrhea [26]. Lidocaine, which blocks the transmission of mucosal afferent neurons, has similar therapeutic effects on toxin-induced diarrhea [26]. Nicotine treatment suppresses visceral hypersensitivity in the dextran sodium sulfate-induced diarrhea [26]. However, the direct toxicity of toxins against neuronal cells has not yet been studied in detail. Here, we investigated whether C. difficile toxin A causes neuronal cell damage and apoptosis.

Our results revealed that toxin A treatment caused significant viability loss and apoptosis in a neuronal cell line (SH-SY5Y). This effect was found to depend on the increased production of reactive oxygen species (ROS) and the subsequent up-regulation of p38 MAPK activity and p21<sup>Cip1/Waf1</sup> expression. These novel findings improve our understanding of the toxicity of C. difficile toxin A toward nervous cells.

**Materials and Methods**

**Preparation of Clostridium difficile Toxin A**

C. difficile strain VPI 10463 (American Type Culture Collection) cells were incubated at 37°C for more than 2 weeks and then centrifuged for 30 min at 6,800 ×g at 4°C. Toxin A was purified as previously described [28].

**Cell Culture and Reagents**

Human neuroblastoma SH-SY5Y cells were maintained in DMEM containing 10% FBS (Invitrogen, USA) in a 37°C humidified incubator with 5% CO<sub>2</sub> [29]. The polyclonal antibody against caspase-3 was obtained from Cell Signaling Technology (USA). The polyclonal antibodies against p21<sup>Cip1/Waf1</sup>, p27<sup>Kip1</sup>, and SP1 were obtained from Santa Cruz Biotechnology (USA), and the antibodies against p-p38 MAPK and p-JNK were purchased from Cell Signaling Technology. The β-actin antibody, propidium iodide (PI), 3-[4,5-dimethylthiazole-2-y]]-2,5-diphenyltetrazolium bromide (MTT) dye, and ROS inhibitors (N-acetyl-L-cysteine (NAC) and sodium formate) were purchased from Sigma-Aldrich (USA).

**Cell Viability**

SH-SY5Y cells (3 × 10<sup>5</sup> cells/well) treated with various agents were incubated with MTT dye for 30 min, the solubilization agent (dimethyl sulfoxide) was added, and the absorbance was determined at 570 nm in a microplate reader (model 3550; Bio-Rad, Canada) [18].

**Analysis of Cell Morphology**

SH-SY5Y cells were seeded to a 6-well plate at 1 × 10<sup>5</sup> cells/well and treated with toxin A (3 nM) for 4 h. Images were taken with an Olympus inverted phase-contrast microscope (200×; Olympus Optical Co., USA) equipped with the Quick Imaging system [17].

**Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay**

Cells were pretreated with NAC for 30 min, exposed to the toxin for 12 h, and then fixed with 4% paraformaldehyde for 20 min at room temperature. Cells with fragmented nuclear DNA were detected by TUNEL assay (Promega, USA), according to the manufacturer’s instructions [30].

**Immunoblot Analysis**

SH-SY5Y cells were washed with cold PBS, then lysed in buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1% Nonidet P-40), and equal amounts of protein were fractionated on SDS-polyacrylamide gels. The appropriate antibodies were applied, and antigen-antibody complexes were detected with the LumiGlo reagent (New England Biolabs, USA) [31].

**Measurement of ROS**

SH-SY5Y cells were incubated for 30 min with 10 μM DCF-DA, and then treated with toxin A for 30 min. Fluorescence intensity was analyzed with a Fluoroscan Ascent FL microplate reader (Thermo Fisher Scientific, USA) using 485 nm excitation and 538 nm emission filters [16].

**Statistical Analysis**

The results are presented as the mean values ± SEM. Data were analyzed using the SIGMA-STAT professional statistics software program (Jande Scientific Software, USA). Analyses of variance with protected t test were used for intergroup comparisons.
Results and Discussion

Clostridium difficile Toxin A Causes Cell Toxicity in Human Neural Cells

*C. difficile* toxin A is known to cause toxicity to various cells, including mucosal epithelial cells and immune cells of the gut [17, 30, 32, 33]. However, the toxicity of toxin A toward nerve cells has not previously been defined. Here, we incubated a human neuroblastoma cell line (SH-SY5Y) with *C. difficile* toxin A (3 nM) for 24 h and used the MTT assay to measure the cell viability. As shown in Figs. 1A and 1B, toxin A treatment dose- and time-dependently decreased neural cell viability. Since toxin A has also been shown to induce cell apoptosis in many cell lines [17, 30, 32], and cell viability loss is often linked to apoptosis [34], we next assessed whether toxin A could induce the DNA fragmentation characteristic of apoptosis [30]. Indeed, SH-SY5Y cells exposed to toxin A for 24 h exhibited marked increases in TUNEL-positive cells (indicating DNA fragmentation) compared with medium-treated control cells (Fig. 1C). The typical shape of the neuronal cells was also dramatically altered by toxin A exposure: the exons were shortened or absent, and the nerve cell body was severely rounded compared with medium-treated control cells (Fig. 1D). The cytotoxic effect of toxin A has been associated with cell rounding in various other cell systems [17, 33]. Our results suggest that, as in other cell types, toxin A triggers viability loss, apoptosis, and morphological changes in neuronal cells.

The toxin A-induced cell rounding observed in various cell types has been associated with cytoskeletal disruption [17, 33] induced by the glucosyltransferase activity of toxin

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Fig. 1. *C. difficile* toxin A causes cytotoxicity against neuronal cells.

(A) Human neuroblastoma cells (SH-SY5Y; 3 × 10^5 cells/well) were treated with the indicated concentrations of toxin A for 24 h, and cell viability was measured by MTT assay (*, p < 0.05). The results represent the mean ± SEM from three independent experiments performed in triplicates. (B) SH-SY5Y cells were incubated with toxin A (3 nM) for 12, 24, and 36 h, and cell viability was measured by MTT assay. The results are expressed as a percentage (*, p < 0.05), and represent the mean ± SEM from three experiments performed in triplicates. (C) SH-SY5Y cells (10^5 cells/well) were incubated with medium (con) or toxin A (Tx, 3 nM) for 24 h. DNA fragmentation was detected by TUNEL staining (white spots indicate apoptosis; 100×), and propidium iodide (PI) staining was to visualize cell nuclei. The presented results are representative of three independent experiments. (D) Light microscopic images (100×) of SH-SY5Y cells incubated for 4 h with medium (con) or toxin A (Tx, 3 nM). The presented results are representative of three independent experiments.
Toxin A binds and glucosylates/inactivates various substrates, including Rho, Rac, and Cdc42, which are Rho family proteins involved in forming the cytoskeleton [11–13]. This causes cytoskeletal disruption and severe cell rounding. In all cell lines tested to date, including those from many different species (rat, mouse, and human), toxin A was found to cause marked changes in cell morphology [11–13, 17]. Thus, toxin A inactivates Rho family proteins of which toxin A enzyme may have a broad spectrum when recognizing and binding substrate proteins.

Toxin A Increases p38 MAPK Phosphorylation/Activity and p21<sup>Cip1/Waf1</sup> Expression

We previously demonstrated that *C. difficile* toxin A-induced cell apoptosis is highly dependent on the p38 MAPK and p21<sup>Cip1/Waf1</sup> pathways in human colonocytes [16, 17]. Given our finding that toxin A reduces cell viability and triggers apoptosis in neuronal cells, we next sought to identify the intracellular molecules responsible for this effect. We treated SH-SY5Y cells with toxin A (3 nM) for 0.5, 1, 2, 4, 6, and 8 h, and measured the levels of phosphorylated (activated) p38 MAPK. As shown in Figs. 2A and 2B, toxin A time- and dose-dependently increased the phosphorylation of p38 MAPK. In contrast, c-Jun N-terminal kinase (JNK) [35] and SP1 [36], which are known to play important roles in the apoptosis of neuroblastoma cells, were not affected by toxin A treatment. Our previous study also demonstrated that toxin A treatment triggered cell cycle arrest (G2-M) with persistent expression of p21<sup>Cip1/Waf1</sup> in intestinal epithelial cells, and that this was critical for toxin A-induced apoptosis [17]. Moreover, the toxin A-induced induction of p21<sup>Cip1/Waf1</sup> is known to depend on p38 MAPK activation [16, 17]. Therefore, we tested whether toxin A affected the protein expression of p21<sup>Cip1/Waf1</sup> in neuronal cells. Indeed, toxin A markedly increased the protein expression of p21<sup>Cip1/Waf1</sup>, but had no effect on p27<sup>Kip1</sup> (another cell-cycle inhibitor protein) (Fig. 2C). Together with the previous reports, our present results show that toxin A treatment commonly activates p38 MAPK and p21<sup>Cip1/Waf1</sup> in many different cell types, including neuronal cells. This suggests that a toxin A-binding cell-surface receptor may be highly conserved across various mammals.

The Toxin A-Induced Activation of p38 MAPK Is Dependent on ROS Production

Many studies have shown that activating the phosphorylation of p38 MAPK is associated with the intracellular level of ROS, which are rapidly produced in response to various stimuli and affect downstream signaling events [16, 37]. Our previous study found that toxin A treatment increased the amount of ROS in human colonocytes, and this was associated with the subsequent...
activation of p38 MAPK [16]. Here, we assessed whether toxin A increased the ROS level in neuronal cells, and whether this affected the activation of p38 MAPK. To address these questions, we incubated SH-SY5Y cells with toxin A (3 nM) for 30 min and used DCF-DA staining to measure intracellular ROS levels. As shown in Fig. 3A, toxin A-treated cells exhibited much more ROS production than medium-treated control cells. Moreover, the toxin A-mediated activation of p38 MAPK was completely blocked by pretreatment with NAC (Fig. 3B), which inhibits the production of the ROS, hydrogen peroxide (H$_2$O$_2$). This is consistent with our previous finding that toxin A mainly produces H$_2$O$_2$ in colonic epithelial cells [16], and that this is critical for the activation of downstream programmed-cell-death-related signaling, such as that involving p38 MAPK and p21$^{Cip1/Waf1}$ [16, 17]. In contrast, the toxin A-mediated activation of p38 MAPK was not affected by sodium formate, which inhibits another ROS, the hydroxyl radical. The present results, along with the previous findings, suggest that toxin A-induced ROS production is essential for the toxin A-induced activation of p38 MAPK. Moreover, our present findings suggest that the ROS newly generated in toxin A-treated neuronal cells is mainly H$_2$O$_2$, and that other oxygen radicals, such as superoxide anions and hydroxyl radicals, do not play a major part in this response. We also examined the direct effect of H$_2$O$_2$ on p38 MAPK activation in neuronal cells. We incubated SH-SY5Y cells with 100 $\mu$M H$_2$O$_2$ [16, 17] for 1 h, and then measured the activating phosphorylation of p38 MAPK. As shown in Fig. 3C, H$_2$O$_2$ time-dependently increased the activating phosphorylation of p38 MAPK.

Taken together, our results suggest that the toxin A-triggered induction of ROS activates p38 MAPK in neuronal cells, and that inhibiting the toxin A-mediated generation of ROS might critically block the enteric nerve cell damage and malfunction seen in C. difficile toxin A-induced gut inflammation. This approach for regulating ROS levels could ameliorate inflammatory responses in the gut.

The NAC-Mediated Down-Regulation of ROS Inhibits Toxin A-Induced Neuronal Cell Apoptosis

Given our observation that the NAC-induced blockade of toxin A-induced ROS production inhibited p38 MAPK activation in neuronal cells, we assessed whether NAC could inhibit the toxin A-induced neuronal cell viability loss and apoptosis. We incubated SH-SY5Y cells with NAC (30 mM) for 30 min, further incubated the cells with toxin A for 12 h, and used the MTT assay to assess cell viability. As shown in Fig. 4A, the marked cell viability reduction seen following toxin A exposure was significantly recovered by NAC pretreatment, whereas NAC alone had no effect on cell viability. Caspase-3, which is known to induce cell apoptosis [17], was also found to be highly activated by toxin A stimulation in our system, but this effect was markedly reduced by NAC pretreatment.
Consistent with this, TUNEL staining showed that toxin A-induced DNA fragmentation was also inhibited by NAC pretreatment (Fig. 4C). These results collectively suggest that the NAC-mediated inhibition of ROS can reduce most of the toxicities observed in toxin A-exposed neuronal cells.

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**References**


