

Botulinum Toxin Complex Increases Paracellular Permeability in Intestinal Epithelial Cells via Activation of p38 Mitogen-Activated Protein Kinase

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ABSTRACT. *Clostridium botulinum* produces a large toxin complex (L-TC) that increases paracellular permeability in intestinal epithelial cells by a mechanism that remains unclear. Here, we show that mitogen-activated protein kinases (MAPKs) are involved in this permeability increase. Paracellular permeability was measured by FITC-dextran flux through a monolayer of rat intestinal epithelial IEC-6 cells, and MAPK activation was estimated from western blots. L-TC of *C. botulinum* serotype D strain 4947 increased paracellular dextran flux and activated extracellular signal-regulated kinase (ERK), p38, but not c-Jun N-terminal kinase (JNK) in IEC-6 cells. The permeability increase induced by L-TC was abrogated by the p38 inhibitor SB203580. These results indicate that L-TC increases paracellular permeability by activating p38, but not JNK and ERK.

KEY WORDS: botulinum toxin complex, *Clostridium botulinum*, intestinal epithelial cells, mitogen-activated protein kinase.

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Clostridium botulinum produces a neurotoxin (BoNT) that prevents neurotransmitter release and in turn causes flaccid muscle paralysis in human and animals. In nerve endings of neuromuscular junctions, BoNT cleaves specific sites on synaptic vesicle fusion proteins, which leads to exocytosis inhibition. BoNT is serologically classified into seven types, A through G. BoNT types A, B, E and F mainly cause human botulism, while BoNT types C and D are causative toxins for animal and avian botulism [12, 17]. Animal botulism is a worldwide problem that causes large economic losses, since it affects cattle and other livestock [18, 26]. An epizooty could also result as a public health problem whenever meat or animal products enter in the food chain. Little is known regarding the epidemiology of the disease, and the factors behind the outbreaks are not well identified.

In culture fluid or contaminated foods, BoNT (150 kDa) associates with non-toxic non-hemagglutinin (NTNHA; 130 kDa) and three hemagglutinins (HA-70, HA-33 and HA-17; 70, 33 and 17 kDa, respectively) to form a large toxin complex (L-TC; 750 kDa) [10]. In food-borne botulism, orally ingested L-TC is absorbed in the small intestine [14, 25]. The free BoNT molecule is susceptible to proteolytic and acidic conditions in the digestive environment and is readily degraded into short peptides or amino acids, whereas BoNT

as part of the L-TC escapes digestion in the gastrointestinal tract because NTNHA and some HAS are resistant to proteolytic degradation and protect BoNT from digestion [8, 16]. L-TC or BoNT has been shown to bind to sugar chains on the surface of intestinal epithelial cells, which leads to their internalization and transcytosis [6, 19]. Because binding and transport of the L-TC to the cells is greater than that of BoNT, non-toxic proteins of the L-TC are thought to have an important role in effective toxin absorption [20]. Recent studies suggested that L-TC passes through the intestinal cell layer not only by an intracellular transport, i.e. transcytosis, but also via paracellular transport. Matsumura *et al.* showed that L-TC type B (L-TC/B) caused an increase in dextran flux, an index of paracellular permeability, in Caco-2 cell monolayers and that the distribution of junctional proteins, such as occludin and ZO-1, was altered by L-TC [15]. Thus, L-TC appears to have a disruptive effect on the paracellular barrier function of epithelial cells. However, the cellular events that are responsible for disrupting this barrier function are unknown.

The purpose of this study is to clarify the intracellular signaling pathways responsible for the increase in paracellular permeability of intestinal epithelial cells that is induced by L-TC. We hypothesized that members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK), may contribute to the permeability increase, because these kinases are known to be involved in regulating cell morphology and integrity [1, 11].

An anaerobic culture of *C. botulinum* serotype D strain 4947 (D-4947) and purification of L-TC from culture su-

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pernatants were performed as described previously [9] with slight modifications. In brief, culture supernatants were subjected to sequential column chromatography using a Toyopearl SP-650S cation exchange column (Tosoh, Tokyo, Japan), a HiLoad Superdex 200 pg gel filtration column (GE Healthcare Biosciences, Buckinghamshire, U.K.), a ceramic hydroxyapatite type 1 column (Bio-Rad, Hercules, CA, U.S.A.) and a Mono S anion exchange column (GE Healthcare Biosciences). Separation of BoNT from L-TC was done as previously described [9].

The rat small intestinal epithelial cell line IEC-6 was purchased from RIKEN BioResource Center (Tsukuba, Japan). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a CO₂ incubator with the culture medium renewed every 2–3 days. For paracellular permeability assay, cells were seeded in culture inserts of a Transwell plate, which is comprised of two compartments separated by a porous polycarbonate membrane (24 wells/plate, Corning, NY, U.S.A.). After 6 days of culture, toxins diluted in 200 μ l DMEM containing 10% FBS were added to the culture inserts. The outer chamber was filled with 900 μ l of the same culture medium. The cells were incubated for the indicated periods and then fixed with 3.7% formaldehyde for 15 min. After rinsing with phosphate buffered saline (PBS), culture inserts with the fixed cells were set in the other 24-well culture plate, which contained 900 μ l PBS in each well. The culture inserts were filled with 200 μ l PBS with 10 μ M fluorescein isothiocyanate (FITC)-dextran (average molecular weight 20 kDa, Sigma, St. Louis, MO, U.S.A.) and incubated for 1 hr at room temperature. Fluorescence intensities of FITC-dextran in PBS in the outer chamber were then measured using a spectrofluorometer with excitation and emission wavelengths of 495 and 510 nm, respectively.

To detect MAPK activation, western blots using antibodies against phosphorylated forms of MAPKs were performed. Confluent IEC-6 cells cultured in dishes (6 cm in diameter) were incubated in culture medium (DMEM with 10% FBS) containing toxins. After rinsing with PBS, the cells were lysed with 300 μ l SDS buffer. Proteins were separated on 10% SDS gels and blotted onto a nitrocellulose membrane. Membranes were incubated with antibodies against phosphorylated or total ERK, p38 or JNK (Cell Signaling Technology, Danvers, MA, U.S.A.) diluted 1:1,000 in TTBS (20 mM Tris-HCl buffer pH 7.5, 150 mM NaCl and 0.1% Tween 20) with bovine serum albumin (BSA) at 4°C overnight. Concentration of BSA in TTBS was 2 or 5% for antibodies against phosphorylated or total MAPKs, respectively. After rinsing with TTBS three times, the membranes were incubated with anti-rabbit IgG-HRP conjugate (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted 1: 5,000 in TTBS with 2% BSA for 1 hr at room temperature. After rinsing, proteins were visualized using chemiluminescence detection. The intensity of detected bands was analyzed with IMAGE J 1.38v software (<http://rsb.info.nih.gov/ij/>).

For fluorescent microscopy, cells were seeded on a glass chamber slide (Nunc A/S, Roskilde, Denmark) and then incubated with 200 nM L-TC diluted in culture medium. Cells

were rinsed with PBS and fixed with 3.7% formaldehyde for 15 min. After rinsing with PBS, cells were incubated with PBS containing Alexa Fluor 546-conjugated phalloidin (5 U/ml; Life Technologies Japan, Tokyo, Japan) for 1 hr. Cells were observed under a fluorescence microscope (Axiovert 40 CFL, Carl Zeiss, Oberkochen, Germany) with standard rhodamine filters.

First of all, we observed the effects of L-TC on the morphology and actin arrangement of IEC-6 cells (Fig. 1A). The control cells were tightly attached, and actin fibers were thickly arranged along the cell contour. Treatment of cells with L-TC caused disruption of cell morphology and a loosening of actin fibers. To investigate whether L-TC affects paracellular permeability, FITC-dextran flux across cell layers was measured after L-TC treatment. As shown in Fig. 1B, L-TC increased paracellular permeability in a time-dependent manner with a peak at 12 hr. To identify which L-TC component may contribute to this permeability increase, we separated the L-TC into BoNT and non-toxic protein complex (NTNHA/HAs) [9] and added these to the culture medium. BoNT did not affect cellular permeability, whereas NTNHA/HAs increased FITC-dextran flux in a manner similar to L-TC, suggesting that the non-toxic proteins are responsible for the permeability increase induced by L-TC. Because L-TC/D binds to sialic acid on the cell surface [20], we tested whether this binding is the first event leading to the observed permeability increase by removing cell surface sialic acid moieties with neuraminidase treatment prior to the permeability assay. The increase in paracellular permeability induced by L-TC was abrogated depending on the neuraminidase concentration (Fig. 1C), indicating that the permeability increase is due to L-TC binding to sialic acid.

We next tested whether L-TC modifies the activity of MAPKs. Fig. 2A, 2C and 2E show time-dependent changes in the phosphorylation of ERK, p38 and JNK, respectively. L-TC induced a gradual increase in ERK phosphorylation that continued throughout the incubation (Fig. 2A) with the degree of phosphorylation dependent on the L-TC concentration used (Fig. 2B). NTNHA/HAs caused ERK phosphorylation in a manner similar to L-TC, whereas BoNT had no influence (Fig. 2A). L-TC caused a rapid increase in p38 phosphorylation that peaked 6 hr after L-TC treatment (Fig. 2C). Similarly, an increase in p38 phosphorylation was observed with NTNHA/HAs treatment, but not BoNT. The phosphorylation of p38 induced by L-TC was also dose-dependent (Fig. 2D). In contrast, L-TC or NTNHA/HAs had little, if any, effect on JNK phosphorylation (Fig. 2E and 2F), which was also unaffected by BoNT (Fig. 2E).

Lastly, we tested whether the MAPKs contribute to the permeability increase induced by L-TC. Figure 3 shows the effects of inhibitors for ERK (PD98059), p38 (SB203580) and JNK (SP600125) at 10 or 20 μ M on FITC-dextran flux through IEC-6 cell monolayers treated with or without L-TC. The increase in FITC-dextran flux induced by L-TC was unaffected by the ERK inhibitor PD98059, suggesting that ERK is not involved in the L-TC-induced permeability increase (Fig. 3A and 3B). In contrast, the p38 inhibitor SB203580 at both 10 and 20 μ M significantly reduced the

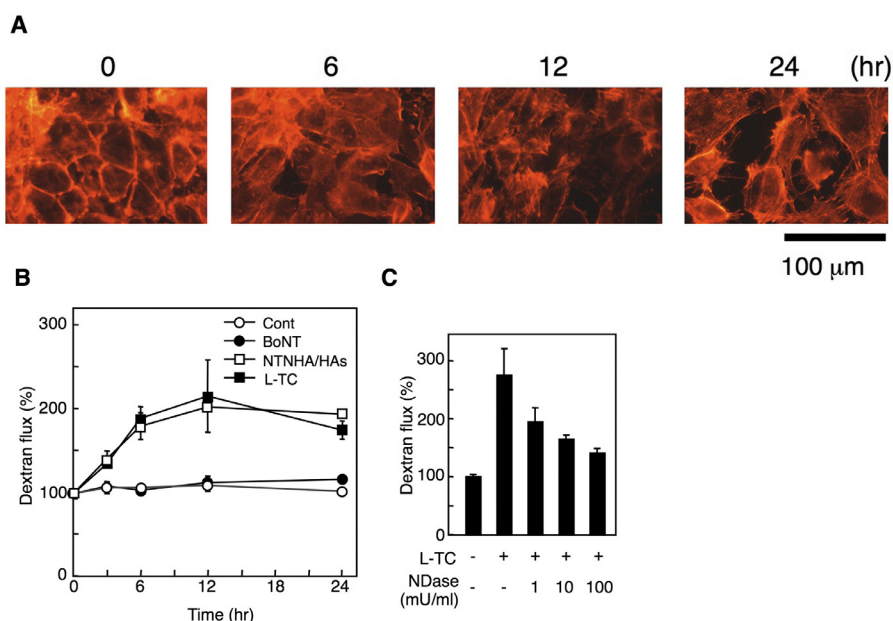


Fig. 1. Effect of toxins on IEC-6 cell morphology and paracellular permeability. (A) Fluorescent microscopy of IEC-6 cells treated with L-TC (200 nM). Actin filaments were visualized with Alexa Fluor 546-conjugated phalloidin. (B) Time-dependent changes in FITC-dextran flux through IEC-6 cell layers. Cells seeded in Transwells were incubated with each toxin (200 nM) and fixed with formaldehyde. FITC-dextran was allowed to pass through the cell layer for 1 hr. Values of FITC-dextran fluorescence intensities were normalized with those obtained in cells without toxin treatment (time 0). (C) Effects of neuraminidase (NDase) treatment on FITC-dextran flux. Cells were treated with NDase for 18 hr and then incubated with L-TC for 24 hr. Values were normalized with FITC-dextran flux of cells incubated with L-TC and without NDase treatment. Data represent mean \pm SEM, $n=3$.

permeability increase (Fig. 3A and 3B), indicating that p38 activation participates in the L-TC-induced increase in paracellular permeability. While inhibition of JNK by SP600125 at 10 and 20 μ M also significantly inhibited the permeability increase (Fig. 3A and 3B), L-TC did not elicit JNK activation (Fig. 2E and 2F). The basal permeability of the cell layers was unaffected by PD98059, SB203580 and SP600125 at 10 μ M, whereas it was slightly but significantly decreased by 20 μ M SB203580 or SP600125 (Fig. 3C and 3D).

In food-borne botulism, absorption of BoNT or L-TC from the intestine is an initial step in oral toxicity. During the past decade, several studies revealed that L-TC and BoNT pass through intestinal cells by transcytosis [6, 13, 20], while a recent study showed that the epithelial barrier was disrupted by L-TC/B and suggested that a paracellular pathway also contributes to toxin translocation from the apical to basolateral space [15]. Here, we show that L-TC/D simultaneously increases paracellular permeability and p38 activity. Furthermore, the permeability increase was not observed when the cells were treated with the p38 inhibitor SB203580. This is the first report demonstrating that p38 contributes to increases in paracellular permeability induced by L-TC.

The increase in paracellular permeability was observed when cells were incubated with L-TC or NTNHA/HAs,

but not BoNT. Similarly, ERK and p38 were activated by L-TC or NTNHA/HAs, but not BoNT. These results suggest that non-toxic proteins contribute to the disruption of barrier function and its underlying mechanism. We recently proposed a 14-mer subunit model of D-4947 L-TC, where L-TC has three "arms" of the HA-33/HA-17 complex exposed on the complex surface [10]. We also reported that L-TC binds and passes through epithelial monolayers much more efficiently than BoNT, due to the higher binding affinity of HA-33 for cells [20]. Thus, we speculate that HA-33 is the most likely protein in L-TC to be responsible for MAPK activation and permeability increase induced by L-TC, although direct evidence for the role of HA-33 in paracellular permeability changes is still needed.

Several microbial toxins exert their pathogenic action by altering the permeability of the intestinal epithelium, and the following two mechanisms have been shown to underlie this alteration. First, some toxins directly interact with proteins present in junctional complexes, such as cadherin and claudin [23, 27]. A disruption or rearrangement of these junctional proteins then leads to a loosening of cell-to-cell connections, including tight junctions and produces a subsequent increase in paracellular permeability. For example, *Clostridium perfringens* enterotoxin has been shown to cause disintegration of tight junctional complexes in MDCK

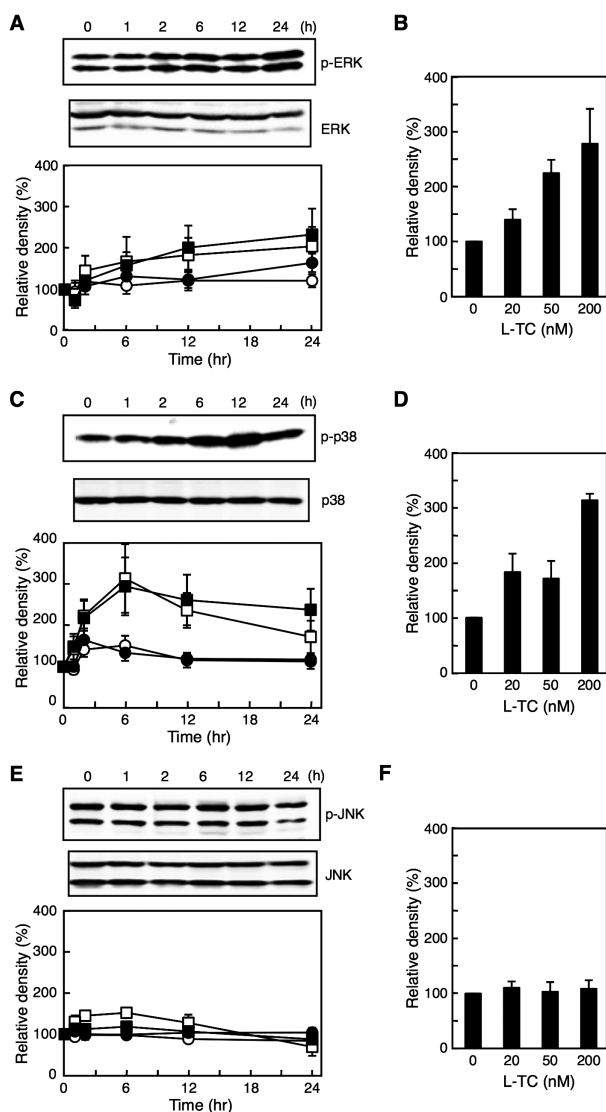


Fig. 2. Toxin-induced phosphorylation of MAPKs in IEC-6 cells. (A, C and E) Time-dependent changes in phosphorylation induced by 200 nM toxin. Upper two panels show representative band patterns of phosphorylated and total MAPKs in IEC-6 cells incubated with L-TC. Open circle, control; closed circle, BoNT; open square, NTNHA/HAS; closed square, L-TC. (B, D and F) Dose-dependent phosphorylation of MAPKs after incubation with L-TC for 12 hr. After toxin incubation, cells were harvested and proteins were subjected to SDS-PAGE and western blotting using antibodies against phosphorylated and total MAPKs. Band densities of phosphorylated MAPKs were normalized to those of total MAPKs, and further normalized with those obtained in cells without toxin treatment (time 0). Data represent mean \pm SEM, $n=3-9$.

cells by directly binding to claudin-3 and -4 [23]. Second, other toxins interact with cellular signaling molecules associated with the arrangement or expression of cytoskeletal and junctional proteins [3, 5, 21]. For example, Chen *et al.* showed that *Clostridium difficile* toxin A increased paracel-

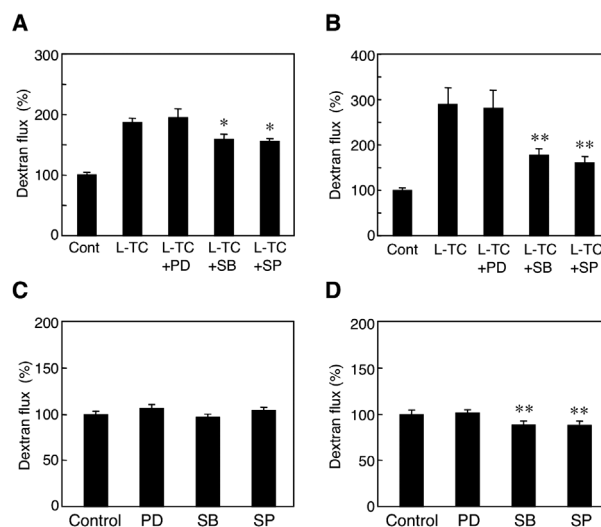


Fig. 3. Effect of MAPK inhibitors on FITC-dextran flux through IEC-6 cell layers treated with (A, B) or without (C, D) L-TC. PD, PD98059; SB, SB203580; SP, SP600125. Each inhibitor at 10 (A, C) or 20 (B, D) μ M was added to the medium 30 min before the experiments. Cells were then incubated with or without L-TC (200 nM) in the presence of each inhibitor for 12 hr. Values were normalized with FITC-dextran flux of cells incubated without inhibitors. Data represent mean \pm SEM, $n=6-15$. **, $P<0.01$ and *, $P<0.05$ compared to L-TC (A, B) or control (D) group (non-repeated ANOVA followed by Bonferroni correction).

lular permeability following phospholipase C and protein kinase C (PKC) activation in colonic T84 cells and that PKC inhibition with specific inhibitors blocked this permeability increase [5]. The present results showed that L-TC induced concomitant increases in FITC-dextran flux and p38 phosphorylation and that the former could be abolished by inhibiting p38, suggesting that p38 activation may be involved in the permeability increase. Thus, the second mechanism mentioned above, a modification of cellular signaling molecules, at least partly plays an important role in the L-TC-induced permeability increase. Recently, Sugawara *et al.* showed that type A and B HA-33 molecules directly interact with E-cadherin, which leads to disruption of tight junctions in several types of epithelial cells [24]. Because the experimental conditions, such as toxin serotypes and cell lines, differ between the previous [24] and present study, it is unclear whether the L-TC/D used here interacted with E-cadherin. Nonetheless, a possibility may be raised that the MAPK activation observed in the present study may be a secondary effect following tight junction disruption caused by the direct interaction of L-TC with junctional proteins. In the previous report, the interaction of HA-33 and E-cadherin required about 1 hr [24], while in this study, p38 activation had already occurred within 1 hr of L-TC treatment. Therefore, the binding or subsequent endocytosis of L-TC likely elicits signals leading to MAPK activation that is independent of tight junction disruption. To fully understand the mechanisms of L-TC-induced increases in paracellular

permeability, it will be necessary to determine the cellular events following L-TC binding to its receptor.

We observed that a JNK inhibitor abrogated the L-TC-induced permeability increase, even though JNK was not activated by L-TC. At this time, the mechanism of the inhibition of permeability increase by SP600125 is unclear. One explanation for this result might be that JNK is constitutively activated to increase cell permeability, but this increase could be offset by other mechanisms. Carrozzino *et al.* [4] demonstrated that addition of SP600125 at 1 μ M enhanced transepithelial electrical activity, an index of paracellular permeability, in 31EG4-2A4 mammary epithelial cells. They also showed that SP600125 or specific silencing of JNK by small interfering RNAs increased claudin mRNAs and suggested that basal JNK activity has a tonic effect on ionic permeability [4]. On the other hand, we did not perform a genetic inhibition of JNK in the present study. Additionally, we used relatively high concentrations of SP600125 (10 and 20 μ M) that were reported to inhibit several kinases other than JNK, such as protein kinases B and C [2]. Thus, for a precise understanding of the effects of JNK demonstrated in this study, it will be necessary to inhibit JNK activity specifically and/or to identify the target of SP600125. MAPKs, including ERK, p38 and JNK, are widely known to play pivotal roles in cell differentiation, growth, migration and death [1, 11] as well as regulating epithelial permeability by affecting expression of junctional proteins and/or remodeling of F-actin [4, 7, 22]. Although we clarified the involvement of p38 in the permeability increase induced by L-TC, the events upstream and downstream of kinase activation are unknown. To establish the signaling cascade involved in the p38-dependent permeability increase, further experiments will be required.

In conclusion, L-TC caused an increase in paracellular permeability in cultured rat intestinal epithelial cells that was associated with ERK and p38 activation. This permeability increase was reduced by an inhibitor of p38, but not ERK. Together, these results suggest that L-TC increases paracellular permeability of intestinal epithelial cells via p38 activation.

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