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Original article

Campylobacter jejuni infection suppressed Cl⁻ secretion induced by CFTR activation in T-84 cells^{*}

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ABSTRACT

Campylobacter jejuni causes foodborne disease associated with abdominal pain, gastroenteritis, and diarrhea. These symptoms are induced by bacterial adherence and invasion of host epithelial cells. *C. jejuni* infection can occur with a low infective dose, suggesting that *C. jejuni* may have evolved strategies to cope with the bacterial clearance system in the gastrointestinal tract. The mucosa layer is the first line of defense against bacteria. Mucus conditions are maintained by water and anion (especially Cl⁻) movement. Cystic fibrosis transmembrane conductance regulator (CFTR) is the main Cl⁻ channel transporting Cl⁻ to the lumen. Mutations in CFTR result in dehydrated secreted mucus and bacterial accumulation in the lungs, and recent studies suggest that closely related pathogenic bacteria also may survive in the intestine. However, the relationship between *C. jejuni* infection and CFTR has been little studied.

Here, we used an ¹²⁵I⁻ efflux assay and measurement of short-circuit current to measure Cl⁻ secretion in *C. jejuni*-infected T-84 human intestinal epithelial cells. The basic state of Cl⁻ secretion was unchanged by *C. jejuni* infection, but CFTR activator was observed to induce Cl⁻ secretion suppressed in *C. jejuni*-infected T-84 cells. The suppression of activated Cl⁻ secretion was bacterial dose-dependent and duration-dependent. A similar result was observed during infection with other *C. jejuni* strains.

The mechanism of suppression may occur by affecting water movement or mucus condition in the intestinal tract. A failure of mucus barrier function may promote bacterial adhesion or invasion of host intestinal epithelial cells, thereby causing bacterial preservation in the host intestinal tract.

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1. Introduction

Campylobacter jejuni is a commensal bacterium in birds and domestic animals that frequently causes gastrointestinal food-borne disease in humans. Infection with *C. jejuni* is a major cause of human bacterial gastroenteritis responsible for more than 400 million cases worldwide each year [1]. *C. jejuni* infection causes digestive symptoms such as abdominal pain, intestinal inflammation, and diarrhea. *C. jejuni* pathogenicity is driven by the production of pro-inflammatory cytokines such as IL-8 in intestinal epithelial cells. Previous studies showed that *C. jejuni* adherence and invasion correlate with activation of MAP kinases leading to production of the pro-inflammatory cytokine, IL-8 [2]. Thus, bacterial internalization into intestinal epithelial cells is important for understanding digestive symptoms induced by *C. jejuni* infection. Interestingly, *C. jejuni* infection can occur with very low infective doses (500–800 bacteria) and long incubation periods (2–7 days) [3]. Those reports suggest that *C. jejuni* may have evolved strategies to cope with the bacterial clearance system. Bacterial internalization into epithelial cells followed by colonization and accumulation may be one such strategy for survival in the gastrointestinal tract.

The mucus condition is a critical determinant of bacterial infection. The mucosa layer of the intestinal tract is the first line of defense against bacteria. It is continuously secreted and is composed of high molecular mass oligomeric mucin glycoproteins. Mucus acts to prevent bacterial attachment and entry into intestinal epithelial cells. A previous study reported that mucin mutant mice have a higher rate of *C. jejuni* colonization [4]. Water flux plays an important role in the maintenance of the mucus barrier function in the intestinal tract. Dehydration of mucus attenuates the mucosa layer movement necessary to clear trapped material.

Anion secretion is closely associated with hydration of the intestinal mucus and clearance of intestinal contents, so that intestinal water balance is regulated by transepithelial ionic movement [5]. Cl^- accounts for 65–70% of all anions in mammals and plays a key role in body fluid homeostasis. Cystic fibrosis transmembrane conductance regulator (CFTR) is the main Cl^- channel expressed in apical membranes of intestinal epithelial cells, and transports Cl^- to the lumen via cAMP or cGMP pathways [6]. Cystic fibrosis (CF) is known to be caused by the loss or dysfunction of CFTR activity resulting from CFTR mutations, leading to aberrant ion and fluid homeostasis in several tissues and organs, including the lung and gastrointestinal tract [7]. The interaction between CFTR and bacterial infection is well understood in the airway. CF patients have dehydrated mucus and attenuated water movement caused by decreased Cl^- secretion leading to stasis, resulting in chronic bacterial infections in lung [8]. CFTR is also highly expressed in the intestinal tract, and CFTR mutations might be associated with intestinal infections. Indeed, a previous study showed that enteropathogenic *Escherichia coli* and *Salmonella enteritidis* suppress Cl^- transport activity in the intestinal tract [9,10]. These reports suggest that Cl^- secretion mediated by CFTR could be critical for bacterial clearance in the intestine, and that CFTR may play a key role in the intestinal host defense mechanism.

The purpose of this study was to examine the influence of *C. jejuni* infection on CFTR activity. We examined Cl^- secretion mediated by CFTR by $^{125}\text{I}^-$ efflux assay and measurement of short-circuit current in *C. jejuni*-infected T-84 cells. Our data showed that forskolin-induced CFTR activation was suppressed by *C. jejuni* infection in T-84 cells. This study may help to elucidate *C. jejuni* survival mechanisms in the intestinal tract.

2. Materials and methods

2.1. Bacterial strains and culture conditions

C. jejuni strains NCTC11168 (ATCC 700819) [11] and 81-176 (ATCC BAA2151) [12] were obtained from American Type Culture Collection (ATCC). NCTC11168 strain was used as a standard strain in this study. The bacteria were cultured in Muller Hinton Broth (DIFCO) under microaerobic conditions (5% O_2 , 10% CO_2 , 85% N_2) at 37 °C for 48 h. The bacteria cells were diluted into fresh MH and grown under microaerobic conditions at 37 °C for 36 h.

2.2. Cell culture

T-84 cells, the human intestinal epithelial cells, were cultured in Dulbecco's modified eagle's medium nutrient mixture F-12 HAM (DMEM/F-12, 1:1; Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS; Hyclone), and 100 $\mu\text{g}/\text{ml}$ gentamicin (Sigma–Aldrich). The cells were seeded on 6-well culture dishes at a density of 3×10^6 cells/well, and cultured for 7 days. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 . A 2 ml aliquot of cell culture medium was changed every 2 days.

2.3. Infection protocol

The culture medium for T-84 cells was replaced with fresh DMEM/F-12 medium (without supplements) before infection. Bacteria were centrifuged and resuspended in phosphate buffered saline (PBS) (pH 7.4) and adjusted for concentration with PBS. The bacterial suspensions were added to each well, and the T-84 cells were infected with multiplicity of infection (MOI) of 20 for 12 h. Infections were carried out at 37 °C in 5% CO_2 .

2.4. Reagents

Forskolin and CFTR_{inhibitor}-172 were obtained from Calbiochem. CPT-cAMP, prostaglandin E_2 (PGE_2), and ATP were obtained from Sigma–Aldrich. The stock of forskolin, CFTR_{inhibitor}-172 and CPT-cAMP were dissolved in DMSO. The stock of PGE_2 and ATP were dissolved in water.

2.5. Efflux assay

The utilization of cellular $^{125}\text{I}^-$ efflux to study Cl^- secretion has been reported previously [13,14]. After infection as described above, the T-84 cells were loaded with radioactive $^{125}\text{I}^-$ by incubating for 1 h at 37 °C with 1 ml HEPES buffer (10 mM HEPES, 145 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2) containing 2 $\mu\text{Ci}/\text{ml}$ Na^{125}I (Perkin Elmer). T-84 cells were washed three times with 1 ml isotope-free HEPES buffer and 1 ml of HEPES buffer was added into each well. The supernatant samples were removed at 2 min intervals and replaced with fresh HEPES buffer. This process was repeated 4–5 times with or without Cl^- secretion agonist (forskolin, CPT-cAMP, PGE_2 , and ATP). After the last supernatant sample collection, the T-84 cells were lysed with 1 ml 0.1 M NaOH for 1 h at 37 °C and recovered. Amounts of $^{125}\text{I}^-$ in recovered supernatant and cell lysis sample were measured using a γ -counter. Total counts were calculated from the sum of counts from cell lysis sample and from all the supernatant samples. Efflux is indicated as the percent of the total count remaining at the end of each efflux period. The percentage efflux was calculated as below: % efflux = (count secreted)/(total count remaining in the cells each minute) \times 100.

2.6. cAMP assay

T-84 cells were cultured on 24-well plates and infected with *C. jejuni* at an MOI of 20 for 12 h. After infection, T-84 cells were stimulated with 10 μ M forskolin or vehicle for 5 min. Intracellular cAMP contents in T-84 cells were measured by an enzyme immunoassay (EIA) system product kit (GE Healthcare Bio-Sciences) as per the manufacturer's instructions.

2.7. Measurement of ion transport

Measurement of short-circuit current to study ion transport has been previously reported [15]. T-84 cells were grown on the transwell for 7 days in order to obtain an electrical resistance greater than 800 Ω /cm². After infection as described above, the T-84 cells were washed with Krebs–Ringer solution (117 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂, 11 mM glucose) and were bathed in Krebs–Ringer solution bubbled with 95% O₂ and 5% CO₂ for 10 min. T-84 cells were mounted in a modified hand-made Ussing chamber for measurement of ion transport. Continuous application of a short-circuit current (I_{sc}) was maintained by a short-circuit current amplifier (NIHON KOHDEN CEZ-9100). I_{sc} was recorded by a polygraph (San-ei, Omni light 8M36). Secretion was stimulated by 10 μ M forskolin on the basolateral side for 10 min. CFTR_{inhibitor}-172 (15 μ M) was added to the apical side after stimulation with forskolin. I_{sc} (ΔI_{sc}) mediated by CFTR was calculated by the difference between CFTR_{inhibitor}-172 treated current (minimum current) and forskolin-stimulated current (maximum current).

2.8. Cytotoxicity assay

T-84 cells were seeded on 24-well plate and infected with *C. jejuni* at an MOI of 20 for 12 h. After infection, supernatants were collected on a new plate, and extracellular lactate dehydrogenase (LDH) was measured using a Cytotoxicity Detection Kit (Promega) following the manufacturer's directions.

3. Results

Infection with *C. jejuni* did not activate CFTR Cl⁻ secretion or cAMP production in T-84 cells.

C. jejuni infection is known to cause diarrhea. Previous reports showed that *E. coli* or *Vibrio cholerae* infection activates Cl⁻ secretion mediated by CFTR via cAMP or cGMP, causing diarrhea. Cholerae toxin (CT) and heat-labile enterotoxin (LT), homologous to CT, produced by *V. cholerae* increase cAMP and activate CFTR. Heat-stable enterotoxin (ST), another major enterotoxin product from *E. coli*, increases cGMP and activates CFTR [16,17]. These reports led us to hypothesize that *C. jejuni* infection might also activate Cl⁻ secretion mediated by CFTR. To investigate whether *C. jejuni* infection would activate Cl⁻ secretion mediated by CFTR, we investigated Cl⁻ secretion in *C. jejuni*-infected cells. In this study, Cl⁻ secretion was measured by ¹²⁵I⁻ efflux from T-84 cells, human intestinal epithelial cells. Cl⁻ secretion in T-84 cells was not activated by *C. jejuni* infection (Fig. 1(a)). Furthermore, production of CFTR activating second messenger, cAMP (Fig. 1(b)) and cGMP (data not shown) was not significantly different between infected cells and no-infected cells. Our data suggested that the mechanism of causing diarrhea induced by *C. jejuni* infection was different from bacterial toxin-induced diarrhea caused by *E. coli* or *V. cholerae*.

Infection with *C. jejuni* suppressed forskolin-activated Cl⁻ secretion mediated by CFTR in T-84 cells.

Inflammatory mediators such as prostaglandin E₂ (PGE₂) promote cAMP production and activate CFTR [18,19]. During *C. jejuni*

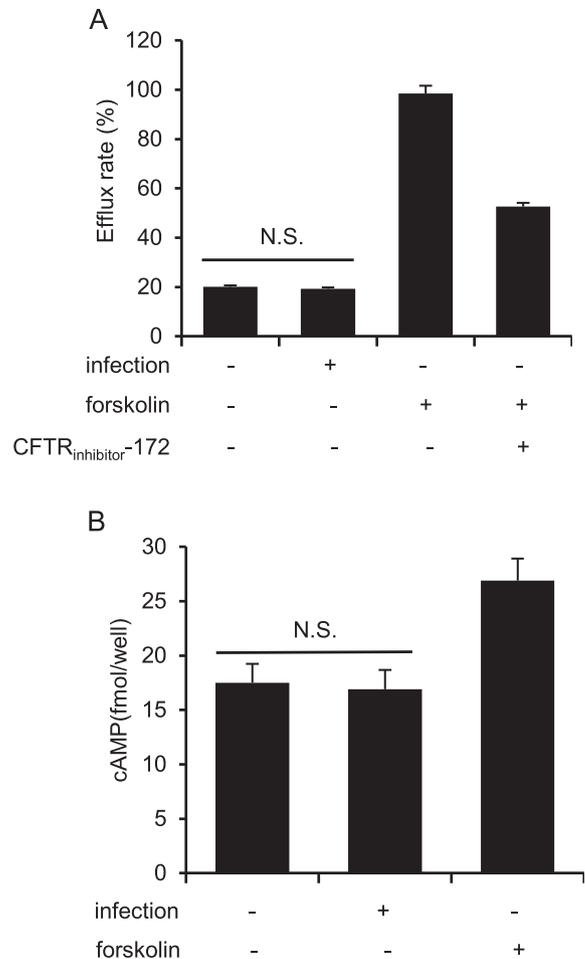


Fig. 1. ¹²⁵I⁻ efflux and intercellular cAMP products are unchanged by *C. jejuni* infection in T-84 cells. T-84 cells were infected with *C. jejuni* (NCTC11168) for 12 h. Cl⁻ secretion was measured using an ¹²⁵I⁻ efflux assay. The cells were pre-loaded with ¹²⁵I⁻ and 6 min efflux was saved for counting (a). Forskolin was used as a positive control in the efflux assay, and CFTR-mediated Cl⁻ secretion was confirmed by CFTR_{inhibitor}-172. Intracellular cAMP production was measured by an enzyme-linked immunosorbent assay (b). T-84 cells were infected with *C. jejuni*, and cells were treated with 10 μ M forskolin for 5 min as positive controls. Results are shown as the mean \pm SD calculated from the results of 3 independent experiments. n.s. not statistically significant.

infection, the inflammatory mediator IL-8 is secreted from intestinal epithelium cells, and production of the cytokine induces inflammation in the intestinal tract [2]. To investigate any interaction between *C. jejuni* infection and CFTR with regards to inflammation, we evaluated ion transport under forskolin, cAMP agonist, stimulated conditions as an inflammatory model by measurement of ion transport with an Ussing chamber. We found that *C. jejuni* infection suppressed CFTR-dependent ion transport (Fig. 2(a–c)). During the infection period, *C. jejuni* infection did not cause release of the cytosolic enzyme lactate dehydrogenase (LDH), a marker of cytotoxicity, from T-84 cells (Fig. 2(d)). These data suggested that suppression of ion transport by *C. jejuni* infections was not related to cell death. Suppression of forskolin-activated Cl⁻ secretion by *C. jejuni* infection was also observed by the ¹²⁵I⁻ efflux assay (Fig. 3(a)). The results of efflux assay agreed with measurements of ion transport. Furthermore, to confirm that the suppression of Cl⁻ secretion was directly induced by *C. jejuni* infection, we checked forskolin-activated Cl⁻ secretion in several infective conditions. *C. jejuni* infection caused bacterial dose-dependent and duration-dependent reduction of CFTR-mediated Cl⁻ secretion (Fig. 3(b and c)). Suppression of forskolin-activated Cl⁻ secretion

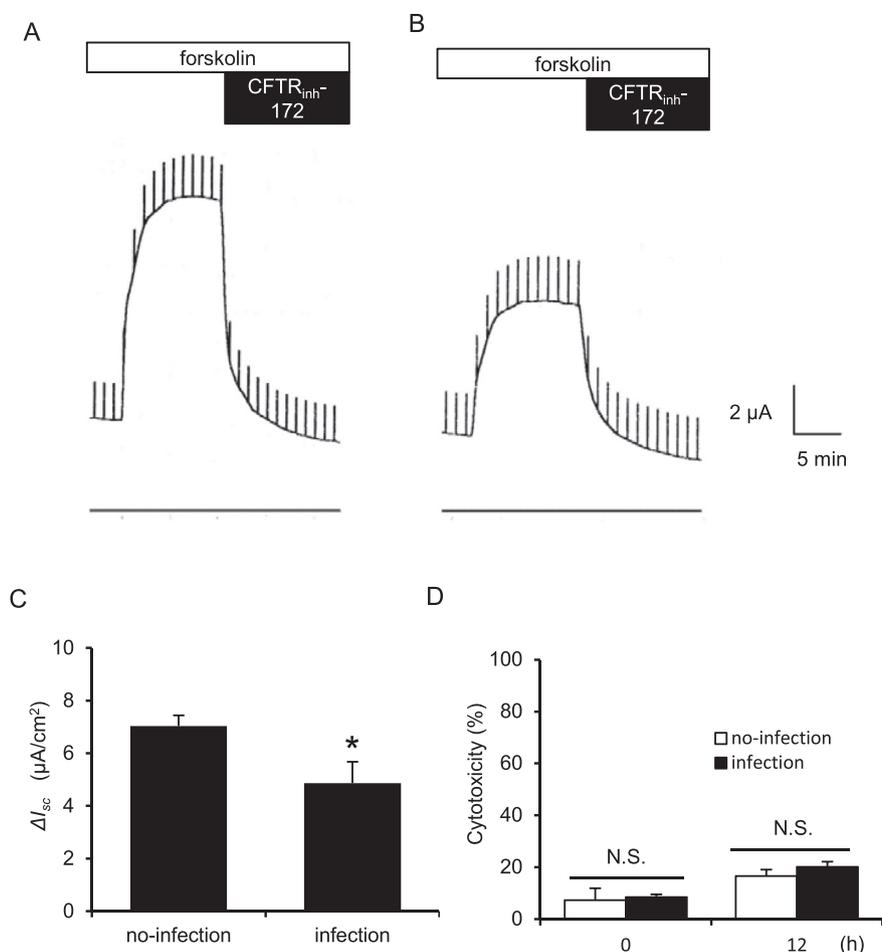


Fig. 2. *C. jejuni* infection suppressed CFTR-mediated ion secretion in forskolin-stimulated T-84 cells. T-84 cell monolayers were grown on transwells, and mounted on an Ussing chamber for measuring ion transport. Short-circuit current (I_{sc}) was measured through stimulation with 10 μM forskolin followed by 13 μM CFTR_{inhibitor}-172 in no-infected T-84 cells (a) and *C. jejuni*-infected T-84 cells (b), and the data were quantified (c). *C. jejuni* infection dependent cell cytotoxicity (d) was assayed by measuring cellular LDH release into the culture medium (d). T-84 cells were infected with *C. jejuni* (black bar) or left uninfected (white bar) for the indicated times, and LDH was measured in the medium. Results are shown as the mean \pm SD calculated from the results of 3 (c) or 4 (d) independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$; n.s. not statistically significant.

was observed not only with the *C. jejuni* NCTC11168 strain, the standard strain in this study, but also with the 81-176 strain (Fig. 3(d)). Our data indicated that *C. jejuni* infection suppressed forskolin-activated Cl^- secretion regardless of the *C. jejuni* strain. These data strengthen the hypothesis that CFTR-mediated Cl^- secretion is silenced during *C. jejuni* infection in the human intestinal tract.

The stimulus of CFTR activator-dependent Cl^- secretion was suppressed by *C. jejuni* infection in T-84 cells.

In order to confirm whether the reduction of CFTR-mediated Cl^- secretion was forskolin stimulation dependent, we evaluated Cl^- secretion stimulated by other CFTR activators in *C. jejuni*-infected T-84 cells. CPT-cAMP is a membrane-permeable activator of CFTR. Under CPT-cAMP stimulus, Cl^- secretion was suppressed by *C. jejuni* infection (Fig. 4(a)). As mentioned above, *C. jejuni* infection would induce inflammation in the intestinal tract. PGE₂, an inflammatory mediator, increases intracellular cAMP and activates CFTR [18,19]. Under PGE₂ stimulus, *C. jejuni* infection suppressed PGE₂-induced Cl^- secretion (Fig. 4(b)). Our data indicated that *C. jejuni* infection induces the suppression of Cl^- secretion activated by all stimulators of CFTR activation used in this study.

Finally, we investigated whether the suppression of Cl^- secretion by *C. jejuni* infection was relevant for another Cl^- channel. We investigated the influence of *C. jejuni* infection on the Ca²⁺-

activated chloride channel (CaCC). CaCC also plays an important role in Cl^- secretion in the intestinal tract like CFTR, and is activated by ATP via G protein coupled receptor (GPCR) pathways [20,21]. We investigated ATP-stimulated Cl^- secretion in T-84 cells infected with *C. jejuni*. We observed that *C. jejuni* infection suppressed Cl^- secretion activated by ATP (Fig. 4(c)). Our data showed that CaCC was suppressed by *C. jejuni* infection, coincident with the attenuation of CFTR in T-84 cells. These results raise the possibility that *C. jejuni* may regulate Cl^- secretion and attenuate water movement in the intestinal tract. We suspect that repression of water movement leads to bacterial accumulation and survival in the intestinal tract.

4. Discussion

CFTR is a key Cl^- channel in the intestinal tract, owing to its close association with water movement in the intestinal epithelium. CFTR is the critical downstream target of cAMP and cGMP-mediated Cl^- secretion in the intestinal tract [6]. Some studies have suggested that water movement induced by CFTR activity may be associated with bacterial survival, and that mucus viscosity defends against microbes and physical injury [8,22].

C. jejuni is major prevalent cause of foodborne diarrheal disease worldwide [23]. Clinical strains of *C. jejuni* cause a variety of

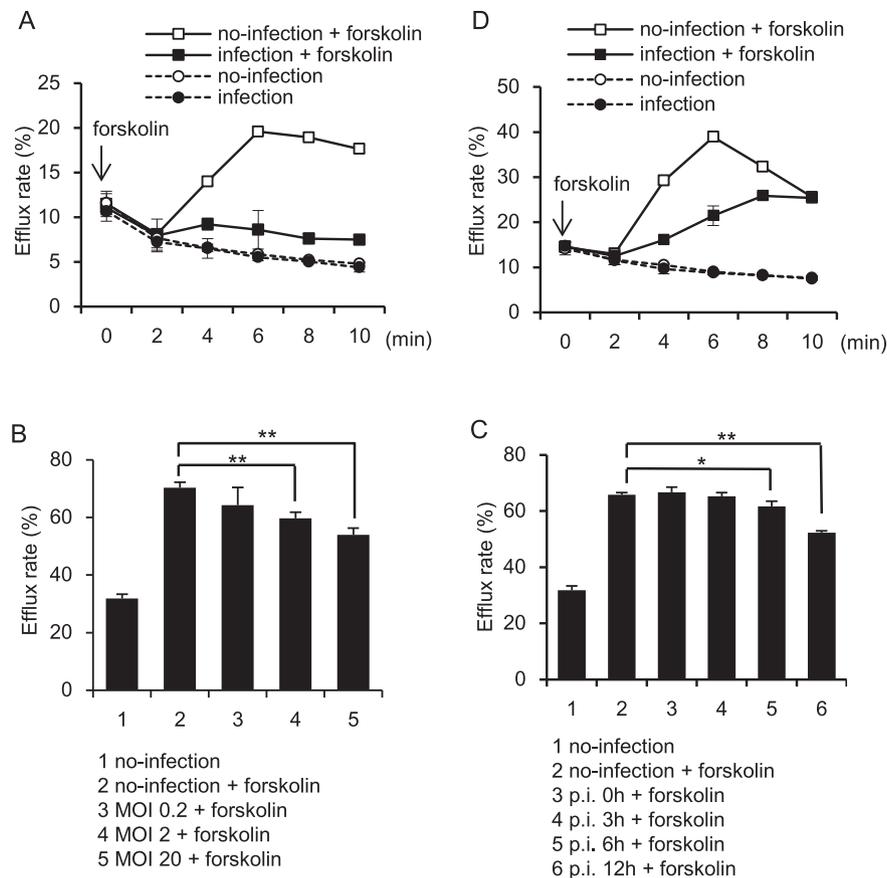


Fig. 3. Forskolin-induced $^{125}\text{I}^-$ efflux was decreased by *C. jejuni* infection. T-84 cells were infected with *C. jejuni* for 12 h and were pre-loaded with $^{125}\text{I}^-$. The $^{125}\text{I}^-$ efflux in T-84 infected with *C. jejuni* was measured at 2 min intervals for a 10 min period. 10 μM forskolin was added 0 min after starting the efflux measurements. The $^{125}\text{I}^-$ efflux in T-84 infected with *C. jejuni* strain NCTC11168 (a) or 81-176 (d) was measured at 2 min intervals for a 10 min period. Relation between *C. jejuni* infection and suppression of Cl^- secretion was validated by comparison of bacterial cell number and infection period. T-84 cells were infected with *C. jejuni* at indicated MOI (b) or for the indicated times (c) and were pre-loaded with $^{125}\text{I}^-$. 10 μM forskolin was added 0 min after starting the efflux measurements. The $^{125}\text{I}^-$ efflux in T-84 *C. jejuni*-infected cells was counted in 6 min efflux. The data were normalized to the forskolin-unstimulated samples. Results were shown as the mean \pm SD calculated from the results of 3 independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$.

symptoms ranging from severe inflammatory bloody diarrhea to no-inflammatory watery diarrhea [24]. We predicted that water movement in the intestinal epithelium cells is associated with *C. jejuni* survival during *C. jejuni* infection. In this study, we investigated the relationship between CFTR and *C. jejuni* infection. First, we investigated Cl^- secretion in steady-state *C. jejuni*-infected T-84 cells, but Cl^- secretion was not changed (Fig. 1(a)). Toxins elaborated by *V. cholerae* or *E. coli* are known to activate CFTR and secrete Cl^- via cAMP or cGMP in intestinal epithelial cells, leading to diarrhea [16,17]. Our data suggested that the mechanism of diarrhea induced by *C. jejuni* infection is different from the secretory diarrhea induced by *V. cholerae* infection or *E. coli* infection. Next, we investigated the effect of *C. jejuni* infection on activated CFTR in T-84 cells. Production of intracellular cAMP, a CFTR activator, was increased by PGE_2 in inflammation [18,19]. *C. jejuni* infection induces IL-8 secretion and induces inflammation in intestinal cells [2]. Therefore, to examine Cl^- secretion during inflammation, forskolin was used as an inflammation model. T-84 cells were stimulated by forskolin after *C. jejuni* infection. We observed that forskolin-stimulated CFTR activity was suppressed in *C. jejuni*-infected T-84 cells (Fig. 2(a–c)). The release of LDH, widely known as a marker of cytotoxicity [25], was measured to evaluate cell death. During the 0–12 h infection period, the release of LDH was not changed (Fig. 2(d)). These data suggested that suppression of Cl^- secretion by *C. jejuni* infection was not related with cell death. Furthermore, reduction of Cl^- secretion was observed in the

presence of other stimulators of CFTR activation (CPT-cAMP, and PGE_2) (Fig. 4(a and b)). Reduction of CFTR-mediated Cl^- secretion was *C. jejuni* dose-dependent and duration-dependent (Fig. 3(b and c)). Also, the diminished responsiveness to forskolin was found not only in *C. jejuni* strain NCTC11168, the standard strain in this study, but also in the 81-176 strain (Fig. 3(a–d)). Finally, in order to determine the effect of *C. jejuni* infection on another Cl^- channel, we investigated the influence of *C. jejuni* infection on CaCC. CaCC is known as a Cl^- channel that secretes Cl^- to the lumen and has an important role in the maintenance of ion balance in the intestinal tract [20,21]. We observed that ATP-activated Cl^- secretion was also suppressed by *C. jejuni* infection. These data suggest that *C. jejuni* infection suppresses Cl^- secretion, thereby attenuating water flux in intestinal epithelial cells.

Mucus is the first barrier for pathogens entering the gastrointestinal tract. Enteric pathogens have to colonize and avoid the mucosa layer prior to causing disease. To establish infection, these bacteria have to circumvent mucus barriers in the gastrointestinal tract. Mucus is particularly important for invasive bacteria, such as *C. jejuni*. The mucosa layer is composed of high molecular mass oligomeric mucin glycoproteins which confer mucus with its water holding capacity [26]. In the epithelial cell surface, including the mucosa layer, liquid balance is modulated by CFTR Cl^- secretion which controls the concentration of mucosa layer contents. CFTR influence on mucus is well-studied for respiratory disease in CF. The absence of CFTR function dehydrates the airway surface,

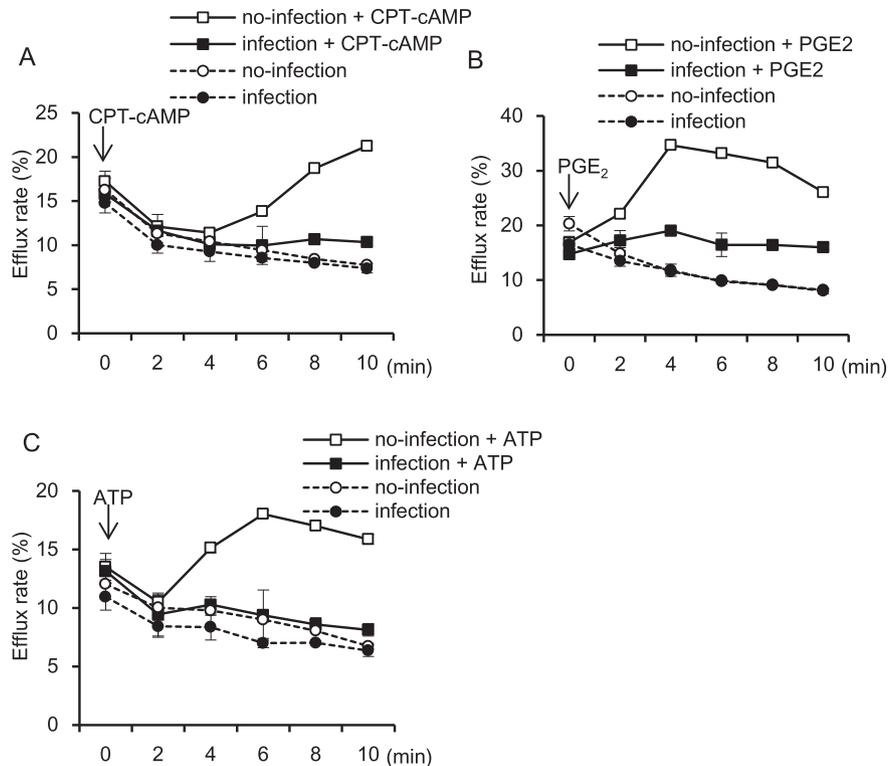


Fig. 4. The stimulus of CFTR activator-dependent $^{125}\text{I}^-$ efflux was suppressed by *C. jejuni* infection in T-84 cells. T-84 cells were infected with *C. jejuni* for 12 h and were pre-loaded with $^{125}\text{I}^-$. The $^{125}\text{I}^-$ efflux in T-84 cells infected with *C. jejuni* was measured at 2 min intervals for a 10 min period. 50 μM CPT-cAMP (a), 10 μM PGE₂ (b) or 200 μM ATP (c) was added 0 min after starting the efflux measurements. Results are shown as the mean \pm SD calculated from the results of 3 independent experiments.

leading to mucus stasis. In CF patient lungs, mucus stasis leads to chronic bacterial infections with bacteria such as *Pseudomonas aeruginosa* [8]. Moreover, chronic infection in CF lung has been considered to be caused by mucus pathology in the airway [27], and it is suggested that destruction of mucus homeostasis by attenuation of CFTR activity might also promote bacterial preservation in the intestinal tract.

In the human intestine, MUC2 is the main secreted mucin constituting the mucus layer. MUC1, MUC3, MUC4, MUC12, MUC13, and MUC17 are the cell surface mucins. During infection, enteric pathogens are known to stimulate mucus secretion. Similarly, expression of MUC1 is upregulated in human colonic biopsies following *C. jejuni* infection [28]. Muc1^{-/-} mice have a higher rate of gastroenteritis in a mouse *C. jejuni* infection model [4]. These data suggest that mucus plays a critical role in preventing *C. jejuni* infection. Mucins are also known as the major chemoattractants for *C. jejuni* [29], and modulation of mucin expression can influence colonization and pathogenicity of *C. jejuni* [30]. Mucus is clearly important for bacterial defense and regulation of bacterial colonization in the intestinal tract. We hypothesized that suppression of CFTR-mediated water movement and mucus dehydration induced by *C. jejuni* infection may contribute to not only defense but also regulation of bacterial colonization in the intestinal tract.

To conclude, our results show that *C. jejuni* infection suppresses forskolin-activated Cl^- secretion. This reaction is predicted to play a key role in *C. jejuni* survival in the intestinal tract. Further investigation of the relationship between *C. jejuni* infection and CFTR would lead to a better understanding of *C. jejuni* survival.

Conflict of interest

None.

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