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To cite this article: S. Krishnan, B. S. Ramakrishna, G. T. Keusch, D. W. K. Acheson, A. B. Pulimood, M. Mathan (1999) Effect of Shiga Toxin on NaCl Transport in Rat Distal Colon, Scandinavian Journal of Gastroenterology, 34:8, 777-783, DOI: 10.1080/003655299750025705

To link to this article: http://dx.doi.org/10.1080/003655299750025705

Published online: 08 Jul 2009.

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Effect of Shiga Toxin on NaCl Transport in Rat Distal Colon

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Background: Shiga toxin causes net fluid secretion in rabbit jejunum by selectively targeting, and inhibiting protein synthesis in, absorptive villous cells. The effect of Shiga toxin on the colon, where it is presumably produced, is not known. This study was undertaken to investigate the effect of Shiga toxin on the rat distal colon.

Methods: Net absorption of water and Na was determined by in vivo perfusion of closed loops of rat colon pre-exposed to Shiga toxin or saline. Unidirectional and net fluxes of $^{22}$Na and $^{36}$Cl were measured in vitro under voltage-clamp conditions across rat distal colon mucosa pre-exposed to Shiga toxin. Shiga toxin binding to sections of rat distal colon was localized by immunohistochemistry. Protein synthesis was measured in surface and crypt colonocytes with $^{3}$H-leucine incorporation.

Results: In the in vivo perfusion studies net absorption of Na and water was increased in Shiga toxin-treated colon compared with controls ($P < 0.01$). In the studies carried out in vitro, $J_{net}^{Na}$ and $J_{net}^{Cl}$ across Shiga toxin-treated mucosa were found to be significantly higher than in control tissue ($P < 0.001$ and $P < 0.01$, respectively). Net absorption of Na or Cl did not increase further in the presence of 25 mM butyrate, indicating the absence of short-chain fatty acids (SCFA)-linked NaCl absorption in Shiga toxin-treated colon. Moreover, Shiga toxin-treated colon failed to respond to theophylline, which induced secretion in the normal colon. Immunohistochemistry showed Shiga toxin binding to crypt cells but not to surface cells in the distal colon. Shiga toxin inhibited protein synthesis (by 27.3%) in crypt cells but not in surface cells ($P < 0.05$).

Conclusions: An unexpected increase in water and NaCl absorption was noted in Shiga toxin-treated rat distal colon, which appears to result from selective effects of the toxin on secretory crypt cells.

Key words: Crypt–surface axis; NaCl absorption; Shiga toxin; short-chain fatty acids

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Shiga toxin, an exotoxin produced by *Shigella dysenteriae* I, inhibits protein synthesis by inactivation of the 60S ribosomal subunit to cause cell death (1). Shiga toxin is considered to be responsible for several of the clinical manifestations of Shigellosis, including acute watery diarrhoea noted in the initial phase of illness (2). Shiga toxin has been noted to induce net secretion of fluid and electrolytes in the small bowel of the rabbit (3, 4). In the rabbit jejunum Shiga toxin-induced fluid secretion has been attributed to specific binding of the toxin to absorptive villous cells, with inhibition of protein synthesis only in villous cells (4). This mode of action contrasts with effects of enterotoxins such as cholera toxin and *Escherichia coli* heat-stable enterotoxin, which act through second messengers to inhibit absorption (in surface cells) or stimulate secretion (in crypt cells) (5). The colon is the site of colonization by *S. dysenteriae*. Colonic absorption of water and sodium is affected in both experimental and human shigellosis (6, 7). Altered colonic absorption in shigellosis may result from epithelial cell damage secondary to invasion of colonic mucosa by the bacteria; from Shiga toxin damage to surface absorptive cells, as observed in the rabbit jejunum; or secondary to the effect of the inflammatory cell infiltrate in the mucosa. The present study was undertaken to examine the effects of Shiga toxin on ion transport in the rat distal colon, with specific reference to its effects on short-chain fatty acid (SCFA)-linked NaCl absorption. Rat distal colon was chosen because of the presence of well-characterized SCFA-linked NaCl absorption in this tissue, in addition to basal electroneutral NaCl absorption.

Materials and Methods

Animals

Adult inbred albino rats (originally of the Wistar strain) of either sex, weighing approximately 200–250 g, were used for all the studies. Rats were anaesthetized using either pentobarbital or ether, as described below, and maintained under anaesthesia for the duration of in vivo perfusion or Shiga toxin incubation. At the end of the perfusion or toxin incubation the rats were killed by section of the inferior vena cava. The experimental protocols were approved by the Research Committee of the Christian Medical College,
Vellore. The Shiga (holo)toxin used in these studies was prepared and purified at the New England Medical Center, USA.

**In vivo perfusion studies.** Rats were anaesthetized with pentobarbital sodium (50 mg/kg body weight) intraperitoneally. The colon was exposed through a midline incision, and the contents flushed out using phosphate-buffered saline (PBS). The distal end of the colon was ligated, and Shiga toxin in PBS (20 μg/ml, 50 μg/ml) or PBS alone (control) was introduced into the colon and the proximal end closed. Rats were maintained under anaesthesia under a heating lamp for 3 h, at the end of which the colon was emptied and filled with 2 ml of Krebs–Henseleit saline containing 4 g/l polyethylene glycol (PEG 4000, Fluka Chemie AG, Switzerland) as a non-absorbable marker. After a 1-h incubation the colonic contents were collected and assayed for Na by flame photometry and for PEG 4000 by turbidimetry (8). The rats were killed; the colon removed, opened, and spread out on graph paper; and its surface area was measured. Net absorption of water and of sodium were calculated by using appropriate equations.

**In vitro flux studies.** Active Na and Cl absorption was studied under voltage clamp conditions across isolated distal colon mucosa in vitro (9). In brief, the rats were anaesthetized with pentobarbital sodium and the abdomen opened. The colon was flushed, ligated at either end, and incubated in vivo with Shiga toxin, 50 μg in 1 ml PBS as described earlier. The animals were maintained under anaesthesia throughout the incubation period. Controls were obtained by instilling in the colon an equivalent volume of PBS. After incubation the rats were killed, the colon excised and stripped of serosal and muscular layers, and approximately 2-cm pieces of distal colon tissue were mounted in Lucite chambers (aperture of 1.13 cm²; CHMI, World Precision Instruments, USA) between serosal and mucosal reservoirs. The spontaneous transepithelial potential difference (PD) was determined, and tissues were voltage-clamped at zero PD, using a DVC-1000 voltage clamp (World Precision Instruments). The short-circuit current (Isc) needed to clamp the voltage at zero was measured. The radioactive isotopes 22Na (Amer sham, Co, UK) and 36Cl (Board of Radiation and Isotope Technology, India) were added to either the mucosal or serosal side, and their unidirectional fluxes during two 15-min time periods were measured. Two solutions were used in these studies, a butyrate-free and a butyrate-containing solution of the following compositions: the butyrate-free solution contained 20 mM NaCl, 2.4 mM K₂HPO₄, 0.4 mM KH₂PO₄, 1.2 mM MgCl₂, 75 mM Na isothionate, 45 mM choline chloride, and 1.2 mM CaCl₂·2H₂O, pH 7.4. Then 10 mM glucose was added to the solution, which was oxygenated before use. Butyrate-containing solution was prepared by substituting sodium butyrate (25 mM) for Na isothionate (25 mM). Na and Cl concentrations were kept constant at 95 and 70 mM, respectively. The butyrate-containing solution was tested, since SCFA-linked NaCl absorption is now recognized as a major absorptive pathway for NaCl absorption from the colon. In both solutions fluxes of Na and Cl were measured before and after the addition of theophylline (10 mM concentration) to the serosal reservoir. The radioactivity of 36Cl and 22Na in the cold side samples was counted in beta (LKB Rackbeta) and gamma (LKB Compugamma) counters, respectively, after addition of scintillant. Conductance (G) was derived from Isc and PD by using Ohm’s law. Net fluxes (J net) were calculated as the difference between mucosal-serosal (J ms) and serosal-mucosal (J sm) fluxes across tissue pairs matched on the basis of less than 10% difference in basal conductance.

**Localization of Shiga toxin-binding in rat distal colon**

The binding of Shiga toxin to colonic epithelium was localized by immunohistochemistry (4). Five-micrometre sections from frozen rat distal colon were incubated with purified Shiga toxin in PBS (1 μg/ml) or PBS alone for 1 h at 37 °C in a humid chamber. Sections were washed with buffer, blocked with methanol and H₂O₂, and then treated with normal swine serum. Sections were overlaid with rabbit polyclonal antiserum to Shiga toxin, incubated for 1 h, and washed with PBS. Swine anti-rabbit antibody conjugated to biotin was then overlaid on the sections, incubated for 50 min, and washed with buffer. Specific Shiga toxin-binding was visualized by using an avidin–biotin complex (Universal Vectastain kit, Vector Laboratories Inc, USA) conjugated to horseradish peroxidase, developed with diaminobenzamidine, washed, and counterstained with haematoxylin and eosin.

**Colonocyte isolation.** Colonocytes were isolated by means of a modification of earlier methods (10, 11). In brief, rats were killed with an ether overdose, and the colon was excised and washed with oxygenated Ca²⁺-free Krebs–Henseleit (Ca²⁺-free KH) solution. The colon was then ligated at one end and distended with Ca²⁺-free KH solution containing 5 mM ethylenediaminetetraacetic acid (EDTA), and the open end sealed. The distended colon was incubated in Ca²⁺-free KH solution in a beaker containing the same solution in a shaking water bath at 37 °C with continuous oxygenation. After incubation for 17 min colonic contents were drained, taking care not to manipulate the colon. This fraction contained predominantly surface cells. The colon was refilled with KH solution for 3 min and gently palpatated with the fingers to loosen the remaining cells. The fraction thus collected contained predominantly crypt cells. Cell fractions were centrifuged at 3000 rpm for 5 min. The resulting cell pellet was washed twice with Ca²⁺-containing KH containing 0.5% bovine serum albumin (Sigma Co., USA) and finally resuspended in the same solution. Sections of the colon processed for light microscopic examination at the end of each step verified the sequential separation of surface and crypt cells.

The purity of the cell fractions was assessed by means of the activity of alkaline phosphatase (12). Cell viability was measured by trypan blue dye exclusion and by lactate...
toxin. To 1 ml of cell suspension, 5 µl of 20 µg/ml Shiga toxin or phosphate-buffered saline alone (control). Values are mean ± standard error. Student t test; * P < 0.001 and ** P < 0.05 compared with control untreated colon. @ P < 0.001 and @@ P < 0.01 compared with 20 µg/ml toxin.

dehydrogenase (LDH) release into the medium (13). The protein content of the suspension was estimated with Lowry’s method (14).

Protein synthesis. The effects of Shiga toxin on protein synthesis in crypt and surface colonocytes were assessed. In brief, cells were suspended in Leibovitz L-15 medium containing 100 units/ml penicillin and 100 µg/ml streptomycin. To 1 ml of cell suspension, 5 µg purified Shiga toxin (or PBS, in the case of controls) was added and mixed thoroughly. The cell suspension was incubated at 37°C for 30 min. After this, 20 µl of 1H-leucine (0.5 mCi/ml, Board of Radiation and Isotope Technology, India) was added, mixed well, and incubated for 2 h. Tubes were then immersed in ice water slurry to arrest metabolism. Cells were centrifuged, washed twice with cold PBS, and solubilized in 0.5 ml 0.5 M KOH. An equal volume of cold 20% trichloroacetic acid (TCA) was added, and the resulting precipitate washed twice with cold 10% TCA. The precipitate was solubilized in 1 ml 0.5 N NaOH and used for protein estimation and for estimation of 3H-leucine incorporation into protein by liquid scintillation spectrometry. Cycloheximide (1 mg/ml) was used as a positive control for protein synthesis inhibition.

Statistics

Statistical analyses were done using SYSTAT (version 3.0) on a personal computer, and all results are expressed as mean ± standard error. The two-tailed Student t test was used to determine the significance of differences between means. A P value of <0.05 was considered statistically significant.

Results

Na and water absorption in vivo

Net absorption of water and Na from Shiga toxin-treated and control rat colon is shown in Fig. 1. Shiga toxin pretreatment significantly increased net absorption of both water and Na (P < 0.05). The increase in absorption was more pronounced at a Shiga toxin concentration of 50 µg/ml compared with 20 µg/ml (Fig. 1) (P < 0.001 and P < 0.01 for water and Na, respectively).

22Na and 36Cl fluxes in vitro

Unidirectional and net fluxes of Na and Cl across rat distal colon are shown in Table I. Basal net Na and Cl fluxes in Shiga toxin-treated tissue were higher than in control tissue.

Table I. Effect of short-chain fatty acids (SCFA) on Na and Cl fluxes in Shiga toxin-treated rat distal colon mucosa in vitro

<table>
<thead>
<tr>
<th>n</th>
<th>Condition</th>
<th>G_0</th>
<th>Change in</th>
<th>I_{ms} Na</th>
<th>I_{am} Na</th>
<th>I_{net} Na</th>
<th>I_{ms} Cl</th>
<th>I_{am} Cl</th>
<th>I_{net} Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>(A) SCFA-free</td>
<td>11.89 ± 0.49</td>
<td>1.80 ± 0.26</td>
<td>4.33 ± 0.41</td>
<td>4.24 ± 0.51</td>
<td>11.66 ± 0.71</td>
<td>7.43 ± 0.69</td>
<td>4.27 ± 0.70</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>(B) 25 mM butyrate</td>
<td>12.49 ± 1.30</td>
<td>0.96 ± 0.22</td>
<td>5.00 ± 0.95</td>
<td>2.84 ± 0.79</td>
<td>11.52 ± 0.77</td>
<td>10.35 ± 1.43</td>
<td>1.17 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>(C) SCFA-free</td>
<td>11.45 ± 1.24</td>
<td>1.73 ± 0.61</td>
<td>5.58 ± 0.69</td>
<td>6.83 ± 1.49</td>
<td>13.88 ± 1.66</td>
<td>8.53 ± 0.82</td>
<td>5.35 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>(D) Butyrate</td>
<td>11.45 ± 1.24</td>
<td>1.73 ± 0.61</td>
<td>5.58 ± 0.69</td>
<td>6.83 ± 1.49</td>
<td>13.88 ± 1.66</td>
<td>8.53 ± 0.82</td>
<td>5.35 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>(E) 25 mM butyrate</td>
<td>11.04 ± 0.96</td>
<td>0.83 ± 0.32</td>
<td>5.63 ± 0.66</td>
<td>5.26 ± 1.81</td>
<td>11.96 ± 2.24</td>
<td>7.99 ± 0.6</td>
<td>3.97 ± 2.2</td>
<td></td>
</tr>
</tbody>
</table>

I_{ms} = mucosa to serosa flux; I_{am} = serosa to mucosa flux; I_{net} = net flux; G_0 = conductance at zero time. Values are expressed as mean ± standard error. Units are in µEq/h/cm² for short-circuit current, Isc, unidirectional and net fluxes, and in µEq/cm² for conductance at zero time, G_0. (I) indicates basal fluxes in absence of theophylline; (II) indicates fluxes after addition of 10 mM theophylline.

* Student paired two-tailed t test, P < 0.001; ** P < 0.01 compared with (A).
@ @ P < 0.01; @@ not significant compared with (C). Unpaired one-tailed t test showed P < 0.05 for both I_{ms}^{Na} and I_{net}^{Na} in C(I) compared with A(I) and I_{net}^{Cl} ** in B(I) compared with A(I). All other values were not significant by unpaired t test compared with A. Post-theophylline values were not significantly different from basal values in each case except in (A) (P > 0.001).

Fig. 1. Net absorption of water (hatched bars) and Na (cross-hatched bars) from rat distal colon. Colonic loops were pretreated with either 20 µg/ml or 50 µg/ml Shiga toxin or phosphate-buffered saline alone (control). Values are mean ± standard error. Student t test; * P < 0.001 and ** P < 0.05 compared with control untreated colon. @ P < 0.001 and @@ P < 0.01 compared with 20 µg/ml toxin.
not exposed to Shiga toxin. In control tissues not exposed to Shiga toxin the presence of 25 mM butyrate increased $J_{\text{net}}^{\text{Na}}$ and $J_{\text{net}}^{\text{Cl}}$ compared with butyrate-free solution. In contrast, in Shiga toxin-treated tissues the presence of 25 mM butyrate did not increase $J_{\text{net}}^{\text{Na}}$ or $J_{\text{net}}^{\text{Cl}}$. $J_{\text{net}}^{\text{Cl}}$ in Shiga toxin-treated tissue was similar to that in control tissue not treated with Shiga toxin and not bathed with butyrate. The mucosa-to-serosa fluxes were lower in butyrate-containing solution than in control. The addition of 10 mM theophylline to the serosal solution did not significantly change net Na and Cl fluxes from basal values in either control or in butyrate–Ringer solution, indicating that the chloride secretory response was abolished. This is in contrast to unexposed tissues, in which theophylline diminished $J_{\text{net}}^{\text{Na}}$ and $J_{\text{net}}^{\text{Cl}}$ in control Ringer solution without butyrate.

**Shiga toxin binding to rat distal colon**

Photomicrographs of representative sections of rat distal colon incubated with antibody to Shiga toxin, with and without prior Shiga toxin exposure, are presented in Fig. 2. Binding of Shiga toxin was noted only in the lower half of crypts. Shiga toxin did not bind to cells in the upper half of crypts or the surface epithelium.

**Protein synthesis in surface and crypt cells**

The procedure followed to isolate surface and crypt cells was validated by histologic examination, which confirmed sequential isolation first of surface and then of crypt cells (Fig. 3). Alkaline phosphatase activity, assayed in crypt and surface cell fractions, indicated a 2.5-fold higher activity in the surface cell fraction than in the crypt cell fraction (50...
units/μg protein compared with 20 units/μg protein). LDH activity in the supernatant after cells were pelleted was less than 5% of the total activity in the cell pellet, indicating good cell viability.

Shiga toxin did not inhibit 3H-leucine incorporation into protein in surface epithelial cells (3.75%; statistically not significantly different). In crypt cells, however, Shiga toxin significantly reduced 3H-leucine incorporation (by 27.3% compared with control; \( P < 0.05 \)). Cycloheximide inhibited 3H-leucine incorporation by 13.6% and 11.9% in crypt and surface cells, respectively, compared with control cells.

Discussion

The present study reports, unexpectedly, that Shiga toxin increased sodium and water absorption in the rat colon. This is in contrast to its action on the rabbit small bowel, where it induces fluid secretion (3, 4). It is also quite unlike the action of bacterial enterotoxins such as cholera toxin or \textit{E. coli} heat-stable enterotoxin, which induce net electrolyte and fluid secretion in the colon (5). Net fluid movement across the colonic mucosa is a result of two opposing unidirectional flows of ions: mucosa to serosa (absorption) and serosa to mucosa (secretion). It is generally held that absorption occurs in colonic surface cells, whereas secretion occurs in crypt epithelium (15, 16). In the case of Shiga toxin-treated rat distal colon, the evidence obtained in this study suggests that secretory crypt cells were ablated by the action of toxin, resulting in increased net absorption of both water and sodium.

In the present study, Shiga toxin was observed to bind only to epithelial cells in the lower parts of crypts in the rat distal colon. Immunohistochemistry did not show binding to the upper part of the crypt or to surface cells. Shiga toxin suppressed protein synthesis in crypt cells but not in surface cells. On the other hand, cycloheximide (a non-specific inhibitor of protein synthesis) caused comparable inhibition of 3H-leucine incorporation in both crypt and surface cells. Further evidence that Shiga toxin selectively affected crypt cell function in the rat colon came from in vitro flux studies. Cl secretion in response to theophylline (which increases mucosal cyclic 3’5’-adenosine monophosphate) was markedly attenuated by Shiga toxin pretreatment compared with control tissues. This indicates defective crypt cell function, since Cl secretion in response to theophylline is believed to originate in the crypt cells (16). The fact that Cl secretion was not totally abolished indicates that some secretory cells were spared. It is likely that these were cells located in the upper crypts that did not bind Shiga toxin.

Studies in the rabbit jejunum showed that Shiga toxin
bound specifically to villous, but not to crypt, cells (4). Using 125I-labeled Shiga toxin binding, it was shown that isolated villous cells had a 60-fold greater number of toxin receptors than isolated crypt cells. In rabbit jejenum, damage to absorptive villous cells led to decreased absorption of sodium and water, resulting in net fluid secretion. This marked difference in response to Shiga toxin between rabbit jejenum and rat distal colon is probably attributable to differences in receptor expression in these two tissues. It is well known that there is great heterogeneity of expression of transport proteins and of receptors between different regions of the intestine and colon and between different species. Receptors for bacterial toxins other than Shiga toxin may also show a gradient of expression along the villus–crypt axis, as shown for E. coli heat-stable enterotoxin (17). Receptors for the latter toxin may also show age-related variations in expression (18). In this study we chose to examine the effect of Shiga toxin in the rat distal colon rather than in rabbit colon because the former epithelium has a well-characterized SCFA-linked NaCl absorptive process in addition to basal electroneutral NaCl absorption. In the human colon, as in the rat distal colon, SCFA are a major stimulus to sodium and fluid absorption (19). However, it is important that findings from this study are not extrapolated to human disease without knowing the distribution of epithelial cell receptors for Shiga toxin within the human colon.

SCFA-linked NaCl absorption is a major absorptive pathway for sodium from human and mammanal colon which has recently assumed prominence (20, 21). This is an active transport process, resulting from the net effect of three different transporters (22). SCFA-linked NaCl absorption is quite different from SCFA absorption per se, the latter being a process that occurs mainly through non-ionic passive diffusion across concentration gradients (23). Specific targeting of colonic crypt cells by Shiga toxin enabled its use to localize SCFA-linked NaCl transport along the crypt–surface axis of the rat distal colon. This study showed the absence of this important transport process in Shiga toxin-treated colon, suggesting that SCFA-linked NaCl transport is a function of the colonic crypt epithelium. There is no information on SCFA-linked NaCl transport in the damaged colon, apart from a study in rabbits infected with Yersinia enterocolitica. In that study SCFA-linked Na absorption in the proximal colon was diminished in infected animals (24). The histologic correlate of decreased SCFA-linked NaCl absorption was dense inflammatory infiltration in the crypt region, with very little inflammation in proximity to the surface epithelium. Although the authors did not specifically remark on this, these results could be consistent with a predominant crypt localization of SCFA-linked Na absorption in the rabbit colon.

The capacity of the colon to absorb sodium and water is a major compensatory mechanism in diarrhoeal disease (21). This capacity is impaired in both human and experimental shigellosis (6, 7). Impaired colonic absorption in shigellosis can result from many factors, including selective effects of Shiga toxin on surface absorptive cells, epithelial cell invasion and destruction caused directly by the organism, or the action of products released from neutrophil polymorphs infiltrating the lamina propria. If, as in the rat colon, Shiga toxin selectively affects crypt cells in the human colon, it is unlikely to be responsible for decreased colonic fluid absorption. In addition to normal colon, SCFA also stimulate sodium and water absorption from the secreting colon (25, 26) and reverse colonic dysfunction in cholera and other infective causes of watery diarrhoea (27). Impairment of SCFA-linked NaCl absorption by Shiga toxin suggests that this pathway may not be useful in reversing colonic fluid malabsorption in shigellosis. SCFA may, however, have beneficial effects independent of SCFA-linked NaCl absorption and have recently been shown to improve colonic inflammation in experimental shigellosis (28).

Acknowledgements

This project was funded by grants from the Rockefeller Foundation, USA, and from the Dept. of Science and Technology, New Delhi, India. Selvi Krishnan was a recipient of the Research Associateship of The Council of Scientific and Industrial Research, Govt. of India.

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Received 10 December 1998
Accepted 13 April 1999


