Enteropathogenic *Escherichia coli* Infection Inhibits Intestinal Serotonin Transporter Function and Expression

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BACKGROUND & AIMS: Serotonin transporter (SERT) plays a critical role in regulating serotonin (5-hydroxytryptamine [5-HT]) availability in the gut. Elevated 5-HT levels are associated with diarrheal conditions such as irritable bowel syndrome and enteric infections. Whether alteration in SERT activity contributes to the pathophysiology of diarrhea induced by the food-borne pathogen enteropathogenic *Escherichia coli* (EPEC) is not known. The present studies examined the effects of EPEC infection on SERT activity and expression in intestinal epithelial cells and elucidated the underlying mechanisms. **METHODS:** Caco-2 cells as a model of human intestinal epithelia and EPEC-infected C57BL/6J mouse model of infection were utilized. SERT activity was measured as Na^+ and Cl^- dependent ^{3}[H] 5-HT uptake. SERT expression was measured by real-time quantitative reverse-transcription polymerase chain reaction, Western blotting, and immunofluorescence studies. **RESULTS:** Infection of Caco-2 cells with EPEC for 30–120 minutes decreased apical SERT activity ($P < .001$) in a type 3 secretion system dependent manner and via involvement of protein tyrosine phosphatases. EPEC infection decreased V_{max} of the transporter; whereas cell surface biotinylation studies revealed no alteration in the cellular or plasma membrane content of SERT in Caco-2 cells. EPEC infection of mice (24 hours) reduced SERT immunostaining with a corresponding decrease in SERT messenger RNA levels, 5-HT uptake, and mucosal 5-HT content in the small intestine. **CONCLUSIONS: Our results demonstrate inhibition of SERT by EPEC and define the mechanisms underlying these effects. These data may aid in the development of a novel pharmacotherapy to modulate the serotonergic system in treatment of infectious diarrheal diseases.**

The gastrointestinal tract contains approximately 90% of the whole body content of serotonin (5-hydroxytryptamine [5-HT]), the majority of which is produced and stored in enterochromaffin cells.[1] 5-HT is a key hormone of the gastrointestinal tract that affects several physiologic processes including absorption or secretion of fluids and electrolytes via its interactions with 5-HT receptor subtypes.[2] The physiologic actions of 5-HT are terminated by the rapid uptake of 5-HT through a highly selective sodium and chloride-coupled 5-HT transporter (SERT).[3–5] This process facilitates 5-HT degradation by the intracellular enzymes. SERT, therefore, has been suggested to play a critical role in regulating 5-HT content and availability in the gut.[6] Our recent studies demonstrated that SERT is apically localized and differentially expressed along the length of the human intestine with highest expression in the ileum.[5]

Impairment of SERT function in pathogenesis of various diarrheal disorders has been increasingly acknowledged. For example, molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter have been described in ulcerative colitis and irritable bowel syndrome.[7,8] Also, animal models of postinfectious irritable bowel syndrome revealed distinct changes in enterochromaffin cell density and decreased SERT expression in the small bowel of parasite-infected mice.[9] These findings suggest that SERT may be an important target for enteric pathogens as well as for the development of therapeutics to modulate diarrhea.

In this regard, enteropathogenic *Escherichia coli* (EPEC) is an important food-borne pathogen and a major cause of infantile diarrhea. EPEC are nontoxigenic, but through a type 3 secretory system (T3SS), the bacterium injects virulence proteins into host cells.[10,11] Until recently, not much was known regarding the pathophysiology of early diarrhea associated with EPEC infection. Recent findings from our laboratory and others demonstrated that diarrhea induced by EPEC is multifactorial and occurs in part from a decrease in intestinal absorption of Na^+, Cl^-, glucose, and butyrate as well as disruption in barrier function.[12,16] However, the involvement of SERT in pathophysiology of EPEC-induced diarrhea is unknown. The aim of the current study was, therefore, to investigate the direct effects of EPEC infection on the expression and activity of SERT in model intestinal epithelial Caco-2 monolayers and in a mouse model.

Abbreviations used in this paper: BFP, bundle-forming pilus; EPEC, enteropathogenic *Escherichia coli*; SERT, serotonin transporter; T3SS, type 3 secretory system.

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Materials and Methods

Cell culture. Fully differentiated Caco-2 confluent monolayers grown on collagen-coated transwell inserts or 24-well plastic supports were used for experiments at days 10–12 postplating.

Mouse model of EPEC infection. The previously published C57BL/6 mouse model of EPEC infection was used.[17] All experiments involving mice were approved by Animal Care Committee of the University of Illinois at Chicago.

Bacterial culture and cell infection. The EPEC strains used were as follows[13,15]: (1) wild-type EPEC strain E2348/69, (2) CVD452 (E2348/69 *escN*:Km), (3) UMD864 (E2348/69 Δ48-759 *espB1*), (4) UMD870 (E2348/69 *espD1*: aph-3[Km]), (5) *espG (*SE1114), (6) *espZ*, (7) *espF* (UMD874), (8) *espH* (SE651*orf18*), and (9) *map* (*orf19*). Cell monolayers were infected at a multiplicity of infection of 100 as described previously.[13,15]

[^{3}H] serotonin uptake in Caco-2 monolayers. 5-HT uptake was performed in Caco-2 cells as described previously.[18] Unless otherwise stated, uptake was initiated by the addition of 0.3 mL of medium containing 25–50 nmol/L [^{3}H] serotonin (Perkin Elmer, Waltham, MA) for 5 minutes. The 5-HT uptake was NaCl dependent with negligible activity seen with the omission of NaCl from the medium.

Na^+-K^+-ATPase activity assay. Na^+-K^+-ATPase activity was measured in a crude membrane preparation from Caco-2 cells as the rate of inorganic phosphate released in the presence or absence of ouabain as described.[19,20]

Cell-surface biotinylation studies. Biotinylation studies in Caco-2 cells were performed using sulfo-NHS-biotin (0.5 mg/mL; Pierce Biotechnology; Rockford, IL) as previously described.[21] Anti-GFP (1:100; Cell Signaling, Beverly, MA) or anti-SERT (1:500, overnight at 4°C; Immunostar, Hudson, WI) antibodies were utilized for Western blotting.

[^{3}H] serotonin uptake in apical membrane vesicles. Apical membrane vesicles from the mouse small intestine were prepared from thawed mucosa as described previously.[22,23] The integrity of the membranes was assessed by measuring initial uptake of D-glucose.[22,23] [^{3}H] serotonin uptake into intestinal apical membrane vesicles was measured using a rapid filtration technique.[5]

Immunofluorescence staining in mouse intestine. Four- to 10-μm frozen sections of small intestinal tissue were fixed with 1% paraformaldehyde and stained as described previously.[5] Tissues were incubated with rabbit anti-SERT antibody (1:100) and anti-villin antibody (1:100) in phosphate-buffered saline with 1% normal goat serum (NGS) for 120 minutes at room temperature. After washing, sections were incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG, Alexa Fluor 488-conjugated goat anti-mouse IgG, and Hoechst 33342 (Invitrogen, Carlsbad, CA) for 60 minutes. Sections were imaged using a Zeiss LSM 510 laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY).

Small intestine mucosal serotonin content. 5-HT content from mucosa was measured from both EPEC-infected and control mice by immediately homogenizing 1 μg/10 μL of mucosa in 0.2 N perchloric acid using the enzyme-linked immunosorbent assay (ELISA)-based commercially available kit (Beckman Coulter, Fullerton, CA).

Statistical Analysis

Results are expressed as mean ± SEM. One-way ANOVA was used for statistical analysis. $P \leq .05$ was considered statistically significant.

Results

EPEC Infection Inhibits SERT Activity

To examine the direct effect of early EPEC infection on SERT, fully differentiated Caco-2 monolayers were infected with wild-type EPEC strain E2348/69. EPEC infection decreased apical 5-HT uptake as early as 15 minutes, reaching maximal inhibition at 30–60 minutes (Figure 1*A*). However, infection of Caco-2 cells with the nonpathogenic *E coli* for 60 minutes had no effect (Figure 1*B*). Previous studies have demonstrated a functional 5-HT uptake at both apical and basolateral membrane domains of Caco-2 monolayers.[18] To examine whether EPEC infection differentially affects apical and basolateral uptake of 5-HT, monolayers grown on transwells were utilized. EPEC infection for 60 minutes decreased apical SERT activity by 53% (Figure 1*C*), whereas basolateral SERT activity was inhibited to a significantly lesser degree (Figure 1*D*). Because our recent studies showed that SERT was predominantly localized to the apical membrane in the human intestine,[5] subsequent experiments examined mechanisms underlying the modulation of SERT activity (represented by apical 5-HT uptake) by EPEC.

SERT is a secondary active transporter that maintains its electrochemical gradient by the action of Na^+/K^+ATPase. It is possible that a decrease in the ionic gradient following EPEC infection might lead to reduced 5-HT uptake. Intriguingly, Na^+/K^+ATPase activity was stimulated by 2-fold in Caco-2 cells infected with EPEC for 60 minutes (Figure 1*E*), indicating that the observed inhibition in SERT activity by EPEC could not be secondary to alterations in Na^+/K^+ATPase activity.

Effects of EPEC Are Functional T3SS Dependent

To elucidate the mechanisms of inhibition of SERT activity by EPEC, Caco-2 cells were exposed to sterile culture supernatant of bacteria grown in Dulbecco's modified Eagle medium.[24] EPEC culture supernatant failed to inhibit SERT activity, unlike infection with whole bacteria

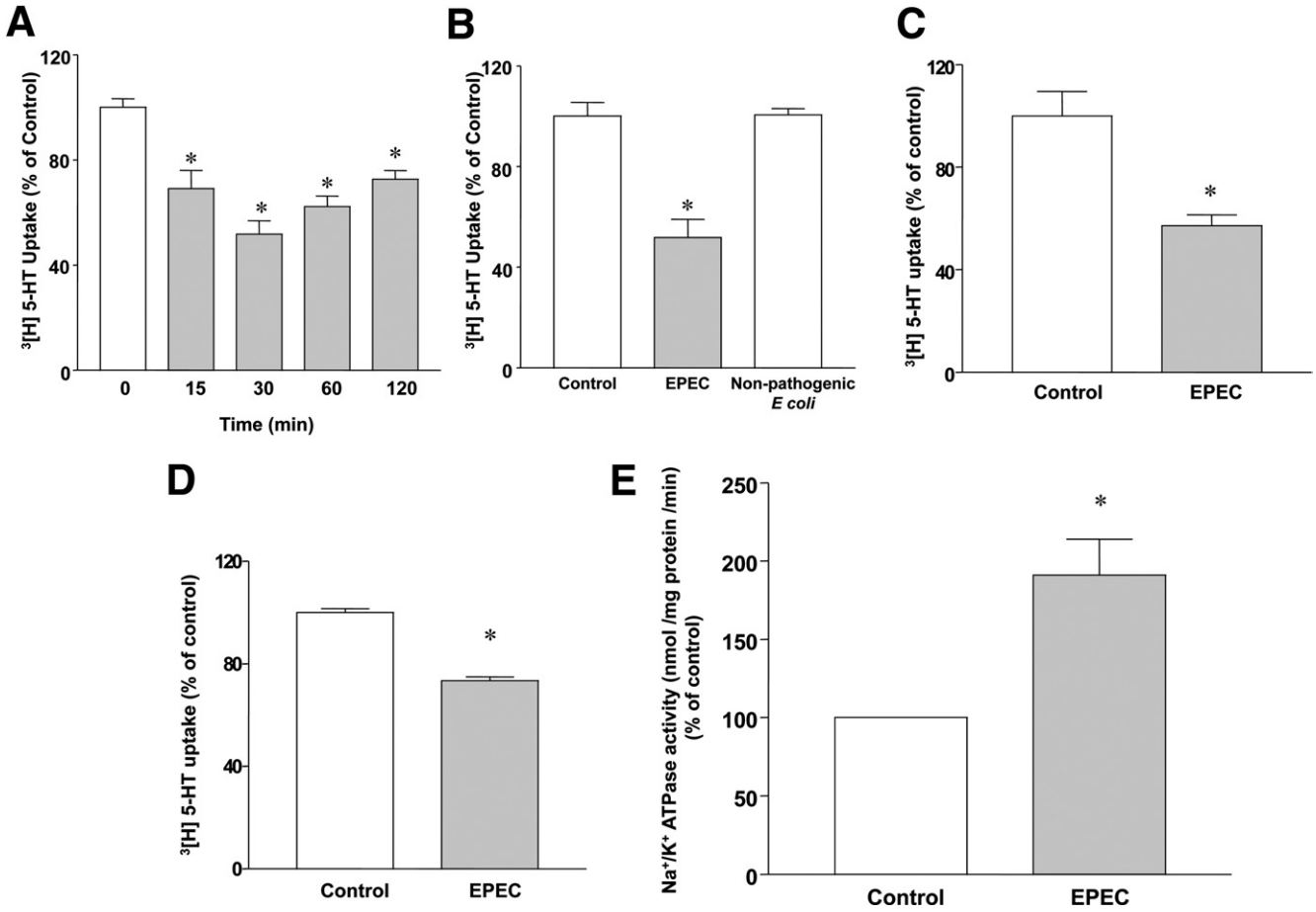

Figure 1. EPEC infection inhibits serotonin uptake in Caco-2 cells. (*A*) Time course of EPEC infection on ^{3}[H] 5-HT uptake. (*B*) Effects of nonpathogenic *E coli* on ^{3}[H]-5-HT uptake. (*C* and *D*) Effects of EPEC infection on apical and basolateral 5-HT uptake. Results are depicted as percent of control. n = 6–9. **P* < .001 vs control. (control values in picomoles/milligrams protein/5 minutes: [(*A*) 0.27 ± 0.014; (*B*) 0.33 ± 0.010; (*C*) 0.22 ± 0.019; and (*D*) 0.43 ± 0.005]). (*E*) EPEC infection increases Na^+/K^+ATPase activity in Caco-2 cells. n = 3. **P* < .001 vs control.

(not shown) indicating that the released soluble components do not mediate SERT inhibition. We next investigated whether attachment of EPEC to host cells via plasmid-encoded bundle-forming pilus (BFP)[25–27] is necessary for SERT inhibition. Previous studies have shown that attachment of *bfp* mutant of EPEC to epithelial cells is significantly lower compared with wild-type EPEC.[17] Infection of Caco-2 cells with *bfp* mutant abrogated the inhibition of SERT activity (Figure 2*A*). EPEC also encodes a T3SS that injects *E coli*-secreted proteins (Esps) into the host cytosol. EscN is the putative ATPase that drives T3SS.[28] Infection of Caco-2 cells with the *escN* mutant had no effect on SERT activity indicating that this response is T3SS dependent (Figure 2*A*). We further investigated the role of the structural components of T3SS, namely EspA (forms a filamentous extension of the T3SS)[29,30] and EspB and EspD (provide a translocation pore).[25,31] Infection of cells with deletion mutants of *espA*, *espB*, or *espD* failed to inhibit SERT activity (Figure 2*B*). These results indicate that the structural components of the T3SS or the secreted effector molecules mediate the inhibitory effect of EPEC on SERT activity.

Several T3SS effector molecules have been previously described.[11,32–34] Deletion of *espF* (involved in barrier disruption),[11] *espG*, or *espG2* (implicated in microtubular network disruption)[32]; *espH* (involved in pedestal formation and filopodia formation)[34]; *espZ*[35] (unknown function); or *map* (alters mitochondrial membrane potential)[33] had no effect on EPEC-mediated inhibition in SERT activity (Figure 2*C*). These studies rule out the involvement of these specific effector molecules in EPEC-induced inhibition of SERT.

EPEC Infection Decreases the Maximal Velocity of 5-HT Uptake

The mechanisms underlying EPEC-mediated inhibition of SERT activity was further examined by performing kinetic studies. EPEC infection significantly decreased SERT activity at all concentrations (25–350 nmol/L) (Figure 3*A*). Analysis of the kinetic parameters by GraphPad Prism revealed that maximal velocity of 5-HT uptake was significantly reduced (V_{max}: in pmol/mg protein/5 min: 1.09 ± 0.19 for control vs 0.35 ± 0.06 in response to EPEC infection, **P* < .001 vs control). In contrast, the apparent

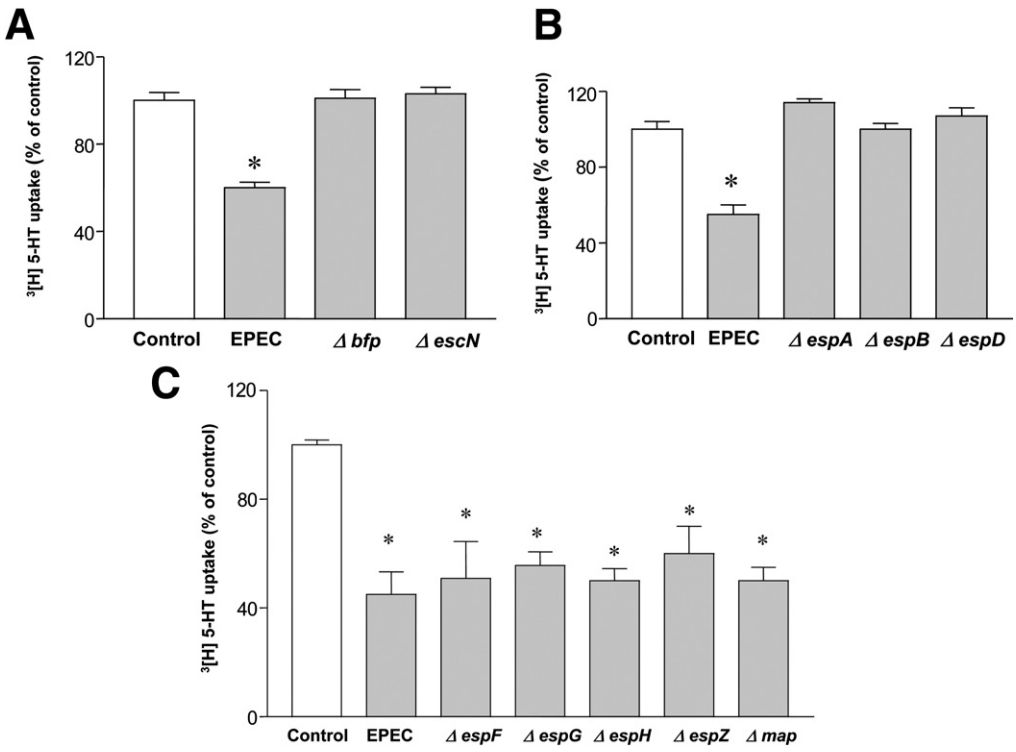

Figure 2. EPEC effects are T3SS dependent. (*A*) bfp And T3SS are essential for EPEC mediated effects. (*B*) Structural components of T3SS are necessary for SERT inhibition. (*C*) Role of effector molecules. n = 6, *$P < .01$ vs control. [control values in picomoles/milligrams protein/5 minutes: [(*A*) 0.39 ± 0.015; (*B*) 0.35 ± 0.017; (*C*) 0.320 ± 0.006]).

Michaelis constant (K_m in nmol/L) was unaltered (control 179 ± 20.8 vs EPEC infected 135 ± 30.4). These results indicated that the number of active SERT available to the substrate is reduced by EPEC with no change in the affinity of the 5-HT for SERT.

To investigate the possibility that EPEC modulates SERT expression, we first examined the SERT messenger RNA (mRNA) levels by real-time QRT-PCR. Infection of Caco-2 monolyers with EPEC for 1–4 hours did not alter the SERT mRNA expression (relative SERT mRNA/histone mRNA expression: control: 1.0; EPEC 1 hour: 1.25 ± 0.15; EPEC 4 hours: 1.34 ± 0.27).

Because SERT mRNA levels remained unaltered, we examined whether EPEC-infected monolayers exhibit reduced SERT levels on the plasma membrane utilizing cell surface biotinylation studies. The anti-SERT antibody used to detect endogenous SERT expression showed multiple bands in Caco-2 cell extracts. However, the expected size band (~70 kilodaltons) of SERT was expressed at similar levels on the plasma membrane of control and

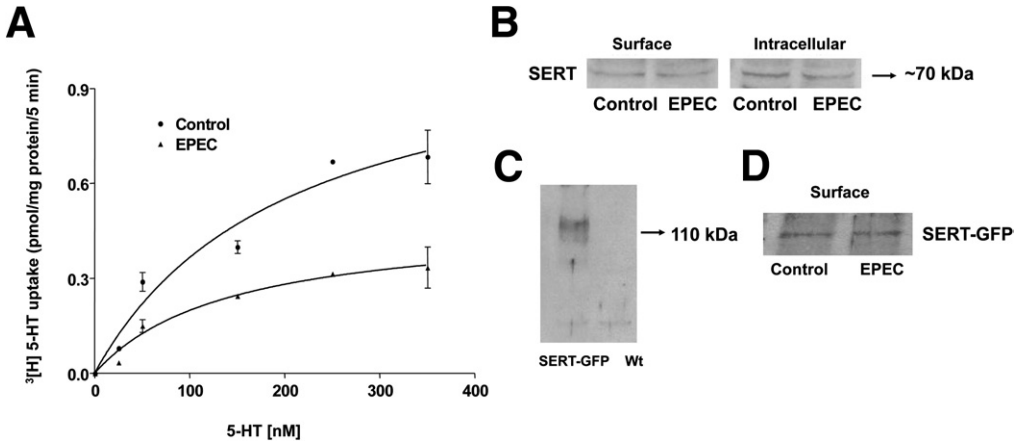

Figure 3. Mechanisms of EPEC-induced inhibition of SERT. (*A*) Kinetic studies of EPEC induced inhibition. A Michaelis-Menten plot from a representative experiment is shown, n = 5. (*B*) Cell-surface expression of SERT. Caco-2 monolayers were infected with wild-type EPEC for 60 minutes and subjected to biotinylation. (*C*) SERT expression in transiently transfected Caco-2 cells. SERT expression in wild-type untransfected and cells transiently transfected with SERT-GFP construct utilizing ant-GFP antibodies. (*D*) EPEC does not alter cell-surface expression of SERT-GFP: Representative blots from 3 different experiments are shown.

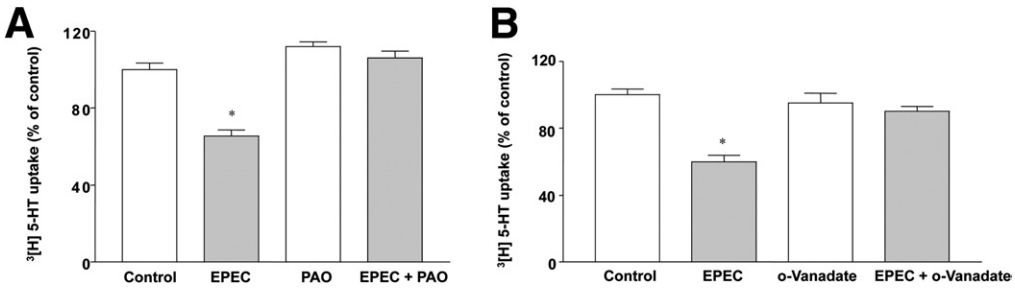

Figure 4. Protein phosphotyrosine phosphatases (PTP) mediate the effects of EPEC. Caco-2 monolayers were pretreated with specific PTP inhibitors. (*A*) Phenylarsine oxide (*PAO*, 10 μmol/L) and (*B*, 5 μmol/L) o-vanadate for 30 minutes and then infected with EPEC in the presence or absence for inhibitors for another 60 minutes. n = 3. *$P < .01$ vs control. (control values in picomoles/milligrams protein/5 minutes: [(*A*) 0.27 ± 0.006; (*B*) 0.38 ± 0.018]).

EPEC-infected cells (Figure 3*B*). Biotinylation studies were also performed in Caco-2 cells transiently transfected with a SERT-(green fluorescent protein) GFP construct (Supplementary Materials) to enhance the specificity of detection. As shown in Figure 3*C*, a single band of ~110 kilodaltons was observed in cells transiently transfected with SERT-GFP fusion construct as compared with untransfected cells. However, EPEC infection of Caco-2 cells did not alter SERT-GFP expression (~110 kilodaltons) on the plasma membrane (Figure 3*D*). Total cellular levels of SERT were also similar in control and EPEC-infected cells (not shown).

Role of Signaling Intermediates

SERT has been shown to be regulated by various signaling pathways such as protein kinase C (PKC) and mitogen-activated protein (MAP) kinases.[36,37] EPEC is also known to induce numerous signaling molecules in host cells.[15,38–40] The inhibition of 5-HT uptake in response to EPEC infection was unaltered in the presence of inhibitors of PKC (Bisindolylmaleimide [BIM], 10 μmol/L) protein kinase A (PKA) (RpcAMP, 20 μmol/L), microtubule (colchicine, 100 μmol/L), phosphoinositide 3 (PI3) kinase (LY294002, 20 μmol/L), intracellular calcium, (1,2-bis(o-Aminophenoxy)ethane-N,N,N′N′ tetracetic acid tetra) acetoxymethylester [BAPTA-AM], 10 μmol/L), tyrosine kinase (herbimycin, 10 μmol/L), pinocytosis (amiloride, 1 mmol/L), or endocytosis inhibitor (nystatin, 5–50 μmol/L) (data not shown).

EPEC infection is also known to induce tyrosine dephosphorylation of various host proteins.[40] To examine the involvement of protein tyrosine phosphatases (PTPs) in EPEC-mediated effects, phenylarsine oxide that cross-links vicinal thiol groups and thereby inactivates phosphatases possessing XCysXXCysX motifs was utilized. EPEC-induced inhibition of 5-HT uptake was abrogated in the presence of 5–10 μmol/L concentrations of phenylarsine oxide suggesting the involvement of PTPases in EPEC-mediated effects (Figure 4*A*). The specificity of action of phenylarsine oxide was examined by using other inhibitors of PTPases including ortho-vanadate (Figure 4*B*) and 3,4 dephostatin (DP) (% of control [EPEC: 60* ± 5; DP: 105 ± 10; EPEC + DP: 83 ± 9] *$P < .001$ vs control), which significantly attenuated the inhibition of SERT.

Effects of EPEC Infection in In Vivo Mouse Model

SERT mRNA expression. Our previous studies demonstrated that SERT is expressed in the human duodenum and ileum with negligible expression in the colon.[5] However, a comprehensive study regarding SERT expression in different regions of the mouse intestine is lacking. SERT mRNA expression was found to be markedly higher in the proximal and distal small intestine compared with colon (Figure 5*A*). EPEC infection of mice significantly decreased small intestinal SERT mRNA lev-

Figure 5. SERT mRNA expression in murine model of EPEC infection. (*A*) SERT mRNA expression in native mouse intestine. (*B*) Time course of EPEC infection on SERT mRNA expression. n = 3–7. *$P < .001$ vs control.

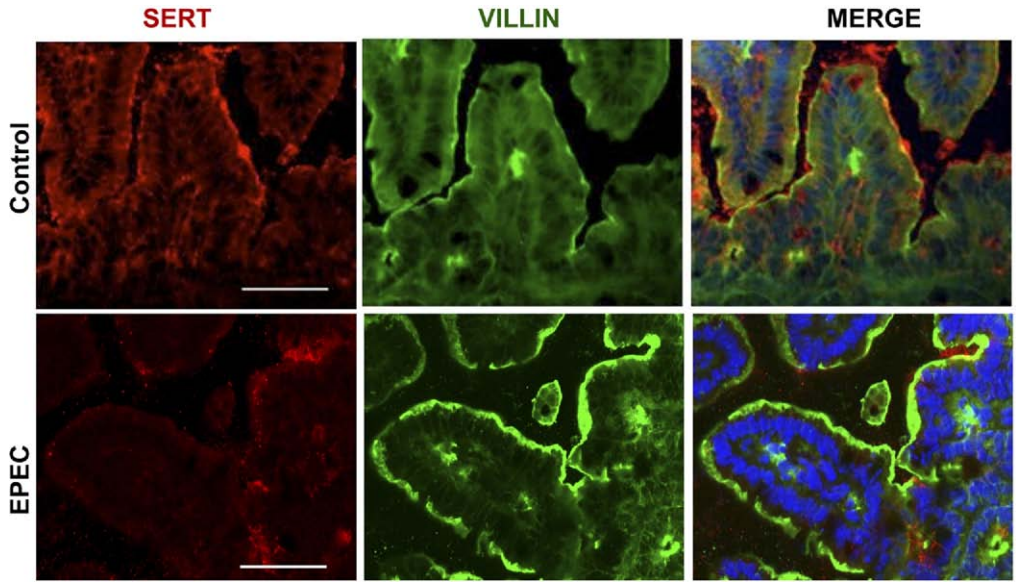

Figure 6. SERT immunostaining in EPEC infected mouse small intestine. Immunofluorescent staining for SERT (*red*) and villin (*green*) with *blue*-counterstained nuclei was performed on OCT sections of control and EPEC-infected mouse distal small intestine. *Scale bar*, 50 μm. A representative of 4 different experiments is shown.

els at 14 hours and 18 hours postinfection, which returned to baseline at 24 hours (Figure 5*B*).

SERT protein expression. Immunofluorescence studies showed that SERT was detected primarily on the apical membranes (Figure 6, *red*) colocalized with the apical membrane marker villin (Figure 6, *green*) in mouse small intestine. EPEC infection of mice for 24 hours, however, led to a significant loss of SERT protein (Figure 6, *red*) on the apical membranes of small intestinal epithelial cells. This decrease in SERT expression was not associated with reduced villin content (structural marker of brush border membrane)[41] (Figure 6). This is important because EPEC is known to efface intestinal microvilli.

SERT function and 5-HT content. Corresponding with decreased SERT expression, EPEC infection for 24 hours significantly decreased initial uptake of ^{3}[H]-5-HT in the purified small intestinal apical membrane vesicles (Figure 7*A*). A decrease in SERT function and expression would result in a decrease in mucosal 5-HT content leading to an increased luminal 5-HT availability. In parallel, a significant decrease was observed in mucosal 5-HT content in the small intestinal mucosa of EPEC-infected mice compared with uninfected controls (Figure 7*B*).

Mechanistic Link Between In Vitro vs In Vivo Mouse Model of EPEC Infection

The differences in data obtained from our in vitro and in vivo models were carefully considered. Most striking was the decrease in SERT mRNA following EPEC infection of mice but not in cultured intestinal epithelial cells. The effect of decreased SERT expression is an increase in luminal 5-HT. However, Caco-2 cells do not secrete 5-HT. We hypothesized that the absence of 5-HT in the in vitro cell culture model may account for the differences in SERT mRNA levels in vitro and in vivo following EPEC infection. To test this hypothesis, Caco-2 cells were treated with different concentrations of 5-HT. SERT mRNA expression was transiently down-regulated by 5-HT (10 μM) in Caco-2 cells at 18 hours, which returned to baseline at 24 hours (Figure 8*A*). Correlative functional studies were performed, whereby, SERT activity was measured in Caco-2 cells treated with 5-HT (10

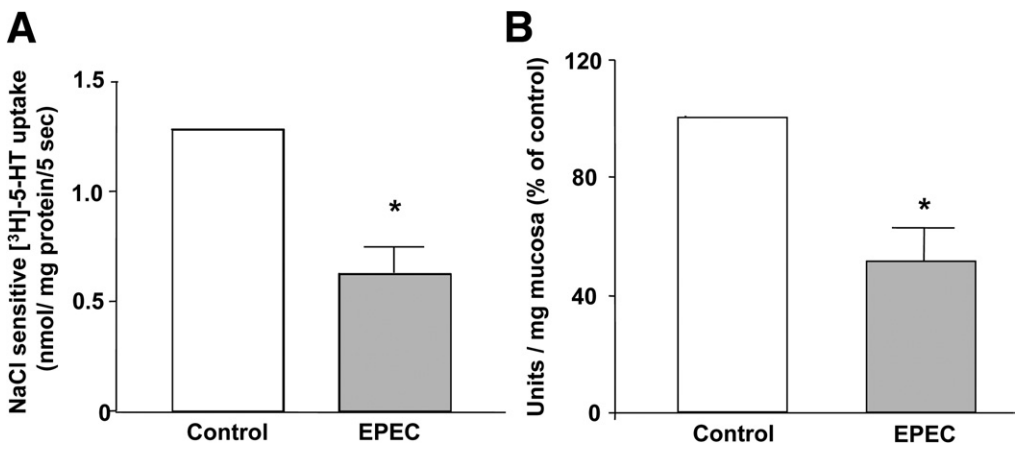

Figure 7. Effects of EPEC infection on SERT function and 5-HT mucosal content. (*A*) EPEC inhibits [^{3}H]5-HT uptake. n = 3. (*B*) 5-HT mucosal content in response to EPEC infection. n = 3. *$P < .05$ vs control.

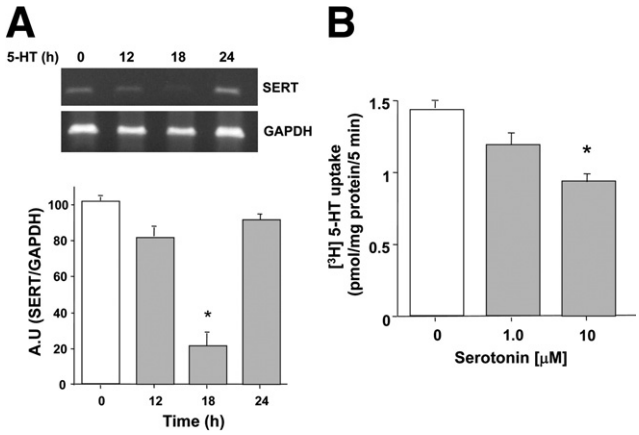

Figure 8. 5-HT inhibits SERT function and expression in Caco-2 cells. (*A*) Semiquantitative RT-PCR was performed with total RNA extracted from control and 5-HT treated Caco-2 cells (n = 3). (*B*) Caco-2 cells were treated with 5-HT (1–10 μmol/L) for 24 hours, and uptake of apical 5-HT was assessed with 200 nmol/L of [^{3}H] 5-HT for 5 minutes (n = 4–6). *$P < .05$.

μmol/L) for 24 hours. As shown in Figure 8*B*, SERT activity was significantly decreased following 5-HT exposure. Previous studies have also shown a decrease in SERT function in response to long-term treatment of Caco-2 cells with 5-HT.[42] These data suggest that increased 5-HT levels in the lumen may contribute to the inhibition of SERT function and expression induced by EPEC infection.

Discussion

Rapid onset of diarrhea is the predominant symptom of EPEC infection. EPEC is nontoxigenic[10] but produces a characteristic attaching and effacing lesion[25] that was originally presumed to cause diarrhea because of loss of overall absorptive surface. However, studies in human volunteers showed that the incubation period between EPEC ingestion and onset of diarrhea is less than 4 hours,[43] suggestive of alterations in ion transport mechanisms rather than a generalized effect of microvilli effacement. In this regard, we have previously shown that EPEC inhibits the absorption of Na^+, Cl^-, and butyrate.[13–15,44] Dean et al[16] reported that EPEC rapidly inactivates the sodium-D-glucose cotransporter (SGLT-1) in human intestinal epithelial cells. Furthermore, infection of susceptible mice with *Citrobacter rodentium* (mouse homolog of EPEC) was associated with impaired intestinal ion transport and fatal fluid loss.[45] In fact, microarray analysis revealed that among the differentially expressed genes in the *C rodentium*-infected susceptible and resistant mice, alterations in intestinal transport genes were overrepresented compared with even the immune response-related genes.[45]

Current studies demonstrating that EPEC infection modulates SERT function represent another novel illustration of epithelial-microbial interactions that underlie the pathophysiology of associated diarrhea. Our in vitro studies utilizing Caco-2 cells delineated the immediate events reducing SERT function following EPEC infection (30 minutes to 2 hours). Because SERT is predominantly expressed in the human small intestine as compared with colon,[6,46] Caco-2 monolayers provided an excellent model system, representing the enterocyte phenotype upon differentiation, to assess SERT function and regulation.[18,42,47,48] Caco-2 cells have also been extensively used previously to study the function and regulation of various small intestinal ion and nutrient transporters.[2,13,49–53] Assessment of the net impact of EPEC infection on SERT was performed utilizing in vivo mouse model of EPEC infection.[13,54] It is impossible to replicate the in vitro models of infection in vivo because of the complex native tissue and requirement of the passage of bacterium through the stomach plus additional barriers to adherence. We selected 14- to 24-h postinfection time period based on our previous studies showing that EPEC colonizes mouse ileum and colon as early as 24 hours.[13,54]

Our findings suggest that infection with EPEC alters SERT function in vitro without altering SERT gene expression, whereas, in vivo, SERT gene expression is also decreased. We have identified a plausible explanation, ie, differences in luminal 5-HT concentrations in the intestinal lumen for the discrepancy between the data from our in vitro and in vivo models. EPEC infection of mice decreased 5-HT mucosal content (which increases 5-HT availability in the gut lumen), a scenario lacking in the in vitro model of infected Caco-2 cells because these cells do not secrete 5-HT. Interestingly, 5-HT treatment of Caco-2 monolayers directly inhibited SERT function via down-regulating SERT mRNA expression in Caco-2 cells at 18 hours that returned to baseline at 24 hours, the same pattern of SERT mRNA expression that occurred in the EPEC-infected mouse model. The transient decrease in SERT mRNA following 5-HT treatment of Caco-2 cells or EPEC infection could be explained by 5-HT receptor desensitization in response to high levels of 5-HT. Another possible contributing factor is an increase in proinflammatory cytokines such as tumor necrosis factor-α, which have been reported previously to down-regulate SERT mRNA in Caco-2 cells.[47] In addition, EPEC has been shown to increase tumor necrosis factor-α expression in the murine model.[17] It is also likely that infection may alter enterochromaffin cell density or function that may contribute to the diarrhea associated with EPEC infection in vivo. Importantly, our data from both in vitro and in vivo models demonstrate EPEC induced inhibition of SERT function (albeit by different mechanisms) that may underlie the diarrheal phenotype of the pathogen.

EPEC-mediated inhibition of SERT could be secondary to modulation of other ion transporters. In this regard, 5-HT is known to modulate both Na^+ and Cl^- transport processes in the intestinal epithelial cells.[2,55] However,

EPEC-mediated effects on epithelial ion transport system appear to be specific. Our previous studies demonstrated that EPEC exhibited differential effects on NHE (Na^+/H^+ exchange) isoform activity, with an inhibition in NHE3 activity via EspF[56] and stimulation in NHE2 activity via PKC.[15] However, the effects of EPEC on Cl^-/OH^- exchange activity were induced by EspG/EspG2.[13] In contrast, the known EPEC effector molecule mediating SERT inhibition has not been determined. Also, EPEC-mediated effects on SERT appear to be not attributed to alterations in the electrochemical gradient because Na^+-K^+-ATPase activity in Caco-2 cells was increased. In parallel, EPEC infection of mice increased Na^+-K^+-ATPase mRNA expression in the small intestine (Supplementary Figure 1).

Notably, our findings for the first time demonstrate the involvement of PTPase in inhibition of SERT by EPEC. These data are in accordance with previous studies that identified tyrosine phosphorylation and dephosphorylation events following EPEC infection in HeLa and Caco-2 cells.[40] *Salmonella typhimurium* is also known to secrete a modular effector protein SptP, homologous to the catalytic domains of PTP.[57] Additionally, *Yersinia* spp has been shown to induce the tyrosine dephosphorylation of host proteins as virulent mechanisms.[58] Interestingly, SERT has been shown to undergo basal phosphorylation that is acutely increased by treatments with PKC activators and protein phosphatase inhibitors.[59] Previous studies utilizing HEK-293 cells have demonstrated that SERT phosphorylation by PKC activation leads to SERT internalization, whereas SERT dephosphorylation by p38 mitogen-activated protein kinase inhibition attenuates SERT insertion to the plasma membrane.[36,37] We previously showed that EPEC infection decreases Cl^- and butyrate absorption in human intestinal epithelial cells via internalization of SLC26A3[13] and monocarboxylate transporter,[44] respectively. However, our current data provide evidence that EPEC-mediated inhibition of SERT activity is independent of membrane trafficking events. Nonetheless, a decrease in V_{max} of the transporter following EPEC infection indicates that EPEC-induced dephosphorylation events may have a direct effect on SERT, resulting in a lowering of the transporter membrane turnover rate or changes in the distribution of the transporter at the plasma membrane level such as via lipid ratfs.

In conclusion, our findings suggest a 2-phase model of SERT inhibition following EPEC infection. In the first phase, SERT function is rapidly reduced via protein tyrosine phosphatases followed by a decrease in SERT expression as evidenced in the in vivo mouse model. Our studies are significant in providing mechanistic insights that may offer novel strategies for treatment of diarrheal diseases. Furthermore, EPEC may provide a prototype for understanding the regulation of SERT, an effective pharmacologic target of gastrointestinal disorders.

Supplementary Data

Note: To access the supplementary material accompanying this article visit the online version of *Gastroenterology* at www.gastrojournal.org and at doi: 10.1053/j.gastro.2009.09.002.

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Conflicts of interest

The authors disclose no conflicts.

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