BACTERIAL PATHOGENICITY

Secretory pathways in *Salmonella* Typhimurium-induced fluid accumulation in the porcine small intestine

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The involvement of 5-hydroxytryptamine (5-HT) and 5-HT₃ receptors and prostaglandin E₂ (PGE₂) in *Salmonella* Typhimurium-induced fluid accumulation in the porcine small intestine was investigated. *Salmonella* Typhimurium (10⁸ and 10¹⁰ cfu) and cholera toxin (CT; 20 µg) were instilled for 8 and 11 h in ligated loops in the porcine jejunum and ileum. Fluid accumulation and concentrations of Na⁺, K⁺, Cl⁻, 5-HT and PGE₂ in the fluid accumulated in the loops were measured. The fluid accumulation was also measured when *Salmonella* Typhimurium (10¹⁰ cfu) and CT (20 µg) were instilled for 8 h in ligated loops in jejunum and ileum in pigs given subcutaneous injections of saline or the 5-HT₃ receptor antagonist ondansetron (200 µg/kg). *Salmonella* Typhimurium (10¹⁰ cfu) and CT both induced fluid accumulation in jejunum and ileum after 8 and 11 h. Both treatments also induced an increase in luminal release of 5-HT and PGE₂. The accumulated fluid was iso-osmotic and hyperosmotic in CT- and *Salmonella* Typhimurium-treated loops, respectively. Ondansetron reduced the Typhimurium-induced fluid accumulation in both jejunum and ileum by c. 40%, while it failed to reduce the response to CT. These results demonstrate that 5-HT and PGE₂ are released and 5-HT₃ receptors activated in the secretory pathway of Typhimurium in the porcine small intestine.

Introduction

Salmonella enterica subspecies *enterica* serotype Typhimurium (hereafter denoted ST)-induced enteritis is an increasing problem in man in many developed countries [1], and a major problem affecting growing pigs in several parts of the world [2]. The mechanisms by which ST causes diarrhoea are poorly understood and have largely been extrapolated from work done in rodents. Bacterial invasion is not correlated with diarrhoea [3, 4], but an inflammatory response with influx of polymorphonuclear leucocytes is needed, as ST fails to induce fluid secretion in the absence of leucocyte influx [5]. Another virulence factor seems to be an enterotoxin with partial resemblance to cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin (LT) [6, 7], but despite the efforts of many groups of investigators a role for an enterotoxin in ST-induced secretion has not been defined. Unlike CT and LT, the

ST enterotoxin is not secreted but must be extracted [8]. Enterotoxigenicity is not the sole criterion of virulence, but there is a correlation between expression of 'cholera-toxin-related antigen' (CTRA) and secretory response in the rabbit ileum [9]. It is assumed that after entering the epithelial cells the bacteria liberate CTRA, thus initiating secretion, and later inflammatory mediators will contribute to the secretory response [1, 10].

CT induces intestinal electrolyte and fluid secretion partly by direct stimulation of the enterocytes via GM1 activating adenylate cyclase, raising cAMP levels, and partly via a secretory reflex arc involving 5-hydroxytryptamine (5-HT) [11–13]. 5-HT is liberated from the enterochromaffin cells [14] and initiates a cascade of reactions involving both the release of eicosanoids, such as prostaglandin E₂ (PGE₂) [13], and activation of the enteric nervous system (ENS) [11, 15]. The reflex arc is assumed to have cholinergic and serotonergic interneurons [16], while the efferent neuron releases vasoactive intestinal polypeptide [11, 17].

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Like CT, ST and cell-free lysates of ST induce fluid secretion and an increase in mucosal cAMP [18] and prostaglandins [19]. The induced fluid secretion and the increase in cAMP and prostaglandins can be reduced by treatment with indomethacin (inhibitor of prostaglandin synthesis), ascribing a role for prostaglandins in the secretory processes at least in rodents [3, 19, 20].

The aim of this study was to investigate a possible role of 5-HT and PGE₂ in ST-induced secretion in the porcine small intestine, which, in addition to its own relevance for veterinary science, is regarded as an appropriate model for man for studying pathophysiological mechanisms of diarrhoea [21]. CT was used as a comparative positive control.

Materials and methods

Animals

Danish Landrace/Yorkshire cross-bred 9–10-week-old pigs (18–20 kg) were used. The animals did not show any clinical signs of diarrhoea or detectable salmonella infection, according to serum antibodies and faecal cultures. The pigs were fasted for 12 h before experiments, but had free access to sterile drinking water containing D-glucose (55 g/L). All experiments followed ethical guidelines approved by the Danish animal experiment inspection.

Bacterial strain and preparation of inoculum

Strain *S. Typhimurium* 3389-1 (DT12) [22] was kindly provided by D. L. Baggesen, Danish National Veterinary Laboratory. The strain was isolated from a clinical case of salmonellosis in pigs. For use as inoculum, the strain was first checked for purity by plating on Blood Agar (Oxoid Blood Agar Base III with sterile calf blood v/v 5%). A single colony was inoculated into 5 ml of LB broth (Bacto Tryptone, Difco, 10 g/L, Bacto Yeast Extract, Difco, 5 g/L, NaCl 5 g/L; pH 7.5) and incubated overnight at 37°C. To obtain cells in logarithmic growth phase, 1 ml of this culture was inoculated into 100 ml of prewarmed LB and incubated with shaking at 37°C until the OD₄₀₅ was 0.3. Cells were concentrated by centrifugation at 4000 rpm for 10 min at 30°C in a Heraeus Megafuge 1.0R with model 3360 rotor. Cells were resuspended in 2 ml of 'Argenzio 4' solution (see experimental procedure) without glucose at the desired density. Viable counts (cfu/ml) of inoculum were determined by a standard plate count technique.

Operative procedure

General anaesthesia was induced by intravenous injection of propofol (Diprivan; Zeneca) 5–7 mg/kg and maintained throughout the experiment by inhalation of isoflurane (Forene; Abbott) in a semiclosed circle

system. At the incision line, a long-acting local analgesic (bupivacaine, Marcain 0.5%; 10 ml) was infiltrated into the skin and underlying tissues to avoid reactions from the nociceptive system. The nociceptive system of the pig is not influenced by isoflurane alone [23], so without local analgesic the sympathetic tone may increase under surgery and possibly alter secretory and absorptive processes in the gut.

Throughout the experiment the condition of the animal was monitored and the rectal temperature was kept at c. 38°C with a heated mattress. Blood pressure was monitored continuously *via* a catheter placed in the artery saphena, from which blood for blood-gas analysis was sampled every hour and the PCO₂ was monitored and kept at c. 40 mm Hg by controlled ventilation (average: rate 12/min, tidal volume 10 ml/kg and fresh O₂ gas flow 200 ml/min). Supporting treatments included a continuous intravenous infusion of Ringer-acetate liq. (160 ml/h; mmol/L: Na⁺ 130, K⁺ 4, Ca²⁺ 2, Mg²⁺ 1, Cl⁻ 112 and Ac⁻ 30).

A midline abdominal incision was made and loops in jejunum and ileum were prepared by ligating between the mesenteric vascular arcades, providing full blood supply during the experiment. The jejunal loops were located 0.3–6.0 m distal to the ligament of Treitz and the ileal loops were placed 6.0–0.1 m proximal to the ileocaecal valve. Loops were 20 cm in length and separated by a distance of 5 cm.

Experimental procedure

Before preparing the loops, and again 6 h later, 10 ml of saline alone was administered by subcutaneous (s.c.) injection. Each loop was inoculated in random order with 10 ml of 'Argenzio-4' test solution [24] (mmol/L: Na⁺ 115, K⁺ 10, Cl⁻ 80, HCO₃⁻ 45, D-glucose 80; pH 7.4) containing either log phase ST 10⁸ cfu/loop (ST10⁸), 10¹⁰ cfu loop (ST10¹⁰), 20 µg of CT, or test solution alone (T, control), or loops were left unfilled (U). After filling, the loops were returned to the peritoneal cavity, and the abdomen was closed. Blood was sampled after 4 h and plasma was frozen until osmolality measurement. Just before removal of the loops, 2-ml samples were collected from the accumulated fluid by syringe. Empty loops were washed with 5 ml of test solution before sampling. The samples were centrifuged at 900 g for 20 min and the supernates were frozen at -20°C until analysis for PGE₂, 5-HT, osmolality and electrolytes. After 8 and 11 h, half the numbers of loops from jejunum and ileum, respectively, were removed. The data from 8-h instillation were used as controls for the ondansetron treatment.

Experiments with ondansetron treatment

The animals were given 10 ml of saline containing the 5-HT₃ receptor antagonist ondansetron (200 µg/kg)

(MW = 366, Glaxo Wellcome, Middlesex) by s.c. injection before surgery and again after 6 h. Loops were prepared and in random order inoculated with 10 ml of Argenzio-4 test solution containing ST 10^{10} , 20 μ g of CT, test solution (T) alone or left unfilled (U). The ondansetron dose of 200 μ g/kg was chosen to ensure maximal antagonism, as 100 μ g/kg maximally reduced CT-induced fluid accumulation in a short-term (4-h) experiment [25]. The loops were returned to the peritoneal cavity and removed after 8 h.

After removal of the loops, the pig was killed by intracardial injection of pentobarbital sodium. Loops were weighed immediately with and without the fluid contents and then dried to constant weight. Fluid accumulation was calculated by simple subtraction. The fluid was centrifuged at 9000 *g* for 20 min and the supernates were frozen at -20°C until osmolality and electrolyte determination. Finally the wet weight: dry weight ratio was calculated to uncover possible differences in intramural fluid accumulation. Fluid accumulation is expressed as g/g dry loop per 8 h and SEM; *n* is the number of observations and *N* is the number of animals.

Analytical procedures

Osmolality (Advanced Wide-Range Osmometer 3W2; Advanced Instruments Inc., Needham Heights, MA, USA) and concentrations of Na^+ , K^+ (FLM3 Flame-photometer; Radiometer, Copenhagen, Denmark) and Cl^- (CMT 10 Chloride titrator; Radiometer) were determined in the accumulated fluid and blood osmolality was determined. PGE $_2$ analysis was performed by radioimmunoassay [26]. 5-HT levels were analysed by enzyme immunoassay (Immunotec, Marseilles, France) and high-performance liquid chromatography (HPLC) in the fluid from ST and CT loops, respectively. Because of the risk of contamination, 5-HT concentrations in ST-containing fluid could not be measured by HPLC.

Statistical analysis

Results are expressed as the means and SEM. The time, segment and dose response data were analysed by three-way ANOVA and the data dealing with the effect of ondansetron were analysed by one-way ANOVA. Pairwise comparison was performed by Student's *t*-test with Bonferroni correction when there were more than two comparisons. Probability values (*p*) ≤ 0.05 were considered significant.

Results

In all experiments, the fluid instilled into T loops was absorbed (Fig. 1) and U loops were empty (data not shown). In this, as in previous studies [25, 27, 28], no indication of any effect in adjacent loops was found.

Fig. 1. *Salmonella* Typhimurium- (10^8 and 10^{10} cfu) and cholera toxin- (20 μ g/loop) induced fluid accumulation in ligated loops in porcine jejunum and ileum after 8 (□) and 11 (▨) h of instillation. Values are mean and SEM. Number of observations are 8–12 in five animals. *Significant compared to control.

ST-induced fluid accumulation (dose- and time-response, segmental differences)

The three-way ANOVA showed no interaction between dose, time or segment and there was no statistically significant time-response. The test revealed differences in response to dose and segment. ST 10^8 cfu failed to induce a significant response (Fig. 1), whereas 10^{10} cfu of ST induced fluid accumulation in jejunum and ileum after instillation for both 8 and 11 h (Fig. 1). The effect of 10^{10} cfu was significantly higher than the effect of 10^8 cfu. Segmental differences were only present in the fluid accumulation after 11 h, showing a higher response in jejunum than in the ileum.

Wet weight: dry weight ratio of ST 10^{10} cfu-treated loops was significantly higher than the wet weight: dry weight ratio of T loops (data not shown).

CT-induced fluid accumulation (time-response and segmental differences)

CT induced fluid accumulation in jejunum and ileum after both 8 and 11 h (Fig. 1). Neither significant time-response nor segmental differences were present in the CT-induced fluid accumulation.

No difference was detected in the wet weight: dry weight ratio between CT-treated and T loops (data not shown).

5-HT and PGE₂ levels in accumulated fluid

The data from 8 and 11 h was mixed, as no time response was evident (data not shown). The 5-HT and PGE₂ levels in accumulated fluid from T (control), CT- and ST-treated loops are shown in Table 1, Table 2 and Table 3. CT- and ST-treated loops had significantly higher levels of 5-HT and PGE₂ in both segments than controls. No segmental differences were present in the 5-HT levels in either CT- or ST-treated loops. The PGE₂ levels in CT-treated loops were also without segmental difference, whereas the levels of PGE₂ were higher in jejunal than in ileal loops after ST stimulation.

Osmolality and electrolyte concentrations

Osmolality and Na⁺, K⁺ and Cl⁻ concentrations in accumulated fluid in CT- and ST-treated loops are

Table 1. Effect of CT on intraluminal 5-HT

Loop	Mean (SEM) 5-HT concentration (nmol/g dry loop)	
	Control	CT
Jejunum	0.33 SEM 0.16 (3)	3.9 SEM 0.74 (5)*
Ileum	0.44 SEM 0.17 (4)	2.7 SEM 0.78 (5)*

The 5-HT in the luminal accumulated fluid was measured by HPLC. Numbers in parentheses indicate the number of observations in three to five animals.

*Significant compared to control.

Table 2. Effect of ST on intraluminal 5-HT

Loop	Mean (SEM) 5-HT concentration (nmol/g dry loop)	
	Control	ST
Jejunum	0.88 SEM 0.23 (7)	3.47 SEM 0.96 (8)*
Ileum	0.95 SEM 0.45 (9)	4.51 SEM 1.12 (10)*

The 5-HT in the luminal accumulated fluid was measured by enzyme immunoassay. Numbers in parentheses indicate the number of observations in three to five animals.

*Significant compared to control.

Table 3. Effect of CT and ST on intraluminal PGE₂

Loop	Mean (SEM) PGE ₂ concentration (ng/g dry loop)		
	Control	ST	CT
Jejunum	4.5 SEM 0.7 (14)	22.3 SEM 4.5 (19)*	22.8 SEM 8.6 (15)*
Ileum	3.3 SEM 0.3 (16)	9.7 SEM 2.4 (18)*	21.1 SEM 8.1 (15)*

Numbers in parentheses indicate the number of observations in three animals.

*Significant compared to control.

Table 4. Osmolality and Na⁺, K⁺ and Cl⁻ concentrations in accumulated fluid after stimulation by CT and ST

Agent, loop	Osmolality (m Osm/kg H ₂ O)	Na ⁺ (nmol/L)	K ⁺ (nmol/L)	Cl ⁻ (nmol/L)	Anion gap
CT, jejunum	282 SEM 6	140 SEM 2	8.8 SEM 0.5	105 SEM 13	44 SEM 13
CT, ileum	278 SEM 9	140 SEM 3	8.7 SEM 0.8	50 SEM 3	99 SEM 5
ST, jejunum	342 SEM 18	127 SEM 2	15.5 SEM 0.9	80 SEM 5	62 SEM 5
ST, ileum	334 SEM 18	131 SEM 3	14.1 SEM 1.1	87 SEM 5	58 SEM 6

mmol/l and osmolality in mOsm/kg H₂O. There were 8–12 observations in four animals. The anion gap was calculated by subtracting the Cl⁻ concentration from the sum of Na⁺ and K⁺ concentrations.

shown in Table 4. Segmental difference was present only in the Cl⁻ concentration and the anion gap in CT-induced fluid accumulation, showing a higher jejunal than ileal Cl⁻ concentration and a higher ileal than jejunal HCO₃⁻ concentration, as the anion gap is assumed mainly to represent HCO₃⁻. The osmolality values in CT-treated loops did not differ from plasma osmolality, which was 262 SEM 4 mOsm/kg H₂O (n = 5, N = 5), whereas the ST-induced accumulated fluid was hyperosmotic. No segmental differences in electrolyte composition were present in ST-induced accumulated fluid, whereas all electrolyte concentrations differed from values in CT-treated loops: there were lower Na⁺ levels and higher K⁺ levels in both segments, whereas Cl⁻ concentration was lower in the jejunum and higher in the ileum when compared with CT-treated loops. The anion gap was also without any segmental difference and in the ileum it was significantly lower than in CT-treated jejunal loops.

Effect of ondansetron

As shown in Fig. 2, ondansetron treatment reduced the ST-induced fluid accumulation by c. 40% in both jejunum and ileum, while ondansetron failed to have an effect on the response to CT. Ondansetron did not affect fluid accumulation in T and U loops (data not shown). The systolic blood pressure was increased from 73.4 SEM 8.7 mm Hg (n = 22, N = 6) to 85.3 SEM 2.5 mm Hg (n = 24, N = 6) by the ondansetron treatment.

Discussion

The main purpose of this study was to test the hypothesis that 5-HT plays a role in ST-induced secretion. The study demonstrated that 5-HT is released to the luminal fluid in the small intestine and that the 5-HT₃ receptor antagonist ondansetron reduces the fluid

Fig. 2. Effect of ondansetron on *Salmonella* Typhimurium- (10^8 cfu) and cholera toxin- ($20 \mu\text{g}/\text{loop}$) induced fluid accumulation in ligated loops in porcine jejunum and ileum after 8 h of instillation: □, CT; ▨, CT + ondansetron; ▤, ST; ▥, ST + ondansetron. Values are mean and SEM. The figures indicate number of observations in five to seven animals. *Significant compared to control.

accumulation induced by ST. These findings support the hypothesis.

The secretory responses to CT and ST are often compared, as they both induce massive electrolyte and fluid secretion, and some strains of ST produce an enterotoxin that resembles CT [6]. CT is the best studied secretagogue, which makes it ideal as a reference, but the gastrointestinal tract, in some regards, exhibits different responses to CT and ST. Unlike CT stimulation, histopathological changes are observed in ST infection [29, 30]. After 8 h of ST instillation in ligated loops in pigs [2] and rabbits [4], mucosal alterations have been reported with massive infiltration of polymorphnuclear leucocytes (PMNL) in the lamina propria, many of which transmigrate the epithelial lining. Crypts are dilated by PMNL debris ('crypt abscess') and lined by attenuated epithelium [2]. The intensity of the reaction is greatest over Peyer's patches [2], in accordance with M-cells having been suggested to be an entrance for the ST infection [31]. Villus atrophy due to extrusion of the villus tip is not present until 12 h after instillation [4]. Besides these histological differences, CT and ST have different cytokine profiles in the induced accumulated fluid [29].

The potency of CT and ST could not be compared with accuracy, as the doses of CT and ST are not directly comparable. For this, further dose- and time-response studies are needed.

As previously reported in rats [13], pigs [32] and man [33, 34], CT induced fluid accumulation and a rise in intraluminal 5-HT and PGE_2 . Both 5-HT and PGE_2

induce secretion by direct stimulation of the enterocytes [35] and by activating the ENS [36]. Furthermore, PGE_2 suppresses ileal Na^+ and water absorption [37, 38]. No intramural fluid accumulation was observed after CT stimulation, consistent with CT inducing secretion without histological changes [30].

The 5-HT receptors participating in fluid and electrolyte secretion include at least G protein-linked 5-HT_{2A}, 5-HT₄ receptors and the 5-HT₃ ligand-gated ion channels [16, 39–41], with some species differences [16, 39, 42–47]. The 5-HT₂ receptors are situated on neurons and enterocytes, the 5-HT₃ receptors are largely located on neuronal structures [16, 48] and 5-HT₄ receptors are located on neurons and intestinal smooth muscles [49].

In this study, treatment with ondansetron – a potent and highly selective 5-HT₃ receptor antagonist [50] – failed to reduce the CT-induced fluid accumulation, which is in contrast to previous findings [25] that showed a 40% reduction in response to CT by ondansetron. The differences may be due to different experimental design, as Grøndahl and co-workers [25] used an instillation time of only 4 h, younger pigs (6–8 weeks, 12–15 kg) and halothane as general anaesthetic. Time dependency in the secretory mechanism of CT has been suggested by Wald and co-workers [51]. Thus 5-HT may be involved mainly in the early phase of the secretion, which could explain the failure of reduction by ondansetron in the present study. The use of different anaesthetics may also influence the effect of ondansetron, as several anaesthetics modulate the 5-HT₃ receptor-mediated functions [52]. Finally, the CT dose ($20 \mu\text{g}$) that induced a submaximal fluid accumulation in the 4-h experiment [25], left the loops completely filled (ballooning) after 8 h. This increased intraluminal pressure may have influenced the secretory response of CT and thereby also the antagonistic effect of ondansetron.

The ondansetron treatment induced an increase in the systolic blood pressure, which is consistent with inhibition of the Bezold-Jarisch reflex, which can be stimulated by 5-HT injections [53, 54].

Analysis of the loop contents demonstrated that CT induced secretion of iso-osmotic fluid and electrolyte composition as previously reported [25, 55]. The lower Cl^- concentration in ileum may reveal a decreased Cl^- secretion in accordance with decreasing amount of Cl^- channels, CFTR, in the aboral direction of the small intestine [56], or an increased $\text{Cl}^-/\text{HCO}_3^-$ exchange in ileum, or both [53]; thus HCO_3^- probably explains the anion gap.

ST induced a dose-dependent fluid accumulation as reported in rabbits [58]. The PGE_2 level in the accumulated fluid was increased, consistent with ST inducing increase of mucosal prostaglandin concentra-

tion after installation in rabbit ileal loops [19]. PGE₂ is involved in the response both *via* inflammation and the enterotoxin, as both ST enterotoxin [20] and non-toxicogenic ST [59] induce secretion involving prostaglandins. The source of the PGE₂ is believed to be the fibroblasts, as cell culture of porcine fibroblasts have been shown to produce PGE in response to inflammatory mediators such as 5-HT, bradykinin, histamine, adenosine and platelet activating factor [35]. Furthermore, Duebbert and Peterson [19] suggest that epithelial prostaglandin production occurs after ST stimulation. The higher amount of PGE₂ and fluid accumulated in the jejunum than in the ileum reveal segmental differences in the secretory response to stimulation with ST, which confirm the importance of studying ST infections in more than one segment [10]. Also, the luminal content of 5-HT was increased after stimulation with ST. The EC cells are supposed to be the major source of the 5-HT release as described for CT [14]. The secretory pathways for PGE₂ and 5-HT are discussed above. In addition, other inflammatory mediators may have contributed to the fluid accumulation, as several have been shown to have both direct and indirect secretory effects on the intestinal epithelium, *via* ENS [35, 60, 61]. Consistent with an inflammatory reaction in the intestinal wall, intramural fluid accumulation was observed in the ST-treated loops.

The hyperosmolality of the accumulated fluid observed in the ST loops is probably due to products of bacteriolysis, contributing to an osmotic component in the stimulated fluid accumulation. A consistently lower Na⁺ concentration was present in ST-treated loops than with CT. Likewise, the high K⁺ concentration could be explained by K⁺ release from bacteriolysis and damaged epithelial cells [2]. No segmental difference was present in the Cl⁻ concentration and anion gap, which may indicate stimulated HCO₃⁻ secretion in both segments.

Ondansetron reduced the ST-induced fluid accumulation by *c.* 40%. This inhibitory effect of ondansetron is probably due partly to inhibition of the secretory effect of the released 5-HT and partly to an inhibition of the secretory nervous reflexes activated by inflammation mediators [59].

It is not possible from these data to determine whether the involvement of 5-HT in the secretory response is mainly through the secretory pathway of an enterotoxin or through inflammatory mediators. Further loop-test studies with cell-free lysates of enterotoxigenic ST and with enterotoxin-negative ST strains are needed.

In summary, these data demonstrate that *Salmonella* Typhimurium – like CT – causes release of 5-HT and PGE₂ into the lumen and fluid accumulation in the porcine small intestine. Furthermore, ondansetron

reduces the fluid accumulation induced by ST by about one-third in both jejunum and ileum. Taken together, these results suggest that 5-HT is released and 5-HT₃ receptors activated in the secretory pathway of ST. Furthermore, the involvement of prostaglandins in ST induced secretion is confirmed in the porcine small intestine.

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