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European Journal of Pharmacology 539 (2006) 116–124
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Mucosally-directed adrenergic nerves and sympathomimetic drugs enhance non-intimate adherence of *Escherichia coli* O157:H7 to porcine cecum and colon

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Received 15 February 2006; received in revised form 27 March 2006; accepted 31 March 2006
Available online 6 April 2006

Abstract

The sympathetic neurotransmitter norepinephrine has been found to increase mucosal adherence of enterohemorrhagic *Escherichia coli* O157:H7 in explants of murine cecum and porcine distal colon. In the present study, we tested the hypothesis that norepinephrine augments the initial, loose adherence of this important pathogen to the intestinal mucosa. In mucosal sheets of porcine cecum or proximal, spiral and distal colon mounted in Ussing chambers, norepinephrine (10 μM, contraluminal addition) increased mucosal adherence of wild-type *E. coli* O157:H7 strain 85-170; in the cecal mucosa, this effect occurred within 30–90 min after bacterial inoculation. In addition, norepinephrine transiently increased short-circuit current in cecal and colonic mucosal sheets, a measure of active anion transport. Norepinephrine was effective in promoting cecal adherence of a non-O157 *E. coli* strain as well as *E. coli* O157:H7 *eae* or *espA* mutant strains that are incapable of intimate mucosal attachment. Nerve fibers immunoreactive for the norepinephrine synthetic enzyme dopamine β-hydroxylase appeared in close proximity to the cecal epithelium, and the norepinephrine reuptake blocker cocaine, like norepinephrine and the selective α_2-adrenoceptor agonist UK-14,304, increased *E. coli* O157:H7 adherence. These results suggest that norepinephrine, acting upon the large bowel mucosa, modulates early, non-intimate adherence of *E. coli* O157:H7 and probably other mucosa-associated bacteria. Sympathetic nerves innervating the cecocolonic mucosa may link acute stress exposure or psychostimulant abuse with an increased microbial colonization of the intestinal surface. This in turn may alter host susceptibility to enteric infections.

Keywords: Intimin; Type III secretion system; Mucosa-adherent bacterium; Norepinephrine; Cocaine; Enteric nervous system

1. Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 is an important human pathogen that has been isolated from contaminated water as well as meat products. It produces hemorrhagic colitis after oral ingestion, and Shiga toxin-producing strains may additionally cause acute renal failure or neurological disturbances especially in young, elderly or immunocompromised individuals (Nataro and Kaper, 1998). *E. coli* O157:H7 possesses a pathogenicity island, termed the locus of enterocyte effacement. This locus encodes a type III secretion system which introduces virulence-associated proteins into host epithelial cells via a hollow filamentous extension of the needle complex encoded by the *espA* gene (Roe et al., 2003). One important protein is the translocated intimin receptor, which is delivered into epithelial cells and interacts with its cognate ligand, intimin, encoded by the *eae* gene in the locus of enterocyte effacement and expressed on the bacterial outer membrane (Campellone and Leong, 2003). Intimin interactions with the translocated intimin receptor and *E. coli* O157:H7-induced changes in the epithelial cytoskeleton contribute to intimate mucosal adherence and the production of characteristic attaching and effacing lesions involving the cecum and large intestine (Moxley, 2004). Intimin also appears to determine the tropism of *E. coli* O157:H7 towards

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doi:10.1016/j.ejphar.2006.03.081

the mucosa of the large intestine (Stevens and Wallis, 2005). In addition to their role in intimin receptor translocation, espA filaments have been shown to act as an initial adhesin of *E. coli* O26:H- (Ebel et al., 1998).

Norepinephrine, at micro- to millimolar concentrations and long (>4 h) exposure periods, has been reported to stimulate *E. coli* O157:H7 growth (Lyte and Nguyen, 1997), epithelial adherence (Vlisidou et al., 2004), and virulence (Lyte et al., 1996). Although the mechanisms underlying this direct interaction of norepinephrine or other biogenic amines with *E. coli* O157:H7 are incompletely defined, they appear to include increased bacterial iron transport (Freestone et al., 2003) and modulation of quorum sensing with potential upregulation of virulence factors (Clarke and Sperandio, 2005). In addition to their actions on enteric bacteria, norepinephrine or other substances released in response to acute stress in the host may act upon the intestinal mucosa to alter interactions between luminal microorganisms and epithelial cells. The results of recent investigations tend to support this hypothesis. Both norepinephrine and adrenocorticotrophic hormone have been reported to enhance *E. coli* O157:H7 adherence to the porcine distal colon through interactions with mucosal α-adrenergic and melanocortin receptors respectively (Green et al., 2004; Schreiber and Brown, 2005).

The main objective of the present study was to test the hypothesis that norepinephrine and other sympathomimetic drugs modulate the initial, loose adherence of *E. coli* O157:H7 and other mucosa-adherent, non-O157 *E. coli*, rather than the *E. coli* O157:H7-mediated processes of epithelial cytoskeletal reorganization and formation of attaching/effacing lesions. To this end, pharmacological experiments were performed using *E. coli* O157:H7 strains manifesting genetically-engineered mutations of the *espA* and *eae* genes. In addition, our initial observations with norepinephrine were extended through the determination of the large intestinal sites and time course of norepinephrine action and a confirmation of the adrenergic receptors mediating bacterial adherence.

2. Materials and methods

2.1. Bacteria

E. coli O157:H7 strains used in this study were from the laboratory of Dr. Mark Stevens. *E. coli* O157:H7 strain 87-170 nal^R (code 95) is a spontaneous Shiga toxin-negative, nalidixic acid-resistant derivative of *E. coli* O157:H7 strain 84-289, which was originally isolated from a food handler in a Canadian nursing home (Tzipori et al., 1987). Strain 85-170 has previously been reported to adhere to porcine ileal explants in an *eae*-dependent manner and to induce the formation of attaching and effacing lesions (Girard et al., 2005). *E. coli* O157:H7 85-170 nal^R harboring a non-polar deletion of *eae* (strain ICC170; code 93) has been described previously (Fitzhenry et al., 2002). Strain 85-170 nal^R *espA*: :kanR (code 99) contains an insertion of the kanamycin resistance gene from plasmid pUC4K in *espA* and was constructed by allelic exchange using the positive-selection suicide vector pCVD442. Mutants lacking *eae* and *espA* were verified by Southern blotting and confirmed to respectively lack intimin or the filamentous type III translocon by Western blotting and immunofluorescence microscopy using specific antisera.

Commensal strains of porcine non-O157 *E. coli* were obtained by plating homogenized colonic mucosa from normal pigs onto Fluorocult agar (EM Science, Gibbstown, NJ) supplemented with 100 μg/ml streptomycin sulfate to isolate *E. coli* strains that were resistant to this antibiotic drug. The selective isolation and differentiation capabilities of Fluorocult medium for *Enterobacteriaceae*, especially *E. coli* O157:H7, which are achieved by a combination of fluorogenic and chromogenic substrates, have been well described to identify relevant bacteria from a variety of sources (Heizmann et al., 1998). Presumptive colonies of *E. coli* that did not have the appearance of *E. coli* O157:H7 were randomly chosen from Fluorocult plates following overnight incubation and were streaked onto Luria-Bertani (LB) agar plates supplemented with 100 μg/ml streptomycin. Following 24 h incubation at 37 °C, individual colonies were picked from these plates and their identities confirmed as *E. coli* using the API-20E Enteric Identification System (BioMerieux, Hazelwood, MO). Colonies were further determined to represent non-O157 *E. coli* with the use of an *E. coli* O157 latex agglutination-based diagnostic test kit (Oxoid, Ogdensburg, NY). One strain of non-O157 *E. coli* (#4) was used in the present study.

Bacteria were stored as glycerol stocks at −80 °C. For each experiment, bacteria were grown to stationary phase following overnight incubation in LB broth at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Animals and tissue preparation

Tissues were obtained from weaned Yorkshire-Landrace pigs of each sex; animals were 6–10 weeks old (10–18 kg body weight) and received food and water ad libitum. Each pig was anesthetized with an intramuscular injection of tiletamine hydrochloride-zolazepam (Telazol®, 8 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA), in combination with xylazine (3 mg/kg), and subsequently euthanized with intravenous Beuthanasia-D® (0.5 ml/kg; Schering-Plough Animal Health, Union, NJ) in accordance with approved University of Minnesota Animal Use and Care Committee protocols. A midline laparotomy was performed to expose the intestine. Explants were obtained from 10 cm of the terminal cecum; the proximal colon starting 15 cm distal to the cecal-colonic junction; the spiral colon starting approximately 120 cm distal to the cecal-colonic junction; and the distal colon above the terminal 10–15 cm of rectum and anus. These tissues were selected for investigation because the large intestine is an important site for *E. coli* O157:H7 colonization and attachment/effacement in swine, particularly the cecum, distal colon and rectum (Wales et al., 2005; Best et al., 2006). Tissues were placed in ice-cold oxygenated (95% O_2, 5% CO_2) physiological tissue preservation solution (ionic composition in mM: Na^+, 130; K^+, 6.0; Mg^{2+}, 0.7; Ca^{2+}, 3.0; HCO_3, 19.6; HPO_4^-, 0.29, $H_2PO_4^-$, 1.3; D-glucose, 11.0) which was maintained at pH 7.4.

2.3. Measurement of transepithelial electrical parameters

Each tissue was stripped of its underlying circular and longitudinal smooth muscle, and the resulting mucosal explant containing the inner submucosal plexus was mounted between two Ussing half-chambers (World Precision Instruments, Sarasota, FL) having a flux area of 1.0 cm^2. D-Glucose and mannitol were added to the contraluminal and luminal bathing medium respectively at a final bath concentration of 10 mM. Both luminal and contraluminal reservoirs contained 10 ml of buffered, standard porcine physiological saline solution similar in composition to porcine extracellular fluid (composition in mM: NaCl, 130.0; KCl, 6.0; $CaCl_2$, 3.0; $MgCl_2$, 0.7; $NaHCO_3$, 20.0; NaH_2PO_4, 0.29; Na_2HPO_4, 1.3) and gassed with 95% O_2 and 5% CO_2 at 39 °C (porcine core temperature). Tissues were voltage-clamped (World Precision Instruments, Sarasota, FL) for measurement of short-circuit current (I_{sc}, in μA/cm^2), a measure of active, electrogenic transepithelial ion transport. Tissue electrical conductance (G_t, in mSiemens/cm^2), a measure of mucosal ionic permeability, was calculated from potential difference and I_{sc} by Ohm's law. Experiments commenced after the Isc had stabilized.

2.4. Drugs

L-(−)-Norepinephrine bitartrate, propranolol hydrochloride, phentolamine mesylate, yohimbine HCl, prazosin HCl, L-phenylephrine HCl, and D,L-isoproterenol HCl were obtained from Sigma Chemical Co. (St. Louis, MO). 5-Bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)quinoxalin-6-amine (UK-14,304) was obtained from Tocris Bioscience (Ellisville, MO) and cocaine HCl (100 mg/ml) was obtained from Roxane Laboratories (Columbus, OH). Stock solutions of drugs were made in distilled water, with the exception of UK-14, 304 which was solubilized in dimethyl sulfoxide, and yohimbine, prazosin and cocaine which were dissolved in ethanol; stock solutions were stored frozen and aliquots were serially diluted with water at each experimental session. Norepinephrine and propranolol were freshly prepared in distilled water prior to each experiment.

2.5. E. coli O157:H7 exposure and measurement of bacterial adherence in tissue mucosal sheets

Fifteen minutes after the addition of drugs to the contraluminal chamber, wild-type or mutated *E. coli* O157:H7 or commensal, enteroadherent *E. coli* in the stationary phase of growth were diluted 1:10 in PBS, and 100 μl of this diluted stock was added to the luminal bath volume of 10 ml (1:100). The exact number of bacteria added was determined by serial spread plating of luminal bathing fluid samples onto Fluorocult *E. coli* O157 agar. The final density of bacteria achieved in the luminal bathing medium varied between 10^6 and 10^7 colony forming units (CFU)/ml.

Following mucosal exposure to the bacteria for 90 min, tissues were removed from Ussing chambers and mucosal adherence of *E. coli* O157:H7 was determined after the method of Knutton et al. (1989). In a previous study of porcine distal colonic explants, intracellular internalization of *E. coli* O157:H7 was negligible (Green et al., 2004). Tissues were removed from Ussing chambers, weighed, and washed three times in PBS (pH 7.4) to remove nonadherent bacteria. They were then homogenized using a Brinkman Polytron (Model PT 10-35; Kinematica AG, Littau, Switzerland) and dilutions of 1:1 and 1:10 were prepared and spreadplated on Fluorocult *E. coli* O157:H7 agar supplemented with 25 μg/ml nalidixic acid to select for *E. coli* O157:H7 nal^R strain 87-170 and derivatives, or with 100 μg/ml streptomycin sulfate to select for streptomycin-resistant non-O157 *E. coli*. Following incubation of plates at 37 °C for 24 h, the number of bacterial colonies (green coloration for *E. coli* O157:H7 and yellow coloration for non-O157 *E. coli*) were enumerated in CFU per gram of tissue and transformed to $\log_{10}$ values.

2.6. Immunohistochemistry

Segments of cecum were pinned mucosa side up on a Silgard-coated surface and fixed in modified Zamboni's fixative (4% paraformaldehyde and 0.2% picric acid) for 2 h at room temperature. The tissue was rinsed extensively in PBS and incubated in 10% sucrose for a minimum of 24 h before cryostat sectioning. Cryostat sections (20 μm) were double-labeled with a rabbit polyclonal antibody against bovine dopamine β-hydroxylase (DBH; 1:1000; Immunostar, Hudson, WI) and a mouse monoclonal antibody against the neuronal marker protein gene product 9.5 (Biogenesis, Poole, England). The staining was visualized with indocarbocyanine (Cy3)-conjugated donkey anti-rabbit and cyanine (Cy2)-conjugated donkey anti-mouse secondary antisera (Jackson Immunoresearch Laboratories, West Grove, PA). Images were collected using a confocal laser-scanning microscope (Bio-Rad MRC 1000) and processed using Adobe Photoshop (version 6.0.1, Adobe Systems, San Jose, CA).

2.7. Statistical analysis

All data are expressed as mean ± standard error of the mean. Statistical analyses of data were performed using the PRISM computer software program (Version 4.0a; GraphPad Software, Inc., San Diego, CA). Single comparisons between control and treatment means were made with a paired or unpaired Student's two-tailed *t*-test when appropriate. Comparisons of multiple means were made by an analysis of variance (ANOVA) with Tukey's test. The minimum level for statistical significance was set at $P<0.05$.

3. Results

3.1. Localization of norepinephrine action on E. coli O157:H7 adherence in the porcine large intestine

Wild-type *E. coli* O157:H7 adhered to mucosal explants from cecum and colon after its exposure to the luminal aspect of

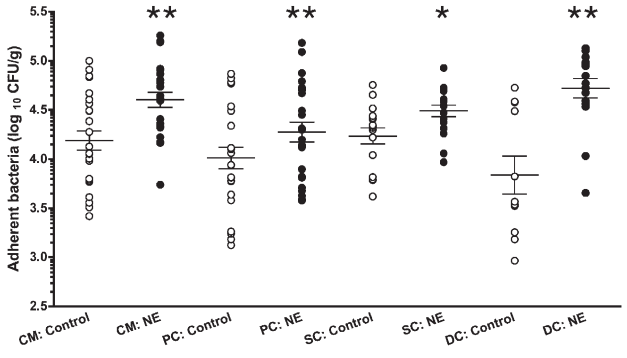

Fig. 1. Effect of norepinephrine (NE) on mucosal adherence of *E. coli* O157:H7 (Shiga toxin-negative nal^R strain 85-170) to porcine cecum (CM), proximal colon (PC), spiral colon (SC) and distal colon (DC). The aligned dot plot represents *E. coli* O157:H7 adherence to individual control tissues untreated with norepinephrine (unfilled circles) and to tissues contraluminally exposed to 10 μM norepinephrine (filled circles) from each intestinal site. Wild-type *E. coli* O157:H7 85-170 nal^R were added to the luminal medium bathing mucosal explants at an inoculum of $2.0\pm0.3\times10^6$ CFU/ml and remained in contact with each tissue for 90 min. Wide horizontal bars indicate mean values and shorter horizontal bars indicate standard error of the mean for each condition. *$P<0.05$ and **$P<0.001$ vs. control mean, *t*-test; $n=11$–24 tissues from 4–6 pigs.

these tissues for 90 min (Fig. 1). Bacterial adherence did not differ significantly among large intestine segments ($F=1.9$; 3,71 *df*, one-way ANOVA). Baseline I_{sc} and G_t were similar among mucosal explants from different large intestine segments as well (Table 1). Added to the contraluminal bath at a concentration of 10 μM, norepinephrine significantly increased *E. coli* O157:H7 adherence over 90 min in mucosal explants from cecum and colon relative to adherence measured in control tissues untreated with norepinephrine (Fig. 1). It also produced a transient increase in I_{sc} which was significantly greater in distal colon explants than in the other segments of the porcine large intestine (Table 2).

3.2. Time course of norepinephrine action on E. coli O157:H7 adherence in porcine cecal mucosa

Bacterial adherence to the cecal mucosa increased as the luminal exposure period to wild-type *E. coli* O157:H7 lengthened; *E. coli* O157:H7 adherence after 90 min of bacterial exposure was significantly greater than after 15, 30 or 60 min of exposure ($P<0.05$, Tukey test). Contraluminal norepinephrine (10 μM) significantly increased cecal adherence of *E. coli* O157:H7 relative to norepinephrine-untreated control tissues at 30–90 min of *E. coli* O157:H7 exposure (Fig. 2).

Table 1
Baseline short-circuit current (I_{sc}) and electrical conductance (G_t) in porcine mucosal explants in the presence of *E. coli* O157:H7 85-170 nal^R

Tissue	I_{sc} (μA/cm^2), mean±S.E.	G_t (mS/cm^2), mean±S.E.	n/N[a]
Cecum	5.3±1.2	19.9±1.9	14/6
Proximal colon	4.3±0.5	19.6±1.6	10/5
Spiral colon	4.5±0.6	19.6±2.7	17/8
Distal colon	5.2±1.0	14.9±2.9	12/6

[a] Total number (n) of tissues tested from N pigs.

Table 2
Effect of 10 μM norepinephrine on short-circuit current in porcine mucosal explants in the presence of *E. coli* O157:H7 85-170 nal^R

Tissue	Peak ΔI_{sc} (μA/cm^2)	n/N[a]
Cecum	37±4	22/8
Proximal colon	14±3	24/8
Spiral colon	17±3	25/8
Distal colon	80±16*	18/6

[a] Total number (n) of tissues tested from N pigs.
* Significantly greater than proximal three segments ($P<0.001$, Tukey test). Mean ΔG_t in response to norepinephrine ranged from 1.9±0.8 (distal colon) to 2.8±0.7 mS/cm^2 (cecum), but did not differ statistically among intestinal segments.

3.3. Effect of norepinephrine action on cecal adherence of E. coli O157:H7 eae and espA mutants

Over a period of 90 min, both *E. coli* O157:H7 85-170 nal^R mutants adhered to the mucosal surface of cecal explants, although the number of the adherent *E. coli* O157:H7 *eae* mutant was significantly less than that of wild-type *E. coli* O157:H7 (Fig. 3). Nevertheless, norepinephrine at a contraluminal concentration of 10 μM increased adherence of both *E. coli* O157:H7 mutants to the cecal mucosa relative to norepinephrine-untreated control tissues (Fig. 3). Norepinephrine significantly enhanced adherence of the *E. coli* O157:H7 *espA* mutant to the distal colonic mucosa as well (Table 3). Moreover, it increased cecal adherence of a non-O157 strain of *E. coli* (Fig. 3).

3.4. Characterization of adrenergic receptors mediating E. coli O157:H7 adherence to cecal mucosa

The effect of norepinephrine on mucosal adherence of *E. coli* O157:H7 in the porcine distal colon was previously found to be

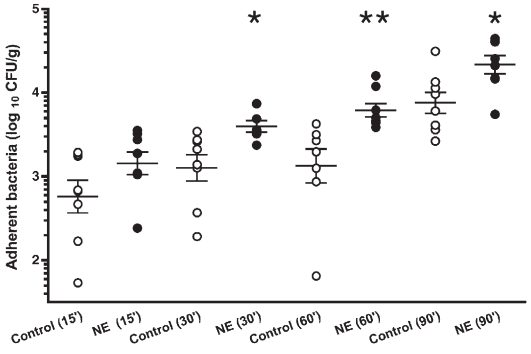

Fig. 2. Time course of the adherence-enhancing effect of norepinephrine (NE) in porcine cecal mucosa. Wild-type *E. coli* O157:H7 85-170 nal^R were added to the luminal medium bathing mucosal explants at an inoculum of $9.9\pm4.2\times10^6$ CFU/ml and remained in contact with each tissue represented in the aligned dot plot for the time periods indicated on the abscissa. In the absence of norepinephrine (unfilled circles), there was a significant time-related increase in bacterial adherence ($F=7.45$, $P=0.0008$, 31 total *df*). In some tissues (filled circles), norepinephrine was added to the contraluminal bathing medium to achieve a bath concentration of 10 μM. Wide horizontal bars indicate mean values and shorter horizontal bars indicate standard error of the mean for each condition. *$P<0.05$ and **$P<0.01$ vs. control mean (unfilled circles), *t*-test; $n=8$ tissues from 8 pigs.

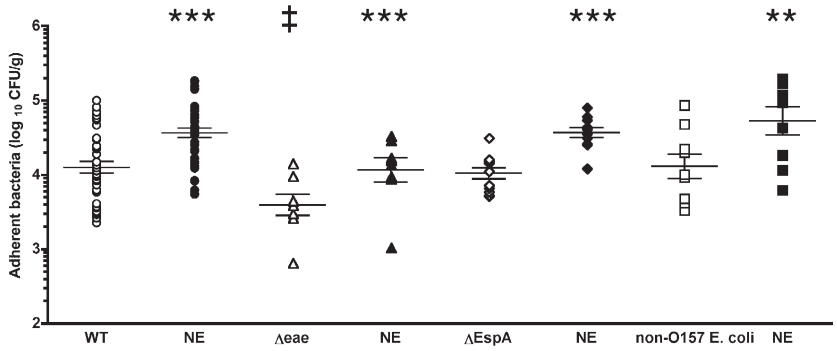

Fig. 3. Norepinephrine-enhanced adherence of *E. coli* O157:H7 does not require intimin or protein translocation into host cells. Bacteria were added to the luminal medium bathing mucosal explants at inocula of $22.4\pm6.0\times10^6$ CFU/ml (*E. coli* O157:H7 nal^R, wild type; $n=5$), $12.6\pm7.6\times10^6$ CFU/ml (O157:H7 nal^R *eae* mutant; $n=5$), $46.8\pm7.6\times10^6$ CFU/ml (85-170 nal^R *espA*::kanR; $n=8$) or $43.1\pm19.3\times10^6$ CFU/ml of a streptomycin-resistant, non-O157:H7 *E. coli* strain isolated from porcine colon ($n=4$). They remained in contact with each tissue represented in the aligned dot plot for 90 min. In some tissues (filled shapes), norepinephrine (NE) was added to the contraluminal bathing medium to achieve a final concentration of 10 μM. Wide horizontal bars indicate mean values and shorter horizontal bars indicate standard error of the mean for each condition. $^‡P<0.05$ vs. wild-type *E. coli* O157:H7 85-170 nal^R strain, Tukey's test. $^{**}P<0.01$ and $^{***}P<0.001$ vs. norepinephrine-untreated control tissues (unfilled shapes), *t*-test; $n=9$–36 tissues from 5–12 pigs.

mediated by α_2-adrenoceptors (Green et al., 2004). To confirm and extend this finding to the porcine cecum, the effects of selective adrenergic receptor antagonists and agonists were examined. In cecal mucosa explants, the effects of norepinephrine (10 μM, contraluminal administration) on wild-type *E. coli* O157:H7 adherence were inhibited significantly by the α-adrenoceptor antagonist phentolamine, but not by the β-adrenoceptor antagonist propranolol (Fig. 4, top). Furthermore, they were inhibited by the α_2-adrenoceptor antagonist yohimbine, but not by the α_1-adrenoceptor antagonist prazosin (Fig. 4, middle).

The highly-selective α_2-adrenoceptor agonist UK-14,304 significantly increased wild-type *E. coli* O157:H7 adherence to the porcine cecal mucosa after its addition to the contraluminal bathing medium at a concentration of 10 μM; in contrast, neither the α_1-adrenoceptor agonist phenylephrine nor the β-adrenoceptor agonist isoproterenol altered *E. coli* O157:H7 adherence (Fig. 4, bottom).

3.5. Localization of adrenergic nerve fibers in cecal mucosa and effects of cocaine on E. coli O157:H7 adherence

The localization of adrenergic nerve fibers in the cecal mucosa was demonstrated using immunohistochemistry. Antisera against dopamine β-hydroxylase (DBH), the last enzyme in the norepinephrine synthetic pathway, labeled nerve fibers in submucosal ganglia and throughout the mucosa. The neuronal localization of DBH staining was confirmed by double-labeling with the neuronal marker protein gene product 9.5 (PGP), which showed overlap of DBH and PGP immunoreactivities (Fig. 5, top).

The psychostimulant drug cocaine is a potent blocker of norepinephrine reuptake into adrenergic nerve terminals (Fleckenstein et al., 2000). At contraluminal concentrations ≥10 μM, cocaine significantly increased *E. coli* O157:H7 85-170 nal^R adherence to the cecal mucosa (Fig. 5, bottom). The estimated 50% effective concentration of cocaine was 14.5 μM based on best fit analysis of the cocaine concentration–effect curve. Cocaine similarly increased *E. coli* O157:H7 adherence in explants of distal colonic mucosa (mean $\log_{10}$ CFU/g *E. coli* O157:H7 85-170 nal^R recovered from 6 pairs of distal colonic mucosa untreated and pretreated with 10 μM cocaine was respectively 2.59 ± 0.18 and 3.04 ± 0.10, $P=0.008$, paired *t*-test).

4. Discussion

Young pigs are an important host for attaching-effacing *E. coli* and have been used in several previous investigations as an animal model for the study of *E. coli* O157:H7 pathogenesis (Wales et al., 2005). The present findings indicate that a strain of *E. coli* O157:H7 is capable of adhering to the mucosae of the porcine cecum and three subregions of the colon after a relatively short (90 min) period of luminal exposure. This is likely to occur at the surface epithelium of these tissues (Green et al., 2004). Adherence of *E. coli* O157:H7 85-170 nal^R was reduced in the absence of the outer membrane adhesin intimin, a finding consistent with those of recent studies using porcine ileal explants cultured in vitro and the same bacterial strains (Girard et al., 2005). In experimental animals inoculated with *E. coli* O157:H7, the microorganism produces attaching-effacing lesions in, and can be cultured from, both the cecum and colon (Francis et al., 1986; Grauke et al., 2002). In explants from all

Table 3
Effect of 10 μM norepinephrine on adherence of an *E. coli* O157:H7 85-170 nal^R *espA* mutant (in $\log_{10}$ CFU/g, mean±S.E.M.) to distal colonic mucosa

Time interval of mucosal *E. coli* O157:H7 exposure (min)	Control	Norepinephrine[a]
30	3.70±0.10	3.82±0.10*
60	3.64±0.08	4.04±0.08*
90	3.89±0.05	4.30±0.05**

n=one pair of tissues from each of 6 pigs/each time point.
$^*P<0.05$ and $^{**}P<0.01$ vs. norepinephrine-untreated control tissues (paired *t*-test).
[a] Added to contraluminal bathing medium.

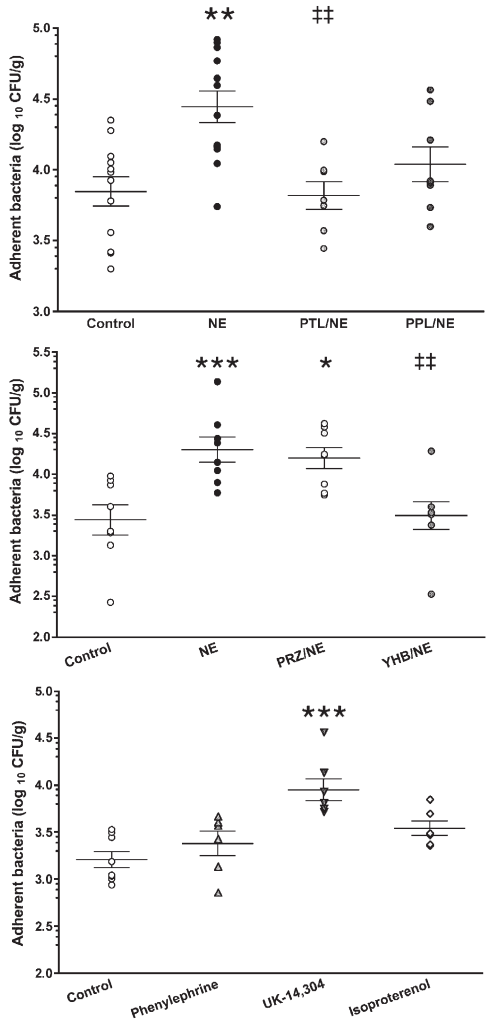

Fig. 4. Characterization of adrenergic receptors mediating mucosal adherence of *E. coli* O157:H7 in porcine cecum. (Top) Effect of 10 μM norepinephrine in the absence (NE) and presence of 0.1 μM phentolamine (PTL/NE) or propranolol (PPL/NE). Bacteria were added to the luminal medium bathing mucosal explants at an inoculum of $16.5 \pm 4.8 \times 10^6$ CFU/ml and remained in contact with each tissue represented in the aligned dot plot for 90 min. All drugs were added to the contraluminal bathing medium. $^{**}P<0.01$ vs. control mean (unfilled circles) and $^{‡‡}P<0.01$ vs. norepinephrine mean (black filled circles), one-way ANOVA with Tukey's test; $n=7$–12 tissues from 7 pigs. (Middle) Effect of 10 μM norepinephrine in the absence (NE) and presence of 0.3 μM prazosin (PRZ/NE) or yohimbine (YHM/NE). Bacteria were added to the luminal medium bathing mucosal explants at an inoculum of $9.0 \pm 2.5 \times 10^6$ CFU/ml and remained in contact with each tissue for 90 min. All drugs were added to the contraluminal bathing medium. $^{*}P<0.05$ and $^{**}P<0.01$ vs. control mean (unfilled circles) and $^{‡‡}P<0.01$ vs. norepinephrine mean (black filled circles), one-way ANOVA with Tukey's test; $n=8$ tissues from 6 pigs. (Bottom) Adherence-promoting effect of selective adrenergic agonists. Bacteria were added to the luminal medium bathing mucosal explants at an inoculum of $8.4 \pm 4.4 \times 10^6$ CFU/ml and remained in contact with each tissue for 90 min. Each agonist was added to the contraluminal bathing medium to achieve a final concentration of 10 μM. $^{***}P<0.001$ vs. control mean (unfilled circles), one-way ANOVA with Tukey's test; $n=6$–8 tissues from 5 pigs. In each panel, wide horizontal bars indicate mean values and shorter horizontal bars indicate standard error of the mean for each condition.

large intestinal segments examined, norepinephrine increased both I_{sc} and mucosal adherence of *E. coli* O157:H7. These effects are similar to those reported previously in the mouse cecum (Chen et al., 2003), a finding suggesting that they are not restricted to a particular host species. Peak I_{sc} responses to norepinephrine, which were highest in porcine distal colonic mucosa, have been attributed to active chloride secretion and are mediated by α_1-adrenoceptors that are likely to be localized on colonic crypt cells (Traynor et al., 1991). As the tissues were voltage-clamped, the potential difference produced by anion secretion was automatically nullified and an ionic gradient to support water flux could not develop. Moreover, the muscarinic cholinergic antagonist carbachol rapidly increases I_{sc} but has no effect on *E. coli* O157:H7 adherence in the porcine distal colon (D.R. Brown, unpublished observations). Therefore, the observed norepinephrine-induced increase in I_{sc} per se would not be expected to alter bacterial adherence.

A previous report (Green et al., 2004) indicated that norepinephrine promotes colonic adherence of both Shiga toxin-producing *E. coli* O157:H7 (strain EDL933) and, as observed in the present study, toxin-negative *E. coli* O157:H7 strains (e.g. strain 700728). Thus, it is unlikely that Shiga toxins play a role in this effect of norepinephrine. *E. coli* O157:H7 infection is generally acknowledged to occur in three stages: initial, loose attachment to host epithelial cells; EspA-mediated protein translocation to host cells; and intimate adherence and pedestal formation (Wales et al., 2005). Three findings strongly suggest that norepinephrine acts at the earliest stage of *E. coli* O157:H7 adherence to the intestinal epithelium of the host. First, norepinephrine increased the number of *E. coli* O157:H7 adhering to the cecal mucosa as early as 30 min after bacterial inoculation of the luminal bathing fluid. Second, norepinephrine increased the cecal adherence of an *E. coli* O157:H7 mutant strain that was incapable of expressing intimin. Third, norepinephrine increased cecal and colonic adherence of an *E. coli* O157:H7 *espA* mutant, which lacks the ability to deliver adherence-promoting type III secreted proteins to host cells. We hypothesize that norepinephrine enhances the early, non-intimate attachment of *E. coli* O157:H7 to the epithelium of the cecum and colon. Although the process of intimate attachment has been well characterized in enterohemorrhagic and enteropathogenic *E. coli*, considerably less is known about the factors mediating early, non-intimate adherence. These include flagellae (Best et al., 2005, 2006), long polar fimbriae (Jordan et al., 2004), and OmpA protein (Torres and Kaper, 2003). It is notable that norepinephrine increased adherence of a non-O157 *E. coli* to the cecal mucosa. In a previous investigation (Green et al., 2004), colonic adherence of a rodent-adapted *E. coli* strain as well as a different non-O157 *E. coli* isolated from pigs was not increased by norepinephrine. It is possible that norepinephrine alters the expression or clustering of host epithelial adhesion molecules that interact with one or more bacterial adherence determinants expressed not only by *E. coli* O157:H7 but also by some non-O157 strains of mucosa-adhering *E. coli* or other species of bacteria.

In the porcine cecum, the adherence-promoting effect of norepinephrine appears to be mediated by α-adrenoceptors. The α-adrenergic antagonist phentolamine, but not the β-adrenoceptor antagonist propranolol, prevented norepinephrine action. In addition, the norepinephrine effect was prevented by the

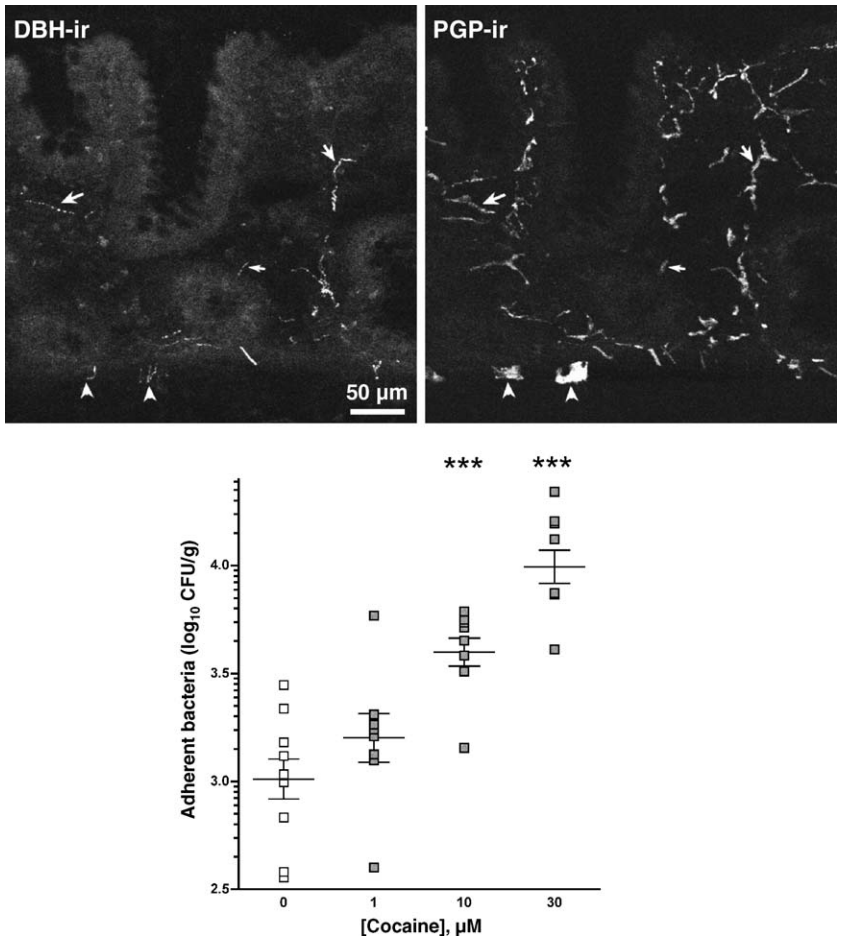

Fig. 5. Endogenous norepinephrine modulates cecal adherence of *E. coli* O157:H7. (Top) Localization of noradrenergic nerve fibers in the mucosa of porcine cecal explant by immunohistochemistry. Double labeling for dopamine β-hydroxylase (DBH; left) and protein gene product 9.5 (PGP; right) demonstrated the presence of DBH-immunoreactive (-ir) nerve fibers within PGP-ir nerve bundles in submucosal ganglia (arrowheads), in the vicinity of crypts (small arrows) as well as within the villous lamina propria. (Bottom) Concentration–effect relationship for the norepinephrine reuptake blocker cocaine in enhancing adherence. Bacteria were added to the luminal medium bathing mucosal explants at an inoculum of $6.1 \pm 1.1 \times 10^6$ CFU/ml and remained in contact with each tissue represented in the aligned dot plot for 90 min. Cocaine was added to the contraluminal bathing medium to achieve the bath concentrations indicated on the abscissa (gray filled squares). Wide horizontal bars indicate mean values and shorter horizontal bars indicate standard error of the mean for each condition. ***$P<0.001$ vs. mean log CFU/g of *E. coli* O157:H7 adhering to cecal mucosa explants not treated with cocaine (concentration = 0, unfilled squares), one-way ANOVA with Tukey's multiple comparisons test; $n = 8–10$ tissues from 5 pigs.

selective α_2-adrenoceptor antagonist yohimbine, and mimicked by the selective α_2-adrenergic agonist UK-14,304. α_2-Adrenoceptors, which are coupled to inhibition of adenylate cyclase, appear to mediate norepinephrine-induced *E. coli* O157:H7 adherence to the porcine distal colonic mucosa as well (Green et al., 2004). In the latter tissue, norepinephrine action remains unaltered in the presence of the axonal conduction blocker saxitoxin, a finding which suggests that the relevant adrenergic receptors are located on non-neuronal cells in the intestinal mucosa, most probably epithelial cells. α_2-Adrenoceptors have been identified previously on both primary and transformed colonic epithelial cells (Senard et al., 1990; Valet et al., 1993).

The present immunohistochemical studies indicate that sympathetic nerve fibers immunoreactive for the norepinephrine-synthesizing enzyme dopamine β-hydroxylase are localized in close proximity to the cecal epithelium. These fibers likely arise from paravertebral ganglia and therefore originate from outside the intestinal wall (Janig and McLachlan, 1987). The psychostimulant drug cocaine, which increases endogenous norepinephrine concentration at neuroepithelial synapses, increased adherence of *E. coli* O157:H7 to the cecal mucosa. The presence of presumptive noradrenergic nerve fibers in the cecal mucosa and ability of cocaine to mimic the effect of norepinephrine on *E. coli* O157:H7 adherence suggest that sympathetic nerves innervating the mucosa may play a role in the short-term regulation of epithelium–microbe interactions. Due to their potent vasoconstrictive activity, cocaine and other psychostimulant drugs such as methamphetamine can produce ischemic colitis (Cappell, 2004), but there appear to be no reports documenting an increased incidence in intestinal infections following the acute administration of these drugs. On the other hand, physical or mental stress appears to alter bacterial adherence to the intestinal mucosa (Alverdy et al., 2005). As acute exposure to stressful stimuli is accompanied by increases in intestinal sympathetic outflow (Bhatia and Tandon, 2005), norepinephrine and α_2-adrenoceptors in the large

intestine may serve to link host responses to stress with an increased susceptibility to enteric infections. Finally, some bacterial strains might alter enteric neurotransmission as a means of competing with other microorganisms in colonizing the intestinal mucosa. For example, *Clostridium difficile* is a noninvasive, enterotoxigenic bacterium that can colonize the large intestine after disruption of the normal microfloral environment by antibiotic treatment. Toxin A from *C. difficile* appears to inhibit transmission in enteric sympathetic nerves (Xia et al., 2000). The cellular mechanisms by which sympathetic nerves modulate bacterial interactions with cecal and colonic epithelia and the potential role that norepinephrine-induced bacterial adherence might play in psychostimulant abuse or disease states affecting the large bowel clearly warrant additional investigations.

Acknowledgments

We thank Melissa A. Casey and H. Noel Opitz for excellent technical assistance. This investigation was supported by National Institutes of Health Grants AI-44918 and MH-50431 (M.L.) and DA-10200 (D.R.B.). M.P.S. gratefully acknowledges the support of the Biotechnology and Biological Sciences Research Council, UK (C518022). Salary support for C.C. was provided by NIH training grant T32 DA-007239.

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