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Article

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Pathogenic *Escherichia coli* increase Cl^- secretion from intestinal epithelia by upregulating galanin-1 receptor expression

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Galanin is widely distributed in enteric nerve terminals lining the human gastrointestinal (GI) tract. We have shown previously that galanin-1 receptors (Gal1-R) are expressed by epithelial cells lining the human GI tract, and upon activation cause Cl^- secretion. Because expression of this receptor is transcriptionally regulated by nuclear factor-κB (NF-κB), which is activated by enteric pathogens as a part of the host epithelial response to infection, we investigated whether such bacterial pathogens could directly increase Gal1-R expression in the T84-cell model system. Pathogenic *Escherichia coli*, but not nonpathogenic *E. coli*, activate a p50/p65 NF-κB complex that binds to oligonucleotides corresponding to a recognition site located within the 5′ flanking region of the human *GAL1R* gene. Pathogenic *E. coli*, but not normal commensal organisms, increase Gal1-R mRNA synthesis and [^{125}I]galanin binding sites. Whereas galanin increases short-circuit current (Isc) approximately 5-fold in uninfected T84 cells, exposure to pathogenic, but not nonpathogenic, *E. coli* results in galanin increasing Isc approximately 20-fold. To confirm the validity of these in vitro observations, we also studied C57BL/6J mice infected with enterohemorrhagic *E. coli* (EHEC) by gavage. Infection caused a progressive increase in both NF-κB activation and Gal1-R expression, with maximal levels of both observed 3 days after gavage. Ussing chamber studies revealed that colons infected with EHEC, but not those exposed to normal colonic flora, markedly increased Isc in response to galanin. These data indicate that pathogen-induced increases in Gal1-R expression by epithelial cells lining the colon may represent a novel unifying pathway responsible for at least a portion of the excessive fluid secretion observed during infectious diarrhea.

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Introduction

Galanin is a neuropeptide found in enteric nerve terminals lining the gastrointestinal (GI) tract of all species studied, including humans (1, 2). When secreted, galanin binds to 1 of 3 separate receptor subtypes expressed by intestinal smooth muscle cells, causing both contraction and relaxation, and acting to modulate intestinal transit (3–5). More recently, we have shown that epithelial cells lining the colon express galanin-1 receptors (Gal1-R) (6), which when activated in the human colon epithelial cell line T84 cause Cl^- secretion (7).

The epithelium lining the colonic lumen is unusual in that it is normally colonized by bacteria, yet only generates an inflammatory response upon contact with pathogens (reviewed in ref. 8). Enteric pathogens primarily cause diarrhea by increasing fluid secretion from epithelial cells lining the GI tract, doing so by utilizing a variety of toxin-dependent and -independent mechanisms (reviewed in ref. 8). Although specific pathways that eventuate in salt and water secretion by intestinal epithelia have been ascribed to individual pathogens, no single process, alone or in association with others, has been shown to fully account for the excessive fluid secretion observed in infectious diarrhea (reviewed in ref. 8). Recently, we and others (9–11) have observed that all enteric pathogens share the feature of activating a common transcription factor: nuclear factor-κB (NF-κB). NF-κB is involved in regulating the expression of many cytokines and chemokines during infection (reviewed in refs. 12, 13). In addition to upregulating the expression of many inflammation-associated genes, we recently demonstrated that NF-κB also increases the transcription of the human *GAL1R* gene (14).

Because enteric pathogens activate NF-κB in epithelial cells lining the colon, and this transcription factor regulates Gal1-R expression, we hypothesized that infection by enteric pathogens would increase Gal1-R expression. Such a process could allow unrelated pathogens to increase intestinal fluid secretion by a

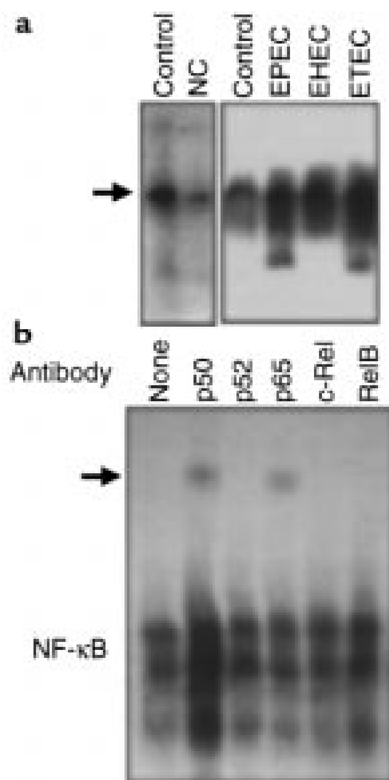

Figure 1
Gel shift (**a**) and supershift (**b**) studies performed on T84-cell nuclear proteins using oligonucleotides directed to the downstream NF-κB recognition site located within the 5′ flanking region of the human *GAL1R* gene. (**a**) Nuclear proteins from control T84 cells (Control), or from T84 cells exposed to normal commensal *E. coli* (NC) or the indicated enteric pathogens, were mixed with ^{32}P end-labeled oligonucleotide corresponding to the NF-κB recognition site located at –809 bp from the site of translation initiation. (**b**) Nuclear proteins from T84 cells exposed to EHEC for 1 hour and combined with ^{32}P end-labeled oligonucleotide in the presence of antibody to the indicated NF-κB subunits. For both **a** and **b**, gels are representative of at least 3 separately performed experiments.

common mechanism, a mechanism that would be coordinated with the well-described increase in cytokines and chemokines. To test this possibility directly, we evaluated the effect of 3 different noninvasive pathogenic strains of *Escherichia coli* on Gal1-R expression, and on this receptor's ability to cause Cl⁻ secretion, in T84 cells. We herein demonstrate that enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC), but not normal commensal *E. coli*, rapidly increase Gal1-R expression in T84 cells. When stimulated with galanin, as occurs in vivo when galanin is released by enteric nerves, increased Cl⁻ secretion is observed. To confirm the validity of these in vitro observations in vivo, we also studied the effects of EHEC infection in C57BL/6J mice. Although epithelial cells lining the murine colon do not normally express Gal1-R, despite ongoing contact with normal colonic flora, infection with EHEC activates NF-κB and causes de novo Gal1-R expression. The colons of EHEC-infected mice increase short-circuit current (Isc) in response to galanin when evaluated in an Ussing chamber. Overall, these observations indicate that enteric pathogen–induced alterations of Gal1-R expression represent a novel unifying pathway accounting for a significant portion of the excessive fluid secretion associated with infectious diarrhea.

Methods

Reagents. Pathogenic bacteria (EHEC, EPEC, ETEC) were generously provided by J. Kaper (Center for Vaccine Development, University of Maryland, Baltimore, Maryland, USA); nonpathogenic *E. coli* were isolated from the fecal flora of normal human volunteers; and T84 cells were provided by K. Barrett (University of California–San Diego, San Diego, California, USA). Specific pathogen–free C57BL/6J male mice (6–8 weeks old) were from The Jackson Laboratory (Bar Harbor, Maine, USA). An antibody to the p65 subunit of NF-κB that only detects the biologically active molecule (15) was from Boehringer Mannheim GmbH (Mannheim, Germany), and was used at a dilution of 1:200 as directed by the manufacturer. All reagents required for immunohistochemistry were from DAKO Corp. (Carpinteria, California, USA). All tissue culture supplies, including Transwells, were from Corning-Costar Corp. (Cambridge, Massachusetts, USA). Galanin was from Bachem California (Torrance, California, USA), while [^{125}I]galanin and all other radionucleotides were from Amersham Life Sciences Inc. (Arlington Heights, Illinois, USA). *Taq* polymerase was obtained from Perkin-Elmer Applied Biosystems (Foster City, California, USA), and *pfu* polymerase was from Stratagene (La Jolla, California, USA); all other enzymes were from Promega Corp. (Madison, Wisconsin, USA). RNA Stat-60 was from Tel-Test Inc. (Friendswood, Texas, USA). All oligonucleotides were synthesized by GIBCO BRL (Gaithersburg, Maryland, USA), while all antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Unless otherwise stated, all other supplies were from Sigma Chemical Co. (St. Louis, Missouri, USA).

Infection of T84-cell monolayers. For these studies, T84-cell monolayers, cultured to confluence in Transwells or 24-well plates, were infected with bacteria in mid-log growth phase at a ratio of approximately 100 organisms per cell for 1 hour as described previously (16). After this time, bacteria were killed by adding gentamicin (50 μg/mL) for 1 hour. This approach was utilized to eliminate confounding factors such as acidic pH and nutrient depletion associated with longer infection times.

Gel shift studies. We previously identified 2 functional NF-κB recognition sites in the 5′ flanking region of the human *GAL1R* gene using the reporter gene for chloramphenicol acetyltransferase (14). The nucleotide sequences of these 2 sites vary significantly from one another, demonstrating only 45% homology. The sequence of the more upstream site, located at –809 bp from the site of translation initiation, is GGG GGG GAT

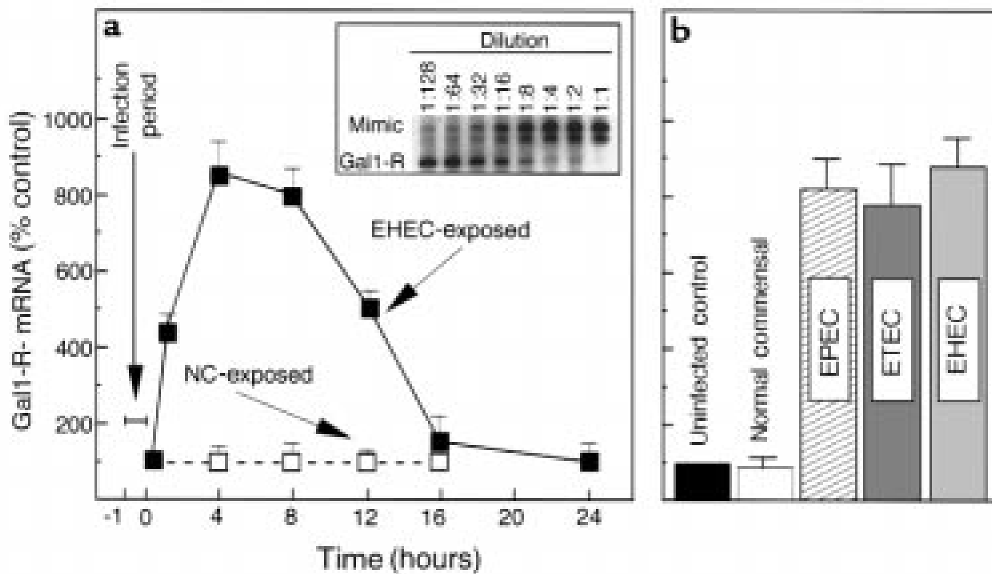

Figure 2
Effect of pathogenic and normal commensal *E. coli* on Gal1-R mRNA expression. (**a**) Time course of alterations in Gal1-R mRNA concentration after a 1-hour infection with EHEC (filled squares) or normal commensal *E. coli* (NC, open squares). Total cellular RNA was extracted from T84 cells cultured to confluence in 24-well plates after exposure to the indicated bacteria, as described in Methods. Gal1-R mRNA amount was determined by performing RT-PCR with serial dilutions of a mimic whose concentration is known (inset). (**b**) Peak increases (at t = 4 hours) in Gal1-R mRNA after infection with the indicated bacteria. Data represent the mean ± SEM for a minimum of 3 separate experiments.

CC; the sequence of the more downstream site, located at –269 bp, is GGG GAT TCC CA (14). To evaluate the contribution of each of these NF-κB sites to regulating Gal1-R mRNA expression, we performed gel shift studies using oligonucleotides complementary to each sequence. For each site, an oligonucleotide was generated containing the appropriate sequence shown above; the sequence was repeated in triplicate to enhance nuclear protein binding capacity. Oligonucleotides were gel purified and then end labeled to yield a specific activity of more than 10^8 cpm/μg. Labeled oligos were combined with nuclear proteins from control T84 cells or from those infected with the indicated organisms for 1 hour. Nuclear proteins were obtained as described previously (9); aliquoted; and stored at –70°C. Binding reactions were performed at room temperature for 30 minutes using 5 μg of nuclear proteins and 0.5 ng (25,000 cpm) of labeled oligonucleotide. Product resolution was accomplished by electrophoresis of the reaction solution on a vertical 5% nondenaturing polyacrylamide gel. Supershift assays were used to determine which specific NF-κB subunits were activated. In these studies, gel shifts were performed as described above, except that rabbit antibodies (1 μg/reaction) against the NF-κB subunits p50, p52, p65, c-Rel, and RelB were added during the binding reaction period.

Quantitative PCR. We determined the amount of Gal1-R mRNA by quantitative PCR, performed using a mimic. We designed a mimic using an unrelated 289-bp segment of DNA, and modified it by altering the 5′ and 3′ ends so that they were homologous to 2 regions within the coding sequence of the human Gal1-R cDNA. These 2 regions are 210 bp apart on the Gal1-R cDNA, and span the area encoding the third intracellular loop. Because this region includes the location of an intron, amplification using primers directed to these 2 regions permits ready differentiation between the mimic (289 bp), Gal1-R cDNA (210 bp), and genomic DNA. In all cases, identical numbers of T84 cells were evaluated, since we extracted total cellular RNA from T84 cells cultured to confluence in 24-well plates. Total RNA was measured and corrected to ensure that identical amounts of message were evaluated per well. Reverse transcriptase in the presence of random hexamers was then used to convert RNA to cDNA, and amplification was performed in the presence of various known concentrations of mimic.

Binding studies. T84 cells were cultured to near-confluence in 6-well plates, and binding studies using [^{125}I]galanin were performed as described previously (7). Nonsaturable binding was less than 15% of total binding in all experiments, with all values in this paper reported as saturable binding.

Creation and evaluation of the Gal1-R antibody. We originally obtained a specific Gal1-R anti-peptide antibody (no. 96125) from John Walsh (CURE/Gastroenteric Biology Center, University of California–Los Angeles, Los Angeles, California), who has extensively characterized this reagent in preliminary studies (17, 18). This antibody is directed to the sequence CNESMGDAKEKN, located in the proximal portion of the Gal1-R COOH-terminus. Because this sequence is located in a region that shares less than 35% homology at the amino acid level with the galanin-2 and galanin-3

receptor subtypes, it is specific for the Gal1-R. In contrast, this antibody is useful across species, because the sequence to which it is directed is 100% conserved for the human, mouse, and rat Gal1-R. Our initial studies indicated that this was a potent and specific antibody; we therefore contracted with Research Genetics (Huntsville, Alabama, USA) to generate a rabbit antipeptide antibody using the same epitope. To evaluate this antibody (no. 85425), we performed immunohistochemistry against mouse pancreas, as physiological studies have indicated that only islet cells, and not acini, express receptors for galanin (19, 20). In all instances, immunohistochemistry was performed using a 3-stage indirect immunoperoxidase technique described previously (21). Control tissues were processed identically, except that they were not exposed to primary antibody; bound antibody was detected by incubating slides with Liquid DAB Substrate-Chromogen System (DAKO Corp.).

To evaluate antibody specificity, we performed Western blot analysis to detect Gal1-R in T84 cells, using a protocol modified from one that has been described previously (22). Specifically, we compared uninfected T84 cells with those that had been infected with EHEC for 1 hour, treated with antibiotics, and then studied 24 hours later.

Infection of C57BL/6J mice by gavage. EHEC were cultured until they were in mid-log growth (OD_{600} 0.1–0.2) as described previously (23). Pathogens were pelleted and resuspended in 1.2 M sodium phosphate (pH 8.0), and approximately 2×10^5 organisms in 200 μL were introduced to a lightly anesthetized animal by gavage, using a Teflon-tipped 10-cm-long needle. Mice were then kept in microisolator cages with free access to food and water. To minimize intestinal fecal contents at the time of study, at 24 hours before sacrifice, animal access to food was eliminated. The Animal Care and Use Committee of the University of Illinois at Chicago approved this study.

Electrophysiological assays for cultured cells. As described previously (7), T84 cells were cultured to confluence in Transwells; only those cells exhibiting high transepithelial resistances consistent with the presence of intact tight junctions were used (i.e., >1,000 $\Omega \cdot cm^2$). Cells were stimulated with the indicated agent, and Isc was determined every 30 seconds in a modified Ussing chamber. Transepithelial electrical resistance was calculated using Ohm's law ($R = V/I$), where potential difference was measured in response to the passage of 25 μA of current using a simplified apparatus described previously (24).

Electrophysiological assays for resected colonic tissues. After sacrifice by CO_2 asphyxiation, proximal mouse colon was resected and mounted immediately in an Ussing chamber. The colon was mounted as a sheet on a ring (4 mm internal diameter) and incubated at 37°C in Ringer's lactate solution that was continually gassed with 95% O_2/5% CO_2. Electrical current (100 μA) was applied across the tissue using Ag/AgCl electrodes connected by salt bridges; resultant potential difference was recorded, and Isc was determined each minute after the development of a stable baseline (~15–30 min).

Statistical evaluations. In all instances, data were generated by directly comparing the effects due to pathogens with those due to nonpathogenic bacteria or controls.

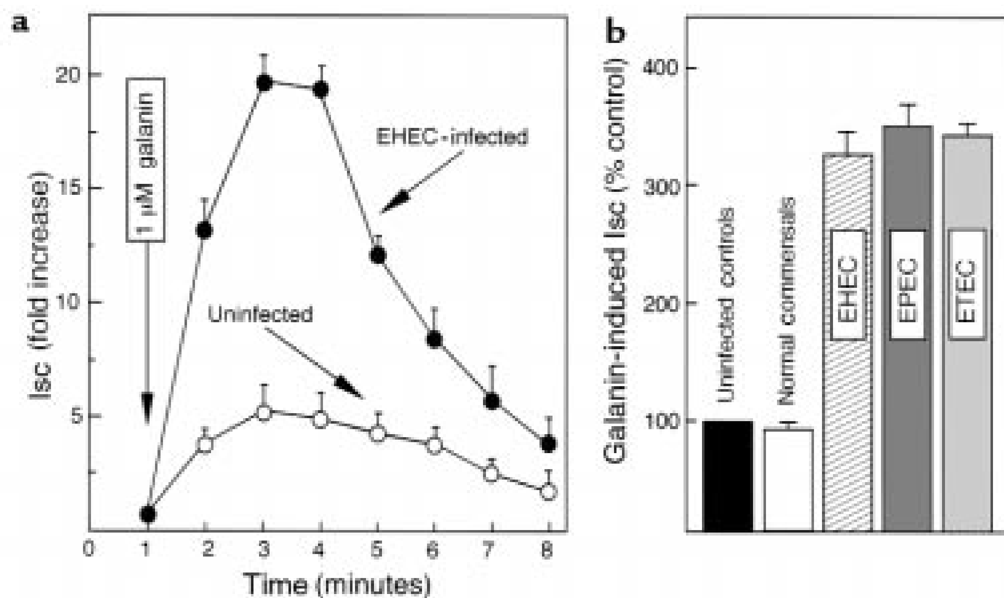

Figure 3
Galanin-induced increases in Isc in T84 cells with or without prior *E. coli* exposure. (**a**) T84 cells were cultured to confluence in Transwells, exposed to EHEC (filled circles) or buffer (open circles) for 1 hour, and treated with gentamicin; the ability of 1 μM galanin to alter Isc was determined 24 hours later. (**b**) Maximal increases in Isc in T84 cells after stimulation with 1 μM galanin in cells exposed to buffer only (Uninfected controls), or 24 hours after a 1-hour infection with normal commensal *E. coli* (Normal commensals) or the indicated pathogenic *E. coli*. Data represent the mean ± SEM for a minimum of 3 separate experiments.

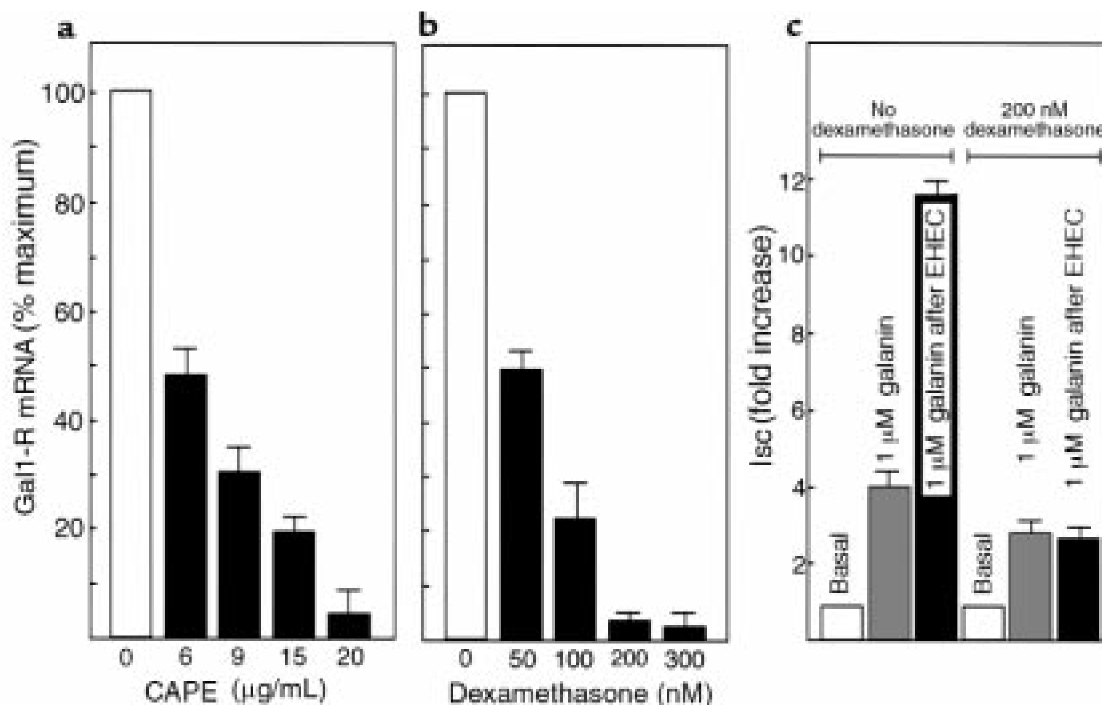

Figure 4
Effect of NF-κB inhibitors on Gal1-R mRNA synthesis and response to galanin after infection with pathogenic *E. coli*. (**a**) Dose-dependent effect of the NF-κB–specific inhibitor CAPE (25) on peak Gal1-R mRNA synthesis 4 hours after a 1-hour exposure to EHEC. Confluent T84 cells were exposed to the indicated concentration of CAPE for 1 hour, infected with EHEC for 1 hour in the continued presence of CAPE, and washed; RNA was extracted 4 hours later. (**b**) Dose-dependent effect of dexamethasone on Gal1-R mRNA synthesis 4 hours after a 1-hour exposure to EHEC. Conditions were identical to those described for **a**. (**c**) Effects of dexamethasone on galanin-induced Isc after infection with EHEC. T84 cells were infected with EHEC for 1 hour in the presence or absence of 200 nM dexamethasone, and the Isc response to 1 µM galanin was determined 24 hours later. Data represent the mean ± SEM for a minimum of 3 separate experiments.

Since n values per experiment varied between conditions, data were evaluated using unpaired Student's t test, with $P < 0.05$ considered significant. In all instances, data are expressed as mean ± SEM.

Results

Infection of T84 cells with pathogenic E. coli activates NF-κB. Nuclear proteins were extracted from uninfected control T84 cells, which were then infected with either nonpathogenic or pathogenic *E. coli*. Gel shift assays were performed using oligonucleotides complementary to either of the 2 NF-κB recognition sites located within the 5′ flanking region of the human *GAL1R* gene. No increase in signal was observed after infection with normal commensal *E. coli*, compared with that seen in uninfected control T84 cells processed in parallel (Figure 1a). Indeed, infection with normal commensal *E. coli* consistently decreased the amount of signal observed compared with control T84 cells processed in parallel. In contrast, infection of T84 cells with all pathogenic *E. coli* tested for 1 hour resulted in a marked increase in NF-κB binding to oligonucleotides representing the upstream NF-κB binding site, located at –809 bp from the site of translation initiation (Figure 1a). However, no significant increase in binding was observed when the oligonucleotide for the more downstream site at –269 bp was evaluated (data not shown).

To identify the subunit composition of the activated NF-κB complex, we performed supershift studies (Figure 1b). When nuclear proteins from T84 cells infected with EHEC were coincubated with the oligonucleotide directed to the upstream NF-κB site, supershifting was observed only when antibodies to p50 or p65 were present (Figure 1b). An identical supershift with p50 and p65 was observed for nuclear proteins from T84 cells infected with EPEC and ETEC (data not shown). These data support the involvement of a p50/p65 NF-κB complex binding to the most upstream recognition site after infection with pathogenic, but not nonpathogenic, *E. coli*.

Kinetics of Gal1-R mRNA synthesis in response to infection. We next determined the time course of Gal1-R mRNA synthesis in T84 cells after a 1-hour infection with various bacteria. Because basal Gal1-R mRNA levels are extremely low (6, 7), we are unable to reliably quantify message amount by Northern analysis. We therefore generated a mimic containing ends complementary to the native Gal1-R mRNA that are 210 bp apart, but which in the mimic are 289 bp apart. By performing PCR with Gal1-R cDNA spiked with various known concentrations of mimic, we precisely determined the amount of message (Figure 2a, inset). Whereas a 1-hour exposure to normal commensal *E. coli* did not increase Gal1-R mRNA quantity, infection with EHEC markedly increased expression

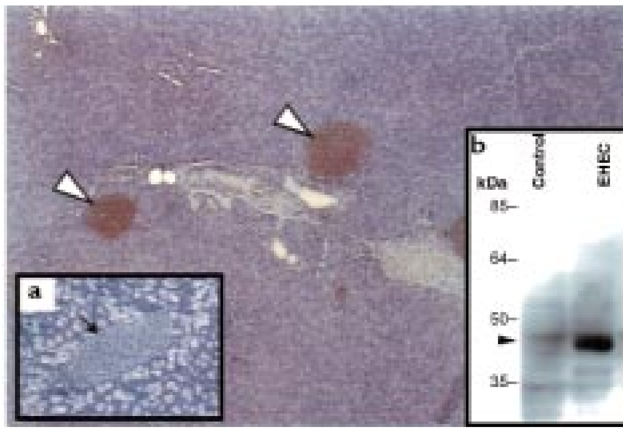

Figure 5
Evaluation of Gal1-R antibody sensitivity and specificity. Immunohistochemistry was performed using Gal1-R antibody (concentration 1:500) on a 5-μm-thick section of formalin-fixed, paraffin-embedded mouse pancreas, as described in Methods. White arrowheads identify the Gal1-R immunostaining islets. Inset a: control tissue processed similarly, except not exposed to primary antibody showing islet detail (arrow). ×100. Inset b: Western blot analysis of uninfected T84 cells (Control) and T84 cells exposed to EHEC for 1 hour and then studied 24 hours later. Proteins (50 μg) were resolved by SDS-PAGE (10% acrylamide), transferred to nitrocellulose, and exposed to antibody overnight at a dilution of 1:1,000 at room temperature. Gal1-R (arrowhead) is identified by reacting with goat anti-rabbit alkaline phosphatase–conjugated IgG, and developing with an Immunoblot AP kit (BCIP-NBT; Cymed, South San Francisco, California, USA).

(Figure 2a). Compared with control cells processed in parallel, EHEC increased Gal1-R quantity in T84 cells about 8-fold, with peak expression observed 4 hours after the 1-hour infection period and return to basal levels approximately 16 hours after initial infection To determine if EPEC and ETEC maximally increased Gal1-R mRNA levels to the same degree, we next determined the amount of message in T84 cells 4 hours after a 1-hour exposure to these pathogens (Figure 2b). Although nonpathogenic *E. coli* did not increase Gal1-R mRNA levels, all 3 pathogenic strains increased message similarly.

Pathogenic E. coli increase Gal1-R binding sites. In contrast to the rapid increase in mRNA, a significant increase in T84 [^{125}I]galanin binding sites was not observed until 4 hours after pathogen infection, with maximal levels reached 12 hours after infection (data not shown). These levels then remained elevated for at least 24 hours after infection, and were elevated to a similar degree after infection with EHEC, EPEC, or ETEC (data not shown). Specifically, basal levels of [^{125}I]galanin binding sites (B_{MAX} = 49 ± 10 fmol/mg protein) were elevated approximately 8- to 10-fold 24 hours after a 1-hour infection period with EHEC (B_{MAX} = 550 ± 20 fmol/mg protein), EPEC (B_{MAX} = 480 ± 50 fmol/mg protein), or ETEC (B_{MAX} = 480 ± 30 fmol/mg protein). In contrast, infection with nonpathogenic *E. coli* did not increase the number of binding sites (B_{MAX} = 45 ± 3 fmol/mg protein).

Functional consequence of increased Gal1-R expression. Commensurate with the increase in binding sites was a marked increase in the ability of galanin to increase Isc, which we have previously shown is due to Cl^- secretion (7). Uninfected T84 cells increased Isc almost 5-fold in response to 1 μM galanin, from 1.8 ± 0.2 to 10.0 ± 0.3 μA/cm^2 (n = 12) (Figure 3a). Maximal increases in Isc were observed at approximately 2 minutes, and returned to baseline at about 10 minutes, after peptide administration. Yet when T84 cells were transiently infected with EHEC for 1 hour and then studied 24 hours later, 1 μM galanin increased Isc almost 20-fold, without altering basal levels (Figure 3a). Specifically, this dose of ligand caused Isc to increase from 1.7 ± 0.4 to a maximum of 31.8 ± 3.7 μA/cm^2 (n = 14). Although EHEC infection potentiated the galanin-induced increase in Isc between 3- and 4-fold, the kinetics of the increase were similar to those observed for uninfected T84 cells processed in parallel (Figure 3a).

The ability of infection to potentiate the galanin-induced increase in Isc was specific to enteric pathogens. When T84 cells were infected with normal commensal organisms for 1 hour, no increase in galanin-induced Isc was observed 24 hours later. In contrast, when T84 cells were infected with EPEC and ETEC, similar increases in galanin-induced Isc were observed (Figure 3b). Specifically, galanin-induced increases in Isc were 3.7-fold after infection with EPEC and 3.5-fold after infection with ETEC, compared with those observed for uninfected control cells processed in parallel. In all cases, the time course of the galanin-induced increase in Isc was similar to that observed for uninfected T84 cells (data not shown).

Activation of NF-κB is critical for pathogen-induced increases in Gal1-R expression. To confirm the role of NF-κB in inducing Gal1-R expression in response to enteric pathogen infection, we evaluated the ability of various inhibitors of this transcription factor to attenuate Gal1-R expression. We first studied the NF-κB–specific inhibitor caffeic acid phenethyl ester (CAPE) (25). Confluent T84 cells were preincubated with varying concentrations of CAPE for 1 hour, and then infected with EHEC for 1 hour in the continued presence of inhibitor. Gal1-R mRNA quantity was determined 4 hours later, the time point at which maximal increases after infection were detected (Figure 2). CAPE inhibited Gal1-R mRNA synthesis in a dose-dependent fashion (Figure 4a). Unfortunately, concentrations of CAPE effective at decreasing Gal1-R mRNA synthesis also decreased T84 transepithelial resistances (data not shown). Even at the lowest concentration of CAPE studied, which decreased Gal1-R mRNA synthesis only by about 50%, transepithelial resistances were significantly diminished to below 100 Ω·cm^2 (data not shown). We were therefore unable to use this specific NF-κB inhibitor in the study of T84-cell electrophysiology.

We next evaluated dexamethasone, commonly used in the treatment of many diverse inflammatory disorders affecting the GI tract, and recently shown to act as an NF-κB inhibitor (26–28). Similar to T84 cells preincubated with CAPE, T84 cells exposed to increasing concentrations of dexamethasone for 1 hour before

infection with EHEC showed a progressive decrease in the Gal1-R mRNA quantity detected 4 hours later (Figure 4b). But unlike CAPE, dexamethasone did not alter T84-cell transepithelial resistances. Resistances were 1,930 ± 50 Ω·cm^2 at the basal level and 2,000 ± 100 Ω·cm^2 immediately after a 1-hour incubation with 200 nM dexamethasone, the lowest concentration to completely inhibit pathogen-induced increases in Gal1-R mRNA. Similarly, there was no difference in transepithelial resistance 24 hours after a 1-hour infection with EHEC, regardless of whether the cells were preincubated with dexamethasone. Specifically, resistance was 2,100 ± 85 Ω·cm^2 in T84 cells 24 hours after infection with EHEC but no exposure to dexamethasone; it was 2,040 ± 70 Ω·cm^2 24 hours after infection with EHEC and a 1-hour dexamethasone preincubation (n = 4 for all conditions). Like CAPE, this concentration of dexamethasone markedly attenuated Gal1-R mRNA synthesis by more than 90% (see Figure 4b), and completely eliminated the EHEC-potentiated increase in galanin-induced Isc (Figure 4c). Specifically, basal Isc increased from 2.1 ± 0.3 to a galanin-induced maximum of 8.4 ± 0.6 μA/cm^2 in control cells, and from 2.0 ± 0.5 to 23.5 ± 2.7 μA/cm^2 24 hours after a 1-hour infection with EHEC (Figure 4c). Preincubation with dexamethasone did not significantly alter the galanin-induced increase in Isc in infected T84 cells. In paired T84-cell monolayers preincubated with 200 nM dexamethasone, but not exposed to EHEC, the galanin-induced Isc increased from 1.6 ± 0.7 to 6.4 ± 0.9 μA/cm^2. However, dexamethasone completely inhibited the ability of EHEC infection to potentiate the galanin-induced increase in Isc. Twenty-four hours after EHEC infection in dexamethasone preincubated T84 cells, galanin increased Isc from 1.8 ± 0.4 to only 5.6 ± 0.3 μA/cm^2, an increase similar to that observed in T84 cells not previously infected with this pathogen. These data support our hypothesis that enteric pathogens increase Gal1-R expression by activating NF-κB.

Characterization of the Gal1-R antibody. To confirm the validity of our in vitro observations, we evaluated an antibody that would allow us to identify Gal1-R expression in different species. The best antibody tested is directed to the Gal1-R COOH-terminus and is based on the design of John Walsh (see Methods). We initially evaluated serial dilutions of this antibody applied to mouse pancreas, because prior studies have shown that islets, but not acini, specifically express galanin receptors (19, 20). Gal1-R antibody at a concentration of 1:500 was optimal for viewing islets (Figure 5), whereas higher concentrations nonspecifically increased background, and lower concentrations diminished the intensity with which islets could be viewed (data not shown). To evaluate the specificity of this antibody, we also performed Western blot analysis on T84 cells that either had or had not been infected with EHEC. Although no specific protein could be identified in uninfected control T84 cells, cells infected with EHEC for 1 hour and then studied 24 hours later showed a dramatic increase in expression of a single protein approximately 40 kDa in size (Figure 5, inset b). This is consistent with our finding of increased numbers of

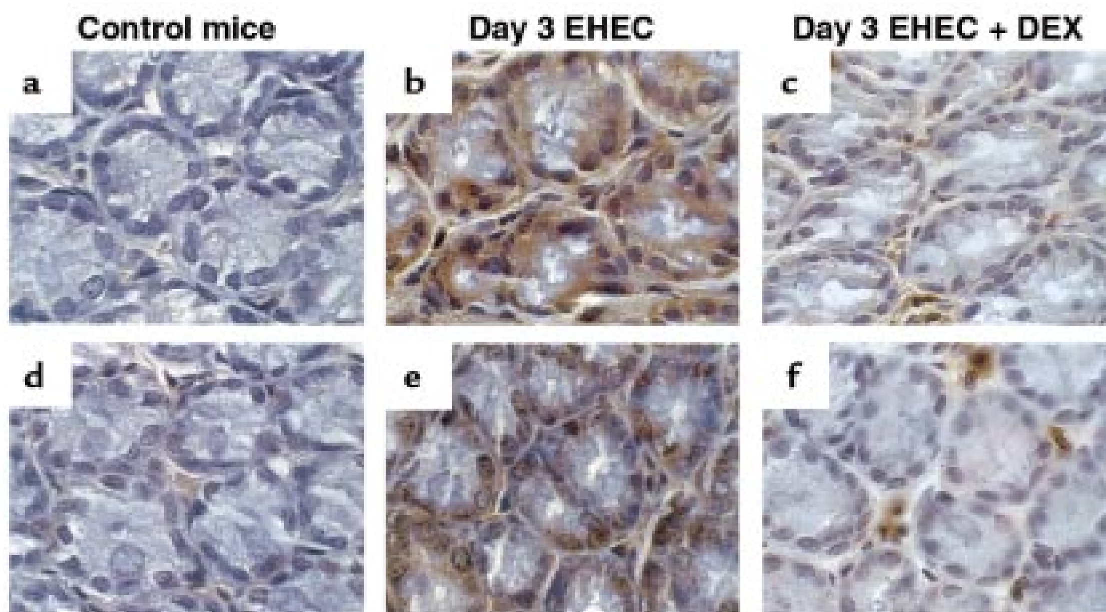

Figure 6
Immunohistochemistry performed on colonic epithelial cells isolated from C57BL/6J mice using antibodies against the Gal1-R (**a**–**c**) or the activated subunit of NF-κB p65 (**d**–**f**). Results from control mice do not show evidence of Gal1-R expression (**a**) or activated NF-κB (**d**). In contrast, 3 days after instilling 2 × 10^5 log-growth EHEC by gavage, both Gal1-R expression (**b**) and NF-κB activation (**e**) are readily apparent. Concomitant parenteral administration of dexamethasone (DEX) during the same period of EHEC infection, however, markedly attenuates Gal1-R expression (**c**) and evidence of NF-κB activation (**f**). Data are representative of 5 separately treated animals per condition. ×400.

Gal1-R binding sites, and increased electrophysiological responsiveness to ligand, after infection with pathogens. This size also is similar to the predicted size of 39.7 kDa for the Gal1-R. These findings establish the sensitivity and specificity of the Gal1-R antibody. We therefore used this antibody to evaluate Gal1-R expression in epithelial cells lining the colon of C57BL/6J mice, the best characterized strain of this species (29).

Murine colonocytes increase Gal1-R expression in response to EHEC infection. Although mouse colon is normally colonized by bacterial flora, murine colonocytes do not basally express Gal1-R, as determined by immunohistochemistry (Figure 4a). Similarly, immunohistochemistry performed using an antibody that only recognizes activated p65 (15) revealed that colonization with normal flora does not show evidence of activated NF-κB (Figure 6d). We then introduced 2×10^5 log-growth EHEC into the stomachs of lightly anesthetized mice by gavage. This resulted in maximal activation of NF-κB (observed primarily in the nucleus; Figure 6e) and Gal1-R expression (Figure 6b) 3 days after infection. All evidence of increased NF-κB activation and Gal1-R expression in murine colonocytes was gone after 6 days, indicating the transient nature of EHEC infection (data not shown).

Consistent with the absence of detectable Gal1-R expression in the colons of control mice, these tissues, when studied in an Ussing chamber, did not respond to galanin (Figure 7). Yet 3 days after gavage with EHEC, murine colonocytes rapidly increased Isc after stimulation with this peptide. Similar to T84 cells, galanin induced a rapid and transient increase in Isc of mouse colonic epithelium 3 days after EHEC infection, with maximal increases observed approximately 2 minutes after ligand administration and return to baseline about 8 minutes later (Figure 7). To confirm the role of NF-κB in the ability of EHEC to increase Gal1-R expression in murine colonocytes, we next treated mice with 0.15 μg/mg of dexamethasone, a dose similar to that used in the treatment of many inflammatory disorders of the human GI tract. When dexamethasone was administered as an intraperitoneal injection every 12 hours, starting with the provision of EHEC by gavage, Gal1-R could not be detected in murine colonocytes 3 days later (Figure 6c). This elimination of Gal1-R expression was associated with the complete absence of activated NF-κB in the nuclei of epithelial cells lining the mouse colon (Figure 6f). Consistent with the absence of Gal1-R in EHEC-infected mice concomitantly given dexamethasone, colonic epithelia failed to increase Isc when stimulated with galanin (Figure 7).

Discussion

The epithelial lining of the colonic lumen is unusual in that it is normally colonized by bacteria, yet can initiate and contribute to the host defense when exposed to pathogens. Until recently, it was believed that enteric pathogens were primarily responsible for the major clinical manifestation of infection, diarrhea, by virtue of their secreting various toxins and/or destroying the host epithelium (reviewed in ref. 8). More recently, it has come to be appreciated that the intestinal epithelium is not simply a passive recipient of microbial attack, but is itself capable of mounting a response to infection by enteric pathogens. This capability, collectively described as the innate intestinal epithelial defense, comprises at least 3 major responses, including the induction of salt and water secretion, secretion of antimicrobial peptides and proteins, and the synthesis of mucins (30). Of these 3 different host epithelial responses to enteric infections, arguably the most important is the ability to increase Cl^- secretion.

Stimulation of colonic epithelial Cl^- secretion leads to increased amounts of luminal water, with the ensuing diarrhea believed to "flush" the pathogens from the GI tract (31). Although it is well established that bacterial products such as cholera toxin (CT) can directly induce Cl^- secretion, it is now appreciated that the host epithelial cell itself can generate this action by increasing local production of prostaglandins (32, 33). Secreted bacterial products, including CT and pathogenic organisms such as *Salmonella*, can increase the synthesis of phospholipase A_2–activating protein (PLAP) (34, 35) and prostaglandin H synthase-2 (PGHS-2) (36), enzymes critical for converting esterified arachidonate present in the epithelial cell membrane to diarrhea-inducing prostaglandins. Thus, prostaglandin synthesis represents a major mechanism whereby the host epithelium is capable of responding to the presence of different pathogens by increasing Cl^- secretion.

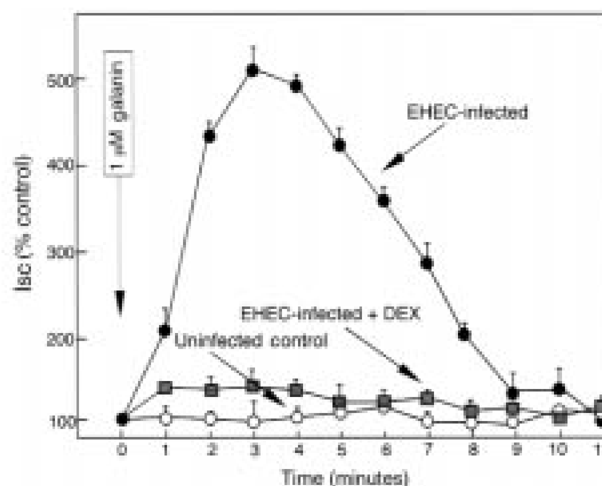

Figure 7

Ability of galanin to increase Isc in murine colonocytes with or without prior EHEC infection. Mouse colonic epithelium was rapidly mounted in an Ussing chamber after sacrifice; after the development of a stable baseline, epithelium was treated with 1 μM galanin as indicated. Whereas control murine colonocytes do not respond to galanin (open circles), those from mice given EHEC by gavage 3 days earlier increase Isc by approximately 5-fold (filled circles). In contrast, concomitant parenteral administration of dexamethasone (DEX) during the same period of EHEC infection completely eliminated galanin's ability to alter Isc (filled squares). Data represent the mean ± SEM for a minimum of 5 separate experiments.

In this study, we provide evidence for a completely novel mechanism of innate intestinal epithelial defense: specifically increased Cl^- secretion by pathogen-induced upregulation of Gal1-R expression. Based on our previously published observations, we initially hypothesized that the Gal1-R, a member of the 7 transmembrane–spanning, G protein–coupled (heptaspanning) receptor superfamily, might be involved in infectious diarrhea. Specifically, we have previously demonstrated that (a) epithelial cells lining the human colon express Gal1-R mRNA (6); (b) activation of Gal1-R expressed by human colonocytes stimulates Cl^- secretion (7); and (c) the human *GAL1R* gene is transcriptionally regulated by the inflammation-associated transcription factor NF-κB, at least in vitro (14). This latter observation was of particular interest since most, if not all, genes involved in the coordinated response to enteric pathogen infections — including those for various chemokines, cytokines, and adhesion molecules — share the common feature of possessing NF-κB recognition sites in their 5′ flanking regions (reviewed in refs. 12, 13, 37). Significantly, we and others have shown that NF-κB is activated in human colonocytes after infection with enteric pathogens including EPEC (9), EHEC (10), enteroinvasive *E. coli* (EIEC) (10), *Salmonella* (10), and *Shigella* (11). Thus, increased NF-κB activity may well represent a central unifying aspect of enteric pathogen–initiated innate intestinal epithelial defense mechanisms. In combination with our studies of the Gal1-R described above, these observations suggest the possibility that infection by enteric pathogens causes diarrhea, at least in part, by increasing Gal1-R expression.

Herein we provide direct evidence in support of this possibility, by showing that in the human colon epithelial cell line T84, noninvasive pathogenic *E. coli* directly activate a p50/p65 NF-κB complex that binds to a recognition site contained in the regulatory domain of the human *GAL1R* gene. NF-κB activation results in increased amounts of Gal1-R mRNA and protein synthesis, ultimately resulting in increased Cl^- secretion. Because normal commensal *E. coli* appear to inhibit NF-κB activation (Figure 1a), and do not act to increase Gal1-R transcription or translation, alterations in the translation and transcription of this protein are specific to infection with enteric pathogens. That these effects are not unique to T84 cells is shown by our murine experiments. Although mouse colon is normally replete with nonpathogenic bacterial flora, there is no evidence of NF-κB activation or Gal1-R expression (Figure 6). Yet EHEC maximally activates NF-κB and increases Gal1-R expression in the colon 3 days after infection (Figure 6). Whereas normal mouse colon is unresponsive to galanin, colons from EHEC-infected mice rapidly increase Isc in response to this peptide. Our murine experiments also support our finding that NF-κB is critical to enteric pathogen–induced increases in Gal1-R expression. Although the specific NF-κB inhibitor CAPE (25) could not be fully evaluated in T84 cells, given its effects on altering transcellular resistances — nor could it be used in whole animals since its toxicity is unknown — this agent completely attenuated pathogen-induced increases in Gal1-R mRNA (Figure 4). Along with our gel shift studies (Figure 1), these data support a critical role for NF-κB in the induction of Gal1-R expression secondary to enteric pathogen infection. We then showed that the Gal1-R mRNA attenuation observed with CAPE could be mimicked by another NF-κB inhibitor, dexamethasone (26–28) (Figure 4). Unlike CAPE, dexamethasone can be used in whole animals. Not only does dexamethasone completely inhibit pathogen-induced increases in Gal1-R mRNA synthesis in T84 cells (Figure 4), it also abrogates the expression of Gal1-R in colonic epithelial cells of mice infected with EHEC (Figure 6). These data support our hypothesis that the diarrhea caused by infection with enteric pathogen, an accepted mechanism of intestinal epithelial host defense, may be mediated, at least in part, by NF-κB increasing Gal1-R expression.

The increased numbers of Gal1-R are presumably activated by ligand known to be present in, and released by, enteric nerves lining the human GI tract (1, 2). However, a recent study has shown that galanin also can be synthesized by inflammatory cells, as well as locally within the dermis and epidermis of rats injected with the inflammation-inducing agent carrageenan (38). This is of particular interest because the human gene for galanin, similar to the gene for the Gal1-R (14), contains a recognition site for the inflammation-associated transcription factor NF-κB (39). Although this investigation was limited to studying alterations in Gal1-R expression and function, these data indicate that increased amounts of ligand also may be present in or around the inflamed intestinal epithelium as exists during infectious colitis. Yet the presence of elevated concentrations of galanin after pathogen exposure is not critical, since we have shown that increased Gal1-R expression nonetheless causes increased Cl^- secretion in response to the same concentration of ligand.

Many other peptide hormones present in enteric nerve terminals are known to cause Cl^- secretion from intestinal epithelial cells, by acting upon specific heptaspanning receptors including bradykinin (40), calcitonin gene-related peptide (41), pituitary adenylate cyclase–activating polypeptide (42), and vasoactive intestinal polypeptide (43, 44). Yet these ligands differ from galanin in that they all cause their secretory effects by increasing intracellular cAMP, whereas we have previously shown that Gal1-R activation causes Cl^- secretion by a cAMP-independent, Ca^{2+}-dependent process (7). More importantly, however, alterations in receptor number and function have not been shown to occur for these other secretagogues during infectious diarrhea. In contrast, we demonstrate that enteric pathogens, but not normal commensal organisms, increase Gal1-R expression in intestinal epithelial cells via the inflammation-associated transcription factor NF-κB, allowing for increased amounts of Cl^- secretion. To our knowledge, these data are the first to show that a receptor for

a particular secretagogue is specifically increased after enteric pathogen infection and, as such, may be an important aspect of a unifying mechanism responsible for a significant component of infectious diarrhea.

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