

**Hemokinin-1 stimulates prostaglandin E₂ production in human colon
through activation of cyclooxygenase-2 and inhibition of 15-
hydroxyprostaglandin dehydrogenase**

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Abbreviations: HK-1, hemokinin-1; SP, substance P; PGE₂, prostaglandin E₂; COX, cyclooxygenase; IR, immunoreactivity; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; NK₁, neurokinin-1; NK₂, neurokinin-2; IBD, inflammatory bowel disease;

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Abstract

Hemokinin-1 (HK-1) is a newly identified tachykinin, originating from the immune system rather than neurons, and may participate in the immune and inflammatory response. In colonic mucosa of inflammatory bowel disease (IBD) patients, upregulation of the *TAC4* gene encoding HK-1 and increased production of prostaglandin E₂ (PGE₂) occur. Our aim was to examine the mechanistic link between human HK-1 (HK-1) and PGE₂ production in normal human colon. Exogenous HK-1 (0.1 μM) for 4h evoked an increased PGE₂ release from colonic mucosal and muscle explants by 10- and 3.5-fold, respectively, compared with unstimulated time controls. The HK-1 stimulated PGE₂ release was inhibited by tachykinin receptor antagonists SR140333 (NK₁) and SR48968 (NK₂) and was also inhibited by COX-2 inhibitor NS-398 but not by COX-1 inhibitor SC-560. A parallel study with substance P showed similar results. Molecular studies with HK-1 treated explants demonstrated a stimulatory effect on COX-2 expression at both transcription and protein level. Notably, this was coupled with an HK-1 induced down-regulation of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) mRNA and protein expression. Immunoreactivity for 15-PGDH occurred on inflammatory cells, epithelial cells, platelets and ganglia. This finding provides an additional mechanism for HK-1 evoked PGE₂ increase, in which HK-1 may interfere with the downstream metabolism of PGE₂ by suppressing 15-PGDH expression. In conclusion, our results uncover a novel inflammatory role for HK-1 which signals via NK₁ and NK₂ receptors to regulate PGE₂ release from human colonic tissue, and may further explain a pathological role for HK-1 in IBD when abnormal levels of PGE₂ occur.

Introduction

Tachykinins are a family of small biologically active peptides, originally identified in neurons. The best known mammalian tachykinins are substance P (SP), neurokinin (NK) A and NKB, which act mainly via NK₁, NK₂ and NK₃ receptors, respectively (Pennefather et al., 2004). Another tachykinin, hemokinin-1 (HK-1, sequence TGKASQFFGLM-NH₂), has now been identified in humans (Kurtz et al., 2002, Page 2004). HK-1 was found in immune cells, rather than in neurons (Zhang et al., 2000), and it shares a similar amino acid sequence and receptor selectivity with SP (Kurt et al., 2002; Pennefather et al., 2004). In the intestine, SP and NKA act as neurotransmitters to modulate gut functions such as motility, secretion, blood flow and inflammation (Holzer and Holzer-Petsche, 1997; Castagliuolo et al., 1997).

SP can also be released from non-neuronal cells (Castagliuolo et al., 1997; Moriarty et al., 2001), and is considered to act as an autocrine, paracrine or endocrine regulator of secretory, immune and inflammatory responses. HK-1 has been implicated in lymphoid (Zhang et al., 2000; Zhang and Paige, 2003, Berger et al., 2010) and myeloid (Janelsins et al., 2009) cell proliferation and development. This leads to the speculation that the intestinal immune and inflammatory actions of SP could be shared by HK-1. However, the actions of HK-1 in the gastrointestinal system remain largely unknown.

It has been hypothesized that tachykinins might be implicated in inflammatory bowel disease (IBD), by interrupting the crosstalk between the neural and immune systems, promoting acute and chronic intestinal inflammation, and thus contributing to the motor, secretory and immunological disturbances which characterize IBD (Holzer, 1998). This hypothesis was supported by evidence that overexpression of SP and its preferred NK₁ receptor occurred in the intestinal mucosa of patients with IBD (Mazumdar and Das, 1992; Yamamoto et al., 1996; Goode et al., 2000). Furthermore, animal studies also suggested a critical role for SP/NK₁ receptor in amplifying intestinal inflammation (Kataeva et al., 1994;

Castagliuolo et al., 1997; Di Sebastiano et al., 1999) and even initiating colitis (Lin et al., 2009). Recently, our molecular studies demonstrated that *TAC4* gene encoding HK-1, together with *TAC1* gene encoding SP and *TACRI* gene encoding NK₁ receptor, were dramatically unregulated in colonic mucosa of ulcerative colitis patients (Liu et al., 2011).

Prostaglandin E₂ (PGE₂) is one of a number of eicosanoids which are synthesised via cyclooxygenase (COX) and metabolized by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Otani et al., 2006). PGE₂ is the major prostaglandin of the intestine and its role during intestinal inflammation is controversial. PGE₂ and prostaglandin EP4 receptor are required for maintaining the mucosal barrier integrity in experimental models of IBD, presumably through the enhancement of epithelial regeneration (Jiang et al., 2007), but at higher levels PGE₂ can reduce colonic mucosal barrier integrity (Lejeune et al., 2010). Studies on EP4 receptor knock-out mice also suggested a protective role for PGE₂ against inflammation (Kabashima et al., 2002). On the other hand, PGE₂ may lead to impaired mucosal healing or excessive fibrosis by inhibiting the migration of the colonic lamina propria fibroblasts from Crohn's disease patients (Rieder et al., 2010). PGE₂ production is increased in IBD (Baumeister et al., 1996), and it has been suggested as a mediator and/or prognostic marker in IBD (Wiercinska-Drapalo et al., 1999; Sheibanie et al., 2007).

PGE₂ and tachykinins have two-way interactions, in that tachykinins can increase levels of PGE₂ in rat intrapulmonary bronchi (Szarek et al., 1998); whereas PGE₂ also stimulates SP release in rat sensory neurons (White, 1996). Although it is clear that abnormal levels of SP, HK-1 and PGE₂ all occur in IBD, and functional interaction between SP and PGE₂ has been reported in human colonic epithelial cells (Koon et al., 2006), a mechanistic link between HK-1 and PGE₂ production remains unknown. Given the potential pathological role of HK-1 in IBD, our aim was to explore the inflammatory role for HK-1 in normal human colonic tissue.

Methods

Patients and specimens

Colon ring segments approximately 5 cm in length were obtained from 20 male and 15 female patients undergoing colectomy for carcinoma (age range 41-72 years). Most specimens were collected from the sigmoid colon (n=27) with some also from ascending colon (n=7), and descending colon (n=1). Segments were taken 10-20 cm away from the site of carcinoma; any specimen appearing macroscopically inflamed or showing abnormal histological features was discarded. Patients who had obstruction or who had undergone radiation or chemotherapy were excluded from our study. This project was approved by the Human Ethics Committees of the University of New South Wales (HREC08310) and South Eastern Sydney and Illawarra Area Health Service (SESAHS 06/69).

Tissue explant preparation

Surgically resected specimens were obtained within 5 min of removal from patients, immediately placed in ice-cold Krebs-Henseleit solution pre-gassed with carbogen (95% O₂ and 5% CO₂) and kept on ice until dissection. Specimens were dissected into smooth muscle and mucosa layers, and kept in Krebs-Henseleit solution at 4°C overnight. For muscle explants, the mucosa, submucosa, serosa and taenia coli were removed, leaving the circular muscle with a thin layer of longitudinal muscle. For mucosal explants, the submucosa was removed. Adjacent explants (3 x 5 mm, approximate weight 60-120 mg) of colonic mucosa or muscle were placed in 2 ml tissue baths containing Krebs-Henseleit solution maintained at 37°C, and aerated with carbogen. Explants were not placed under tension, and were allowed to equilibrate for 1 h before being washed and exposed to different treatments before aliquots of bath fluids were collected and snap-frozen for later PGE₂ measurement. At the end of treatments, explants were snap-frozen for RNA or protein

extraction as described below.

Tachykinin-stimulated PGE₂ release in colonic tissue explants

Time-course experiments were initially performed, where mucosal and muscle explants were incubated in the presence or absence of human HK-1 (0.1 μ M) for 0, 1, 2, 4 and 8 h. In concentration-response experiments, mucosal and muscle explants were challenged with HK-1 (10 pM to 10 μ M) for 4 h (found to be the optimum time period).

The effects of selective tachykinin receptor antagonists were examined. The NK₁ receptor antagonist SR140333 (0.1 μ M) or NK₂ receptor antagonist SR48968 (0.1 μ M) was added 1 h before the initiation of HK-1 or SP. For experiments investigating COX inhibitors, non-selective COX inhibitor indomethacin (1 μ M), selective COX-1 inhibitor SC-560 (0.1 μ M), or selective COX-2 inhibitor NS-398 (60 μ M) were added 30 min before the addition of HK-1 or SP. These concentrations of SC-560 and NS-398 were based on their COX isoform selectivity (Barnett et al., 1994; Johnson et al., 1995). Some tissue explants were also pre-incubated with the nerve blocking agent, tetrodotoxin (TTX, 0.1 μ M), for 30 min. Following the pretreatment with inhibitors, explants were exposed to HK-1 or SP for 4 h.

PGE₂ ELISA

PGE₂ levels released into bath fluid were measured by using PGE₂ ELISA kit according to the manufacturer's instructions. Results (in pg/mg tissue) were expressed as mean \pm standard error (SEM).

Quantitative real-time PCR

After incubation, total RNA was extracted from mucosal and muscle explants using the Trizol method followed by a DNase treatment, to remove contaminating DNA. SuperScript

III First Strand Synthesis kit was used to generate cDNA according to the manufacturer's recommendations, which was then subjected to real-time quantitative PCR using Maxima SYBR Green qPCR Master Mix and a thermal cycler (Eppendorf, Hamburg, Germany). The forward primer (fp) and reverse primer (rp) for each gene used in PCR was: COX-1 (official gene name *PTGS1*), fp 5'-ctc cca gga gta cag cta cga-3' and rp 5'-cca gca atc tgg cga gag a-3'; COX-2 (*PTGS2*), fp 5'-ggc ttc cat tga cca gag cag-3' and rp 5'-gcc gag gct ttt cta cca ga-3'; 15-PGDH (*HPGD*), fp 5'-ttg gaa gac tgg aca ttt tgg-3' and rp 5'-cct tca cct cca ttt tgc tt-3'; GAPDH fp 5'-cat gag aag tat gac aac agc ct-3' and rp 5'-agt cct tcc acg ata cca aag t-3'. The PCR cycle consisted of 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 68°C for 20 s. In the final step, the melting curve analysis was carried out during gradual temperature elevation from 60°C to 95°C. Experiments were conducted in duplicate, using GAPDH as a housekeeping gene (HKG) and a control smooth muscle sample as a calibrator in each PCR run (Liu et al., 2011). After amplification, the mRNA level for each gene was expressed as fold change, in which each target gene was normalized to GAPDH, and expressed relative to the calibrator using the formula:

Fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [Ct_{(target)} - Ct_{(HKG)}]_{sample} - [Ct_{(target)} - Ct_{(HKG)}]_{calibrator}$ (Pfaffl, 2001).

Western blot

Protein was extracted from human colonic mucosal strips after incubation in lysis buffer (10 mM Tris-HCl, 1 mM EGTA and 10 mM EDTA) in the presence of a protease inhibitor cocktail (Roche, Castle Hill, NSW, Australia). Proteins (20 µg) in the tissue lysates were subjected to 10% SDS-PAGE gel and transferred to PVDF membrane. The membrane was loaded onto blot holders of the SNAP i.d. Protein Detection System (Millipore, Billerica, MA, USA) and subjected to immunodetection according to the manufacture's protocol.

Primary antibodies used were mouse monoclonal anti-COX-1 (1:1000), rabbit polyclonal anti-COX-2 (1:200), rabbit polyclonal anti-15PGDH (1:200) and rabbit polyclonal anti-GAPDH (1:8000). Secondary antibodies used were horseradish peroxidases (HRP)-conjugated goat anti-rabbit and mouse IgG (1:2000), respectively. The proteins were detected using the Enhanced Chemiluminescence (ECL) reaction kit (Millipore). Bands of COX-1, COX-2, 15PGDH and GAPDH were scanned and subjected to densitometry analysis, and the results were expressed relative to GAPDH.

Immunohistochemistry

Full-thickness pieces of normal human colon (n=6) were fixed in Zamboni's solution. Paraffin-embedded sections were cut and mounted on poly-L-lysine coated slides. Reduction of peroxidases was accomplished by incubating in H₂O₂ (3%) for 5-10 min at room temperature followed by nonspecific protein blocking (2% horse or goat serum) for 30 min. Sections were incubated with primary antibodies overnight at room temperature. Primary antibodies used were mouse monoclonal COX-1 antibody (1:500), mouse monoclonal COX-2 antibody (1:100) and rabbit polyclonal 15-PGDH antibody (1:50). Sections were then washed thoroughly and incubated with biotinylated horse anti-mouse or goat anti-rabbit secondary antibodies (1:2000) for 2 h, followed by avidin-biotinylated enzyme complex for 1 h. Immunoreactivity was visualized by reaction with the substrate diaminobenzidine. Each experiment included a negative control incubated with secondary antibody alone; these consistently exhibited no immunoreactivity.

Immunohistochemistry was also performed in tissue explants (n=7) incubated in the presence or absence of HK-1 (0.1 μ M) for 4 h. The experimental procedures were the same as mentioned above.

Statistical analyses

Data for PGE₂ ELISA and Western blot densitometry were normally distributed, and expressed as mean \pm SEM. The means were compared using unpaired or paired Student's *t* test. In addition, one way analysis of variance (ANOVA) was used with a post (Newman-Keuls) test to determine the difference between groups. Real-time PCR data were non-parametric and expressed as median. Thus, Wilcoxon test was used to compare pair-matched medians. All data analyses were performed using GraphPad Prism (Version 5, GraphPad Software, San Diego, CA, USA). *P* values of less than 0.05 were considered to indicate a significant difference.

Materials

HK-1 (American Peptides, Sunnyvale, CA, USA) and SP (Auspep, Melbourne, VIC, Australia) were reconstituted in 0.01 M acetic acid containing 1% β -mercaptoethanol and stored as aliquots at -20°C. PGE₂ ELISA kit was purchased from R&D System (Minneapolis, MN, USA), DNase treatment kit from Promega (Madison, WI, USA), and Trizol kit and SuperScriptIII cDNA Synthesis Kit from Invitrogen (Carlsbad, CA, USA). Maxima SYBR Green qPCR Master Mix was purchased from Fermentas (Burlington, ON, Canada). Indomethacin and TTX were purchased from Sigma-Aldrich (Sydney, NSW, Australia), NS-398, N-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide) and SC-560, 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole from Cayman Chemical (Ann Arbor, MI, USA), which were then dissolved in DMSO. Antibodies for COX-1 (monoclonal, Cat. No 160110), COX-2 (monoclonal, Cat. No 160112; polyclonal, Cat. No 160107) and 15-PGDH (polyclonal, Cat. No 160615) were obtained from Cayman Chemical (Ann Arbor, MI, USA). Anti-GAPDH antibody (polyclonal, Cat. No G9545) and secondary antibodies (anti-mouse IgG, Cat. No A6782; anti-rabbit IgG, Cat. No A4914) for Western

blot were purchased from Sigma-Aldrich and biotinylated secondary antibodies (anti-mouse IgG, Cat. No BA-2000; anti-rabbit IgG, Cat. No BA-1000) for immunohistochemistry from Vector Labs (Burlingame, CA, USA). SR140333, ((S)-1-(12-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)-piperidin-3-yl]ethyl)-4-phenyl-1-azoniabicyclo[2.2.2]octane, and SR48968, N-[(2S)-4-(4-acetamido-4-phenylpiperidin-1-yl)-2-(3,4-dichlorophenyl)butyl]-N-methylbenzamide were gifts from Sanofi-Synthelabo Recherche, Montpellier, France. All the drug solutions were stored as aliquots at -20°C, except NS-398 which was stored at room temperature. All other reagents were of analytical grade.

Results

Time course of HK-1-induced PGE₂ production in mucosa and muscle

To investigate whether HK-1 causes PGE₂ production in colonic muscle and mucosa, paired tissue explants were incubated with or without HK-1 (0.1 μM) for 0 h to 8 h. HK-1 induced PGE₂ release into bath fluid, in a time-dependent manner (Figure 1A and B). There was a trend for an increase in PGE₂ release with the time in unstimulated tissues, but this was not significant. After incubation with HK-1 for 1 and 2 h, the PGE₂ levels were no different from unstimulated paired time controls. In the mucosa, PGE₂ levels showed a 10-fold increase ($p<0.01$) after 4 h and a 2-fold increase ($p<0.05$) after 8 h, compared with paired time controls (Figure 1A). In muscle, there was a 3.5-fold increase in PGE₂ release ($p<0.01$) after 4 h, whereas there was no significant change after 8 h (Figure 1B). Thus, 4 h was considered as the optimal time point and used for all subsequent experiments for mucosa and muscle.

Concentration-response relationship of HK-1-induced PGE₂ production in mucosa and muscle

After 4 hours incubation with HK-1, PGE₂ release from both mucosa and muscle showed a bell-shape curve in response to HK-1 in the 0.1 pM to 10 μM range, with the maximum response at 0.1 μM HK-1 (Figure 2A, B). In mucosal explants, the effects of HK-1 at 0.01, 0.1 μM and 1 μM (Figure 2A) were significantly different from the non-stimulated control, whereas the effect of HK-1 in muscle was statistically different from control only at 0.1 μM (Figure 2B). In all subsequent experiments, 0.1 μM was used as the optimal concentration of HK-1.

Effect of inhibitors on responses to HK-1 and SP in mucosal explants

Firstly, we investigated whether PGE₂ production was dependent upon the activation of tachykinin receptors. HK-1-induced PGE₂ production was inhibited to a similar extent by both the NK₁ receptor antagonist SR140333 (44% of inhibition) and the NK₂ receptor antagonist SR48968 (51% of inhibition) (Figure 3 A). In parallel, SP (0.1 μM) also stimulated PGE₂ production, and this was inhibited by both SR140333 and SR48968 in a virtually identical manner compared with HK-1 (Figure 3 B). The NK₁ and NK₂ antagonists alone did not affect the basal level of PGE₂ release at 4 h (n=7, data not shown).

To determine which COX isoform was involved in HK-1- and SP-induced PGE₂ production, mucosal strips were pretreated with the non-selective COX inhibitor indomethacin, the COX-1 selective inhibitor SC-560 or the COX-2 selective inhibitor NS-398. Both HK-1- and SP- induced PGE₂ production was unaffected by SC-560 (Figure 4). However, it was notable that indomethacin (n=8, *p*<0.05) and NS-398 (n=8, *p*<0.05) not only blocked, but significantly reduced HK-1-stimulated PGE₂ release below unstimulated baseline levels (Figure 4).

To determine the role of neurons in PGE₂ release, mucosal explants were incubated in the presence and absence of TTX (0.1 μM). TTX alone significantly increased PGE₂ release by 217 ± 78% over the basal level (n=8, *p*<0.05), but TTX did not affect HK-1 induced PGE₂ production (205 ± 138%, n=8).

HK-1- regulated COX and 15-PGDH gene expression in mucosal and muscle explants

Paired explants were incubated in the presence and absence of HK-1 (0.1 μM) for 4 h, and expression of COX-1, COX-2 and 15-PGDH mRNA in the explants was then determined using real-time RT-PCR. Although HK-1 had no effect on levels of COX-1 mRNA (Figure 5 A, B), it enhanced the expression of COX-2 mRNA by 2.4-fold and 1.5-fold, in mucosal and

muscle explants, respectively (Figure 5 C, D), compared with non-stimulated paired controls. No significant upregulation of COX-2 expression induced by HK-1 was seen at earlier incubation time points (data not shown).

Exposure of colonic mucosa to HK-1 (0.1 μ M) significantly reduced 15-PGDH mRNA expression by 1.7-fold compared with non-stimulated control samples (Figure 5E). We also attempted to demonstrate the expression of 15-PGDH mRNA in muscle explants, but expression was too low (data not shown).

HK-1-regulated COX and 15-PGDH protein expression in mucosal explants

Western blot was used to determine protein expression of COX-1, COX-2 and 15-PGDH in paired mucosal explants, after incubation with or without HK-1 (0.1 μ M) for 4 h (Figure 6A). COX-1 protein expression was unaltered by HK-1, but COX-2 expression was significantly increased with 2.7-fold change (Figure 6A, B). Notably, HK-1 caused a 1.6-fold reduction ($p<0.05$) of 15-PGDH protein expression (Figure 6A, B).

Localization of COX-1, COX-2 and 15-PGDH immunoreactivity in unstimulated normal human colon

At the antibody concentrations used here, the immunoreactivity (IR) of COX-1 in epithelial, stromal, intestinal smooth muscle and vascular smooth muscle cells was more pronounced than that of COX-2 and 15-PGDH (Table 1, Figure 7, 8). COX-1 IR was widespread in human colonic mucosa, muscle and ganglia, as well as vascular smooth muscle (Figure 7). COX-2 IR was generally weaker than COX-1, except in submucosal ganglia where intensity was similar. In the epithelium, COX-2 IR was localized primarily at the cell membrane, whereas COX-1 IR showed a cytoplasmic and nuclear expression (Figure 7, 8).

Immunostaining for 15-PGDH was mainly cytoplasmic, of moderate intensity similar to that of COX-2. 15-PGDH staining was highly localized, to regions of the mucosa and to submucosal and myenteric ganglia (Figure 7, 8). Notably, nuclear staining of 15-PGDH was also observed in epithelial cells (Figure 7C, 8C) and myenteric ganglia (Figure 7L). Furthermore, weak 15-PGDH staining was seen on goblet cells (Figure 8C).

In blood vessels, prominent immunostaining of granulocyte- and monocyte-like cells was observed with COX-1, whereas immunostaining with COX-2 and 15-PGDH was moderate. Platelets showed immunoreactivity for all three enzymes (Figure 8 G, H, I).

HK-1-induced alteration of COX and 15-PGDH immunoreactivity in human colonic mucosa

The immunoreactive staining for COX-1, COX-2 and 15-PGDH appeared less dense in surface epithelial cells of HK-1 treated tissue explants compared to non-treated control tissues (Figure 9). COX-1 IR in other mucosal structures seemed unaffected by HK-1 (Figure 9A,B). HK-1 evoked a clear increase of COX-2 IR and reduction of 15-PGDH IR in crypt epithelial cells and immune cells in lamina propria (Figure 9C-F), whereas changes were less noticeable in smooth muscle and submucosal regions.

Discussion

SP and the NK₁ receptor are upregulated in IBD human intestine and in experimental animal models of IBD (Goode et al., 2000; Liu et al., 2011). Studies also demonstrate that PGE₂ plays an important role in the pathogenesis of IBD (Sheibanie et al., 2007). HK-1 has been implicated in immune/inflammation, but little has been reported about its effects in the human colon. In the current work, we investigated the effect of human HK-1 on PGE₂ production in the normal human colon, and precisely defined its actions on COX and 15-PGDH. To our knowledge, this is the first study simultaneously evaluating the pro-inflammatory effects of HK-1 and SP in normal human gut. This extends previous observations showing that exogenous SP stimulates PGE₂ production in human colonic epithelial cells (Koon et al., 2006), and provides an original finding that similar to SP, HK-1 has the ability to induce PGE₂ release in human colon.

The effect of HK-1 to enhance PGE₂ release in both colonic mucosa and muscle was time- and concentration- dependent. Unusually, a bell-shaped concentration curve was observed, with the maximum response at 0.1 μM HK-1, and a similar phenomenon has been seen in studies with SP in human polymorphonuclear leucocytes (Gallicchio et al., 2009). This bell-shape response indicates that at higher concentrations, desensitization may occur; tachykinins may bind to other tachykinin receptor subtypes, or may activate different mechanisms which have inhibitory effects on PGE₂ production. Although significant PGE₂ production was elicited in both muscle and mucosa, the greater effects in mucosa (10-fold change) suggest a prominent role for HK-1 in that location. In colonic inflammation, immune cells in the lamina propria could be the primary source of HK-1, since endogenous HK-1 is preferentially expressed in lymphocytes and macrophages in mice (Zhang et al., 2000; Zhang and Paige, 2003) and humans (Klassert et al., 2008). This is in line with an increased expression of *TAC4* gene encoding HK-1 in colonic mucosa in ulcerative colitis

(Liu et al., 2011).

HK-1 shows strong preference for the NK₁ compared with NK₂ or NK₃ receptor (Bellucci et al., 2002). Our present findings show that HK-1 and SP signal via both NK₁ and NK₂ receptors to induce PGE₂ release in colonic mucosa. It has been previously reported that SP/NK₁ receptors causes PGE₂ release from rat intrapulmonary bronchi (Szarek et al., 1998) and porcine jejunum (Thorboell et al 1998). A recent study has demonstrated that SP stimulates PGE₂ release in human colonic epithelial cells stably transfected with NK₁ receptors (Koon et al., 2006), but such interactions have not been extensively studied in native human intestine. The pathological implication of NK₁ receptor in inflammation has been demonstrated by the beneficial effects of NK₁ antagonists in experimental models of colitis (Cutrufo et al., 1999; Di Sebastiano et al., 1999), and by our previous work showing an upregulation of *TACR1* gene in colonic mucosa of IBD patients (Liu et al., 2011).

In addition to the activation of the NK₁ receptor pathway, we provide new findings that NK₂ receptors also influence the SP and HK-1-evoked PGE₂ production in the human colon. A relationship between NK₂ receptors and PGE₂ release has been previously shown in alveolar macrophages (Brunelleschi et al., 1992) urinary bladder (Tramontana et al., 2000), as well as in normal human colon (Burcher et al., 2008). A relationship between NK₂ receptors and inflammation was shown in human IBD by Renzi et al. (2000) who demonstrated marked increases in the expression of NK₁ receptors in epithelia and in vascular endothelia, whereas NK₂ receptors were increased in inflammatory cells of lamina propria and activated eosinophils around mucosal crypts. The simultaneous involvement of both NK₁ and NK₂ receptors have also been reported in animal models of inflammation (Cutrufo et al., 1999) and hypersecretion (Turvill et al., 2000). Our study showing the active role of NK₁ and NK₂ receptors in PGE₂ production may help to explain the significance of upregulated NK₁ and NK₂ receptors in the pathophysiology of IBD. Although NK₁ and NK₂

receptors have significant roles in gut pathophysiology, few drugs targeting these individual receptors have entered the clinic for human use. A promising NK₂ receptor antagonist is being tested for irritable bowel syndrome and may have an effect in inflammation (Quartara et al., 2009). Our study implies that a combined use of both receptor antagonists may be more therapeutically beneficial.

The steady-state cellular levels of PGE₂ depend on the relative rate of COX-dependent biosynthesis and 15-PGDH-mediated catabolism. In this study, we demonstrated that HK-1-induced PGE₂ release requires the involvement of COX-2 rather than COX-1. Furthermore, HK-1 induces PGE₂ release at the same concentrations and incubation times found to induce COX-2 gene and protein expressions, which were both upregulated to a similar extent. Strikingly, we have also provided new insights on the effect of HK-1 on expression of 15-PGDH, the key enzyme responsible for PGE₂ inactivation. Coupled with the marked upregulation of COX-2, HK-1 significantly inhibited 15-PGDH expression at both mRNA and protein level. This finding suggests that HK-1 may have dual effects on PGE₂ production, not only by increased PGE₂ synthesis through upregulation of COX-2, but also by decreased 15-PGDH expression and, hence, reduced degradation of PGE₂. This, we believe, is the most novel finding of our study. This is in line with a previous study showing a marked reduction of 15-PGDH in colonic mucosa from IBD patients (Otani et al., 2006).

Our immunohistochemical data in colonic mucosa showed COX-2 as well as COX-1 expression primarily in epithelial cells (upper crypts) and immune cells in lamina propria, with some immunoreactivity also localized to submucosal ganglia. They also revealed that 15-PGDH has a similar pattern of immunoreactivity as COX-1 and COX-2, and represent another novel finding that 15-PGDH is also expressed in neuronal regions. In line with our findings that HK-1 increased PGE₂ in muscle explants, we also found expression of COX-1, COX-2 and 15-PGDH in myenteric ganglia, as well as a weaker expression in longitudinal

and circular muscle. Our previous studies have shown that indomethacin enhances contractile responses to NKA in human colonic muscle strips from control and diverticular disease patients, suggesting NK₂ receptor-stimulated release of prostanoids which relax smooth muscle (Burcher et al., 2008). COX-1 and COX-2 have been shown to be present in the neuromuscular compartment of human colon where they modulate cholinergic excitatory control of colonic motility (Fornai et al., 2005). Further studies using confocal microscopy are necessary to confirm which neuronal and other markers are colocalized with COX and 15-PGDH. Surprisingly, our data showed that TTX enhanced basal PGE₂ release, but had no effect on HK-1 stimulated PGE₂ production. This suggests that there is an inhibitory neuronal component which can reduce PGE₂ release. However, the lack of effect of TTX on HK-1 induced response is suggestive of epithelial and immune cells as the major cellular source of HK-1-COX-2-derived PGE₂.

In summary, our study has demonstrated a novel role for HK-1 as an important stimulant for PGE₂ release in human colon, and explored the mechanism involved. This is the first demonstration that HK-1, acting via both NK₁ and NK₂ receptors, upregulates COX-2 expression, an effect shared by SP. We further demonstrate that HK-1-induces 15-PGDH downregulation and this represents an additional mechanism by which HK-1 increases PGE₂ production. This study reveals a mechanistic link between HK-1 and PGE₂ in humans, and provides new insights into a potential functional role of HK-1 in the pathophysiology of colonic inflammation.

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Authorship Contributions

Participated in research design: Dai, Liu

Conducted experiments: Dai

Contributed new reagents or analytic tools: Perera, King

Performed data analysis: Dai, Burcher, Liu

Wrote or contributed to the writing of the manuscript: Dai, Burcher, Southwell, Liu

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Footnotes

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This manuscript represents partial fulfillment for Ms. Dai's PhD thesis in Pharmacology.

Legends for Figures

Figure 1. Time-course study of HK-1-induced PGE₂ release. Adjacent human colonic (A) mucosal or (B) muscle explants were stimulated in the absence (open bars) or presence (closed bars) of HK-1 (0.1 μM) for up to 8 h. Results are expressed as mean ± SEM and represent 4-7 (A) and 4-5 (B) independent experiments. Data were analyzed by paired *t* test. * *p* < 0.05, ** *p* < 0.01, comparing non-stimulated versus HK-1-stimulated PGE₂ release from the same time period.

Figure 2. Concentration-response relationship of PGE₂ release by HK-1. Adjacent human colonic (A) mucosal or (B) muscle explants were exposed to Krebs-Henseleit (open bars) or five different concentrations (0.1 pM to 10 μM) of HK-1 (closed bars) for 4 h. Results are expressed as mean ± SEM and represent 10-11 (A) and 5-6 (B) independent experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test. * *p* < 0.05, ** *p* < 0.01, versus non-stimulated samples.

Figure 3. Effect of tachykinin receptor antagonists on (A) HK-1- and (B) SP-stimulated PGE₂ release from human colonic mucosa, after 4 h incubation. The HK-1- and SP-evoked release was significantly attenuated by SR140333 (0.1 μM) and SR48968 (0.1 μM) respectively. Results show mean ± SEM, from 14-15 (A) and 8-9 (B) independent experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test. * *p* < 0.05, ** *p* < 0.01 for effect of antagonist on HK-1- or SP-evoked release.

Figure 4. Effects of COX inhibitors on (A) HK-1- and (B) SP-stimulated PGE₂ release from human colonic mucosa, after 4 h incubation. Indomethacin (1 μM) or NS-398 (60 μM) completely blocked HK-1- or SP-stimulated PGE₂ release, whereas SC-560 (0.1 μM) was ineffective. Results, expressed as mean ± SEM, represent 7-8 (A) and 5-6 (B) independent experiments. Data were analyzed by One-way ANOVA followed by a Newman-Keuls multiple comparison test. * $p < 0.05$ versus HK-1- or SP-evoked release.

Figure 5. Scatter plot depicting mRNA expression of (A, B) COX-1, (C, D) COX-2 and (E) 15-PGDH in response to HK-1 stimulation (0.1 μM). Paired human colonic mucosal (A, C, E; $n=9-11$) or muscle (B, D; $n=7$) explants were exposed to Krebs-Henseleit (open bars) or 0.1 μM HK-1 (closed bars). Results are normalized to GAPDH and expressed as fold increases over values of the calibrator. Bars show the median values. * $p < 0.05$, ** $p < 0.01$ versus paired controls in each group (Wilcoxon test).

Figure 6. Protein expression of COX-1, COX-2 and 15-PGDH in response to HK-1 stimulation, using western blot. (A) Gel depicting protein bands of COX-1, COX-2 and 15-PGDH in paired human colonic mucosal explants exposed to Krebs-Henseleit or HK-1 (0.1 μM). GAPDH was used as internal control. (B), protein bands were subjected to densitometric analysis. Results were normalized to GAPDH controls and shown as relative density. HK-1 (closed bars) was without effect on COX-1 protein expression, but elicited significant stimulatory effect on COX-2 expression, and had an inhibitory effect on 15-PGDH protein expression, compared with unstimulated controls (open bars). Data, expressed as mean ± SEM, represent 7 independent experiments in duplicate. * $p < 0.05$, ** $p < 0.01$ with respect to unstimulated controls (paired t -test).

Figure 7. Immunohistochemical localization of COX-1 (A, D, G, J), COX-2 (B, E, H, K) and 15-PGDH (C, F, I, L) protein expression in normal human colonic mucosa (A, B, C), submucosal ganglia (D, E, F), submucosal blood vessel (G, H, I) and myenteric ganglia (J, K, L). In general, COX-1 IR was greater than COX-2 and 15-PGDH IR. IR for all three enzymes was observed on epithelial cells, stromal cells, smooth muscle cells, ganglionic cell bodies, and some lamina propria immune cells. 15-PGDH appeared to stain the nuclei of some epithelial cells. Boxes indicate regions shown at higher power in Figure 8. Labels: bv, arterial blood vessel; cm, circular muscle; g, gland; lp, lamina propria; lm, longitudinal muscle; mg, myenteric ganglion; mm, muscularis mucosae; sm, submucosa; smg, submucosal ganglion;. Scale bars, 50 μ m.

Figure 8. High power photomicrographs showing COX-1 (A, D, G), COX-2 (B, E, H) and 15-PGDH (C, F, I) immunoreactivity in mucosal and submucosal regions. (A-F) are enlargements of regions outlined in Figure 7, and (G-I) show blood cells in lumen of submucosal blood vessel. Immunoreactivity for the three enzymes was observed on epithelial cells (arrowheads) in upper crypts (A-C); immune cells (arrowed) in the lamina propria (D-F) and on submucosal vascular leukocytes (arrowed) and platelets (arrowheads) (G-I). Nuclei of large lymphocytes were strongly stained by COX-1 (*). Goblet cells (gc) were weakly immunostained by 15-PGDH (C) but not by COX. Arrows identify COX-1 and 15-PGDH stained granulocyte-like and monocyte-like cells in lamina propria and in lumen of blood vessels; COX-2 staining was weaker. Scale bars, 10 μ m.

Figure 9. Immunohistochemical localization of COX-1 (A, B), COX-2 (C, D) and 15-PGDH (E, F) protein expression in non-HK-1 treated mucosal explants (A, C, E) and HK-1 treated mucosal explants (B, D, F). The immunoreactive staining for COX-1, COX-2 and 15-PGDH appeared less dense in surface epithelial cells of HK-1 treated tissues. COX-1 IR in other mucosal structures seemed unaffected by HK-1 (A, B). HK-1 induced an increase of COX-2 IR (C, D) and reduction of 15-PGDH IR (E, F) in crypt epithelial cells and immune cells in lamina propria. Labels: g, gland; lp, lamina propria. Scale bars, 50 μ m.

Table 1. Immunoreactivity of COX-1, COX-2 and 15-PGDH in normal human colon

| Cell type | COX-1 | COX-2 | 15-PGDH |
|--|-------|-------|---------|
| Epithelial cells in apical surface of crypts | +++ | + | ++* |
| Glandular epithelium | +++ * | + | + * |
| Stromal cell mucosa | ++ | +/- | +/- |
| Immune cell in lamina propria | + | + | + |
| Muscularis mucosae | ++ | +/- | +/- |
| Submucosal ganglia | ++/+ | ++/+ | ++/+ |
| Submucosal blood vessel wall | ++ | + | + |
| Leukocytes | +++ | + | + |
| Platelets | + | + | + |
| Circular muscle | ++ | +/- | +/- |
| Myenteric ganglia | +++ | ++ | ++ * |
| Longitudinal muscle | ++ | +/- | +/- |

Intensity of staining: +++, strong; ++, moderate; +, weak; -, absent.

Most immunoreactivity was cytoplasmic; * indicates that staining also seen on nuclei.

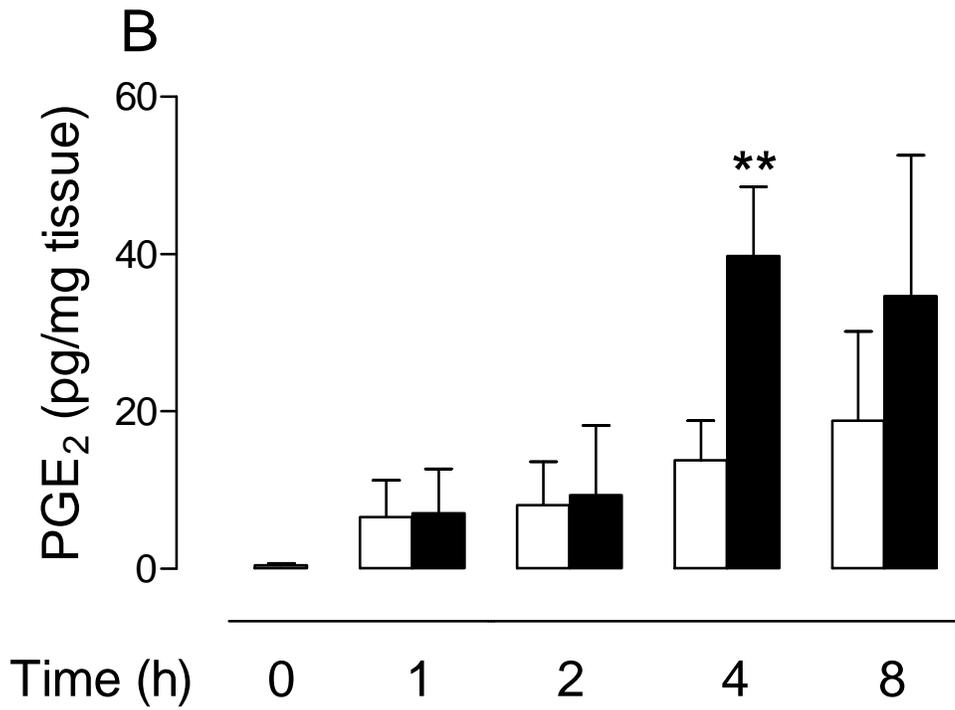
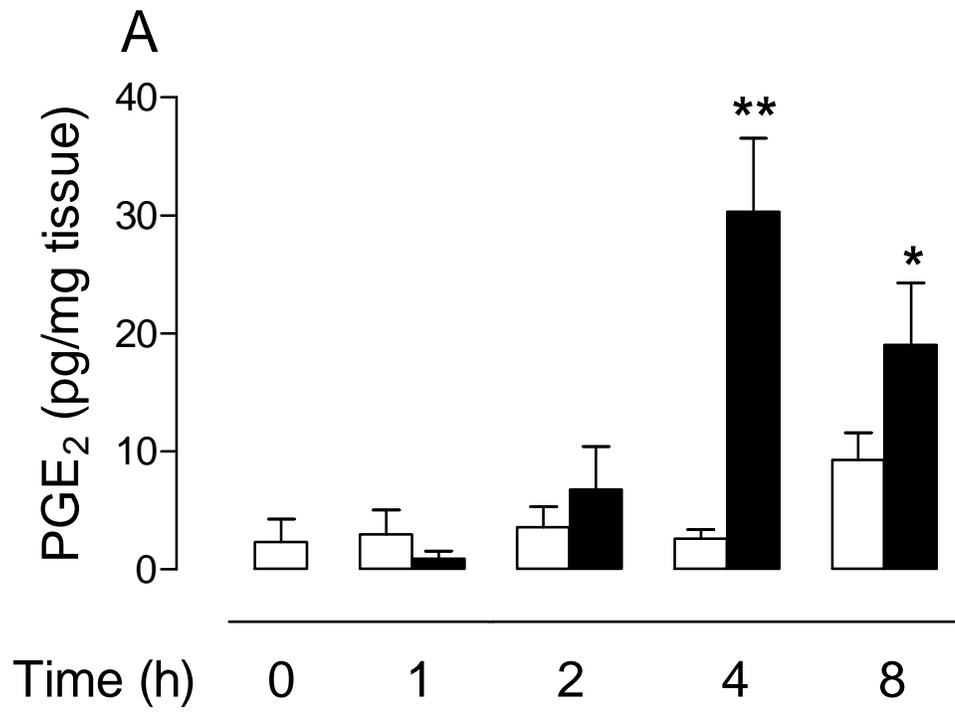


Figure 1

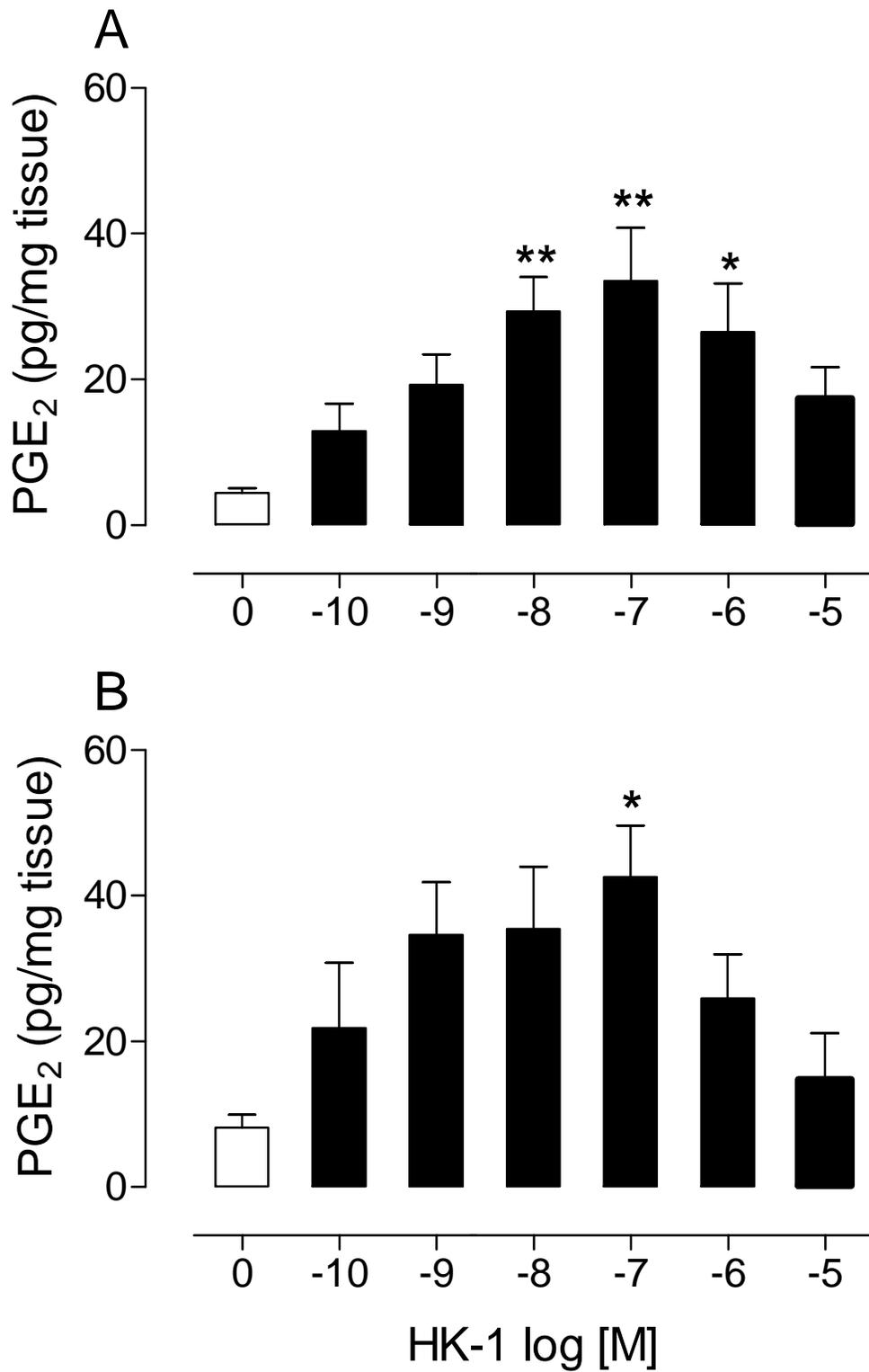


Figure 2

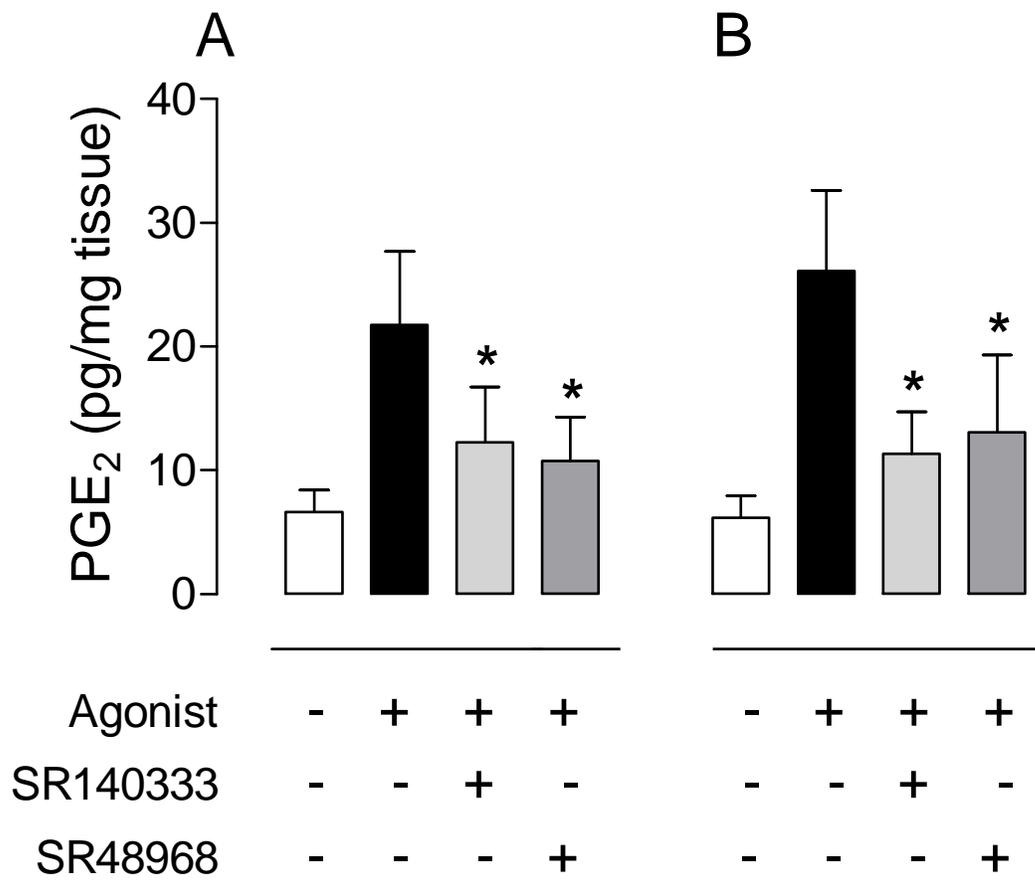


Figure 3

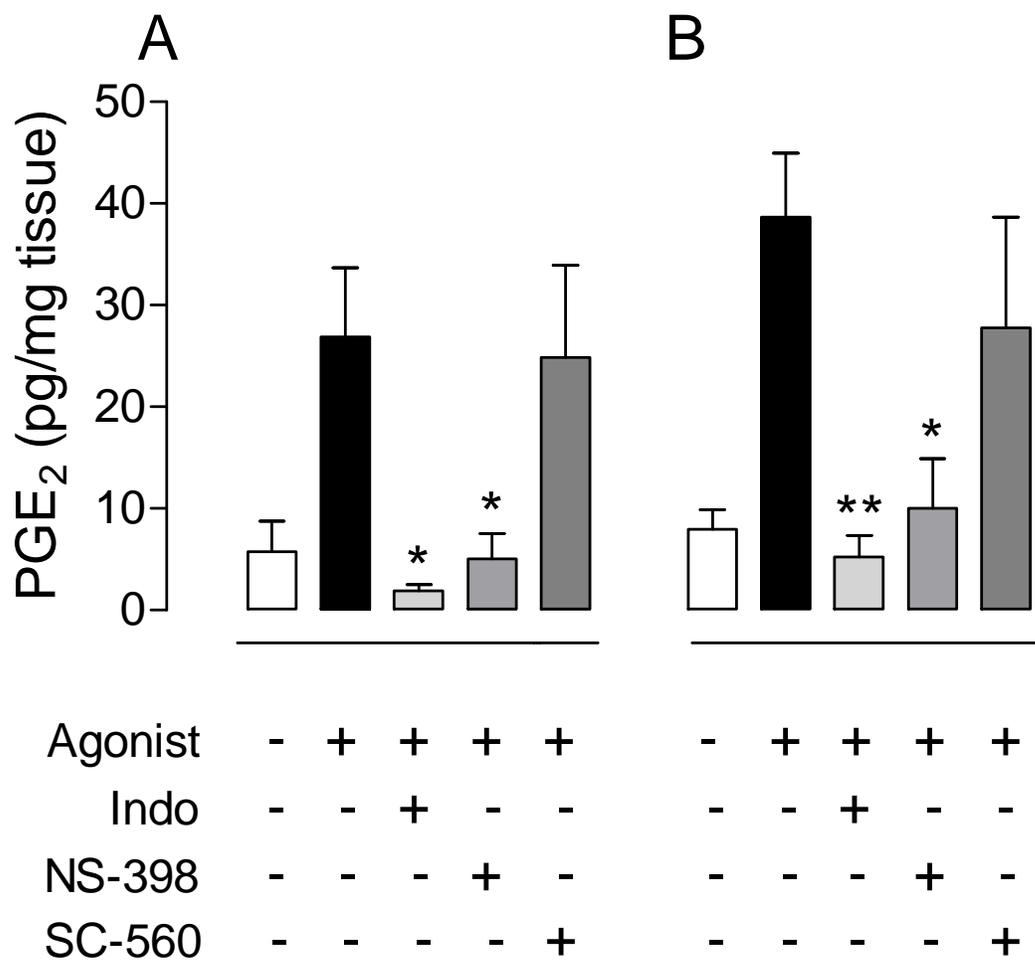


Figure 4

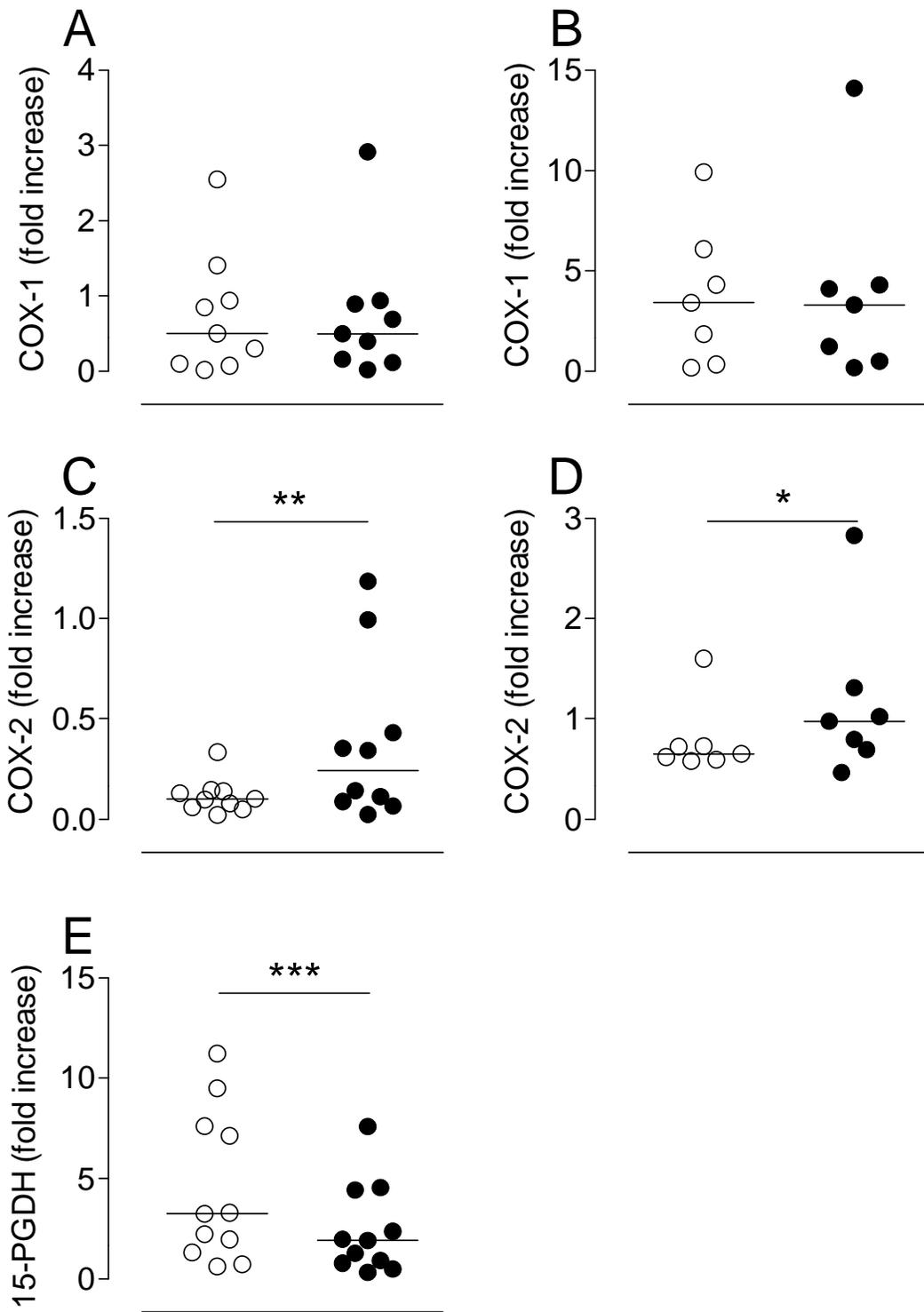


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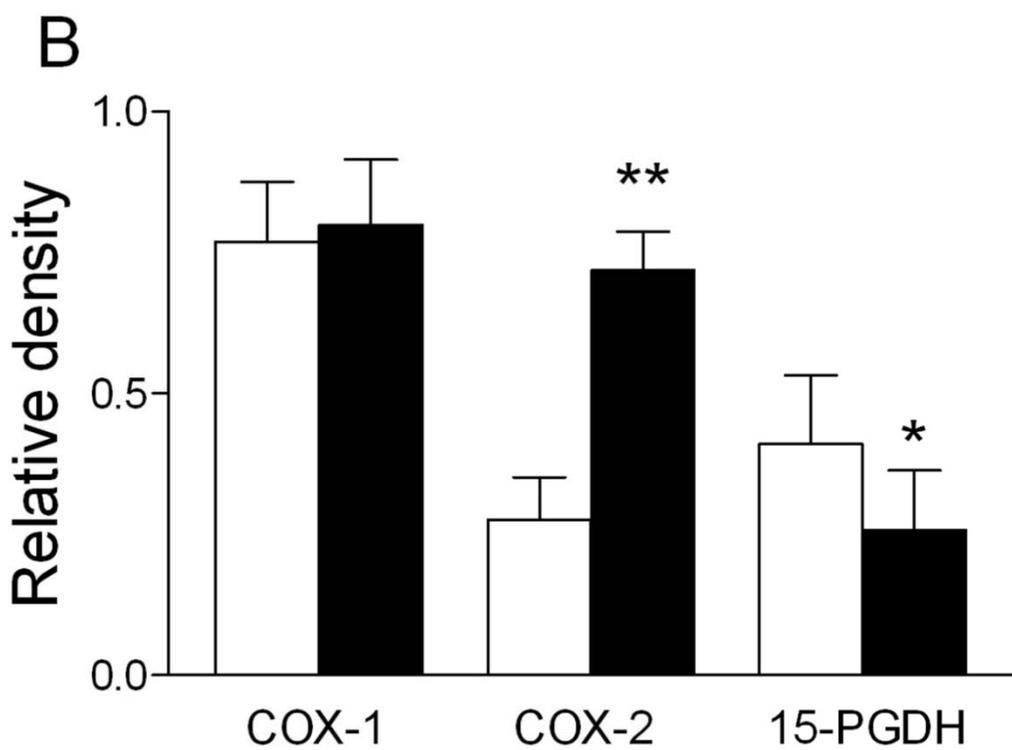
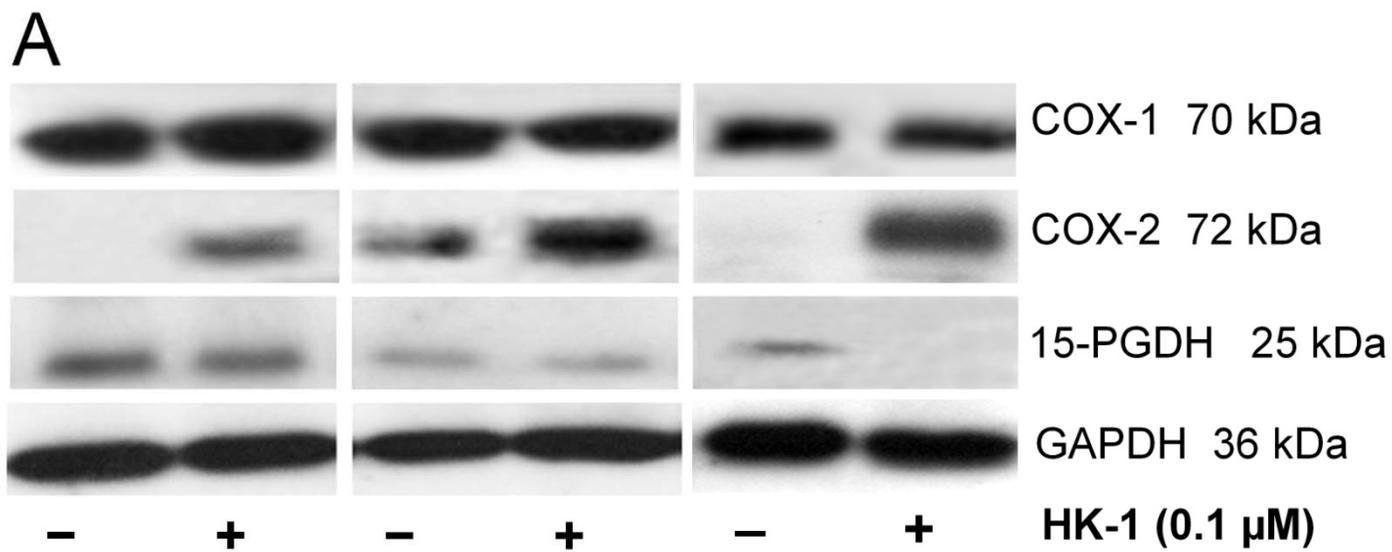


Figure 6

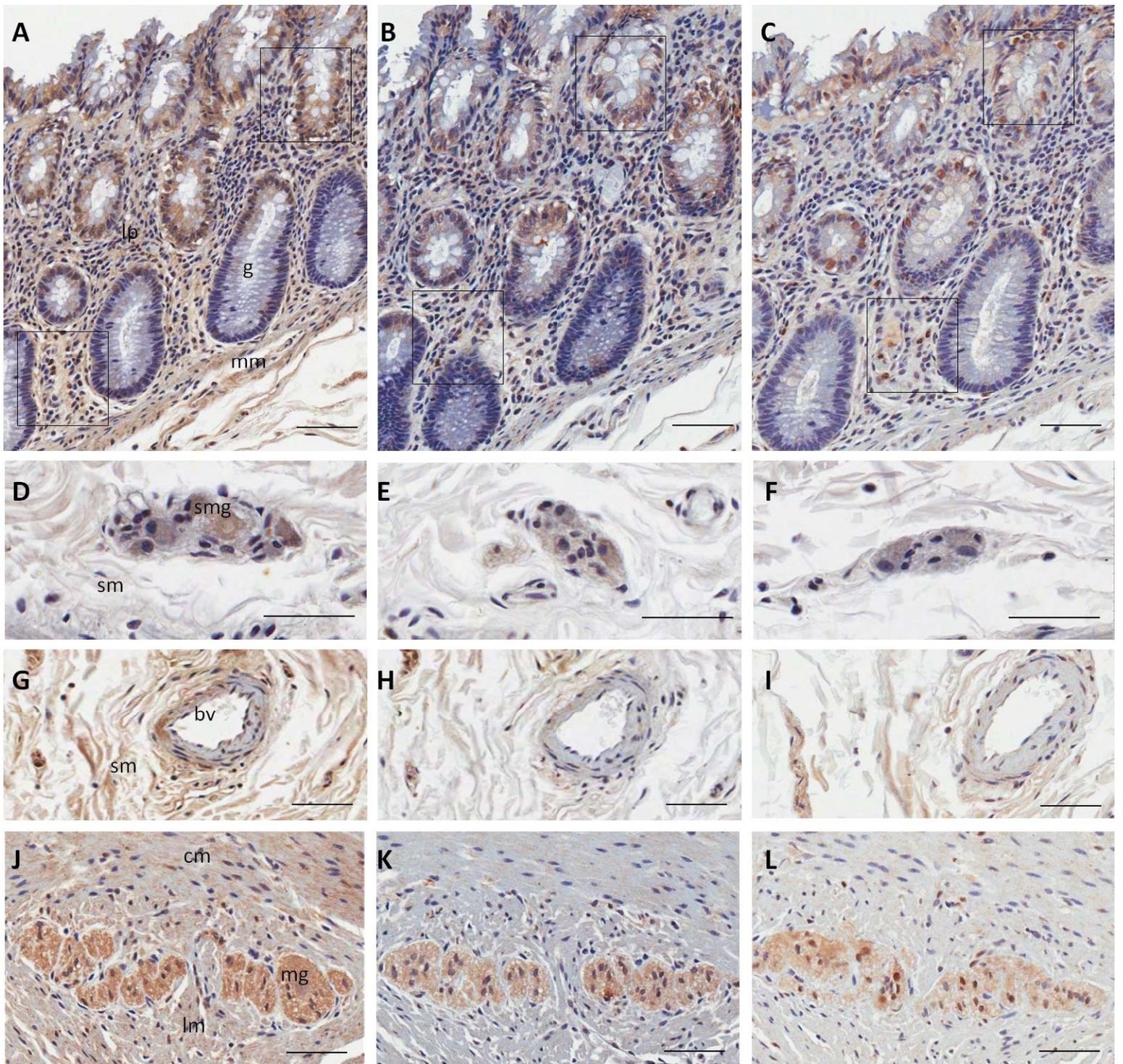


Figure 7

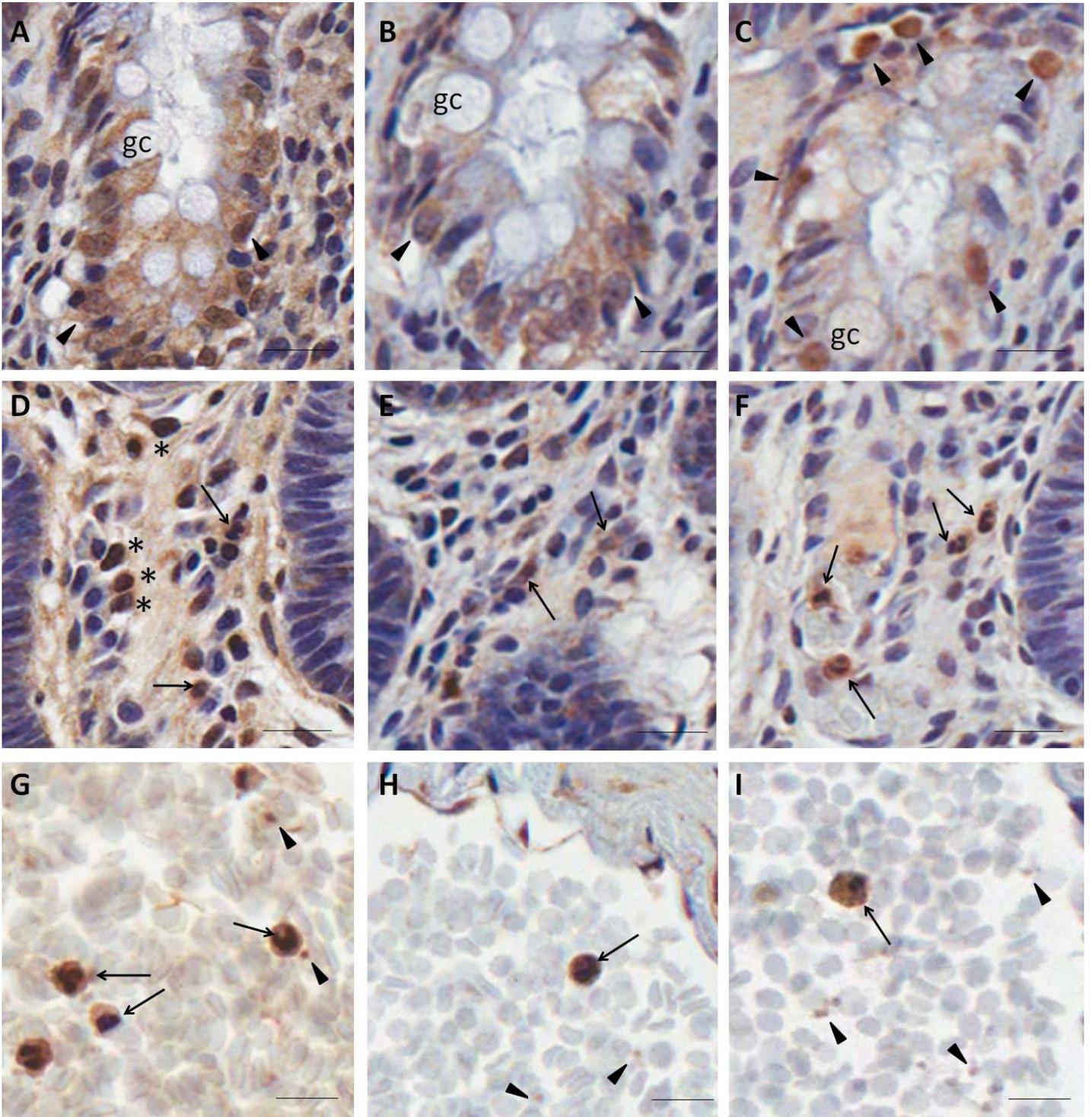


Figure 8

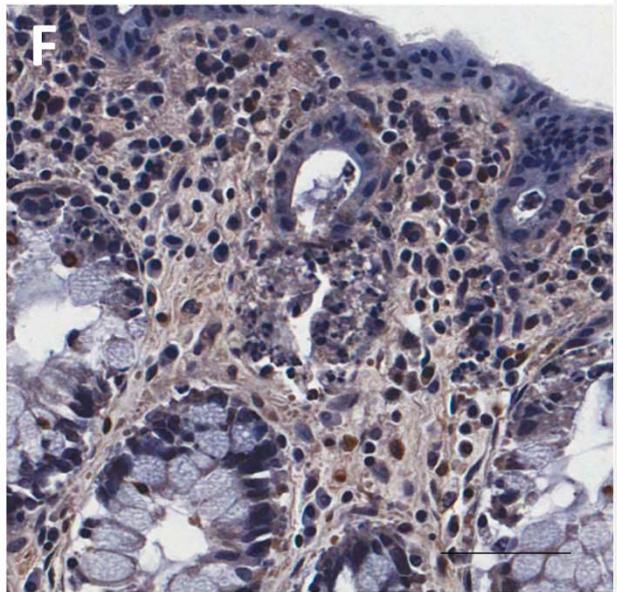
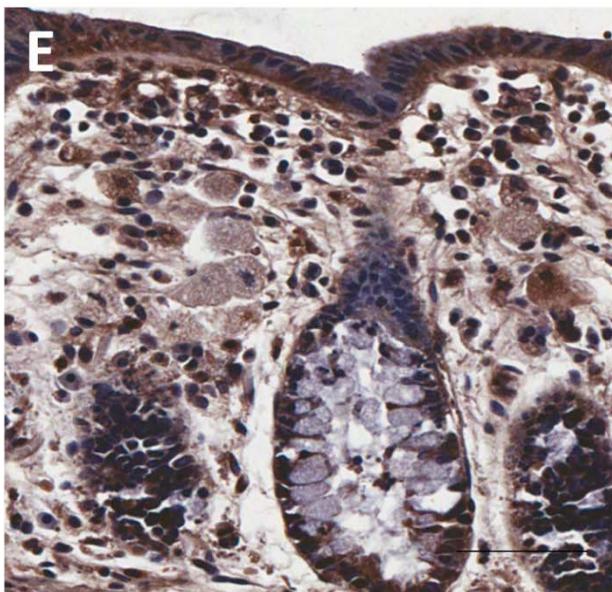
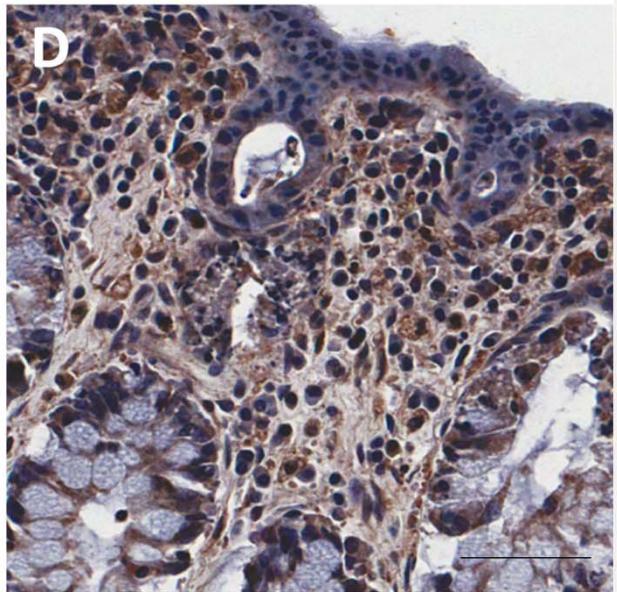
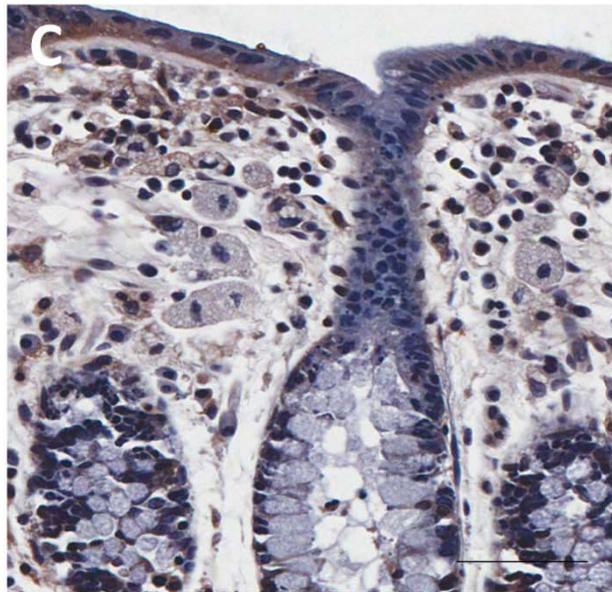
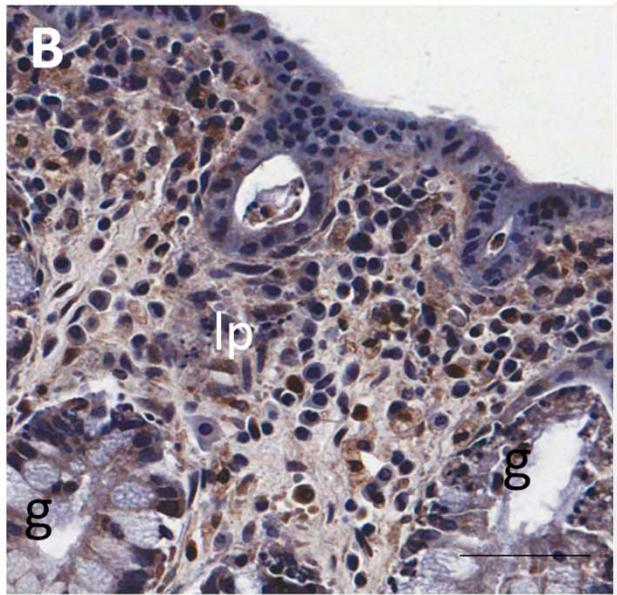
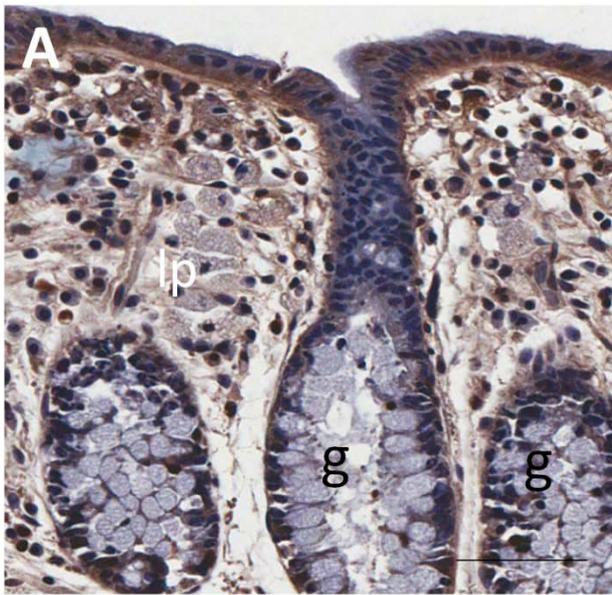


Figure 9