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Urocortin 3 expression at baseline and during inflammation in the colon: Corticotropin releasing factor receptors cross-talk

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Abstract

Urocortins (Ucn1–3), members of the corticotropin-releasing factor (CRF) family of neuropeptides, are emerging as potent immunomodulators. Localized, cellular expression of Ucn1 and Ucn2, but not Ucn3, has been demonstrated during inflammation. Here, we investigated the role of Ucn3 in a rat model of Crohn's colitis and the relative contribution of CRF receptors (CRF₁ and CRF₂) in regulating Ucn3 expression at baseline and during inflammation. Ucn3 mRNA and peptide were ubiquitously expressed throughout the GI tract in naïve rats. Ucn3 immunoreactivity was seen in epithelial cells and myenteric neurons. On day 1 of colitis, Ucn3 mRNA levels decreased by 80% and did not recover to baseline even by day 9. Next, we ascertained pro- or anti-inflammatory actions of Ucn3 during colitis. Surprisingly, unlike observed anti-inflammatory actions of Ucn1, exogenous Ucn3 did not alter histopathological outcomes during colitis and neither did it alter levels of pro-inflammatory cytokines IL-6 and TNF- α . At baseline, colon-specific knockdown of CRF₁, but not CRF₂ decreased Ucn3 mRNA by 78%, whereas during colitis, Ucn3 mRNA levels increased after CRF₁ knockdown. In cultured cells, co-expression of CRF₁ + CRF₂ attenuated Ucn3-stimulated intracellular Ca²⁺ peak by 48% as compared to cells expressing CRF₂ alone. Phosphorylation of p38 kinase increased by 250% during colitis and was significantly attenuated after Ucn3 administration. Thus, our results suggest that a balanced and coordinated expression of CRF receptors is required for proper regulation of Ucn3 at baseline and during inflammation.

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Disclosure

The authors have nothing to disclose.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.peptides.2014.01.007>.

Keywords

Crohn's colitis; Inflammation; RNAi; Ca²⁺ signaling; MAPK

1. Introduction

Corticotropin-releasing factor (CRF) and urocortins (Ucn1–3) comprise a family of neuropeptide hormones that are important mediators of stress response. These neuropeptides mediate their effects via at least two known G protein-coupled receptors, CRF₁ and CRF₂. CRF is primarily responsible for regulating and/or initiating stress responses via activation of the hypothalamic–pituitary–adrenal axis [31], whereas the urocortins play a vital role in the recovery response to stress [32].

Inflammation is an essential defense mechanism and a major event in the response to infection, stress and injury. Inflammation is also a key event in the progression of inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis. Although Ucn3 was first believed to be a central regulator of the peripheral stress response, accumulating evidence demonstrates localized, cellular expression of Ucn3 in response to GI inflammation [3,4,8,13,19,23]. Ucn1 is expressed throughout the GI tract; Ucn1 expression increases in a rat model of Crohn's colitis and CRF₂ is important for resolution of inflammation [3]. Exogenous Ucn1 significantly ameliorates colonic inflammation in a murine model of Crohn's colitis, which is accompanied by decreased mRNA expression of inflammatory cytokines including TNF- α and IL-6 [13]. Urocortin2 (Ucn2) is a potent immunomodulator and a vasodilator that induces vascular relaxation and has positive effects on the heart via CRF₂, thus providing protection against heart failure [1,41]. Ucn2 and its high-affinity receptor CRF₂ are expressed in the GI tract under basal conditions and during Crohn's colitis [4].

Ucn3, a member of the CRF family, is a 38 amino acid peptide that in vitro binds to CRF₂ with high affinity, but does not bind to CRF₁ [25]. Central actions of Ucn3 include decrease in ethanol and food intake [40]. Peripheral actions also inhibit feeding behavior and delay gastric emptying in a dose-dependent manner [29,43].

Although Ucn3 mRNA and peptide expression have been reported in pituitary gland, brain, GI tract, pancreas, heart, kidney, and skeletal muscle in humans and rodents [16,25–27,36,42], its systematic localization in the rat GI tract at baseline or during inflammation has not been examined. Moreover, whether Ucn3 exerts pro- or anti-inflammatory effects during Crohn's colitis or if its expression is altered after the knockdown of CRF₁ and CRF₂ by RNA interference (RNAi) at baseline or after induction of colitis has not been studied before. Therefore, we sought to characterize Ucn3 expression at baseline and under inflammatory conditions in the rat colon. We also sought to ascertain the role of Ucn3 and the relative influence of CRF receptors on the development and progression of inflammation in the colon by eliminating CRF₁ and CRF₂ expression using RNAi. The results presented in this manuscript indicate that unlike Ucn1 and Ucn2, Ucn3 follows different kinetics during colitis and that expression of Ucn3 is dependent upon coordinated expression of both CRF receptors, but like Ucn1 and Ucn2, it is ubiquitously expressed in the GI tract. Thus, a

balanced and coordinated expression of CRF receptors is required for proper regulation of Ucn3 at baseline and during inflammation.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (Simonsen Laboratories), weighing 260–280 g were used for our experiments. The rats were individually housed in hanging wire cages in a room that was temperature- and light-controlled. The rats had ad libitum access to food and water, unless otherwise stated, and were given at least 3 days to acclimate to the housing facility before any experiments were performed. All procedures were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

2.2. Basal and TNBS-induced colitis studies

A rectal enema of 2,4,6-trinitrobenzenesulfonic acid (30 mg TNBS) in 50% ethanol was used to induce colitis [7,30], and groups of rats were euthanized on days 1, 3, 6 and 9 after TNBS enema. 50% ethanol was used as a vehicle as this concentration of ethanol is needed to break the mucosal barrier to induce colitis with TNBS (hapten-induced colitis). Rats treated with 50% ethanol were euthanized on days 1 and 3. Naïve and saline-treated rats served as additional controls ($n = 4-7$) and were euthanized on days 3 and 6. The entire affected region of the distal colon was dissected, cleaned, and the middle piece was removed for H&E/immunohistochemical staining, with regions of gross inflammation included for microscopic review. The remaining tissue was pooled and snap frozen in liquid nitrogen for RNA and protein isolation.

2.3. Urocortin 3 treatment

To examine if Ucn3 alters inflammation during TNBS-colitis, Ucn3 (American Peptides, 3 doses of 30 µg/kg) were injected intraperitoneally in rats (Fig. S1). The rats were euthanized on days 3 and 6 after TNBS enema and Ucn3 treatment, and colon tissue was harvested as described [4].

2.4. Immunohistochemistry (IHC)

Regions of the GI tract were cleaned, fixed in 4% paraformaldehyde, postfixed in 30% sucrose, embedded in OCT compound (Sakura Finetek), sectioned (4–6 µm), and thaw-mounted onto Superfrost Plus (Fisher) slides. Sections were incubated overnight at 4 °C with rabbit anti-Ucn3 at a 1:1000 dilution, washed and incubated with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase at a 1:300 dilution. Diaminobenzidine tetrachloride was used for visualization and hematoxylin was used as a counterstain. Sections incubated with secondary antibody, but no primary antibody served as a negative control. A specific antiserum against human Ucn3 was raised in a rabbit injecting tyrosyl-Ucn3 (Sawady Technology, Tokyo, Japan; Custom Synthesis) conjugated with bovine serum albumin (Sigma Chemical Co., St. Louis, MO by carbodiimide (Peptide Institute), as previously reported [34]. The RIA using this Ucn3 antiserum showed 100% cross reaction with human SCP (Peptide Institute) and mouse/rat Ucn-3 (Phoenix Pharmaceuticals, Inc.), but less than 0.001% with CRF, Ucn1, human SRP (Peptide

Institute), and other peptides tested. Serial sections were stained with hematoxylin and eosin and evaluated by a pathologist blinded to the study.

Sections were also stained with hematoxylin and eosin and evaluated by a pathologist in a blinded manner. Histological grading of sections was based on the following characteristics: intra-abdominal adhesions, mucosal ulceration, submucosal thickening, ulcer size, presence or absence of necrosis, immune cell infiltration, edema, and formation of granulation tissue. Grade score reflects the degree of acute inflammation, with higher grade representing more extensive ulceration and necrosis and lower grade representing resolution of inflammation to scar or normal mucosa [3,11].

2.5. Semiquantitative RT-PCR

Total RNA was isolated from distal colon using RNA Stat-60 (Tel-Test) according to the manufacturer's protocol. First-strand cDNA was synthesized from 2 µg of total RNA by using random hexamers and MMLV-RT (Applied Biosystems) in a 20 µl reaction volume. 5 µl of the RT reaction was used as a template for each PCR reaction at 63 °C for 30–35 cycles using rat Ucn3 (forward primer: 5'-ATGCTGATGCCCCACTTACTTCCTG-3', and reverse primer: 5'-CCAATCTGTGCCATGAGTTGAGC-3'), or TNF-α, IL-6 and cyclophilin [4]. PCR product from colon was sequenced to confirm identity. Cyclophilin was selected as an unrelated housekeeping gene for normalization. Band intensities of Ucn3 were quantified from an agarose gel relative to cyclophilin band intensities using NIH ImageJ64.

2.6. RNAi studies

Long double-stranded RNA (dsRNA) for the knockdown of CRF₁ and CRF₂ was transcribed in vitro and were specific for either CRF₁ (dsCRF₁) or CRF₂ (dsCRF₂) and dsRNA against one receptor did not alter expression of the other receptor, but specifically knocked down expression of the cognate protein as shown by us previously [3].

2.7. Western blot analysis

Tissue samples were homogenized in lysis buffer containing protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail (Sigma), and 0.04% Triton X-100. Proteins (40 µg) were separated by SDS-PAGE and transferred to PVDF membranes (Millipore) and blocked for 1 h at room temperature (*LI-COR*). Membranes were incubated with antibodies to phospho-p38 and p38 (Cell signaling, rabbit, 1:1000) overnight at 4 °C. Membranes were washed for 30 min (1× PBS, 0.1% Tween 20) and incubated with goat anti-rabbit secondary antibodies conjugated to IRDye800 (*LI-COR*) (1:10,000, 1 h, room temperature). Blots were analyzed and bands were quantified with the Odyssey Infrared Imaging System (*LI-COR*).

2.8. cDNA construction and transient transfections

HA-tagged CRF₁ pcDNA5.1 was purchased from Missouri S&T cDNA Resource Center (www.cdna.org). FLAG-tagged CRF₂ was cloned in pcDNA5.1. Both constructs were confirmed by sequencing before transfection studies. HEK-293 cells were transiently transfected using Lipo-fectamine™ 2000 according to the manufacturer's guidelines (Invitrogen) with HA-CRF₁ + FLAG-CRF₂ together or individually on coverslips as

previously described by us. Two days after transfection, the cells were fixed using 4% paraformaldehyde (PFA) and incubated overnight at 4 °C with primary antibodies (rabbit anti-HA11, 1:1000), or (mouse anti-FLAG 1:1000; from Sigma–Aldrich), washed and incubated with secondary antibodies (goat anti-rabbit, or -mouse, IgG coupled to rhodamine red-X (RRX) or fluorescein isothiocyanate (FITC), Jackson ImmunoResearch Laboratories). Cells were observed with a Zeiss laser-scanning confocal microscope (LSM Meta 510; Carl Zeiss, Thornwood, NY) using a Fluar Plan Apochromat ×63 oil immersion objective (NA 1.4).

2.9. Measurement of $[Ca^{2+}]_i$

HEK-293 cells were transiently transfected with HA-CRF₁ + FLAG-CRF₂ together or individually and grown on a 96-well plates (25,000 cells were seeded per well; $n = 3$ per condition, repeated twice). $[Ca^{2+}]_i$ with or without stimulation with 100 nM Ucn3 was measured using Fura-2AM on a Flex station microplate reader as described previously [15]. Results are expressed as increase in Ca^{2+} influx peak above baseline values and area under the curves (AUCs). AUCs were calculated using SoftMax Pro 5.0 and represents Ca^{2+} influx × time.

2.10. Statistical analysis

Data were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison tests. Regression analysis was performed using Ucn3 and Ucn2 mRNA as response variable and using histopathology scores as explanatory variables in order to evaluate correlations between response and explanatory variables in control and rats with colitis. Results are represented as means ± SEM. A p value of 0.01 was considered statistically significant.

3. Results

3.1. Ucn3 is ubiquitously expressed in the normal GI tract

Ucn3 mRNA was present throughout the GI tract from the stomach to the rectum, with the exception of the caecum, as shown by RT-PCR (Fig. 1A). mRNA levels were highest in the duodenum and stomach, moderate in the proximal and terminal ileum, and proximal and distal colon, low in the jejunum and rectum, and undetectable in the caecum. The pancreas and kidney, which do not belong to the GI tract, but support digestion, showed high expression of Ucn3 (Fig. 1A). In agreement with the mRNA results, immunoreactive Ucn3 (Ucn3-IR) was also ubiquitously distributed throughout the GI tract (Fig. 1B). Analysis of Ucn3-IR in the distal colon revealed cell-specific distribution. Higher magnification revealed that within the colon, epithelial cells were positive for Ucn3-IR (Fig. 1C), as were colonic crypts (Fig. 1C). Lamina propria showed few support cells that were positive for Ucn3-IR (Fig. 1C). Neurons of the submucosal and myenteric plexus, nerve fibers in the submucosa, endothelial cells lining the blood vessels, and immune cells in Peyer's patch of distal colon stained positive for Ucn3-IR, but goblet cells did not (Fig. 1C). Thus, Ucn3 is ubiquitously present in the GI tract similar to Ucn1 and Ucn2 [3,4]. In the stomach, Ucn3-IR was present in the fundus, main body, the corpus, and the pyloric stomach (Figs. 1B and S2A), but was undetected in controls (Fig. S2B). In the corpus, parietal, but not chief cells

showed Ucn3-IR. Robust Ucn3-IR was present in the pyloric pits, at the base of the crypts and in the submucosa (Fig. S2A).

3.2. Modulation of Ucn3 mRNA in TNBS-induced colitis

Ucn1 and Ucn2 show a biphasic response during colitis [3,4], but expression pattern of Ucn3 during colitis is unknown. Our analysis of Ucn3 mRNA expression during TNBS-induced colitis showed that treatment with 50% ethanol (vehicle) resulted in significant increase in Ucn3 compared to baseline. Average Ucn3 mRNA expression dropped by 80% compared to vehicle control on day 1, further dropped by 88% compared to vehicle on day 3, and remained low on day 6 (Fig. 2A and inset; $p < 0.01$). On day 9, Ucn3 level recovered to baseline levels, but still remained 56% below vehicle (Fig. 2A). Furthermore, Ucn3 mRNA levels showed a positive correlation with histology scores ($r^2 = 0.49$; Fig. S3). In agreement with our RT-PCR findings, Ucn3-IR was reduced in the colonic crypts 3 and 6 days after colitis induction (Fig. 2B) when compared to vehicle control. However, Ucn3-IR was more prominent in the support cells of lamina propria. Colitis resulted in edema of the submucosa that was characterized by infiltrated mixed immune cells positive for Ucn3-IR (Fig. 2B).

3.3. Effects of Ucn3 on body, spleen, thymus weights and colon length

Others and we have reported that Ucn1 actions are anti-inflammatory in the periphery [13,20], but the role of Ucn3 in mediating inflammation in the gut remains unknown. Rats with TNBS colitis failed to gain weight throughout the course of our study (Fig. S4). Exogenous Ucn3 treatment during TNBS colitis did not further change body weight gain or loss in the early phase (days 1–3), but by day 6, rats treated with Ucn3 showed a significant decrease in body weight gain compared with TNBS treatment alone, or vehicle controls (Fig. S4).

The spleen plays an important role in defending the body against foreign microbes. Under stress, the spleen often enlarges. As expected, TNBS treatment alone resulted in a 23% increase of spleen weight on day 3 (Fig. S5A). Exogenous Ucn3 treatment had no significant effect on spleen weight at baseline or during colitis (Fig. S5A).

The thymus is the major site of T cell production and a key organ of the immune system. Unlike the spleen, the thymus often shrinks during stress and that shrinkage is directly proportional to the degree of stress and increase in plasma adrenocorticotrophic hormone and corticosterone levels [33]. TNBS treatment resulted in significant decrease of thymus weight on days 3 and 6 (Fig. S5B; $p < 0.01$). Surprisingly, Ucn3 treatment prevented colitis-associated decrease in thymus weights (Fig. S5B). Importantly, thymus weights of rats treated with saline alone or with saline + Ucn3 were comparable (Fig. S5B).

Gross examination of the colon revealed that TNBS colitis resulted in shortening and thickening of the colon compared to vehicle control (Fig. S5C). However, treatment with Ucn3 did not result in significant change in the colon length (Fig. S5C).

3.4. Ucn3 does not affect histopathological outcomes during TNBS-induced colitis

Histological examination of rats treated with TNBS alone or with Ucn3 + TNBS revealed severe transmural colon inflammation with mucosal necrosis. On day 3, inflammatory cells were mixed, with abscess formation. In the patches of viable mucosa, there were no focal crypt abscesses or granulomas (Fig. 3A). By day 6, the acute inflammation shifted to early chronic. There were no obvious differences in edema or fibrosis between these groups. Naïve rats and those treated with saline + Ucn3 showed normal colon histology (Fig. 3B).

Alterations in the levels of Th1-type pro-inflammatory cytokines such as TNF- α and IL-6 are a hallmark of Crohn's disease. TNBS colitis resulted in significant increases in TNF- α and IL-6 mRNA expression on days 3 and 6 and Ucn3 treatment did not alter the levels or kinetics of TNF- α or IL-6 mRNA levels during colitis or at baseline (Fig. S6). Thus, unlike Ucn1, Ucn3 does not alter histopathological outcome during colitis or cytokine signaling associated with Crohn's colitis.

3.5. Altered Ucn3 mRNA levels after CRF receptor knockdown at baseline

We previously reported that knockdown of CRF₂, but not CRF₁, affects Ucn1 mRNA levels in the colon during colitis, whereas the baseline Ucn1 expression was not altered by the knockdown of either CRF₁ or CRF₂ in the colon [3]. We were therefore surprised to find that knockdown of CRF₁, but not CRF₂, resulted in a 78% decrease of Ucn3 baseline levels when compared with Ucn3 levels in naïve or saline-treated rats (Fig. 4A; $p < 0.01$). Because this was an unexpected finding, we next determined if expression of Ucn2 mRNA was also altered after receptor colon-specific knockdown. Ucn2 mRNA levels were not affected by knockdown of either CRF₁ or CRF₂ (Fig. 4B). Thus, CRF₁ at baseline appears to derepress Ucn3, but not Ucn2 or Ucn1, whereas during colitis, Ucn2 and Ucn3 show varying degrees of association with inflammation (Fig. S3).

3.6. CRF receptor knockdown in the colon alters Ucn3 expression during colitis

Although TNBS treatment alone significantly decreased Ucn3 mRNA levels (Fig. 2A), levels increased significantly in rats treated with TNBS + siCRF₁ ($p < 0.01$), on day 3, but not in those treated with TNBS + siCRF₂ (Fig. 5A). By day 6, however, Ucn3 mRNA levels remained high in rats treated with TNBS + siCRF₁, but decreased by 88% ($p < 0.01$) in rats treated with TNBS + siCRF₂ as compared with TNBS alone (Fig. 5B). In contrast to Ucn3 levels, Ucn2 levels decreased by 63% in the TNBS + siCRF₁ group and by 71% in the TNBS + siCRF₂ group on day 3 (Fig. 5C; $p < 0.01$). On day 6 of TNBS-induced colitis, Ucn2 levels decreased, in agreement with its biphasic expression, but remained unaltered in rats treated with siCRF₁ + TNBS or siCRF₂ + TNBS (Fig. 5D). Thus, a coordinated expression of both CRF receptors is essential for regulation of urocortins during inflammation.

3.7. CRF₁ cross-talk with CRF₂ attenuates Ucn3-mediated intracellular calcium signaling

Because knockdown of CRF₁, but not CRF₂ at baseline decreased Ucn3 mRNA, we next addressed possible mechanisms of cross-talk between the two CRF receptors. HEK-293 is an established model system for investigating the signaling of CRF₁ and CRF₂ receptors and

naturally expresses these two receptors at low levels [10]. HEK cells were transiently transfected with either both receptors together (CRF₁ + CRF₂) or with individual CRF receptors. Confocal microscopy demonstrated equivalent level of cell surface expression of CRF receptor (s) in HEK cells transfected with individual CRF receptors or co-transfected with both receptors (Fig. 6A). As expected, challenge with 100 nM Ucn3 elicited a robust intracellular Ca²⁺ response in the presence of CRF₂, but not CRF₁ (Fig. 6B). When CRF₁ and CRF₂ were co-transfected, Ucn3-mediated CRF₂ intracellular Ca²⁺ peak was attenuated by 48% (Fig. 6B, $p < 0.01$). The kinetics of Ca²⁺ flux were similar, however, the area under curve was decreased by 51% in cells co-transfected with CRF₁ + CRF₂ as compared to CRF₂ alone (Fig. 6C, $p < 0.01$), suggesting that CRF₁, when co-present with CRF₂, opposes CRF₂-mediated Ca²⁺ signaling in the presence of Ucn3.

3.8. Modulation of p38 phosphorylation during TNBS induced colitis

Upon receptor-binding, urocortins signal via downstream activation of mitogen-activated protein kinases (MAPK) that includes increased phosphorylation of ERK, JNK, and p38 kinases. Regulation of ERK and JNK kinases during colitis has been reported [34], but whether Ucn3 modulates the p38 kinase signaling pathway is unknown. We found that phospho~p38 levels were elevated by 252% ($p < 0.05$) over baseline on day 3 of colitis and fell to basal levels by day 6 (Fig. 7). Ucn3 injection significantly decreased phospho~p38 by 57% on day 3 of TNBS-induced colitis (Fig. 7; $p < 0.05$) compared to TNBS treatment alone. The phospho~p38 levels decreased further on day 6 of colitis in rats treated with Ucn3 + TNBS, but were comparable to the level in rats treated with TNBS alone.

4. Discussion

Accumulating evidence demonstrates localized, cellular expression of urocortins in response to GI inflammation. Others and we have shown that Ucn1, Ucn2 and CRF₂ are required for normal resolution of GI inflammatory diseases like colitis [3–5,19,21–23,37,39]. Loss of CRF₂ leads to increased inflammation, delayed healing, and exacerbates the inflammatory insult [3,14,17].

It is known that infusion of Ucn1 can ameliorate both colonic and pancreatic inflammation [13,20]. Whether Ucn3 is involved in the pathogenesis of GI disorders, such as Crohn's colitis has not been studied before. We therefore sought to characterize Ucn3 expression at baseline and under inflammatory conditions in the rat colon. We found Ucn3 expression throughout the GI tract; although the lamina propria of the duodenum, jejunum and colon stained positive for Ucn3-IR, epithelial cells, colonic crypts of proximal and distal colon were strongly positive for Ucn3-IR, suggesting a role in gut motility and absorption. Peyer's patch and lymphoid follicles in the lamina propria and sub-mucosa of colon were positive for Ucn3-IR, suggesting a role in regulating inflammation. Ucn3-IR was observed in the parietal, but not the chief cells of the body of the stomach, but its functional significance in the stomach remains to be established. Ucn3-IR was also found in the myenteric nerve plexus of the stomach, which may explain why exogenous Ucn3 increases gastric emptying.

Our finding that Ucn3 treatment significantly prevented body weight gain during colitis can be explained by previous studies that show Ucn3 decreases food intake [43] and gastric

emptying [29] and/or due to its natriuretic action under stress [35]. In our study, Ucn3 did not ameliorate or exacerbate inflammation as assessed by histopathology and changes in inflammatory cytokines such as TNF- α and IL-6; nonetheless, the significant effect of Ucn3 on thymus weight suggests the presence of some important actions of Ucn3 on thymus-related immunity, which needs further investigations. Furthermore, the dose we used in our study is in the range that was effective in delaying gastric emptying in rats, but had no effect on colonic transit [29], thus it is possible that a much higher dose of Ucn3 than Ucn1 is required to have an effect on inflammation. In our study, after TNBS colitis was induced, Ucn3 mRNA levels decreased significantly in the early phase, but Ucn3 levels recovered in the late phase. This pattern contrasts with our findings for Ucn1 and Ucn2, which showed a biphasic expression pattern in the early and mid phase of colitis [3,4]. Vehicle had an effect on regulating the expression of not just Ucn3, but Ucn1 and Ucn2 as well [3]. Therefore, we used tissue from naïve and/or saline-treated animals as additional controls. Furthermore, while Ucn1-IR was elevated, Ucn3-IR in GI tissues from patients with ulcerative colitis did not differ significantly from those of non-IBD patients; tissue from Crohn's patients was not examined [36]. Moreover, Ucn3 mRNA showed a correlation with histology score ($r^2 = 0.495$), suggesting that changes in Ucn3 seen during colitis are secondary to changes in expression of other urocortins (Ucn1–2) or CRF₂, creating a feedback loop. Thus, unlike Ucn1 that reduces inflammation during colitis [13] or acute pancreatitis in a gender-specific manner [20], Ucn3 does not appear to have an overt role in the initiation or prevention of inflammation.

Our finding that colon-specific knockdown of CRF₁, but not CRF₂, at baseline significantly altered Ucn3, but not Ucn2 expression is striking, because neither Ucn3 nor Ucn2 bind CRF₁ in an in vitro binding assay [25]. In contrast, knockdown of CRF₂, but not CRF₁ during colitis decreased Ucn3 levels. Thus, local and transient knockdown of CRF₁ represses Ucn3 at baseline, but allows for an uncontrolled increase in expression during inflammation and/or colitis. While a thorough analysis of the mechanistic basis of CRF₁ responses in the gut is beyond the scope of this study, we addressed one possible signaling mechanism by which the presence of CRF₁ may oppose responses of CRF₂ in the presence of a specific ligand. We postulate that CRF₁ and CRF₂ directly or indirectly interact at the plasma membrane and exist as dimers, to coordinate receptor cross-talk in the presence of multiple ligands, both at baseline and during inflammation. In agreement with our hypothesis, Ucn3 evoked a robust intracellular Ca²⁺ response in the presence of CRF₂ alone, but when the two receptors were present at the same time, CRF₁ attenuated CRF₂-mediated Ca²⁺ signaling in response to Ucn3 challenge. CRF₁ can heterodimerize with other GPCRs to mediate signaling; CRF₁ endocytosis and recycling was shown to enhance 5-HT_{2R}-mediated signaling during anxiety-related behavior [28]. Recently, the CRF-CRF₁ system was shown to interact with the nectin–afadin complex to mediate effects of stress on memory [45]. These findings suggest a yet another mechanism by which CRF₁ and CRF₂ cross-talk is essential in keeping the Ucn2/Ucn3 system under homeostasis at baseline and during recovery from an inflammatory insult. We thus propose that CRF receptors do not work in isolation in the gut, and that a coordinated expression of both receptors is key in mediating signaling and actions of the CRF and urocortin peptides [2] and important for a comprehensive response to stress and recovery from insult.

Upon receptor-binding, urocortins signal via downstream activation of MAPKs that includes phosphorylation of ERK, JNK and p38 kinases. The regulation of ERK and JNK kinases during colitis has been reported [3,6,38]. Colitis induced phosphorylation of p38 kinase. Exogenous Ucn3 did not alter baseline phosphorylation of p38 kinase, but significantly attenuated p38 phosphorylation during colitis in the early phase. Studies using the p38 inhibitors have indicated that phospho-p38 increases significantly in IBD tissue [9,44]. p38 is also required for the phosphorylation of heat shock proteins (Hsp) such as Hsp27 and Hsp70, which plays a role in protection against cellular damage and recovery response to stress [12,18,24]. Thus, multiple signals feed to activate the MAPK/p38 kinase pathways that may modulate different aspects of inflammation. Our results provide a basis to delineate the role Ucn3 might play in healing from stressful and inflammatory insults.

In conclusion, our study delineates the role of Ucn3 in Crohn's colitis and shows that a balanced and coordinated expression of CRF receptors is required for proper regulation of urocortins. Thus, when designing therapeutic interventions for either receptor, it may be important to keep this CRF receptor cross-talk in mind.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

CRF₁/CRF₂	corticotropin-releasing factor receptor 1 and 2
GI	gastrointestinal
IR	immunoreactivity
TNBS	2, 4, 6-trinitrobenzenesulfonic acid
Ucn 1, 2, 3	urocortin 1, 2 & 3

Fig. 1.

Ubiquitous expression of Ucn3 in the GI tract of naïve rats. (A) RT-PCR confirmed the presence of Ucn3 in the GI tract from the stomach to the rectum with the exception of caecum, where no Ucn3 was detected; cyclophilin, a housekeeping gene, was present in all regions examined. M, marker; S, stomach; D, duodenum; J, jejunum; PI, proximal ileum; TI, terminal ileum; PC, proximal colon; DC, distal colon; R, rectum, Ce, caecum; -ctrl; negative control, K; kidneys, P; pancreas. (B)

Immunohistochemistry confirmed the presence of Ucn3-immunoreactivity (Ucn3-IR) throughout the GI tract. In duodenum, jejunum, proximal colon, and the distal colon, Ucn3-IR was present in epithelial lining cells of the villi and the crypts, stromal cells of the lamina propria (LP). Ucn3-IR was also present in the smooth muscle cells of the circular muscle (CM), but absent in

goblet cells (G). G: goblet cells; LM: longitudinal muscle; PP: Peyer's patch; SM: Submucosa. (C) The presence of Ucn3-IR in the crypts, endothelial cells of the blood vessels, and the neurons of the myenteric plexus (MP).

Fig. 2.

Modulation of Ucn3 mRNA during colitis. (A) Semiquantitative RT-PCR revealed that treatment with 50% ethanol (vehicle) resulted in significant increase in Ucn3 (a: $p < 0.01$: basal Ucn3 vs. vehicle). Ucn3 mRNA levels decreased significantly compared to vehicle-treated controls (b: $p < 0.01$: vehicle vs. 1d, 3d, 6d and 9d), or basal (c: $p < 0.01$: basal vs. 3d and 6d) and recovered to basal levels by day 9. Ucn3 mRNA levels were normalized against cyclophilin (Cyclo) mRNA, a housekeeping gene. Inset shows the kinetics of Ucn3 mRNA over days after TNBS treatment. (B) The presence of Ucn3-IR in the distal colon (DC) in vehicle- and TNBS-treated rats. CM; circular muscle; G; goblet cells; LM: longitudinal muscle; MP; myenteric plexus SM; sub mucosa. Inset: higher magnification images of the crypts and myenteric plexus showing expression of Ucn3-IR.

Fig. 3.

Ucn3 administration does not ameliorate or exacerbate histopathology. Histological examination of rats treated with (A) TNBS alone, or (B) with Ucn3 + TNBS revealed severe transmural colon inflammation with major mucosal necrosis on days 3 and 6. No obvious differences were seen in the quantity of edema or fibrosis between the two groups. Naïve rats and those treated with saline + Ucn3 showed normal colon histology. CM: circular muscle; LM; longitudinal muscle; SM: sub mucosa.

Fig. 4.

Altered Ucn3 and Ucn2 levels at baseline after CRF₁ or CRF₂ knockdown by RNAi: (A) Knockdown of CRF₁ (dsCRF₁) at baseline resulted in a 78% decrease of Ucn3 mRNA levels (a: $p < 0.01$: basal vs. dsCRF₁). Knockdown of CRF₂ (dsCRF₂) did not significantly change Ucn3 levels from baseline but were different from dsCRF₁ group (b: $p < 0.01$: dsCRF₁ vs. dsCRF₂). (B) Knockdown of CRF₁ (dsCRF₁) or CRF₂ (dsCRF₂) at baseline did not result in a significant decrease of Ucn2 levels.

Fig. 5.

Modulation of Ucn3 and Ucn2 mRNA during colitis after CRF₁ or CRF₂ knockdown by RNAi: (A) three days after induction of colitis, Ucn3 mRNA levels in rats treated with dsCRF₁ increased compared with those treated with dsCRF₂ or TNBS alone (a: $p < 0.01$: 3d TNBS vs. 3d TNBS + dsCRF₁; b: $p < 0.01$: dsCRF₁ vs. dsCRF₂). (B) Six days after induction of colitis, Ucn3 levels remained high in rats treated with siCRF₁ (a: $p < 0.01$), but decreased significantly in those treated with dsCRF₂ + TNBS group compared with TNBS alone (b: $p < 0.001$). (C) dsCRF₁ or dsCRF₂ resulted in a significant decrease of Ucn2 levels compared with 3d TNBS alone (a: $p < 0.01$: 3d TNBS vs. 3d TNBS + dsCRF₁/dsCRF₂). (D) Ucn2 levels dropped by 50% after 6 days of

TNBS treatment alone, but on day 6, remained unaltered from day 3 levels for $\text{dsCRF}_1 + \text{TNBS}$ or $\text{dsCRF}_2 + \text{TNBS}$. Additionally, *Ucn2* mRNA levels on day 6 were comparable in all groups examined.

Fig. 6.

CRF₁ attenuates Ucn3-mediated CRF₂ intracellular calcium signaling. HEK cells were transiently transfected with either both CRF₁ + CRF₂ together or with individual CRF receptors: (A) equivalent levels of cell surface expression of CRF₁ or CRF₂ in HEK cells transfected with individual receptors (top panel), or co-transfected together (bottom panel). Merge image (yellow) shows that both CRF₁ and CRF₂ receptors co-localize at the cell surface (bottom panel). DAPI: nuclear stain. (B) Challenge with 100 nM Ucn3 elicited a robust intracellular Ca²⁺ response in the presence of CRF₂, but not CRF₁ receptor. When CRF₁ and CRF₂ receptors were co-transfected, Ucn3-stimulated Ca²⁺ peak responses were attenuated by 48% (**p* < 0.01) compared to CRF₂ alone. (C) Kinetics of Ucn3-mediated Ca²⁺ responses in CRF₂ alone transfected cells (left) vs. CRF₁ + CRF₂ co-

transfected cells (right); representative tracing are shown. Area under the curves (AUCs) was calculated and shows 51% decreases between the two groups ($p < 0.01$).

Fig. 7.

Modulation of p38 phosphorylation. Three days after TNBS colitis, p38 was activated by 250% (p~p38) (a: $p < 0.05$) compared to baseline. Ucn3 treatment significantly attenuated p~p38 at 3 days compared with 3 days of TNBS alone (b: $p < 0.05$).