Urocortin 1 modulates immunosignaling in a rat model of colitis via corticotropin-releasing factor receptor 2

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Urocortin 1 modulates immunosignaling in a rat model of colitis via corticotropin-releasing factor receptor 2. Am J Physiol Gastrointest Liver Physiol 300: G884–G894, 2011. First published February 17, 2011; doi:10.1152/ajpgi.00319.2010.—Urocortins (UCNs) and their receptors are potent immunoregulators in the gastrointestinal (GI) tract, where they can exert both pro- and anti-inflammatory effects. We examined the contribution of Ucn1 and its receptors to the pathogenesis, progression, and resolution of colitis. Trinitrobenzene sulfonic acid was used to induce colitis in rats. Ucn1 mRNA and immunoreactivity (IR) were ubiquitously expressed throughout the GI tract under basal conditions. During colitis, Ucn1 mRNA levels fell below basal levels on day 1 then increased again by day 6, in association with an increase in the number of Ucn1-IR inflammatory cells. Ucn1-IR cells were also numerous in proliferating granulation tissue. In contrast to Ucn1 expression, average phosphorylated ERK1/2 (pERK1/2) expression rose above controls levels on day 1 and was very low on day 6 of colitis. Knockdown of corticotropin-releasing factor 2 (CRF2) but not CRF1 by RNA interference downregulation of the Th1-driven autoimmune response. In mice, Ucn1 agonists downregulated the production of inflammatory mediators by endotoxin-activated macrophages and protected against lethality after cecal ligation and puncture, or after bacterial endotoxin were injected. In contrast, the number of Ucn1-positive cells in the lamina propria of patients with ulcerative colitis was positively correlated with inflammation severity and was decreased after glucocorticoid treatment. Likewise, the expression of Ucn1 mRNA in synovia of patients with rheumatoid arthritis was greater than that of patients with osteoarthritis. The complexity of Ucn1 activity in the GI tract can be partially attributed to the differential distribution of its two receptors, CRF1 and CRF2. CRF1, which is associated with the central nervous system response to stress-induced secretion of CRF, is expressed at low levels in the periphery. In contrast, CRF2 is associated with stress-coping behavior and is robustly expressed in the periphery and throughout the GI tract. CRF receptors belong to the family of G protein-coupled receptors, which can be classified according to their interaction with β-arrestins (βARRs). Class “A” receptors (CRF1/2, β2-adrenergic and μ-opioid receptors) show preferential binding to βARR2 over βARR1, interact transiently with βARRs, and usually do not colocalize with βARRs in endosomes. Class “B” receptors (e.g., neurokinin 1 receptor) show equal affinity for βARR1 and βARR3, form sustained interactions, and internalize as a stable complex colocalizing in endosomes for extended periods. CRF1/2 couple to Gαq/adenylyl cyclase/cAMP (18, 20, 49) and Gαi (39), and activated receptors can initiate multiple signaling cascades, such as mobilization of intracellular Ca2+ (5, 16, 19, 40), and activation of kinase signaling pathways that include the protein kinase C (PKC), p44/p42, and p38 mitogen-activated protein kinase (MAPK).

In models of hypoxia and ischemia-reperfusion of the heart, when Ucn1 binds to CRF receptors it can activate a myriad of signaling cascades downstream resulting in activation of phosphoinositide-3-kinase, PKC, and MAPKs. The extracellular signal-related kinase (ERK) is part of a larger family of

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MAP kinases. Its phosphorylation (pERK1/2) and activation are mediated by the MAP kinase MEK1/2 after mediators, including Ucn1, activate MEK1/2. Ucn1 has been shown to protect the rodent heart after reperfusion injury by activating this pERK1/2-dependent signaling pathway (44). Repeated colorectal distention results in altered phosphorylation of pERK1/2 in laminae I and II of spinal cord (32). Although the role of Ucn1-mediated changes in pERK1/2 during cardioprotection is well defined (12), its role in inflammatory bowel disease or inflammation is less well studied.

Distribution of Ucn1 in the GI tract has not been systematically described. Since IBD can affect any segment of the GI tract, and if Ucn1 is a key player in the pathophysiology of this disease, its localization in the GI is the first step in this characterization and is therefore of interest and importance. Next, in a rat model of colitis, we sought to ascertain the role of Ucn1 and its two receptors in the development and progression of inflammation. To analyze the relative influence of CRF1 and CRF2 on the development and progression of inflammation in the colon, we eliminated their expression before we induced colitis using RNA interference (RNAi). Our results show for the first time that, during colitis in rats, average Ucn1 mRNA levels fall on day 1 of treatment and then rise again, whereas pERK1/2 levels show an inverse relationship with Ucn1 mRNA levels. This inverse relationship is crucial for a normal immune response.

**MATERIALS AND METHODS**

**Animals.** Adult male Sprague-Dawley rats (Simonsen Laboratories) weighing 260–280 g were used for all experiments. The rats were housed in a room that was temperature (22°C) and light controlled (12-h light:12-h dark cycle starting at 7 AM) and were given at least 3–5 days to acclimate to the housing facility before any experiments began. They had ad libitum access to food and water and were handled daily to avoid handling act as a stressor, unless otherwise stated. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**TNBS model.** A rectal enema of 30 mg 2,4,6-trinitrobenzenesulfonic acid (TNBS) in 50% ethanol in a final volume of 250 μl was used to induce colitis as described previously (10, 34). A 50% ethanol enema served as a vehicle control. Rats that received a saline enema served as a vehicle control. Rats that received a saline enema and naive rats served as additional controls. Groups of rats were then euthanized and portions were used for myeloperoxidase (MPO), protein, and RNA analyses. Tissue for MPO was weighed before being snap frozen in liquid nitrogen, whereas tissue meant for RNA and protein was prompted frozen in liquid nitrogen.

**Immunohistochemistry and histological evaluation.** Regions of the GI tract were cleaned, fixed in 4% paraformaldehyde, and postfixed in 30% sucrose, embedded in OCT compound (Tissue-Tek, Sakura Finetek), sectioned (4–6 μm), and thaw-mounted onto Superfrost Plus (Fisher) slides. Sections were incubated overnight at 4°C with either a rabbit anti-Ucn1 (Sigma, and a generous gift of Dr. Kazuhiro Takahashi, Sendai, Miyagi, Japan [48]) at a 1:1,000 dilution or rabbit anti-PGP9.5 (Biogenesis) at 1:200 dilution. Sections were washed and incubated with or without goat anti-rabbit secondary antibody conjugated to horseradish peroxidase at a 1:300 dilution (for Ucn1) or goat anti-rabbit (1:200) conjugated to Rhodamine Red-X (Jackson ImmunoResearch). For Ucn1-stained sections, diaminobenzidine tetrachloride was used for visualization and hematoxylin was used as a counterstain. Blocking peptide for Ucn1 (Sigma) was used to preabsorb the primary antibody and served as a negative control (Supplemental Fig. S1; the online version of this article contains supplemental data). Specificity of this Ucn1 antibody and absence of its cross-reactivity with CRF1, Ucn2, and Ucn3 has been previously described (2). Sections were also stained with hematoxylin and eosin and evaluated by a pathologist in a blinded fashion. Histological grading of sections included crypt destruction, immune cell infiltration, edema, and formation of granulation tissue (13).

**Semiquantitative RT-PCR.** Total RNA was isolated from snap-frozen tissue by using TRIzol (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. First-strand cDNA was synthesized from 2 μg of total RNA by using random hexamers and Moloney murine leukemia virus-RT from GeneAmp (Applied Biosystems) in a 20 μl reaction volume. Five or 10 μl of the RT reaction was used as template for each PCR reaction at 67°C for 30–35 cycles using either rat Ucn1 (forward primer: 5’-CTCCTGTAGGCTTGGCTGTCTCT-3’; reverse primer sequence was 5’-GCCCACTGGAATGATAGTGC-3’), TNF-α, or cyclophilin (6). PCR product from colon was sequenced to confirm identity. RT-PCR for TNF-α was performed with conditions and primer sequences described previously (6). Cyclophilin was selected as an unrelated housekeeping gene for normalization. PCR products were analyzed by agarose gel electrophoresis, and band intensities of Ucn1 and TNF-α were quantified relative to cyclophilin band intensities via ImageJ64 (National Institutes of Health [NIH]).

**Synthesis of long double-stranded RNA/siRNA.** The cloned cDNAs for CRF1 or CRF2 were used as templates to transcribe sense and antisense RNAs in vitro using the Megascript RNA kit (Ambion) as described previously (19). HaeIII-digested (HaeIII 4) epitope-tagged full-length CRF1 or CRF2 were cotransfected into HEK cells seeded on coverslips, along with 5 μg of small interfering (si)CRF1, or siCRF2, and 5 μl of Lipofectamine 2000. Three days after transfection, cell were fixed and then incubated overnight at 4°C with rabbit anti-HA primary antibody (1:500, Sigma). Cells were then washed and incubated with goat anti-rabbit secondary antibody (1:200) conjugated to Rhodamine Red-X (Jackson ImmunoResearch). Immunofluorescence and confocal microscopy were used to confirm knockdown of receptor expression and cross-reactivity.

**RNAi studies.** Rats were anesthetized with 2% isoflurane, and a midline laparotomy was performed. The descending colon was exposed, and a marking suture was placed in the colon wall at the splenic flexure. Fifteen micrograms of double-stranded RNA (dsRNA) for CRF1 (siCRF1) or CRF2 (siCRF2) were mixed with 1.5 μl of Lipofectamine 2000 and injected intramuscularly at two sites into the colon wall as described previously (10). Rats were euthanized at 3 days after RNAi treatment to assess effect of CRF receptor knockdown under basal conditions. Groups of rats with simultaneous RNAi injections and TNBS treatment were euthanized 3 or 6 days after.

**Western blot analysis.** Tissue samples were homogenized in Tris-HCl, pH 7.4 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 polyvinylidene difluoride membranes (Immobilon-FL, Millipore) and blocked for 1 h at room temperature ( Odyssey Blocking Buffer, LiCOR). Membranes were incubated with antibodies either to pERK1/2 (E-4, mouse, 1:1,000) and ERK2 (C-14, rabbit, 1:5,000;
Santa Cruz) or to goat CRF-RI/II (C-20, 1:1,000; Santa Cruz) and mouse β-actin (1:10,000; Sigma) overnight at 4°C. Membranes were washed for 30 min (1× PBS, 0.1% Tween 20) and incubated with secondary antibodies goat anti-mouse or donkey anti-goat conjugated to AlexaFluor680 (Invitrogen) and goat anti-rabbit or donkey anti-mouse conjugated to IRDye800 (Rockland Immunocchemicals) (both 1:20,000, 1 h, room temperature). Blots were analyzed with the Odyssey Infrared Imaging System (LI-COR).

MPO assay. MPO activity was measured using a modified 3,3',5,5'-tetramethylbenzidine (TMB) assay (47). Snap-frozen tissue was homogenized in hexadecyltrimethylammonium bromide and 50 mM KH₂PO₄ (pH 6.0). This suspension was then sonicated and centrifuged. The supernatant was freeze-thawed and assayed using the TMB Liquid Substrate System (Sigma). Human MPO with known activity was used as a standard. MPO activity was then normalized to the weight of tissue sample used.

Statistical analysis. Data were analyzed first by one-way ANOVA followed by Newman-Keuls multiple comparison. Results are represented as means ± SE. A P value of ≤0.05 was considered statistically significant.

RESULTS

Ucn1 is ubiquitously expressed in the normal GI tract. RT-PCR revealed that Ucn1 mRNA was present throughout the GI tract from the esophagus to the rectum (Fig. 1A). In agreement with the RNA data, Ucn1-IR was also ubiquitously distributed throughout the GI tract (Fig. 1B). Because of our

Fig. 1. Urocortin 1 (Ucn1) is ubiquitously present throughout the gastrointestinal (GI) tract. A: RT-PCR confirmed presence of Ucn1 in all regions of the GI tract from the esophagus to the rectum. M, marker; O, esophagus; D, duodenum; J, jejunum; Ce, cecum; St, stomach; PI, proximal ileum; TI, terminal ileum; PC, proximal colon; DC, distal colon; R, rectum. B: immunohistochemistry confirmed presence of Ucn1-immunoreactivity (IR) throughout the various regions of GI tract. Muc, mucosa; Mus, muscularis; PP, Peyer’s patch. C: in the distal colon, Ucn1-IR was present in columnar epithelial lining cells, the cuboidal epithelial cells of the crypt base, mononuclear cells of the lamina propria, and the nuclei of the endothelial cells of the lymphatics, blood vessels, and smooth muscles. Muc, smooth muscle cells; MP, myenteric plexus; LP, lamina propria; Epi, epithelium; C, crypt. D: Ucn1-IR in distal colon, 3 days after TNBS exposure. All layers are swollen with edema and infiltrated by strongly Ucn1-IR mixed inflammatory cells. Epithelial lining cells are immature (cuboidal) rather than mature columnar cells, and brush border and goblet cells are lost. The LP and the myenteric plexus are edematous and inflamed, with scattered neurons that remain positive for Ucn1-IR.
interest in inflammatory diseases of the bowel, we focused our detailed analysis for the spatiotemporal distribution of Ucn1 in the bowel. Robust levels of Ucn1-IR were present in the terminal ileum, proximal colon, and distal colon. Higher magnification revealed that within the colon and the ileum epithelial cells were strongly positive for Ucn1-IR (Fig. 1C and Supplemental Fig. S1). Support cells in the muscularis and endothelial cells that line the lymphatic and blood vessels were also positive (Fig. 1C). Immune cells of the Peyter’s patches were strongly immunoreactive for Ucn1 within all cells of the aggregate (Fig. 1B, PP) and scattered immune cells of the lamina propria were positive (Fig. 1C). Neurons of the submucosal and myenteric plexus stained strongly positive throughout the GI tract (Fig. 1B).

Time course of spatiotemporal changes in Ucn1-IR during colitis. TNBS exposure led to a full acute inflammatory reaction by day 3, which progressed to granulation tissue by day 6 (Fig. 2A). Increased immune cell infiltration, submucosal thickening, and edema were evident after TNBS treatment compared with vehicle controls, resulting in average elevated histological scores in all groups on all days examined (Fig. 2B) during colitis with both days 3 and 6 (*P < 0.0001) being statistically significant from vehicle controls.

Next, we ascertained whether Ucn1-IR in the distal colon was altered during the acute-to-mid phase of colitis (1–6 days). All cell types that had been Ucn1 positive at baseline remained positive on day 3 and throughout the course of the study (Fig. 1D and data not shown). Ucn1-IR immune cells were abundant in ulcerating and inflamed tissue. In the regions where colonic mucosa was not necrotic, columnar luminal absorptive cells remained positive for Ucn1-IR, as did the less mature, proliferative cuboidal cells. By day 6, submucosa was thickened by granulation tissue infiltrated with a mix of Ucn1-IR immune cells, proliferating fibroblasts, angioblasts, and myoblasts. The neural plexus remained strongly Ucn1 positive throughout the acute and healing stages; the number of visible proliferating neurons and their extensions increased dramatically in the late granulation tissue (Supplemental Fig. S3). After TNBS treatment, but before the Ucn1-IR-positive inflammatory cells accumulated, massive colonic neural plexus and neural fiber disruption were evident when the tissue was stained with a neuronal marker, PGP9.5 (Supplemental Fig. S3). The drop in the number of surviving neurons on day 6 was striking, but by late phase the neuronal architecture appeared to return to normal organization, with the mesenteric nerve plexus beginning to align parallel to the musculature; however, intramuscular and lamina propria nerve fibers remain unorganized (Supplemental Fig. S3).

Kinetics of Ucn1 mRNA in TNBS-induced colitis. After colitis was induced with TNBS, average Ucn1 mRNA expression dropped by half on day 1 compared with vehicle or naïve controls. The levels then rose again by approximately twofold over day 1 levels on day 6 (P < 0.0001, 1 day vs. 6 days), a period midinflammation in which acute inflammation is transitioning to granulation tissue (Fig. 3A). Because ethanol (50%) used as vehicle control has been shown to alter expression of other genes (10) and the signaling cascade or can cause acute colonic damage on its own (34), we also examined the effect of 50% ethanol on Ucn1 mRNA levels. We found that it did not significantly alter Ucn1 mRNA expression compared with levels in the colons of naïve rats on the days tested (Fig. 3A).

pERK1/2 levels inversely correlate with Ucn1 expression levels during the course of inflammation. In patients with Crohn’s disease, activation of JNK and ERK is increased (9, 43). pERK1/2 has been observed after CRF receptors are activated (32, 40). Increases in Ucn1 expression during the course of colitis is expected to activate Ucn1 receptors. We therefore determined the time course of pERK1/2 activation between 1 and 6 days after a single bolus of TNBS. We found that the ratio of pERK1/2 over total ERK increased over approximately twofold in the acute phase of colitis on days 1 and 3 (Fig. 3B), whereas as total ERK remained unchanged.

![Fig. 2. Microscopic evaluation of the progression of inflammation during colitis. A: representative histological samples of inflammation progression over time. 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. B: graphic representation of average and SE of the grade score from each group. *P < 0.0001 vehicle (Veh) vs. 3 and 6 days (3d and 6d, respectively) 2,4,6-trinitrobenzenesulfonic acid (TNBS).](image-url)
when Ucn1 levels are high.pression: high on expression falling by 10-fold over that in saline controls. Thus
expression intact (Fig. 5, control, left). In cells cotransfected with CRF2, robust expression of either receptor was evident by immunofluorescence (Fig. 5B, control, left). In cells cotransfected with CRF1 sequences, CRF1 expression was significantly reduced (Fig. 5B, middle and top, C, left), whereas cotransfection with CRF2 sequences left CRF1 expression intact (Fig. 5B, right, top). Similarly, in cells cotransfected with CRF2 and CRF3 sequences, CRF2 expression was markedly inhibited (Fig. 5B, right, top and C, right), whereas cotransfection with CRF1 sequences did not inhibit CRF2 expression (Fig. 5B, middle, top). In vitro, RNAi resulted in 30–70% knockdown of the target receptor depending on transfection efficiency of the receptor and dsRNA (Fig. 5C). Since transfection of HEK cells with HA-tagged CRF receptors is a heterologous expression system, compensatory changes in receptor expression are not expected, but downregulation of receptor due to cross-reactivity of sequences, if present, would be visible. We next determined that dsRNA sequences used in HEK cells against CRF receptors also knocked down protein expression in a site-specific manner using RNAi. We have previously described the extent of spread of RNAi’s effect in colon (10). Days 3 and 6 after colitis induction were the time points chosen to ascertain whether CRF receptor knockdown ameliorates or exacerbates colitis because these are the days most representative of acute inflammation and early healing. We found that 3 days after simultaneous administration of TNBS and TNBS + CRF1 or TNBS + CRF2 siRNAs, rats continued to lose body weight in a manner similar to what occurred after TNBS treatment alone (P < 0.001), whereas rats treated with vehicle gained weight, as expected (Supplemental Fig. S2). Gross examination of the dissected colon revealed that TNBS colitis resulted in shortening and thickening of colon on days 3 and 6 and that the ulceration encompassed a significant length of the shortened distal colon (Fig. 4, A and B). Administration of TNBS + siCRF1 also resulted in shortening and thickening of colon that was similar to that of TNBS alone at all time points. However, TNBS + siCRF2 dramatically and significantly (P < 0.0001) restricted the macroscopic spread of ulceration on days 3 and 6 compared with TNBS alone and TNBS + siCRF1-treated rats (Fig. 4, A and B).

Knockdown of CRF2, but not CRF1, alters edema progression at the microscopic level. Macroscopic differences were seen in the lateral spread of ulceration, but microscopically, inflammation developed and progressed to granulation tissue in affected regions of the colon in all groups. Treatment with TNBS after knockdown of CRF1 did not alter the development of colitis, and histologically the changes were similar to those seen in rats treated with TNBS alone. Although TNBS treatment of CRF2 knockdown animals showed development and progression of inflammation in the affected colon, there was an unusual abundance of submucosal edema that was persistent at day 6 (Fig. 4A). In rats treated with TNBS alone and with TNBS + siCRF1, acute inflammation resolved at a similar rate. We confirmed that sequences used to knock down CRF2 do not also knock down CRF1, or vice versa, since these receptors share 70% identity at protein level. In vivo, it is difficult to determine unequivocally whether changes in receptor expression are due to compensatory effects of knockdown, progression of inflammation itself, or both. In HEK cells cotransfected with control siRNA and either full-length HA-tagged CRF1 or CRF2, robust expression of either receptor was evident by immunofluorescence (Fig. 5B, control, left). In cells cotransfected with CRF1 and siCRF1 sequences, CRF1 expression was significantly reduced (Fig. 5B, middle, top, and C, left), whereas cotransfection with siCRF2 sequences left CRF1 expression intact (Fig. 5B, right, top). Similarly, in cells cotransfected with CRF2 and siCRF2 sequences, CRF2 expression was markedly inhibited (Fig. 5B, right, top and C, right), whereas cotransfection with siCRF1 sequences did not inhibit CRF2 expression (Fig. 5B, middle, top). In vitro, RNAi resulted in 30–70% knockdown of the target receptor depending on transfection efficiency of the receptor and dsRNA (Fig. 5C). Since transfection of HEK cells with HA-tagged CRF receptors is a heterologous expression system, compensatory changes in receptor expression are not expected, but downregulation of receptor due to cross-reactivity of sequences, if present, would be visible. We next determined that dsRNA sequences used in HEK cells against CRF receptors also knocked down protein expression in rat colon tissue. As shown in Fig. 5D, RNAi was successful in silencing protein expression of CRF1/2 in the rat

between groups by TNBS treatment. Saline treatment did not cause changes in pERK1/2 or total ERK levels (Fig. 3B). By day 6, pERK1/2 levels were very low (P < 0.0001), with expression falling by 10-fold over that in saline controls. Thus pERK1/2 levels appear to inversely correlate with Ucn1 expression: high on day 1 when Ucn1 levels are low, and low on day 6 when Ucn1 levels are high.

Colon-specific CRF2, but not CRF1, is required for macroscopic spread of ulceration during colitis. To further examine the role of Ucn1 receptors in the initiation and progression of inflammation of the distal colon, we inhibited colonic expression of CRF1 or CRF2 protein expression in a site-specific
colon, whereas CRF$_{1/2}$ expression was clearly evident in control colon tissue and in one rat (rat 3) in which the injection of siCRF$_2$ failed. The blot was also probed with β-actin, a housekeeping gene to control for equal loading of tissue lysate.

Knockdown of CRF$_2$, but not of CRF$_1$, induces Ucn1 mRNA expression. Knockdown of CRF$_1$ or CRF$_2$ in rat colon under basal (no TNBS) conditions did not affect Ucn1 mRNA expression compared with naive rats. Three days after colitis was induced, Ucn1 mRNA levels were significantly increased in rats in the TNBS + siCRF$_2$ group when compared with basal knockdown of either CRF$_1$ or CRF$_2$ (Fig. 6A). Ucn1 mRNA levels were also significantly increased 3 days after colitis in the TNBS + siCRF$_2$ group when compared with basal knockdown of either CRF$_1$ or CRF$_2$ (Fig. 6A). Ucn1 mRNA levels were also significantly increased compared with all control groups and was also significantly different from siCRF$_1$ + TNBS group, whereas Ucn1 levels in TNBS + siCRF$_2$ group did not significantly change from days 3 to 6 (Fig. 6A).

Knockdown of CRF$_2$, but not of CRF$_1$, attenuates activation of ERK1/2 phosphorylation. Knockdown of CRF$_1$ or CRF$_2$ in rat colon under basal conditions did not affect pERK1/2 levels compared with basal or vehicle-treated rats (Fig. 6B). TNBS treatment resulted in increased activation of pERK1/2 on day 3 compared with all control groups (vehicle and basal knockdown of CRF$_{1/2}$). Unlike Ucn1 levels on day 3, pERK1/2 levels were reduced by 50% in the TNBS + siCRF$_2$ group, whereas after TNBS + siCRF$_1$ treatment pERK1/2 levels were reduced by 18–20% compared with TNBS treatment alone (Fig. 6B). By day 6, pERK1/2 levels were at their nadir in the TNBS-alone and TNBS + siCRF$_1$ groups compared with 3-day TNBS treatment, but in the TNBS + siCRF$_2$ group pERK1/2 levels remained unchanged from those on day 3 (Fig. 6B).

Knockdown of CRF$_2$, but not of CRF$_1$, markedly induced TNF-α mRNA expression. Three days after colitis was induced, TNF-α mRNA levels rose approximately twofold over control levels, a rise that was similar to that observed on day 3 in rats treated with TNBS + siCRF$_1$ (Fig. 6C). However, 3 days after TNBS + siCRF$_2$ treatment, TNF-α mRNA levels increased sixfold. Importantly, knockdown of either CRF$_1$ or CRF$_2$ in basal colon did not change TNF-α expression (Fig. 6C). Six days after colitis progressed, TNF-α expression increased by 3.5- to fourfold in the TNBS and TNBS + siCRF$_1$ groups, respectively, but remained unchanged from day 3 in TNBS + siCRF$_2$ group.

Changes in MPO activity after RNAi-mediated knockdown of CRF$_1$ and CRF$_2$. Colitis and TNBS treatment is known to result in a marked increase in transmural neutrophil infiltration, which is often reflected by a marked increase in MPO activity. As expected, 3 days after TNBS treatment, MPO activity

Fig. 4. Local elimination of CRF$_2$ but not CRF$_1$ expression restricts macroscopic spread of ulceration after TNBS treatment. A and B: 3 and 6 days after TNBS + small interfering (si)CRF$_2$ treatment, ulceration was contained and the observed spread was 40–52% less than that seen in rats treated with TNBS + siCRF$_1$ or TNBS alone (*P < 0.001).
Fig. 5. Peripheral elimination of CRF2 but not CRF1 alters edema accumulation and progression, but not development of colitis at the microscopic level. A: in all treatment groups and at both time points examined, acute inflammation developed as expected and evolved into granulation tissue at a similar rate. In contrast to rats treated with TNBS + siCRF1 or TNBS alone, tissue sections from rats treated with TNBS + siCRF2 revealed an unusual abundance of submucosal edema that did not resolve by day 6. SM, submucosa; GT, granulation tissue. B: specific knockdown of corticotropin-releasing factor (CRF) receptors by RNA interference (RNAi). Robust expression of either receptor is evident in HEK cells cotransfected with control siRNA and either full-length hemagglutinin (HA)-tagged CRF1 or CRF2, (left, top). In cells cotransfected with CRF1 and siCRF1 sequences, CRF1 expression is significantly reduced (middle, top) but unaffected in cells cotransfected with siCRF2 (right, top). In cells cotransfected with CRF2 and siCRF2 sequences, CRF2 expression is markedly reduced (right, top), and remains unaffected in cells cotransfected with siCRF1 (middle, top), indicating specificity of sequences used. DAPI, 4,6-diamidino-2-phenylindole. C and D: Western blot revealed that both in vitro in HEK cells (C) and in vivo in rat colon (D) tissue, specific double-stranded RNA (dsRNA) against CRF receptors was effective in knocking down expression of cognate receptors (30–70% knockdown was achieved in transfected cells depending upon transfection efficiency). In 1 rat (rat 3), injection of siCRF2 was not successful because the solution leaked, and we thus predicted that CRF2 would not be silenced. As expected, expression of CRF2 was intact in rat 3, whereas in colon lysates from other animals CRF2 or CRF1 was undetectable after RNAi treatment compared with control colon tissue. Rat 3 was therefore eliminated from the entire study (D, right, lane 3).
increased dramatically compared with naive and siCRF1 and siCRF2 knocked-down controls (Fig. 6D). Knockdown of either receptor in basal colon did not alter MPO activity from that of vehicle-treated rats. Conversely, in rats treated with TNBS + siCRF1 or TNBS + siCRF2, MPO activity decreased by 22 and 37%, respectively on day 3 (P < 0.005). By day 6, MPO activity decreased by 53% in rats treated with TNBS alone compared with day 3 (P < 0.05). Surprisingly, 6 days after TNBS + siCRF1 treatment, MPO activity in this group fell by 74%, reaching its lowest level among all treatment groups. In sharp contrast, MPO activity remained elevated and unchanged from day 3 in rats treated with TNBS + siCRF2.

**DISCUSSION**

Little is known about localization of the neuropeptide Ucn1 throughout the GI tract and about how the levels of urocortins and their receptors change during the normal progression of colitis. Similarly to what we previously found with Ucn2 mRNA expression (6), Ucn1 mRNA and protein immunoreactivity were abundantly expressed throughout the GI tract under basal conditions, primarily in mucosal epithelium and neural plexuses. Immunohistochemical analysis revealed that many cell types within the colon constitutively express Ucn1, including neural, epithelial, endothelial, smooth muscle, and immune cells of the Peyer’s patches. Basal levels of Ucn1 expression have also been reported in epithelial, endothelial, and smooth muscle cells of the prostate (1) and in parietal cells of the stomach and crypt cells of the small and large intestine, as well as in the submucosal and myenteric plexuses (17, 26, 37). Reports of mucosal Ucn1 expression in the colon have been contradictory, with one report finding Ucn1 expression in few cells (28), whereas others have reported strong Ucn1 expression both in the lamina propria cells and in the neurons of the enteric plexuses (4, 36, 42). In agreement with the latter findings, we find strong expression of Ucn1 in both the mucosa and in the enteric plexuses. Therefore, the effects of Ucn1 agonist and antagonist therapy must be considered in the context of Ucn1’s ubiquitous expression even under basal conditions.

We monitored the kinetics of Ucn1 mRNA expression in the early-to-mid phase of inflammation. As inflammation progressed, average Ucn1 mRNA levels dropped below baseline levels on day 1, then rose again by day 6: a period of transition between acute inflammation and early granulation tissue. Abundant Ucn1-IR cells in proliferating neural plexus and...
other mesenchymal cells within the healing granulation tissue were evident. Patients with Helicobacter pylori infection and gastritis exhibit high Ucn1 levels that are further increased if the patients respond to H. pylori eradication treatment, but stay unchanged in nonresponders, leading to the conclusion that Ucn1 actions are anti-inflammatory (7). Recently, intraperitoneal delivery of Ucn1 was shown to have an anti-inflammatory effect in a murine model of colitis (15). High levels of Ucn1 mRNA levels that we observed on day 6, and the left shift in the kinetics of Ucn1 mRNA levels after local elimination of CRF2 and TNBS treatment, suggest that a balanced increase in Ucn1 levels is essential to resolve inflammation.

In the colon of naïve animals, pan-neuronal staining with anti-PGP9.5 showed an abundance of neurons that were also positive for Ucn1-IR at basal or under unoinflamed conditions. Massive colonic neural plexus and neural fiber disruption before the Ucn1-IR positive inflammatory cells accumulated after TNBS treatment possibly explain the drop in Ucn1 mRNA levels below basal levels on day 1. The drop in the number of surviving neurons on day 6 was striking given that Ucn1 expression is high at day 6. This would argue that infiltrated immune cells and early proliferative tissue that express Ucn1 are a major source of Ucn1 during inflammation. The nerve bundles that survive during the course of inflammation continue to express Ucn1. In the late phase, neuronal architecture appeared to return to normal organization, with the mesenteric nerve plexus beginning to align parallel to the musculature; however, intramuscular and lamina propria nerve fibers remain unorganized. These undulations in Ucn1, Ucn2, and CRF2 mRNA expression kinetics (6, 27, 29, 35) may be explained by variability in the number of nerve fibers and plexus seen in this phase and suggest there is a modulation in degree and type of inflammatory response as healing begins that cannot be attributed simply to a pro- or anti-inflammatory effect.

The early drop in Ucn1 that we found after acute TNBS treatment, together with the acute rise in Ucn2 mRNA we previously reported (6), are similar to results by others who found that Ucn1 mRNA levels decreased and Ucn2 mRNA levels increased, in human aortic endothelial cells stimulated with TNF-α (23). In our previous study, TNF-α and MPO levels in tissue homogenate rose during this acute phase (days 1–3) and found CRF2 levels to be decreased in the acute phase of TNBS-induced colitis (6). Coordinated expression of the uroctins and their receptors appears to be finely tuned during this acute phase. Whether the early drop in Ucn1 levels below basal levels on day 1 of inflammation is necessary for the development of a normal acute reaction remains to be determined.

Activation of CRF2 by Ucn1 is known to activate a number of signaling pathways including JNK, MAPK, and pERK1/2. JNK activation does not appear to regulate Ucn1-mediated fluid leak during LPS-induced inflammation (11) in the gut. In the heart, stress-induced Ucn1 stimulates pERK1/2 activation in the cardiomyocytes, and antagonism of CRF2 with astressin-2 abolishes Ucn1-dependent activation of ERK1/2 in those cells (21). In our study, activation of pERK1/2 exhibited opposite relationship with Ucn1 levels: elevated in early inflammation and low at day 6. These findings suggest that time lags exist between measurable Ucn1 and CRF2 mRNA levels and measurable pERK1/2 phosphorylation cycles, again making definitive cause and effect relationships difficult to determine in the clinical setting.

To further evaluate the role of CRF1 and CRF2 during inflammation, we examined the effect of eliminating their expression in a local and site-specific manner using RNAi. Knockdown of CRF1 or CRF2 by itself in basal colon tissue did not alter expression of Ucn1 or TNF-α, and neither did it alter pERK1/2 or MPO activity from basal levels. We found no microscopic evidence of differences in either the development or the progression of inflammation in the TNBS-alone or TNBS + CRF1/2 knockdown groups. The composition of the mixed-cell infiltrate over time and the lymphocyte subtypes that were present or absent after local knockdown of CRF receptors remain to be determined; however, TNF-α levels and MPO activity did not change from baseline. The macroscopic lateral spread of inflammation was decreased in CRF-R2 knockout animals. Transiently eliminating expression of CRF2 resulted in abnormally dense submucosal edema on day 3 that did not resolve by day 6 of TNBS treatment. Bowel motility and microvascular permeability are both dependent on Ucn1 and CRF2 (11, 31). It is therefore interesting that in our previous study (11), local Ucn1 exposure promoted microvascular permeability by twofold, and synergized the increase in mesenteric venule permeability and release of TNF-α was associated with LPS-induced inflammation. Possibly, pathways of smooth muscle contraction and edema reabsorption are abnormal in CRF2 knockout animals, leading to abnormal lateral spread of TNBS, inflammation, or both.

Our findings that local knockdown of CRF2 increases interstitial edema are supported by a very recent study that reported that increased edema in CRF2−/− mice after dextran sulfate sodium (DSS)-induced colitis (22). In contrast to our findings for local deletion of CRF2 in TNBS colitis, genetic deletion of CRF2 in CRF2−/− mice resulted in increased intestinal inflammation and angiogenesis in response to DSS-induced colitis. In that study (22), vascular endothelial growth factor (VEGF) produced by smooth muscle cells of the intestine may have contributed to increased angiogenesis during DSS-induced colitis because intestinal enterochromaffin cells do not produce VEGF.

Eliminating local expression of CRF1 (TNBS + siCRF1) had little effect on the histological or biochemical markers of inflammation compared with what was observed for TNBS alone. This is in contrast to results from a recent study of CRF1−/− mice, in which inflammation and angiogenesis were decreased in a murine model of DSS-induced colitis (22). It is possible that global deletion of CRF receptors and accompanying developmental compensations have a more significant effect on intestinal inflammation than just local elimination and may account for some of the differences between our study and that of Im et al. (22).

Consistent with the hypothesis that ERK1/2 phosphorylation values reflect CRF2 activation, pERK1/2 levels were decreased at day 3 and remained unchanged at day 6 in the TNBS + siCRF2 rats. This pattern was in contrast to that of pERK1/2 levels in rats treated with TNBS alone or with TNBS + siCRF1; in both groups, pERK1/2 levels increased at day 3 and fell sharply at day 6. As though to compensate for the loss of CRF2 on day 3, TNBS + siCRF2 knockout animals had dramatically increased Ucn1 mRNA and TNF-α values that had not changed by day 6, whereas, in TNBS-alone or
TNBS + siCRF₁ rats, Ucn1 mRNA and TNF-α levels started lower and gradually increased by day 6. MPO levels also remained high in the CRF₂ siRNA-treated animals. However, the increases in Ucn1 and TNF-α expression and attenuated pERK1/2 activation did not necessarily reflect a more rapid progression toward healing. It might reflect compensatory reactions to the loss of a critical receptor, in this case CRF₂ and not CRF₁. Studying the cause-and-effect relationship is complicated by autocrine/paracrine cascades that may trigger differing molecular pathways within the same cell, depending on the inflammatory disease and disease stage (acute vs. chronic). Evaluation of agonist or antagonist therapeutics must take these factors into account. We conclude that the expression of urocortins and their receptors and their influence on individual cell types vary according local milieu. The pro- or anti-inflammatory effects of the urocortins and their receptors are spatiotemporal and must be evaluated within that context.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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