Peptides 54 (2014) 58–66

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

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

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ARTICLE INFO

Article history:
Received 13 December 2013
Received in revised form 11 January 2014
Accepted 13 January 2014
Available online 22 January 2014

Keywords:
Crohn's colitis
Inflammation
RNAi
Ca^{2+} signaling
MAPK

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 (CRF_1 and CRF_2) 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 CRF_1, but not CRF_2 decreased Ucn3 mRNA by 78%, whereas during colitis, Ucn3 mRNA levels increased after CRF_1 knockdown. In cultured cells, co-expression of CRF_1 + CRF_2 attenuated Ucn3-stimulated intracellular Ca^{2+} peak by 48% as compared to cells expressing CRF_2 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.

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, CRF_1 and CRF_2. 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 Ucns were first believed to be central regulators of the peripheral stress response, accumulating evidence demonstrates localized, cellular expression of Ucns 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 CRF_2 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 CRF_2, thus providing protection against heart failure [1,41]. Ucn2 and its high-affinity receptor CRF_2 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 CRF_2 with high affinity, but does not bind to CRF_1 [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

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http://dx.doi.org/10.1016/j.peptides.2014.01.007

inflammation has not been examined. Moreover, whether Ucn3 exerts pro- or anti-inflammatory affects during Crohn's colitis or if its expression is altered after the knockdown of CRF_1 and CRF_2 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 CRF_1 and CRF_2 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′-ATGCTGATGCCCACTTACTTCCTG-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 CRF_1 and CRF_2 was transcribed in vitro and were specific for either CRF_1 ($dsCRF_1$) or CRF_2 ($dsCRF_2$) 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 phospo-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 CRF_1 pcDNA5.1 was purchased from Missouri S&T cDNA Resource Center (www.cdna.org). FLAG-tagged CRF_2 was cloned in pcDNA5.1. Both constructs were confirmed by