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Corticotropin releasing factor in the rat colon: Expression, localization and upregulation by endotoxin

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ABSTRACT

Little is known about CRF expression and regulation in the rat colon compared to the brain. We investigated CRF gene expression, cellular location, and regulation by endotoxin and corticosterone in the male rat colon at 6 h after intraperitoneal (ip) injection. CRF mRNA level, detected by reverse transcription-polymerase chain reaction (RT-PCR) was 1.3-fold higher in the distal than proximal colon and 3.4-fold higher in the proximal colonic submucosa plus muscle layers than in mucosa. CRF immunoreactivity was located in the epithelia, lamina propria and crypts, and co-localized with tryptophan hydroxylase, a marker for enterochromaffin (EC) cells, and in enteric neurons. Lipopolysaccharide (LPS, 100 μg/kg, ip) increased defecation by 2.9-fold and upregulated CRF mRNA by 2.5-fold in the proximal and 1.1-fold in the distal colon while there was no change induced by corticosterone as monitored by quantitative PCR. LPS-induced increased CRF mRNA expression occurred in the submucosa plus muscle layers (1.5-fold) and the mucosa of proximal colon (0.9-fold). LPS increased significantly CRF immunoreactivity in the submucosal and myenteric plexuses of proximal and distal colon compared to saline groups. These results indicate that in rats, CRF is expressed in both proximal and distal colon and more prominently in enteric neurons of the submucosa plus muscle layers and subject to upregulation at the gene and protein levels by LPS through corticosteroid independent pathways. These data suggests that colonic CRF may be part of the local effector limb of the CRF_1 receptor mediated colonic alterations induced by acute stress.

1. Introduction

CRF, characterized by Vale et al. in 1981 as a novel 41-amino-acid hypothalamic peptide [44], was established to mediate stress-evoked activation of the hypothalamo–pituitary–adrenal (HPA) axis through the activation of pituitary CRF_1 receptor [4,10]. Subsequently, activation of CRF-CRF_1 receptor signaling was shown to participate in various components of the adaptive response to stressful events [4,10]. In particular, CRF injected centrally recapitulates stress-related colonic functional alterations (increased motility, permeability, secretion, transit, bacterial translocation, secretion and hypersensitivity to colorectal distension) and CRF_1 receptor antagonists alleviate acute stress-induced colonic responses in conscious rodents [40].

In addition to the centrally mediated CRF actions, recent experimental and clinical studies indicate that peripheral injection of CRF or CRF_1 agonists mimic the effects of acute stress on the colon as shown by the increase in myenteric neuronal activity, motility, transit, mucus secretion, macromolecular permeability and visceral pain [7,11,25,26,36,46,47,55]. These actions of exogenously administered CRF into the circulation or isolated colon in rats or colonic biopsy specimens in humans are largely mediated by the activation of CRF_1 receptor prominently located in myenteric neurons and immune cells [9,22,29,34,46,55]. The relevance of the peripheral activation of CRF receptor signaling is also supported by the dampening of acute stress-induced stimulation of colonic secretory motor function by peripheral injection of peptide CRF receptor antagonists with poor brain penetrance in rats and humans [39,41,50]. However, compared to the brain, less is known on CRF gene expression and CRF localization and its regulation by stressors in the rat colon. So far, CRF mRNA has been reported to be expressed in the mice and rat ileum [24,51], rat cecum [45] and human colonic mucosa [18] and rapidly upregulated in rat and mice ileum in response to toxin A that was perfused in the ileal loop [24,51].

Therefore in the present study, we investigated CRF expression at the gene and protein levels in the proximal and distal colon in naïve rats and in response to peripheral injection of lipopolysac-

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charide (LPS) and corticosterone. Gene expression was assessed by reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR [53]. The cellular location and identity (endocrine vs neuronal) of CRF positive cells were characterized using immunohistochemistry and double immunostaining with tryptophan hydroxylase (TPH) as an enterochromaffin cell marker [21,54] and Hu C/D as a neuronal marker [27] in the whole thickness tissue sections. CRF immunoreactivity change in response to LPS was also evaluated using digital computer-assisted image analysis in whole-mount preparation of enteric plexuses (submucosal and myenteric).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (265–297 g, Harlan Laboratory, San Diego, CA) were group housed under controlled conditions (22–24 °C, lights on from 6:00 AM to 6:00 PM) with free access to standard rodent chow (Prolab RMH 2500, PMY Nutrition International, Brentwood, MO) and tap water. Studies were conducted under the approved protocols of the Department of Veterans Affairs Animal Component of Research Veterans Affairs Greater Los Angeles Healthcare System (04012-06 and 9906-820). All experiments were performed between 9:00 AM and 1:00 PM to avoid confounding variables of diurnal rhythm influence on the HPA axis under basal and stress conditions.

2.2. CRF expression in the colon of naïve rats

2.2.1. CRF mRNA expression in the proximal and distal colon by RT-PCR

Three naïve rats were decapitated and proximal (1 cm from cecum) and distal (2 cm to the anus) segments of the colon were collected. In the proximal colon segment, half of it was separated into mucosa and submucosa plus muscle layer as previously described [55]. All colonic samples were snapped frozen in dry ice and stored at −70 °C until used. Total RNA was extracted using RNA-Bee™ (TEL-TEST, Friendswood, TX), following manufacturer's recommended protocol. RNA pellets were resuspended in DEPC-treated water and further digested with DNase I for 60 min at 37 °C (Promega, Madison, WI). Total RNA (5 μg) was denatured at 65 °C for 5 min and used to synthesize first-strand cDNA in a total volume of 20 μl reaction by ThermoScript™ RT-PCR system (Invitrogen, CA). cDNA (1 μl) was amplified for CRF directly by the polymerase chain reaction (PCR) with 33 cycles at 92 °C for 40 s, 57 °C for 40 s, 72 °C for 2 min, and a final extension step at 72 °C for 5 min using specific CRF primers for rat (Table 1). PCR for the housekeeping gene, rat acidic ribosome protein (ARP) served as the internal control as in our previous study [55]. PCR products were separated by 1% agarose gel electrophoresis, and visualized with ethidium bromide. The gel images were acquired by Kodak EDAS 290 system and quantitative densitometry was performed with NIH Image software (Scion Corporation, Frederick, MD). In all samples, the intensity of the bands was normalized to that of ARP in each sample respectively and results were expressed as the fold change in reference to the control group. Then the PCR product corresponding to the predicted CRF was extracted with QIA quick Gel Extraction kit (QIAGEN, Hilden, Germany). The DNA fragment was inserted into pCR2.1 vector and transformed into bacterial competent cells (TA Cloning kit, Invitrogen). The plasmid with positive insert was sequenced in both directions to confirm its identity to rat CRF using the Big Dye Terminator (ver. 3) in Cycle Sequencing System (Applied Biosystems, Foster City, CA).

Table 1
Sequences of oligonucleotide primers used for RT-PCR and real-time quantitative PCR.

cDNA	Direction	Primer (5′-3′)	Size (bp)
For RT-PCR			
rCRF	Sense	TGATCCGCATGGGTGAAGAATACTTCCTC	394
	Antisense	CCCGATAATCTCCATCAGTTTCCTGTTGCTG	
rARP	Sense	GTTGAACATCTCCCCCTTCTC	402
	Antisense	ATGTCCTCATCGGATTCCTCC	
For real-time PCR			
rCRF	Sense	TCTCTCTGGATCTCACCTTCCACC	77
	Antisense	AGCTTGCTGAGCTAACTGCTCTGC	
rGAPDH	Sense	AGACAGCCGCATCTTCTTGT	142
	Antisense	TGATGGCAACAATGTCCACT	

r: rat.

2.2.2. CRF immunohistochemistry in the whole thickness sections of proximal colon and double labeling with tryptophan hydroxylase or Hu C/D

The proximal colon was collected from 3 naïve rats, fixed in formalin, embedded in paraffin and sectioned at 5 μm thickness. Immunohistochemistry was performed using avidin–boiotin-peroxidase technique. Sections were deparaffinized in xylene and hydrated in descending grades of ethanol. After washing 2 times (5 min each) in phosphate buffer saline (PBS), slides were placed in a plastic Coplin jar filled with 10 mM citrate buffer (pH 6.0), boiled for 8 min, and followed by cooling to room temperature. Sections were washed twice in PBS. Endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in PBS at room temperature. Slides were incubated overnight at 4 °C with rabbit anti-rat/human CRF antibody C70 (1:20,000, gift from Dr. Wylie W Vale, Clayton Foundation Laboratories, Salk Institute, San Diego, CA) diluted in PBS containing 0.3% Triton X-100, followed by incubation with biotinylated donkey anti-goat IgG (1:1000; Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. Sections were subsequently processed for avidin–biotin-peroxidase procedure using diaminobenzidine as a chromogen, and then counterstained with hematoxylin. Immunohistochemical control was routinely performed following the same procedures, except that the primary antibody was pre-absorbed with CRF antigen peptide (20 μg/ml, rat CRF, gift from Jean Rivier, Clayton Foundation Laboratories, Salk Institute).

For the double immunostaining, sections were deparaffinized in xylene, hydrated in descending grades of ethanol and an antigen retrieval procedure was conducted as described above, then sections were washed three times at 10-min intervals with PBS, and incubated in 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 0.3% Triton X-100/PBS for 30 min at room temperature followed by two-overnight incubations with the mixture of rabbit anti-rat CRF antibody (1:5000, gift from Dr. Wylie Wale) and mouse anti-TPH (1:40, Novocastra Lab Ltd., Newcastles, UK), or mouse anti-Hu C/D (1:200, Molecular probes, Eugene, OR). Sections were washed with PBS three times at 10-min intervals and incubated for 2 h at room temperature with the mixture of FITC-conjugated donkey anti-rabbit IgG and Rhodamine Red™-X-conjugated donkey anti-mouse IgG (1:100, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After three washes in PBS, sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich, Saint Louis, MO), mounted on slides with anti-fade mounting media (Vector Laboratory Inc., Burlingame, CA) and visualized by standard fluorescence microscopy. The double labeling of CRF/TPH was assessed in at least 20 TPH immunoreactive (ir) positive cells for each rat and expressed in percentage of the total number of TPH positive cells respectively. The means of the values from each animal were used to calculate the group mean.