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Original article

Corticotropin-releasing hormone and urocortin expression in peripheral blood cells from experimentally infected cattle with *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Urocortin (UCN) is a new neuropeptide of the corticotrophin-releasing hormone (CRH) family which plays an important role in immune responses. *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) is the etiological agent of paratuberculosis (Johne's disease). The role of UCN or CRH in the pathogenesis of *Map*-infection is unknown. In the present study, we first cloned the bovine UCN gene and demonstrated the profile of UCN or CRH expression in peripheral blood cells from *Map*-infected cattle and uninfected controls by real-time reverse transcription-polymerase chain reaction (RT-PCR) and ELISA analysis. These data are the first observations of the characteristic kinetics of these neuropeptides in *Map*-infection. UCN or CRH expression in non-stimulated blood samples from infected cattle was higher than that in similarly treated samples from uninfected controls; however, exposure to *Map* lysate and live *Map* resulted in down-regulated expression of UCN in infected cattle compared to their counterparts from uninfected controls. These results have provided a direction in understanding the pathogenesis of paratuberculosis and improving diagnostic methods for *Map*-infection.

Keywords: Urocortin; Corticotrophin-releasing hormone; *Mycobacterium avium* subspecies *paratuberculosis*; Paratuberculosis; Neuropeptide; Bovine

1. Introduction

Urocortin (UCN), a new 40-amino-acid mammalian neuropeptide of the corticotropin-releasing hormone (CRH) family, was recently identified from the rat midbrain. Rat UCN shares 45% identity with rat CRH [1] and 95% identity within the mature peptide region to human UCN [2]. UCN and CRH are major regulators of the hypothalamic-pituitary-adrenal (HPA) axis [2–3], and play a dual role in the pathophysiology of inflammation both centrally and peripherally.

Central neuroendocrine UCN and CRH secreted from the hypothalamus play an indirect immunosuppressive and anti-inflammatory role through endogenous glucocorticoid release by activation of the HPA axis [4–5]. An intracerebroventricular injection of UCN produced a marked decrease in the proliferative response of splenocytes to a mitogen, which suggested the potent suppressive effects of UCN on splenic lymphocyte activity [6]. In contrast to the central effect, UCN and CRH have also been shown to be present at peripheral sites of inflammation where they have a direct immunostimulatory role as autocrine or paracrine inflammatory mediators, and

Abbreviations: CRH, corticotropin-releasing hormone; ELISA, enzyme-linked immunosorbent assay; GSP, gene specific primer; HPA, hypothalamic-pituitary-adrenal; IFN-γ, interferon gamma; IL, interleukin; LPS, lipopolysacharide; *Map*, *Mycobacterium avium* subspecies *paratuberculosis*; NGSP, nested gene specific primer; NUP, nested universal primer; PBMC, peripheral blood mononuclear cell; PPD, purified protein derivative; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor alpha; UCN, urocortin; UPM, universal primer mix.

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have been shown to promote local immune responses [7–10]. Recent reports described the local production of peripheral UCN and CRH at different inflammatory sites in several experimental models or clinical inflammation, including carrageenin-induced granulomatous tissues [9], streptococcal cell wall- and adjuvant-induced arthritic joints of rats [7], the joints of patients with rheumatoid arthritis and osteoarthritis [8,10] and the colonic mucosa of patients suffering from ulcerative colitis [11]. UCN along with CRH may participate in the pathophysiology of many inflammatory conditions. The roles of CRH and UCN, their cooperation in systematic and local host defense systems, and their interrelationships are mostly unclear.

Mycobacterium avium subspecies *paratuberculosis* (*Map*) is the etiological agent of severe enteritis in ruminants, known as paratuberculosis (Johne's disease) [12]. This infection is characterized by chronic granulomatous enteritis, persistent diarrhea, progressive wasting, and finally death. Paratuberculosis has resulted in significant economic losses to the dairy and cattle industries [13]. Furthermore, the bacteria are speculated to be the cause of human Crohn's disease, an incurable chronic inflammatory bowel disease [14]. *Map* is unlike other mycobacteria in Th1 immune responses. After initial bacterial challenge, animals induce Th1 immune responses and control bacterial growth first, then decrease Th1 immune responses during the sub-clinical stage and generally with no subsequent increases. Coussens et al. found that stimulation with *Map* tended to reduce interferon gamma (IFN-γ), interleukin-1α (IL-1α), and interleukin-6 (IL-6) expression, while consistently enhancing interleukin-10 (IL-10) expression in peripheral blood mononuclear cells (PBMCs) from sub-clinically infected cattle, despite a continued inflammatory reaction at sites of the infection [15]. Recently, our group proved that a more sustained IL-10 expression induced by *Map* antigens had a suppressive effect on *Map*-specific T cell proliferation, IFN-γ secretion and Th1 immune responses [16]. This specific immune suppression in the sub-clinical stage of paratuberculosis is a key phenomenon since it obstructs the immune diagnosis and is considered to be a cause of super-chronic infection.

We hypothesized that CRH and UCN are immune suppressive mediators related to the pathogenesis of *Map*-infection, since previous findings suggested that CRH and UCN played important roles in down-regulating immune responses, particularly in cell-mediated immune responses [17]; however, the existence of UCN in cattle has not been confirmed thus far, and no information is available on immune responses of CRH and UCN to *Map*-infection. In the present study, we described the cDNA cloning of bovine UCN, and evaluated CRH and UCN expression during *Map*-infection.

2. Materials and methods

2.1. Preparation of PBMCs

PBMCs were isolated from jugular blood samples using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation based on the manufacturer's instructions. Cells resuspended in RPMI 1640 medium (Sigma Chemical Corp., St. Louis, MO.) containing 10% fetal calf serum were plated at 3×10^6 cells per 90 mm diameter tissue culture plates (Sumilon; Sumitomo Bakelite Corp., Tokyo, Japan). After 1 h culture, cells were stimulated with 10 μg/ml of lipopolysaccharide (LPS) (Sigma Chemical Corp.), and then cultured for another 6 h.

2.2. RNA isolation

Total RNA was isolated from LPS-stimulated PBMCs using trizol reagent (Invitrogen Life Technologies, Corp., Carlsbad, CA) according to the manufacturer's protocol.

2.3. Cloning of bovine UCN

To determine the nucleotide sequence of bovine UCN mRNA, cDNA was cloned using reverse transcription-polymerase chain reaction (RT-PCR) in conjunction with 5′- and 3′-rapid amplification of cDNA ends (RACE) using a SMART™ RACE cDNA amplification kit (BD Biosciences Clontech, Inc., Mountain View, CA) according to the manufacturer's protocol. The first-strand cDNA was synthesized with total RNA as described above using PowerScript™ reverse transcriptase according to the manufacturer's protocol. Briefly, the first-strand cDNA synthesis for 5′-RACE was performed using an oligo (dT) primer and adapter oligonucleotide, termed the 5′-cDNA synthesis (5′-CDS) primer and SMART II A oligonucleotide, respectively. For 3′-RACE, the 3′-cDNA synthesis (3′-CDS) primer, having an adapter portion at its 5′-end, was used. To amplify the first-strand cDNA, external gene specific primers (GSP) and internal nested gene specific primers (NGSP) were designed to produce an overlapping region between the 5′- and 3′-RACE PCR fragments based on the conserved nucleotide sequences of human, mouse and rat UCN. The 5′-RACE PCR was performed with 5′-GSP or 5′-NGSP and universal primer mix (UPM, primers for adapter oligonucleotide sequence) or nested universal primer (NUP) using Advantage 2 polymerase (BD Biosciences Clontech, Inc.) under the following conditions: 1 cycle of 94 °C for 1 min; 30 cycles of 94 °C for 30 s; 55 °C for 30 s; 72 °C for 1 min; and 5 min of final extension at 72 °C. For 3′-RACE PCR, 3′-GSP or 3′-NGSP and UPM or NUP were used under the following conditions: 1 cycle of 94 °C for 1 min; 35 cycles of 94 °C for 1 min; 60 °C for 1 min; 72 °C for 2 min; and 5 min of final extension at 72 °C. After agarose gel electrophoresis of the RACE-PCR products, the predicted size fragments were purified using Microcon® centrifugal filter (Millipore Corp., Billerica, MA) and subcloned into pCR®2.1 vector using TOPO TA cloning kit (Invitrogen Life Technologies, Corp.). The oligonucleotides used for cloning are detailed in Table 1.

2.4. DNA sequencing and analysis

Double-strand DNA sequencing was performed using the ABI PRISM BigDye™ terminator cycle sequencing kit