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Corticotropin-releasing hormone and urocortin promote phagocytosis of rat macrophages through convergent but distinct pathways

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ABSTRACT

Aims: Phagocytosis plays essential roles during inflammation and immune response. This study aims to explore the underlying mechanism of corticotropin-releasing hormone (CRH) and urocortin (UCN)-promoted phagocytosis of rat macrophages.
Main methods: To induce phagocytosis, rat macrophages were incubated with carboxylated fluorescent microspheres. The phagocytosis activity was evaluated by flow cytometric analysis. Actin reorganization was determined by immunostaining with TRITC-labeled phalloidin and transmission electron microscopy (TEM) analysis. Protein expressions of p-RhoA, p-Rac1, p-extracellular signal-related kinase (ERK)1/2 and GAPDH were examined by Western blotting. Protein kinase C (PKC) and protein kinase A (PKA) activities were examined using PreTag non-radio activity assay.
Key findings: Administration of CRH or UCN alone significantly enhanced phagocytosis of microspheres by rat macrophages, as well as actin reorganization. Ligation of CRH and UCN with CRH receptor increased the phosphorylation of both RhoA and Rac1. Inhibition of RhoA/Rac1 signal pathway suppressed CRH- or UCN-enhanced phagocytosis and actin reorganization. Blockage of PKA signal by MDL-12330A decreased CRH or UCN-promoted p-RhoA and p-Rac1 expressions. Blockage of PKC signal by cholerythine choride decreased CRH or UCN-promoted p-Rac1 expression and UCN-promoted p-RhoA expression, but increased the CRH-induced p-RhoA expression. ERK1/2 was also activated and served as upstream factor of RhoA/Rac1 signal pathway.
Significance: The results reveal that CRH and UCN promote phagocytosis of rat macrophages through convergent but dissociable pathways. PKA/PKC–ERK1/2-RhoA/Rac1 signal pathway plays an essential role in CRH- and UCN-enhanced phagocytosis.

Introduction

Monocyte/macrophages are recognized as a critical cell type for host defense against invading pathogens [1]. Phagocytosis, a mechanism of ingesting large particles (≥0.5 μm) by phagocytes, such as macrophages, plays an important role in inflammation and immune response [1,7,11]. Macrophage phagocytosis is regulated by a variety of distinct receptors, including pattern-recognition receptors (eg. mannose receptor), opsonic receptors (eg. Fc receptor), scavenger receptors (eg. CD36, P2X7) [24] and apoptotic corpse receptors (eg. T cell immunoglobulin mucin family) [6]. These receptors trigger the activation of complex signaling pathways which contribute to the local rearrangement of the actin microfilament cytoskeleton, extension of the plasma membrane and particle engulfment ([6]). Despite the fact that the mechanism of phagocytosis remains unclear, it has been widely accepted that the GTPases of the Rho family contribute to the phagocytosis process by controlling actin filament polymerization and rearrangement [4].

The corticotropin-releasing hormone (CRH) and CRH-related peptide urocortin (UCN) bind to CRH receptors, which couple to multiple G proteins, and trigger the downstream signaling cascades [5,10]. CRH functions as a hypothalamic peptide to initiate adrenocorticotropin release from the anterior pituitary and thereby the endocrine limb of the stress response [20]. Peripheral secretion of CRH and UCN is involved in the modulation of the peripheral immune response [2]. However, the role of CRH and UCN on the regulation of macrophage phagocytosis is still poorly understood. A recent report shows that CRH promotes growth of rat brain norepinephrine neuronal processes through Rho GTPase regulators of the actin cytoskeleton [18]. Therefore, it is possible that CRH and UCN may contribute to the regulation of phagocytosis process through the activation of the Rho GTPase pathway.

In this study, we investigated the potential role of CRH and UCN in regulating phagocytosis of rat macrophages. The involvement of Rho proteins, such as RhoA and Rac1, in CRH and UCN-regulated phagocytosis was also determined. Our study provides basic evidence for better understanding the mechanism of CRH and UCN-regulated macrophage phagocytosis.

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Materials and methods

Reagents

CRH, UCN, W56 (specific inhibitor for Rho–ROCK signal pathway), Y27632 (specific inhibitor for Rac1), antalarmin (specific inhibitor for CRHR1), astressin (non-specific inhibitor for CRHR1/R2), MDL-12330A (specific inhibitor for PKA), cholerythine choride (CR) (specific inhibitor for PKC), PD98059 (specific inhibitor for MEK1–ERK1/2), bovine serum albumin (BSA), DMSO and TRITC-labeled Phalloidin were purchased from Sigma, USA. Carboxylate-modified fluorescent microspheres, 2 nm in diameter (F-8827) were obtained from Molecular Probes, Inc., Eugene OR. Fetal bovine serum (FBS) was bought from HyClone (USA). DMEM medium was from GIBCO (USA) and Triton-X 100 was from Amresco (USA). Primary antibodies, including rabbit anti-phosphorylated-RhoA, mouse anti-phosphorylated Rac1, mouse anti-p-ERK1/2 and anti-GAPDH (V-18), and horseradish peroxidase (HRP)-labeled goat anti-rabbit, goat anti-mouse IgG secondary antibodies were purchased from Santa Cruz, USA. Polyvinylidine fluoride (PVDF) membranes were bought from Millipore, USA.

Animals

Male and female clean grade Sprague–Dawley (SD) rats, weighing 220–240 g, were provided by Laboratory Animal Center, Institute of Surgery Research, Daping Hospital, Third Military Medical University (Chongqing, China). Animals were routinely housed and had free access to water and food. All efforts were made to minimize animal suffering and to reduce the number of animals used. All animal procedures and the study were approved by the Ethics Committee of Daping Hospital, Third Military Medical University.

Primary culture of rat macrophages

Isolation and culture of rat macrophages were carried out as previously described methods [16] with modification. Briefly, animals were anesthetized by intramuscular injection of 3% pentobarbital sodium (30 mg/kg). The peritoneal cavity was washed with 20 ml cold Ca^{2+}- and Mg^{2+}-free D-Hank's solution. After a gentle massage of the abdominal wall, the peritoneal fluid, containing resident macrophages, was collected and centrifuged at 250 g for 10 min. The supernatant was removed and the total peritoneal cells were resuspended in DMEM culture medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Using trypan blue dye exclusion assay, the survival cells were identified and the survival rate was over 95%. Cells were seeded onto 6-well plates and incubated in culture medium at 37 °C in a 5% CO_2 incubator for 2 h. Cells were washed twice with phosphate buffered saline (PBS), and adherent macrophages were maintained in fresh culture medium at 37 °C in a 5% CO_2 incubator until use.

Phagocytosis assay and flow cytometric analysis

Rat macrophages were seeded onto 6-well plate at a density of 4–5 × 10^5 cells/well. Cells were incubated with different concentrations (0.1 nM, 1 nM, 10 nM or 100 nM) of CRH or UCN in serum-free solution for 1 h. Then, cells were treated with 2 μl carboxylate-modified yellow-green (YG) microspheres (9 × 10^6 beads, diameter 2 μm) for an indicated time period at 37 °C in dark with gentle shaking to ensure adequate contact and avoid non-specific adhesion. After washing with pre-cold PBS cells were analyzed by FACSCalibur flow cytometer (Becton Dickinson) with excitation/emission wavelength settings of 488/530 nm. A total of 10,000 events were acquired in each run. Log mean fluorescence intensity (MFI) was evaluated by Cell Quest software. The percentage of phagocytosis (PP) was determined by the following formula: PP = M1 + M2 + M3 + M4, where M1, M2, M3, and M4 correspond to the number of cells with one, two, three and four microspheres. The relative mean fluorescence intensity (RMFI) was also calculated (RMFI = MFI of treatment group/MFI of control).

Immunostaining

Cells were fixed with 4% paraformaldehyde (PFA) for 20 min, washed with PBS, permeabilized with 0.2% Triton X-100 for 5 min at room temperature, blocked with 0.5% BSA for 30 min, and stained with TRITC-labeled phalloidin (200 ng/ml) at 37 °C in dark for 45 min. After washing with PBS three times, cells were visualized under fluorescent microscope (Leica, Germany).

Transmission electron microscopy (TEM) analysis

Treated cells were fixed with 2.5% glutaraldehyde for 2 h and post-fixed with 1% OsO_4 for another 2 h. After dehydrating in series of graded acetone washes, samples were embedded. Ultrathin sections (70 nm thick) were stained with uranyl acetate and lead citrate and examined under a JEM-1400 transmission electron microscope (Japan).

Western blotting

In order to determine the protein expressions of target genes, western blotting was carried out. In brief, total protein was extracted from cells by RIPA buffer. Protein concentration was measured using a BCA Protein Assay reagent kit (Pierce, USA) following the manufacturer's instructions. Equal amounts of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a PVDF membrane. Membranes were blocked with Tris buffered saline plus Tween-20 (TBS-T) containing 5% w/v dried skim milk, and incubated with primary antibodies at 4 °C overnight. The primary antibodies used were anti-p-RhoA (1:500 dilution), anti-p-Rac1 (1:300 dilution), anti-p-ERK1/2 (1:200 dilution) and anti-GAPDH (1:700 dilution). After washing with TBS-T, membranes were incubated with HRP-labeled IgG secondary antibodies (1:700 dilution). Immunoreactivity was visualized by gel imaging system (Bio-Rad, USA) using enhanced chemiluminescence (ECL) detection reagent (Pierce, USA). To quantify the protein level, the expression bands of target proteins were analyzed by Quantity One software. GAPDH was used as an internal control. Data were calculated from three independent experiments.

Measurement of PKA and PKC activities

PKC and PKA activities were examined using the respective PreTag nonradioactivity assay kits according to the manufacturer's instructions (Promega, USA).

Statistical analysis

Data were analyzed with Statistical Package for the Social Sciences (SPSS) version 11.0 software and were expressed as means ± standard deviation (SD). Paired Student t-test was used for comparison between different groups. Statistical differences between values were determined by ANOVA. A $P < 0.05$ was considered to be statistically different.

Results

CRH and UCN enhanced phagocytosis of microspheres in rat macrophages

As evaluated by flow cytometry analysis, CRH (ranged from 0.1 nM to 100 nM) and UCN (ranged from 1 nM to 100 nM) significantly enhanced the uptake of fluorescent beads by rat macrophages (Fig. 1). Thirty minutes after phagocytosis, the percentage of phagocytosis and the relative mean fluorescence intensity of YG beads were statistically elevated after 1 h of CRH or UCN administration ($P < 0.05$ compared with control) (Fig. 1A, B). With prolonged duration of microsphere