Enhanced Intracellular Calcium Induced by Urocortin Is Involved in Degranulation of Rat Lung Mast Cells

Yuqing Wu^{1,2}, Jue Hu¹, Rongjian Zhang¹, Chenghua Zhou², Youhua Xu¹, Xiaowei Guan¹ and Shengnan Li¹

¹Department of Pharmacology, Nanjing Medical University, Nanjing, ²Department of Pharmacology, Xuzhou Medical College, Xuzhou, P R. China

Key Words

Urocortin (UCN) • Lung • Mast cell • Degranulation • Intracellular calcium

Abstract

Corticotropin-releasing factor (CRF), which activates the hypothalamic-pituitary-adrenal axis under stress, also has proinflammatory peripheral effects possibly through mast cells. The purpose of this study was to investigate the effect of urocortin (UCN), a 40-amino-acid CRF family peptide, on degranulation and intracellular calcium of rat lung mast cells. The activation and degranulation of mast cells were observed by Toluidine blue staining and transmission electron microscope. The intracellular calcium was investigated using confocal laser scanning microscopy and flow cytometry. The results indicated that all the three different concentrations of UCN (0.1, 1 and 10 μ M) significantly induced the activation and degranulation of rat lung mast cells *in vitro*. This effect was markedly blocked by selective CRF receptor 1 (CRF-R1) antagonist antalarmin, but not by specific CRF receptor 2 (CRF-R2) antagonist antisauvagine-30 (anti-Svg-30). The results also showed that UCN caused a rapid peak increase in $[Ca^{2+}]_i$ at point of

300s after UCN treatment, followed by a decrease to a sustained plateau phase. The peak increase in $[Ca^{2+}]_i$ induced by UCN was significantly inhibited by antalarmin, but not by anti-Svg-30. This effect of UCN on $[Ca^{2+}]_i$ in rat lung mast cells was also found by flow cytometry. Regression analysis revealed a positive correlation between mast cells degranulation extent and the maximum value of $[Ca^{2+}]_i$ ($P < 0.01$). Taken together, our present study suggested that UCN induced the increase of $[Ca^{2+}]_i$ and degranulation of rat lung mast cells through CRF-R1. These findings may have implications for the pathophysiology of allergic and inflammatory lung disorders such as asthma, which is closely associated with mast cell activation and degranulation.

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Introduction

Urocortin (UCN), a recently identified 40-amino-acid mammalian peptide from rat midbrain sharing 45% sequence homology with corticotropin-releasing factor (CRF) [1], is a new member of the CRF family. By regulating hypothalamic-pituitary-adrenal axis, centrally

produced CRF exerts profound immunosuppressive effect [2]. CRF and UCN have also been shown to be present at many peripheral tissues where, they act as locally expressed autocrine or paracrine proinflammatory factors [3-5].

Recent studies showed human cord blood-derived cultured mast cells(hCBMC) and leukemic mast cells (HMC-1) are immunocytochemically positive for CRF and UCN. The ability of these mast cells to synthesize CRF and UCN was confirmed by showing mRNA expression with RT-PCR. Stimulation of IgE-sensitized hCBMC with anti-IgE results in secretion of more CRF and UCN [6]. Moreover, CRF [7, 8] and UCN [9] induce mast cell degranulation and vascular permeability in rodent skin, but absent in W/W^v mast cell deficient mice [7]. High numbers of activated mast cells are present in endometriosis sites that were strongly positive for CRF/UCN. CRF and UCN may activate mast cells and contribute to the fibrosis and inflammation in endometriosis [10]. Systemic or uterine stressful conditions may increase the incidence of spontaneous abortions by inducing the local release of CRF and UCN, which then activate endometrial mast cells to secrete abortogenic tryptase and IL-8 [11]. These findings indicate that mast cell is not only a potential source of CRF and UCN which could have both autocrine and paracrine functions but also the target of them [12, 13].

Our group recently found that both UCN mRNA and peptide were obviously expressed in rat lung by RT-PCR and immunohistochemistry [14]. The expression of UCN was significantly up-regulated in inflammatory airway of asthmatic rats [15]. We also found that rats receiving inhalation aerosol of UCN had a significant elevation of lung vascular permeability by the Evans blue (EB) technique. Enhanced pulmonary vascular permeability induced by UCN was markedly inhibited by pretreatment with the mast-cell stabilizer cromolyn [14]. It suggested that peripherally produced UCN in lung as a possible autocrine and paracrine immune-inflammatory mediator may participate in the pathophysiology of allergic asthma through activating mast cells.

Although both many previous studies and our results have indicated the stimulating effect of urocortin on mast cells in various inflammatory conditions in vivo, to date, there is no direct evidence that UCN induces activation and degranulation of mast cells in vitro. To further determine whether peripherally produced UCN in lung participate in the pathophysiology of mast cell related lung disorders, we have undertaken this study to examine the effect of UCN on activation and degranulation of mast

cells isolated from rat lung in vitro. Furthermore, we also examined the Ca²⁺ mechanism of UCN on mast cell activation and degranulation.

Materials and Methods

Rat lung mast cell preparations

Healthy male Sprague-Dawley (SD) rats 6 to 8 week of age and weighing 250 to 300 g were provided by Experimental Animal Center of Nanjing Medical University. The study was carried out in agreement with the official recommendations of The Chinese Community guidelines. Rats were killed by decapitation and bloodletting. Rat lung mast cells were isolated and purified according to the method previously reported [16, 17]. Mast cell counts were obtained using toluidine blue staining and cell viability was determined using trypan blue exclusion. Cell viability was consistently greater than 98%. The purity range of partially purified mast cells was about 80%.

Toluidine blue staining for mast cell

Freshly purified mast cells were suspended in sterile RPMI-1640 (2x10⁵ cells/ml) at 37°C in 5% CO₂. The medium was supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Cells were divided into seven groups treated with normal saline (control), UCN (0.1, 1 and 10 µM), antalarmin and 10 µM UCN, anti-Svg-30 and 10 µM UCN, and compound 48/80 respectively. Antalarmin and anti-Svg-30 were administered 20 min before 10 µM UCN treatment. After UCN treatment 30 min and compound 48/80 treatment 5 min, the cultured cells were collected. Cytospin slides were prepared and fixed in 10% neutral formalin. Mast cells were stained with 0.25% acidified (pH=2) toluidine blue (Sigma, St. Louis, MO, USA) for 15 min at room temperature to identify mast cells [18]. Mast cells were observed at 400x under light microscope. Mast cell activation and degranulation was defined as the presence of extruded granules close to the surface of the cell in question or staining of about half or less of the cell section with toluidine blue [19]. To quantitate the incidence of activation, the degranulated cells were counted. The data were presented as the percentage of degranulated cells to the total number of mast cells.

Electron Microscopy for mast cell

The same cells culture and administration as Toluidine blue staining were performed. The collected mast cells from different groups were centrifuged and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for at least 24 hours. The pellets was rinsed with 0.1 M cacodylate buffer and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer at 4°C for 1 h, dehydrated in an acetone series, and embedded in Epon 812. Thin sections were stained with uranyl acetate and diluted lead citrate and observed in transmission electron microscope. To quantitate the degranulation extent, the degranulated granules were counted. Degranulation extent was indicated as the percentage of degranulated granules to the total number of granules in a

Fig. 1. Effect of urocortin on activation and degranulation of rat lung mast cells in vitro. A: Treatment with normal saline for 30 min. B: Treatment with 10 μ M UCN for 30 min. C: Pretreatment with 10^{-4} M antalarmin 20 min before 10 μ M UCN treatment. D: Pretreatment with 10^{-4} M Anti-Svg-30 20 min before 10 μ M UCN treatment. E: Treatment with Compound 48/80 for 5 min. (Toluidine blue staining, $\times 400$).

mast cell. In each section three cells were counted and the data was presented as mean of three values.

Determination of $[Ca^{2+}]_i$ Level by laser scanning confocal microscope

The isolated and purified mast cells from rat lung were suspended in RPMI 1640 culture medium. The cell suspension was added in a 6-well culture plate which was placed a 24 mm glass coverslips treated with poly-L-lysine in every well. After mast cells were fixed on coverslips, the culture medium was discarded and the coverslips were washed three times. Then the mast cells on coverslip were incubated with 6 μ M Fluo 3-AM in PBS containing Ca^{2+} and Mg^{2+} for 40 min in the dark at 37°C. Extracellular Fluo 3 was removed by washing three times. Intracellular Ca^{2+} imaging was carried out by means of a LSM 510 invert confocal laser scanning microscope (Carl Zeiss Jena, Germany). The Fluo-3 dye was excited with a 488 nm wavelength argon laser beam and the emission fluorescence was monitored at 525 nm. $[Ca^{2+}]_i$ was expressed as a fluorescence intensity relative to basal fluorescence.

Determination of $[Ca^{2+}]_i$ Level by Flow cytometry

The isolated and purified mast cells from rat lung were incubated with Fluo-3 AM (5 mM) in PBS containing Ca^{2+} and

Mg^{2+} for 30 min at 37°C. Then the cell suspension was centrifuged at 1500 rpm for 5 min, and the supernatant was discarded. Cells were washed and next resuspended with 1 ml PBS containing Ca^{2+} and Mg^{2+} . $[Ca^{2+}]_i$ of 10,000 cells was analyzed by flow cytometry. The mean fluorescence intensity of cell populations was recorded.

Statistics analysis

All values were expressed as mean $\pm$ SD. The significance for the difference between the groups was analyzed with SPSS 10.0 by one-way ANOVA. Differences were considered significant when $P < 0.05$.

Results

Toluidine blue staining for mast cell

As shown in Fig. 1 and 2, there was very few mast cells degranulation in NS control group ($8.58 \pm 1.73\%$). UCN (0.1, 1 and 10 μ M) induced a marked increase of mast cells activation and degranulation ($61.15 \pm 8.44\%$, $41.97 \pm 11.09\%$ and $40.25 \pm 11.87\%$). In the light microscope, a large number of the degranulated cells

Fig. 2. Effect of urocortin on activation and degranulation of rat lung mast cells in vitro by Toluidine blue staining. To quantitate the extent of activation and degranulation, the degranulated cells were counted. The data were presented as the percentage of degranulated cells to the total number of mast cells. * $P < 0.05$, ** $P < 0.01$, vs control; ## $P < 0.01$, vs UCN $10 \mu\text{M}$; $n = 6$.

Fig. 3. Effect of urocortin on granules extrusion of rat lung mast cells in vitro. A: Treatment with normal saline for 30 min. B: Treatment with $10 \mu\text{M}$ UCN for 30 min. C: Pretreatment with 10^{-4} M antalarmin 20 min before $10 \mu\text{M}$ UCN treatment. D: Pretreatment with 10^{-4} M Anti-Svg-30 20 min before $10 \mu\text{M}$ UCN treatment. E: Treatment with Compound 48/80 for 5 min. (Transmission electron microscope observation, $\times 6000$ to 8000).

showed a swollen appearance and were stained pink instead of dark blue. The extruded granules close to the surface of the cell were seen (Fig. 1B). It was statistically significant when compared with that from control group ($P < 0.01$). Enhanced mast cells degranulation induced by C48/80 was performed as a positive control (Fig. 1E). We then investigated whether the effect of UCN was mediated by specific CRF receptors. In the present study

we found that treatment with selective CRF-R1 antagonist antalarmin (10^{-4} M) 20 minutes before the administration of UCN ($10 \mu\text{M}$) markedly inhibited the response of mast cells to UCN ($17.57 \pm 8\%$ vs $61.15 \pm 8.44\%$, $P < 0.01$). However, the enhanced degranulation of mast cells induced by UCN ($10 \mu\text{M}$) was not inhibited by specific CRF-R2 antagonist anti-Svg-30.

Fig. 4. Effect of urocortin on granules extrusion of rat lung mast cells in vitro by transmission electron microscope observation. To quantitate the degranulation extent, the degranulated granules were counted. Degranulation extent was indicated as the percentage of degranulated granules to the total number of granules in a mast cell. In each section three cells were counted and the data was presented as mean of three values. * $P<0.05$, ** $P<0.01$, vs control; # $P<0.05$, ## $P<0.01$, vs UCN 10 μM ; $n=6$.

Fig. 5. Fluorescence microphotograph of rat lung mast cells. Intracellular Ca^{2+} imaging was carried out using a Ca^{2+} -sensitive indicator Fluo 3-AM by means of a laser scanning confocal microscope after different treatment.

Electron Microscopy

As shown in Fig. 3 and 4, there was very few granules extrusion from mast cells in NS control group ($5.85 \pm 2.42\%$). The cytoplasm of the cell is tightly packed with granules having a uniformly dense and osmiophilic structure. Few altered granules can be observed (Fig. 3A). UCN (0.1, 1 and 10 μM) induced a marked increase of mast cells granules extrusion ($50.97 \pm 15.5\%$, $40.17 \pm 10.24\%$ and $36.05 \pm 9.99\%$). It was statistically significant when compared with that from control group ($P<0.01$). Enhanced mast cells degranulation induced by C48/80 was performed as a positive control (Fig. 3E). Furthermore, we found that treatment with selective CRF-R1 antagonist antalarmin (10^{-4} M) 20 minutes before the administration of UCN (10 μM) markedly inhibited the granules extrusion of mast cells to UCN ($9.93 \pm 3.57\%$ vs $50.97 \pm 15.5\%$, $P<0.01$). However, specific CRF-R2 antagonist anti-Svg-30 failed to suppress the degranulation of mast cells induced by UCN (10 μM). The results obtained by electron microscopy analysis were consistent with those by Toluidine blue staining for mast cells.

Determination of $[\text{Ca}^{2+}]_i$ Level by Confocal laser scanning microscope

To clarify the effect of UCN on intracellular Ca^{2+} in rat lung mast cells, LSCM was performed. As shown in Fig. 5 and 6, in Ca^{2+} -containing medium, UCN caused a rapid peak increase in $[\text{Ca}^{2+}]_i$ at 300s after administration, followed by a decrease to a sustained plateau phase. Although no obvious effect of anti-Svg-30 on enhanced $[\text{Ca}^{2+}]_i$ induced by UCN in rat lung mast cells, antalarmin

Fig. 6. Effect of urocortin on intracellular Ca^{2+} in rat lung mast cells in Ca^{2+} -containing medium. (A) Tracing showing the effect of normal saline on Ca^{2+} response. (B) Tracing showing the effect of UCN (10 μM) on Ca^{2+} response. (C) Cells were pretreated with antalarmin (10⁻⁴ M) for 20 min before UCN stimulation. (D) Cells were pretreated with anti-Svg-30 (10⁻⁴ M) for 20 min before UCN stimulation. (E) Tracing showing the effect of C48/80 on Ca^{2+} response.

significantly attenuated this effect of UCN. C48/80 induced a rapid peak increase in $[\text{Ca}^{2+}]_i$ at 10s after administration, and the maximum value of $[\text{Ca}^{2+}]_i$ was seen at 50s (Fig. 5B and 6E). In control group, intracellular fluorescence intensity was steady and less than 50. At 300s after UCN treatment fluorescence intensity in rat lung mast cells come to the maximum value (85.25 ± 4.49 , $P < 0.01$, vs control). The increased fluorescence intensity of intracellular Ca^{2+} induced by UCN was markedly inhibited by antalarmin from (85.25 ± 4.49) to (43.85 ± 4.83) ($P < 0.01$), while not by anti-Svg-30 (90.63 ± 4.96) (Fig. 7A and 8A). After C48/80 treatment, the maximum value of fluorescence intensity (127.58 ± 8.25 , $P < 0.01$, vs UCN) was observed at 50s (Fig. 7B and 8B). It suggested that the increase of $[\text{Ca}^{2+}]_i$ induced by UCN is later and weaker than that induced by C48/80.

Determination of $[\text{Ca}^{2+}]_i$ Level by Flow cytometry

The effects of UCN on $[\text{Ca}^{2+}]_i$ Level obtained by flow cytometry were consistent with those by laser scanning confocal microscope. The fluorescence intensity was recorded at point of 300s after UCN administration and 50s after C48/80 stimulation. As shown in Fig. 9, the fluorescence intensity of $[\text{Ca}^{2+}]_i$ Level was largely raised by UCN from control level (91.63 ± 16.61) to (352.23 ± 49.68) ($P < 0.01$). The increased fluorescence intensity of intracellular Ca^{2+} induced by UCN was significantly downregulated by pretreatment with antalarmin from (352.23 ± 49.68) to (88.49 ± 19.89) ($P < 0.01$), while not by anti-Svg-30 (367.29 ± 52.61). C48/80 was used as a positive control. After stimulation with C48/80 for 50s, the fluorescence intensity reached (489.45 ± 68.86) ($P < 0.01$, vs control).

Fig. 7. (A) The fluorescence intensity of intracellular Ca^{2+} at 0s, 300s and 400s time point in rat lung mast cells in different groups. ** $P < 0.01$, vs 0s; ## $P < 0.01$, vs 300s. (B) The fluorescence intensity of intracellular Ca^{2+} at 0s, 50s and 200s time point in rat lung mast cells in different groups. ** $P < 0.01$, vs 0s; ## $P < 0.01$, vs 50s. The statistical analysis was performed in the same group.

Discussion

Mast cells are critical in allergic and inflammatory diseases such as asthma by releasing vasoactive molecules, proteases and cytokines. Urocortin (UCN), a new member of CRF family, has proinflammatory peripheral effects possibly through mast cells. We recently reported enhanced expression of UCN in asthmatic rat lung [15]. It was also found that rats receiving inhalation

Fig. 8. (A) Effect of urocortin on fluorescence intensity of intracellular Ca^{2+} at 0s, 300s and 400s time point in rat lung mast cells. * $P < 0.05$, ** $P < 0.01$, vs control; ## $P < 0.01$, vs urocortin. (B) Effect of C48/80 on fluorescence intensity of intracellular Ca^{2+} at 0s, 50s and 200s time point in rat lung mast cells. ** $P < 0.01$, vs control. The statistical analysis was performed at the same time point.

aerosol of UCN had a significant elevation of lung vascular permeability possibly through activating mast cells [14]. In the present study we further investigate the effect of UCN on rat lung mast cell activation and degranulation in vitro.

Both the toluidine blue staining and electron microscopy observation results indicated that although the extent of degranulation was weaker than that induced by C48/80, different concentrations of UCN (0.1, 1 and

Fig. 9. Effect of urocortin on intracellular Ca^{2+} in rat lung mast cells in Ca^{2+} -containing medium. Fluorescence intensity was recorded using a Ca^{2+} -sensitive indicator Fluo 3-AM by flow cytometry. (A) The fluorescence intensity was recorded at point of 300s after administration. (B) The fluorescence intensity was recorded at point of 50s after C48/80 stimulation. In antalarmin and anti-Svg-30 groups, cells were pretreated with antalarmin (10^{-4} M) and anti-Svg-30 (10^{-4} M) for 20 min respectively before UCN stimulation. ** $P < 0.01$, vs control; ## $P < 0.01$, vs urocortin.

10 μM) all caused significant activation and degranulation of rat lung mast cells in vitro. We then investigated whether the effect of UCN was mediated by specific CRF receptors. In the present study we found that treatment with selective CRF-R1 antagonist antalarmin (10^{-4} M) 20 minutes before the administration of UCN (10 μM) markedly inhibited the response of mast cells to UCN. However, the enhanced degranulation of mast cells induced by UCN (10 μM) was not influenced by specific CRF-R2 antagonist anti-Svg-30. It suggested that the effect of UCN inducing activation and degranulation of rat lung mast cells in vitro is mediated by CRF-R1.

It has been demonstrated that both CRF-R1 and CRF-R2 were expressed in human mast cells [20, 21].

Recent study reported CRF-induced secretion of vascular endothelial growth factor (VEGF) from mast cells was blocked by the CRF-R1 antagonist antalarmin, but not the CRF-R2 antagonist astressin 2B [21]. CRA1000, a selective CRF-R1 antagonist, reduced the number and activation of mast cells in stress-induced exacerbation of chronic contact dermatitis in rats [22]. CRF and UCN triggered mast cells degranulation and increased vascular permeability in rat skin at least through CRF-R1 [23, 24]. All the results above suggested that CRF-R1 may mediated the activation and degranulation of mast cells. Our result that rat lung mast cells degranulation induced by UCN in vitro is mediated by CRF-R1 was consistent with previous reports.

The features of the exocytotic process of mast cells have been described: 1) an increase in intracellular calcium ion concentration ($[\text{Ca}^{2+}]_i$) and 2) the disassembly of actin filaments at the cell surface. An $[\text{Ca}^{2+}]_i$ increase plays a crucial role in the stimulus-secretion coupling in a variety of cells [25, 26], including mast cells [27-29]. In order to clarify the Ca^{2+} mechanism of UCN on mast cell activation and degranulation we further examined the effect of UCN on $[\text{Ca}^{2+}]_i$ in rat lung mast cells. The results by laser scanning confocal microscope showed that UCN induced a rapid peak increase in $[\text{Ca}^{2+}]_i$ at 300 s after administration, followed by a decrease to a sustained plateau phase. This $[\text{Ca}^{2+}]_i$ increase induced by UCN was significantly blocked by selective CRF-R1 antagonist antalarmin, while not by anti-Svg-30. The similar results about changes of $[\text{Ca}^{2+}]_i$ were also obtained by flow cytometry. It suggested that the enhanced $[\text{Ca}^{2+}]_i$ triggered by UCN in rat lung mast cells was mediated mainly by CRF-R1. Compared to UCN, C48/80 induced a rapid peak increase in $[\text{Ca}^{2+}]_i$ at 10s after administration, and the maximum value of $[\text{Ca}^{2+}]_i$ was higher and seen at 50s after administration. It suggested that the increase of $[\text{Ca}^{2+}]_i$ induced by UCN is later and weaker than that induced by C48/80. Regression analysis revealed a positive correlation between mast cells degranulation extent and the maximum value of $[\text{Ca}^{2+}]_i$ ($P < 0.01$). Previous studies reported that both CRF and UCN elevated $[\text{Ca}^{2+}]_i$ levels in rat pancreatic beta-cells [30] and single human skin cells [31] mainly by activation of CRF-R1. This increase in $[\text{Ca}^{2+}]_i$ levels mediated by CRF-R1 was also observed in rat lung mast cells in our present study.

CRF-R1 belongs to the family of seven transmembrane segment proteins coupled to the Gs signaling system. The CRF-R1 signaling pathway involves cAMP and calcium activated metabolism. It was

demonstrated that the phenotypic effects of CRF in human skin cells are largely mediated by CRF-R1alpha through increases in concentrations of cAMP, inositol triphosphate (IP₃), or Ca²⁺ with subsequent activation of protein kinases A (PKA) and C (PKC) dependent pathways [31]. Cutaneous expression of the CRF/proopiomelanocortin (POMC) system appears to generate a function analogous to the hypothalamic-pituitary-adrenal (HPA) axis, representing general mechanism of cutaneous response to stressful stimuli [32, 33]. The present study indicated that UCN significantly triggered the activation and degranulation of rat lung mast cells and markedly induced the increase of intracellular calcium through activation of CRF-R1. Our results suggest that UCN may activate CRF-R1 coupled to the Gs signaling system in lung mast cells, which leads to an increase in intracellular calcium. The enhanced intracellular calcium in lung mast cells triggers the activation and degranulation

of mast cells, which release various inflammatory factors. These results imply that UCN may be one of the most potent triggers of rat lung mast cell degranulation and involved in pathophysiology of allergic and inflammatory lung disorders such as asthma, which is closely associated with mast cell activation and degranulation.

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