

Expression of urocortin in rat lung and its effect on pulmonary vascular permeability

Yuqing Wu, Yinyan Xu, Hong Zhou, Jin Tao and Shengnan Li

Department of Pharmacology, Nanjing Medical University, Nanjing, 210029, P R China
(Requests for offprints should be addressed to S Li; Email: njmusnli@126.com)

Abstract

Urocortin (UCN), a newly identified, 40-amino-acid, corticotropin-releasing hormone (CRH) structurally related peptide, has been demonstrated to be expressed in the central nervous system and many peripheral tissues of rats and man. This study aimed to investigate the expression profile of UCN in rat lung and the effect of UCN on lung vascular permeability. The expression of UCN mRNA was detected by reverse transcriptase PCR (RT-PCR). UCN peptide was measured by immunohistochemistry and Western blot analysis. We found that both UCN mRNA and peptide were obviously expressed in rat lung. Immunohistochemistry results showed that UCN peptide is mainly expressed in bronchial epithelium mucosa and alveolar epithelium. We also found that rats receiving inhalation aerosol of UCN had a significant elevation of lung vascular permeability compared with rats receiving vehicle and ovalbumin (OVA) by the Evans blue (EB) technique. UCN aerosol inhalation resulted in

obvious pulmonary congestion and edema observed under light microscope by hematoxylin and eosin (HE) staining. The nonselective peptide CRH receptor antagonist astressin markedly reduced lung vascular permeability triggered by UCN. Enhanced pulmonary vascular permeability induced by UCN was markedly inhibited by pretreatment with the mast-cell stabilizer cromolyn and histamine-1 (H₁) receptor antagonist azelastine respectively, but not by the leukotriene receptor antagonist montelukast. In summary, in the present study, we demonstrated for the first time that UCN is expressed in rat lung and contributes to an increase in lung vascular permeability through activation of CRH receptors. Mast cells and histamine may be involved in this effect of UCN. Peripherally produced UCN in lung may act as an autocrine and paracrine proinflammatory factor.

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Introduction

Corticotropin-releasing hormone (CRH) is a 41-amino-acid neuropeptide, best known for its role in regulating the hypothalamic-pituitary adrenal axis in mammals (Vale *et al.* 1981, Rivier *et al.* 1983, Shibahara *et al.* 1983, Schulte *et al.* 1985, Chrousos 1995). By stimulating adrenocorticotropin hormone (ACTH) release from the pituitary (Vale *et al.* 1981, Linton *et al.* 1985) and, thus, cortisol secretion, centrally produced CRH exerts profound immunosuppressive effects (Chrousos 1995). More recently, a new member of the CRH family, urocortin (UCN), was cloned and shown to be expressed in the central nervous system of rats (Vaughan *et al.* 1995) and man (Donaldson *et al.* 1996). Rat UCN is a 40-amino-acid peptide with 45% sequence identity to CRH (Vaughan *et al.* 1995), 95% amino-acid identity with human UCN (Donaldson *et al.* 1996) and 63% identity with the fish peptide, urotensin (Vaughan *et al.* 1995). UCN has central effects similar to those of CRH, including stimulation of ACTH secretion and appetite suppression (Spina *et al.* 1996).

There are two receptors for CRH and UCN, the CRH receptor types 1 (CRH-R1) and 2 (CRH-R2) (Liaw *et al.* 1996, Vale *et al.* 1997). CRH has low affinity for CRH-R2 and high affinity for CRH-R1, whereas UCN has high affinity for both (Kishimoto *et al.* 1995). UCN has fourfold higher affinity than CRH for CRH-R1 and 40-fold higher affinity for CRH-R2 (Vaughan *et al.* 1995).

Besides central nervous system distribution and effects, CRH and UCN have also been shown to be present in many peripheral tissues, where, in contrast to their systemic, indirect immunosuppressive effects, they act as autocrine or paracrine proinflammatory factors (Karalis *et al.* 1991, Schulte *et al.* 1995). Indeed, many recent studies report the local production of immune CRH and UCN in various inflammatory sites, including carrageenin-induced granulomatous tissues (Karalis *et al.* 1991) and streptococcal cell wall- and adjuvant-induced arthritic joints of rats (Crofford *et al.* 1992), and the joints of patients with rheumatoid arthritis (RA) and osteoarthritis (OA) (Crofford *et al.* 1993, Masataka *et al.* 2001, Uzuki *et al.* 2001), as well as in some neurogenic skin disorders (Katsarou-Katsari *et al.* 2001),

autoimmune thyroiditis and ulcerative colitis (Chrousos 1995, Saruta *et al.* 2004). Moreover, systemic administration of anti-CRH serum reduced carrageenin-induced subcutaneous inflammation (Karalis *et al.* 1991). UCN along with CRH may, therefore, participate in the pathophysiology of many inflammatory conditions.

Recent studies show that human cord blood-derived, cultured mast cells (hCBMC) and leukemic mast cells (HMC-1) are immunocytochemically positive for CRH and UCN. The ability of these mast cells to synthesize CRH and UCN was confirmed by showing mRNA expression with reverse transcriptase PCR (RT-PCR). Stimulation of IgE-sensitized hCBMC with anti-IgE results in secretion of more CRH and UCN (Kempuraj *et al.* 2004a). Moreover, CRH (Michael *et al.* 2003, Theoharides *et al.* 1998) and UCN (Singh *et al.* 1999a) induce mast cell degranulation and vascular permeability in rodent skin, but not in W/W^v mast cell-deficient mice (Theoharides *et al.* 1998). High numbers of activated mast cells are present in endometriosis sites strongly positive for CRH/UCN. CRH and UCN may activate mast cells and contribute to the fibrosis and inflammation in endometriosis (Kempuraj *et al.* 2004b). Systemic or uterine stressful conditions may increase the incidence of spontaneous abortions by inducing the local release of CRH and UCN, which then activate endometrial mast cells to secrete abortogenic tryptase and interleukin (IL)-8 (Bhuvaneshwari *et al.* 2003). These findings indicate that mast cells are not only a potential source, but also the target of CRH and UCN, which could have both autocrine and paracrine functions (Theoharides *et al.* 2004). This finding that mast cells secrete CRH/UCN and are activated by it reinforces the bidirectional relationship between CRH/UCN and mast cells in inflammatory conditions (Theoharides & Cochrane 2004), especially in allergic inflammatory disorders exacerbated by stress. Furthermore, the expression of both UCN mRNA and peptide in human lymphocytes and Jurkat T lymphoma cells has been proven (Bamberger *et al.* 1998).

CRH has been detected in many peripheral tissues, including fetal lung (Keegan *et al.* 1994, Muglia *et al.* 1994). Although UCN has also been demonstrated to be considerably expressed peripherally, the expression of UCN and its biologic actions in lung have never been reported. In this study, we investigated and reported the expression profile of UCN in rat lung tissues at both mRNA and peptide level by RT-PCR, immunohistochemistry and Western blotting. On the basis of the already mentioned role of UCN in inflammation, we evaluated the effect of UCN on lung vascular permeability.

Materials and Methods

Animals

Healthy male Sprague-Dawley (SD) rats 6–8 weeks of age and weighing 250–300 g were provided by the

Experimental Animal Center of Nanjing Medical University. All the rats were housed three per cage, given free access to food and water, and maintained in animal facilities with filtered air on a diurnal lighting cycle. All the studies were carried out in agreement with the official recommendations of the Chinese Community guidelines.

Collection of tissues

Six rats were killed by decapitation and exsanguinated. After midline thoracotomy, lung tissues were collected from rats and either flash frozen in liquid nitrogen for RNA and protein extraction, or fixed in 10% neutral formalin, embedded in paraffin, and sectioned onto poly-L-lysine-coated slides for immunohistochemical analysis and hematoxylin and eosin (HE) staining.

Semiquantitative RT-PCR for UCN mRNA

Total RNA was extracted from 30 mg lung tissues with RNeasy Mini Kit (Qiagen), according to the manufacturer's guidelines, and quantified by UV absorption. The following procedures and chemicals for RT-PCR were used. For cDNA synthesis (Qiagen), 20 µl reverse transcription mixture containing total RNA 2 µg, each dNTP mixture 0.5 mmol/l, Oligo-dT primer 0.5 µmol/l, RNase inhibitor 10 U, and Omniscript reverse transcriptase (Qiagen) 4 U were well mixed and incubated at 37 °C for 60 min. Specific primers for rat GAPDH (an internal standard) and UCN were synthesized from published sequences (Baigent & Lowry 2000):

- GAPDH primers – forward primer: 5'-TCC CAG AGC TGA ACG GGA AGC TCA CTG-3'; reverse primer: 5'-TGG AGG CCA TGT AGG CCA TGA GGT CCA-3'

- UCN primers – forward primer: 5'-GCT ACG CTC CTG GTA GCG TTG CTG CTT CTG-3'; reverse primer: 5'-GCC GAT CAC TTG CCC ACC GAG TCG AAT ATG-3'.

All primers were synthesized by Takara Co (Dalian, China) with the product sizes 339 and 356 bp respectively. PCR reaction constituents were as follows: 2 µl cDNA mixture was subjected to amplification in 30 µl final volume with MgCl₂ 1.5 mmol/l, each dNTP 0.2 mmol/l, 12 pmol of each primer, and 2 U of Taq DNA polymerase in the reaction buffer. PCR reaction conditions were as follows: 95 °C for 5 min and then 94 °C for 30 s, 52 °C for 40 s, 72 °C for 40 s, for 35 cycles; and 72 °C for 10 min to end the reaction. PCR products of 10 µl were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. The density of each band was measured by UVP gel analysis system. Control PCR reactions were used to overcome the possibility of amplifying genomic DNA in these circumstances.

Immunohistochemistry for UCN peptide

Immunohistochemical staining was performed with the avidinbiotin peroxidase complex. Lung tissue specimens were preserved in 10% neutral formalin, embedded in paraffin, serially sectioned onto microscope slides at a thickness of 5 μm , and then deparaffinized. The slides were immersed for 10 min in 3% hydrogen peroxide to deplete endogenous peroxidase activity. Nonspecific binding sites were saturated by exposure to 0.2% BSA and normal goat serum for 20 min. Rabbit polyclonal, UCN-specific antibody (Sigma; 1:100 dilution) that reacts specifically with urocortin and does not cross-react with structurally related CRH family peptides, including CRH, sauvagine and urotensin, or control normal rabbit IgG, was applied to tissue sections and incubated in a humidified chamber at 37 °C for 60 min. Then the slides were washed with PBS for 10 min. Biotinylated goat antirabbit IgG was applied to tissue sections, and the slides were incubated at room temperature for 30 min. They were then washed with PBS for 10 min, followed by incubation with prepared streptavidin biotinylated peroxidase complex for 45 min and washed with PBS for 10 min. Finally, color was developed by adding 3,3'-diaminobenzidine to the sections. The sections were counterstained with hematoxylin, dehydrated and observed under light microscope. Positive staining was indicated by brownish deposits.

Western blot analysis for UCN peptide

The lung tissues from rats were homogenized and centrifuged in ice-cold lysis buffer containing protease inhibitor phenylmethylsulfonyl fluoride. The pellets were discarded. The protein content of the crude preparations was measured by the Bradford method with lyophilized BSA (Bio-Rad) as standard. The protein sample (50 μg) was separated on a 15% SDS-polyacrylamide gel for 70 min and electrophoretically transferred onto a nitrocellulose membrane in Tris-glycine transfer buffer containing 20% methanol in a Trans-Blot cell (Bio-Rad) for 35 min. A low-molecular-weight marker (Rainbowmarker; Amersham-Buchler) served as standard. Membranes were blocked in 5% instant nonfat dry milk in Tris-buffered saline, washed in Tris-buffered saline, and probed with rabbit-raised antibody against UCN (Sigma; 1:1000 dilution) that reacts specifically with urocortin and does not cross-react with structurally related CRH family peptides including CRH, sauvagine and urotensin. The immunoblots were subsequently washed and incubated in horseradish peroxidase-coupled antirabbit IgG antibody (1:5000) for 1 h. The bound antibodies were visualized by enhanced chemiluminescence with the ECL system (Amersham Pharmacia Biotech) and exposure to X-OMAT film (Kodak). Western blots were repeated five times with qualitatively similar results.

Detection of Evans blue (EB) extravasation

To investigate the effect of UCN on pulmonary microvascular permeability, 10^{-6} , 10^{-7} and 10^{-8} M UCN was inhaled by aerosol respectively at flow rate of 0.5 ml per min for 10 min. Rats in negative control groups were treated with normal saline (NS) and ovalbumin (OVA) respectively, and the values were used as normal control for all the EB extravasation experiments. Rats in positive control were treated with histamine. To study the effects of cromolyn, astressin, azelastine and montelukast on EB extravasation triggered by 10^{-6} M UCN, different concentrations of cromolyn, astressin, azelastine and montelukast were inhaled by aerosol respectively 20 min before treatment of UCN. Eight rats in each group were used for experiments. EB, a nonradioactive diazo dye that binds tightly to albumin, is a sensitive marker for early pulmonary edema (Udaka *et al.* 1970, Patterson *et al.* 1992). Prolonged perfusion with EB does not alter vasoreactivity or water content in isolated, perfused rat lungs (Dallal & Chang 1994). A solution of 1% EB (containing 100 U heparin per ml solution) was prepared in sterile water and stored at 4 °C. After inhalation aerosol of UCN, the rats were injected intravenously with 1% EB (2 ml/kg) via tail vein. The animals were anesthetized 60 min later by intraperitoneal injection with sodium pentobarbital (30 mg/kg). After midline thoracotomy, the superior and inferior vena cava were ligated, the aorta was transected, and 20 ml normal saline solution were injected into the right ventricle at a pressure of 20 cmH_2O to wash out the pulmonary intravascular content. A sample of lung tissue was weighed, immersed in *N,N*-dimethylformamide, and homogenized. The homogenate was incubated at 50 °C for 36 h and centrifuged at 3900 *g* for 10 min. The optical density of 1 ml supernatant was measured at 620 nm wavelength, and the EB concentration was determined from a standard curve of EB-*N,N*-dimethylformamide solutions. Microvascular permeability was expressed as micrograms of EB per gram lung tissue.

Histopathologic observation

In this study, we investigated the effect of UCN on lung congestion and edema induced by increased lung vascular permeability. The animals were killed by decapitation 60 min after inhalation aerosol of UCN. The lower lobes of the right lungs were excised, fixed in 10% neutral-buffered formalin for at least 24 h, and processed routinely. After being embedded in paraffin, the tissues were cut into 5- μm -thick sections and stained with HE before examination under light microscope.

Statistical analysis

All values were expressed as mean \pm S.D. The significance for the difference between the groups was analyzed with

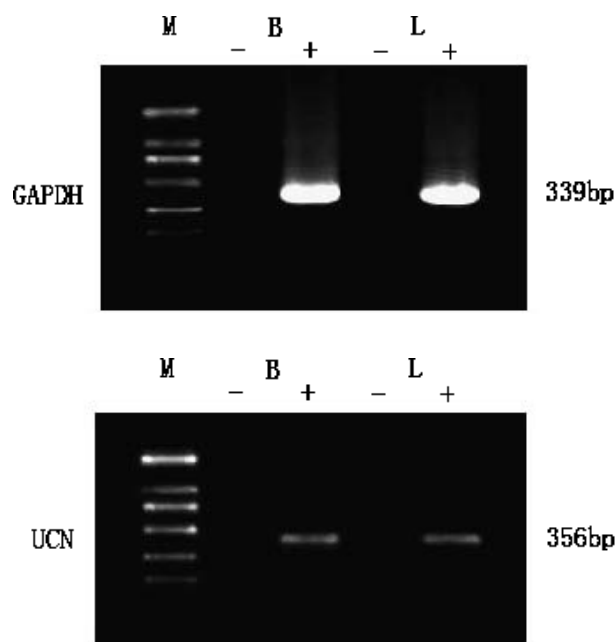


Figure 1 Semiquantitative RT-PCR carried out on samples of brain (B) and lung (L) to detect the presence of GAPDH and UCN transcripts in these tissues. Thirty-five PCR cycles were carried out for all reactions. M, DNA ladder marker; -, control reaction without reverse transcriptase; +, PCR reaction with the addition of reverse transcriptase. The brain provided a positive control test.

SPSS 10.0 by one-way ANOVA. Differences were considered significant when $P < 0.05$.

Results

Semiquantitative RT-PCR

The results of RT-PCR with UCN primers in this study demonstrated that UCN mRNA is expressed in rat lung tissues. The predicted size band was detected in all the lung samples from rats. Positive control performed with total RNA of rat brain also showed the expected band. Negative control performed with no reverse transcriptase added to the reaction yielded no detectable band, indicating no evidence of either contamination or the presence of genomic DNA. Primers specific for GAPDH generated the expected 339-nucleotide band in all cases. The brightness of the GAPDH bands was similar, showing that similar amounts of mRNA were used for each sample (Fig. 1). The gel bands illustrated in Fig. 1 represent the majority of the results obtained and could be reproduced on at least four occasions. Brain mRNA provided an ideal positive control for all the PCR reactions. The brightness of the UCN bands amplified from lung and brain mRNA appeared to be similar.

Immunohistochemistry for UCN in lung tissues

For immunohistochemistry, the lung tissues were immunostained with specific antibody to UCN. The results showed that UCN peptide was mainly expressed in bronchial epithelium mucosa and alveolar epithelium. The cytoplasm was uniformly stained brown, indicating that bronchial epithelium mucosa and alveolar epithelium cells contain UCN peptide (Fig. 2B). UCN immunoreactivity was also detected in lung vascular smooth muscle cells and vascular endothelial cells (Fig. 2D). However, no staining for UCN in airway smooth muscle cells was found (Fig. 2B). Negative controls stained by normal rabbit serum instead of UCN antiserum revealed no positive immunostaining (Fig. 2A and C).

Western blot analysis

Subsequently, to validate our findings, we further investigated the expression of UCN peptide in rat lung tissue by Western blot analysis. In this study, UCN peptide expression was found in all six rat lung specimens. As shown in Fig. 3, rat UCN peptide was detected in lung as a single band of approximately 5 kDa by Western blot analysis with a UCN-specific antibody. No band was found with normal serum instead of UCN-specific antibody. The band illustrated in Fig. 3 represents the majority of the results obtained and could be reproduced on at least four occasions.

Effect of UCN on EB extravasation

The effect of UCN on pulmonary microvascular permeability was assessed by extraction of extravasated EB. There was very little EB extravasation from pulmonary capillaries in NS and OVA control groups (22.5 ± 9.73 ; 28.17 ± 9.62 $\mu\text{g/g}$ lung tissue). Inhalation aerosol of UCN (10^{-7} and 10^{-6} M) induced a marked increase in lung vascular permeability, as shown by tissue extravasation of i.v. injected EB. There was more EB exudation from lung blood vessel, with increasing doses of UCN (69.5 ± 12.91 ; 81.67 ± 22.46 $\mu\text{g/g}$ lung tissue). It was statistically significant when compared with that from control group ($P < 0.01$). Enhanced lung vascular permeability induced by histamine was performed as a positive control. The results indicate that UCN may be one of the most potent rat lung vasodilators. Inhalation aerosol of UCN at a concentration as low as 10^{-8} M did not enhance lung vascular permeability markedly (33.0 ± 12.52 $\mu\text{g/g}$ lung tissue, $P > 0.05$, vs control) (Fig. 4).

Effect of atressin on pulmonary microvascular permeability triggered by UCN

There was very little EB extravasation from pulmonary capillaries in NS and OVA control groups. However, rats treated

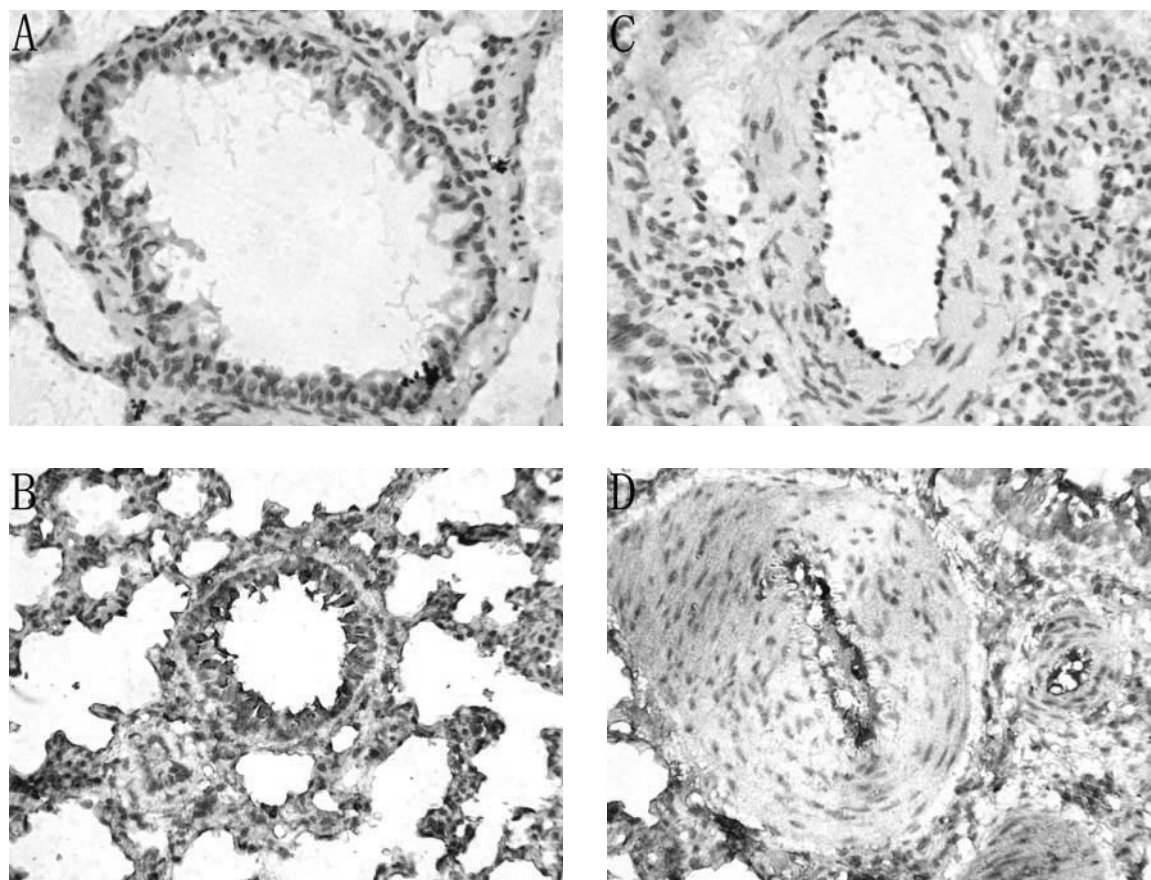


Figure 2 Immunohistochemistry for UCN in rat lung tissues. Immunoreactivity appears brown as a result of DAB colorimetric reaction. $\times 400$. (A) Bronchus and lung alveolus stained with normal rabbit IgG. (B) Bronchus and lung alveolus stained with rabbit anti-UCN antibody. (C) Lung blood vessel stained with normal rabbit IgG. (D) Lung blood vessel stained with rabbit anti-UCN antibody.

with inhalation aerosol of UCN at a concentration of 10^{-6} M increased lung vascular permeability significantly. We then investigated whether the effect of UCN was mediated by specific CRH receptors, but we did not find any effect of the nonselective peptide CRH receptor antagonist astressin alone on EB extravasation (data not shown). However, inhalation aerosol of different concentration of astressin (10^{-7} M, 10^{-6} M and 10^{-5} M) 20 min before the inhalation aerosol of UCN (10^{-6} M) markedly inhibited the response to UCN (Fig. 5).

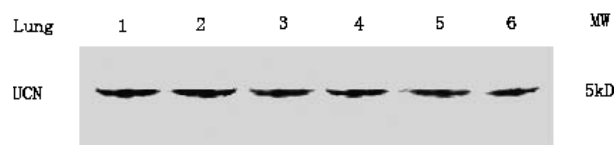


Figure 3 Expression of UCN peptide in rat lung tissues by Western blot analysis. Lanes 1–6 represent six rats respectively. MW, molecular weight.

Effect of cromolyn on pulmonary microvascular permeability triggered by UCN

It was hypothesized that UCN-induced vascular permeability may be secondary to the release of vasodilatory molecules from lung mast cells. To investigate whether mast cells are involved in the effect of UCN on lung vascular permeability, we used the mast-cell stabilizer cromolyn. UCN at a concentration of 10^{-6} M induced an increase in EB extravasation. This effect was attenuated by inhalation aerosol of different concentration of cromolyn (10^{-6} M, 10^{-5} M and 10^{-4} M) 20 min before the inhalation aerosol of UCN (10^{-6} M). It showed that cromolyn inhibited EB extravasation induced by UCN. However, cromolyn at a concentration as high as 10^{-4} M could not inhibit the effect of UCN on lung vascular permeability entirely. There is a statistical difference between NS or OVA control and cromolyn (10^{-4} M) plus UCN (10^{-6} M) group ($P < 0.05$) (Fig. 6).

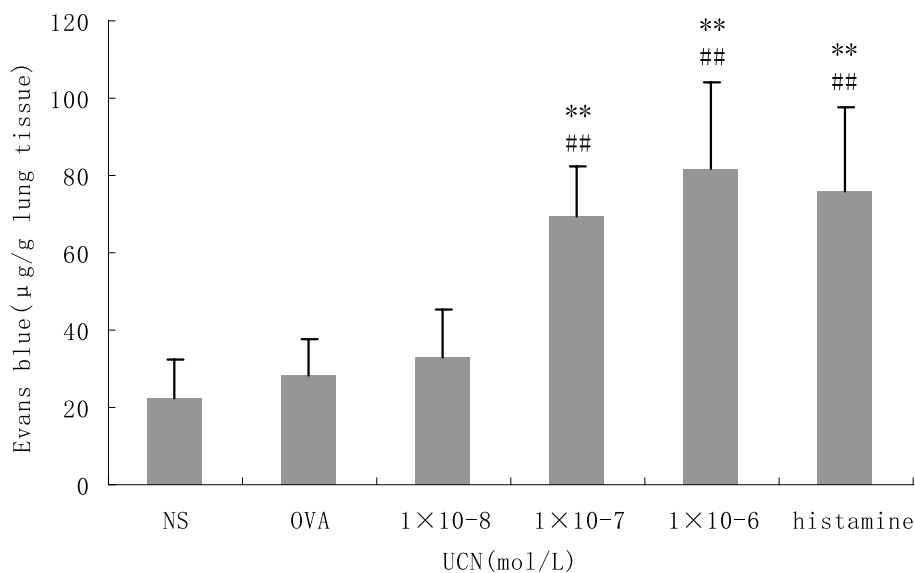


Figure 4 Pulmonary vascular permeability (μg EB/g lung tissue) measured by EB technique after inhalation aerosol of increasing concentrations of UCN. NS, normal saline; OVA, ovalbumin. Data are expressed as mean ± s.d. (n=8). **P<0.01, vs NS control; ##P<0.01, vs OVA control.

Effect of azelastine on pulmonary microvascular permeability triggered by UCN

To examine whether the mast cell-derived vasodilatory molecule histamine mediated the effect of UCN, the H₁ receptor antagonist azelastine was administered by aerosol before the treatment with UCN. EB extravasation in response to UCN

(10⁻⁶ M) was reduced by pretreatment with azelastine. The content of exudated EB was reduced from 81.67 ± 22.46 to 52.5 ± 16.26, 47.17 ± 17.33 and 44.67 ± 15.2 μg EB/g lung tissue by pretreatment with 10⁻⁶, 10⁻⁵ and 10⁻⁴ M azelastine respectively (P<0.05, P<0.01). These results suggest that histamine is the major molecule mediating UCN-induced EB extravasation. However, azelastine at a

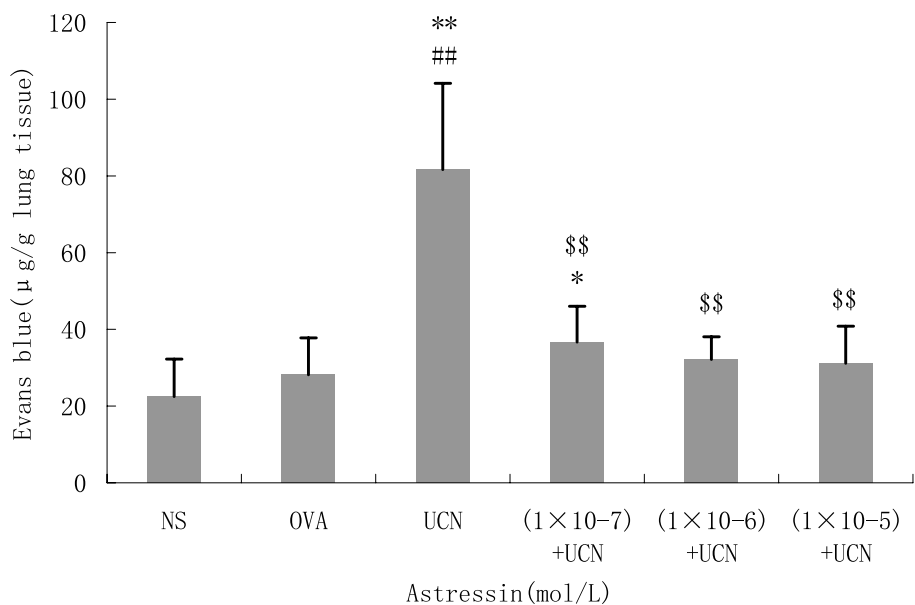


Figure 5 Effect of astressin on pulmonary vascular permeability (μg EB/g lung tissue) induced by inhalation aerosol of 10⁻⁶ M UCN measured by EB technique. Data are expressed as mean ± s.d. (n=8). *P<0.05, **P<0.01, vs NS control; ##P<0.01, vs OVA control; \$\$P<0.01, vs UCN group.

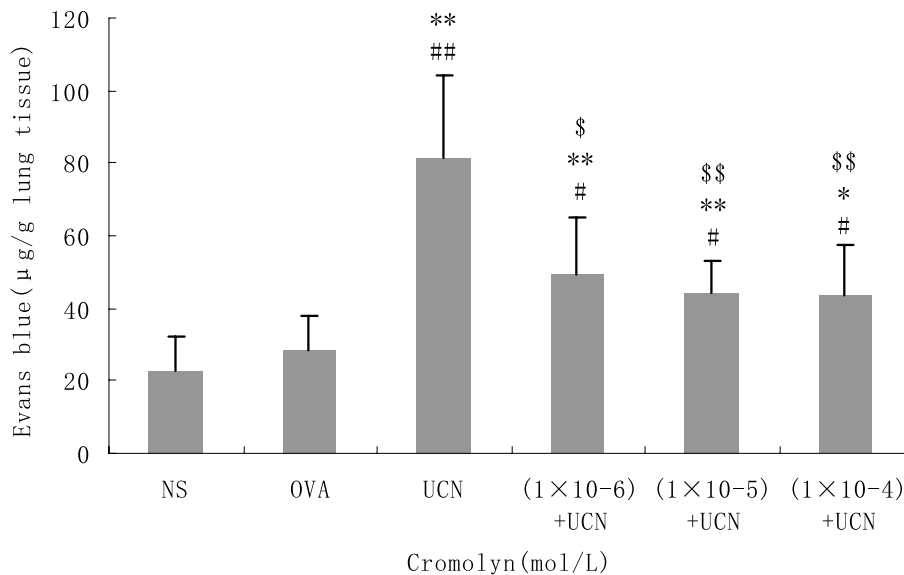


Figure 6 Effect of cromolyn on pulmonary vascular permeability ($\mu\text{g EB/g lung tissue}$) induced by inhalation aerosol of 10^{-6} M UCN measured by EB technique. Data are expressed as mean \pm s.d. ($n=8$). * $P<0.05$, ** $P<0.01$, vs NS control; # $P<0.05$, ## $P<0.01$, vs OVA control; \$ $P<0.05$, \$\$ $P<0.01$, vs UCN group.

concentration as high as 10^{-4} M could not entirely inhibit enhanced lung vascular permeability triggered by UCN. It was statistically significant when compared with NS or OVA control group ($P<0.05$) (Fig. 7).

Effect of montelukast on pulmonary microvascular permeability triggered by UCN

As demonstrated in Fig. 8, inhalation aerosol of UCN increased EB extravasation from pulmonary capillaries significantly. To examine whether the vasodilatory molecule leukotriene mediated the vasodilatory effect of UCN, the leukotriene receptor antagonist montelukast was administered by aerosol before treatment with UCN. Although EB extravasation from pulmonary capillaries in response to UCN (10^{-6} M) was a little reduced by pretreatment with 10^{-6} , 10^{-5} and 10^{-4} M montelukast respectively, there was no statistically significant difference between pretreatment with and no pretreatment with montelukast ($P>0.05$). Our results show that montelukast cannot markedly inhibit the increased lung vascular permeability induced by inhalation aerosol of UCN. These results suggest that leukotriene is not the major molecule mediating UCN-induced EB extravasation.

Histopathologic observations in lung tissue

Observed under light microscope by HE staining, rat lung tissue sections in normal control showed normal alveolar architecture (Fig. 9A). After inhalation aerosol of UCN, marked lung histopathologic abnormalities were observed,

such as congestion, edema and alveolar septum thickening in interstitial tissue (Fig. 9B). This suggests that inhalation aerosol of UCN increases lung vascular permeability significantly. After pretreatment with astressin, cromolyn and azelastine, pulmonary congestion and edema induced by UCN were obviously attenuated (Fig. 9C–E). Pretreatment with montelukast did not significantly lighten the pulmonary congestion and edema triggered by UCN (Fig. 9F).

Discussion

UCN was identified initially as a neuropeptide expressed in the central nervous system (Vaughan *et al.* 1995). In this study, we demonstrated for the first time the expression of UCN mRNA and peptide in rat lung tissues. The expression of UCN was predominantly detected in bronchial epithelium mucosa and alveolar epithelium. Immunohistochemistry of UCN in serial tissue sections revealed that UCN peptide is mainly expressed in cytoplasm. In addition, the present results showed that inhalation aerosol of UCN can increase pulmonary vascular permeability significantly in rats through activation of CRH receptors. Mast cells and histamine may also be involved in this process as important effector cells and an inflammatory mediator.

UCN has been identified outside the central nervous system in many peripheral tissues such as gastrointestinal tract, testis, cardiac myocytes, thymus and spleen (Oki *et al.* 1998, Okosi *et al.* 1998, Baigent & Lowry 2000).

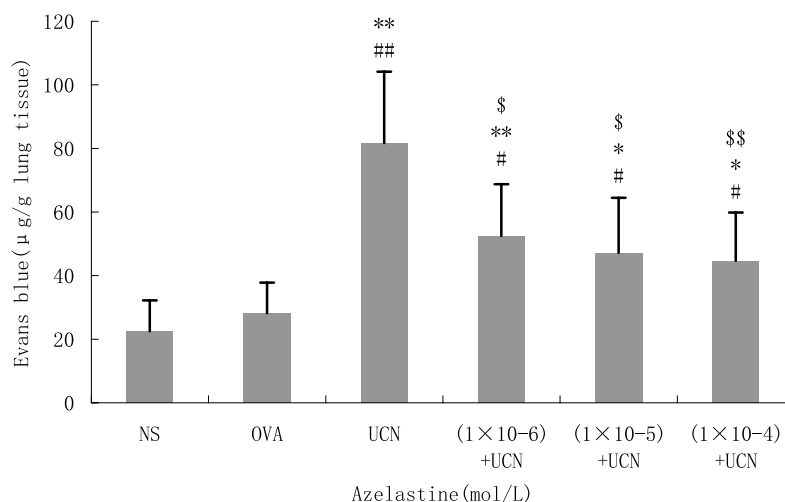


Figure 7 Effect of azelastine on pulmonary vascular permeability ($\mu\text{g EB/g lung tissue}$) induced by inhalation aerosol of 10^{-6} M UCN measured by EB technique. Data are expressed as mean \pm S.D. ($n=8$). * $P<0.05$, ** $P<0.01$, vs NS control; # $P<0.05$, ## $P<0.01$, vs OVA control; \$ $P<0.05$, \$\$ $P<0.01$, vs UCN group.

Lower levels of UCN mRNA have been detected in the kidney and liver (Kageyama *et al.* 1999), and UCN has been detected in the human skin (Slominski *et al.* 2000), placenta and fetal membranes (Petraglia *et al.* 1996). However, the expression profile of UCN in lung has never been reported. In the present study, we found that amplification of cDNA with UCN-specific primers was detected in all rat lung samples by RT-PCR analysis. This is the first report that UCN mRNA is stably expressed in rat lung tissues. To further validate our findings, Western blotting and immunohistochemistry analysis were performed to investigate whether UCN

peptide is expressed in rat lung tissues. The expected results were also obtained. The expression of UCN peptide was found by Western blot analysis in all six rat lung specimens. The band size obtained in experiments was consistent with the expected size of UCN peptide. The results of immunohistochemistry showed that UCN peptide is mainly expressed in bronchial epithelium mucosa and alveolar epithelium. The vascular smooth muscle cells and blood vessel endothelial cells were also positively stained. However, staining for UCN in airway smooth muscle cells was not found. These results suggest that bronchial epithelium mucosa and alveolar epithelium

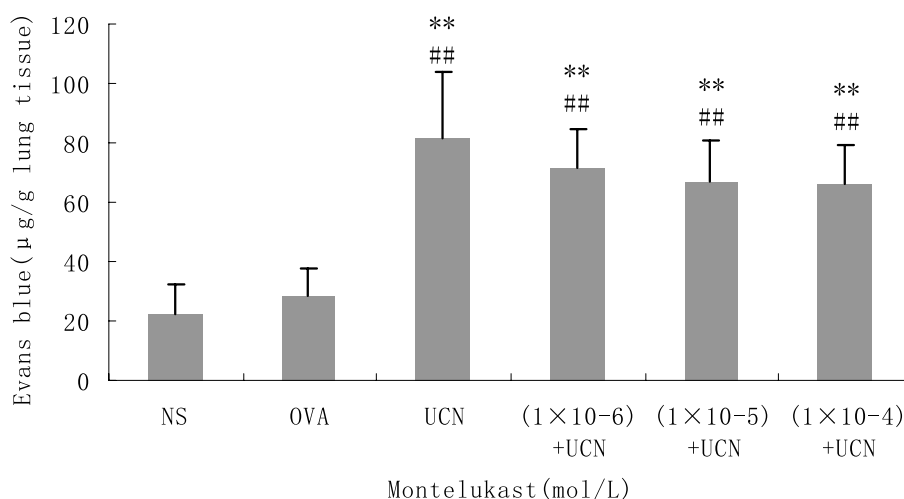


Figure 8 Effect of montelukast on pulmonary vascular permeability ($\mu\text{g EB/g lung tissue}$) induced by inhalation aerosol of 10^{-6} M UCN measured by EB technique. Data are expressed as mean \pm S.D. ($n=8$). ** $P<0.01$, vs NS control; ## $P<0.01$, vs OVA control.

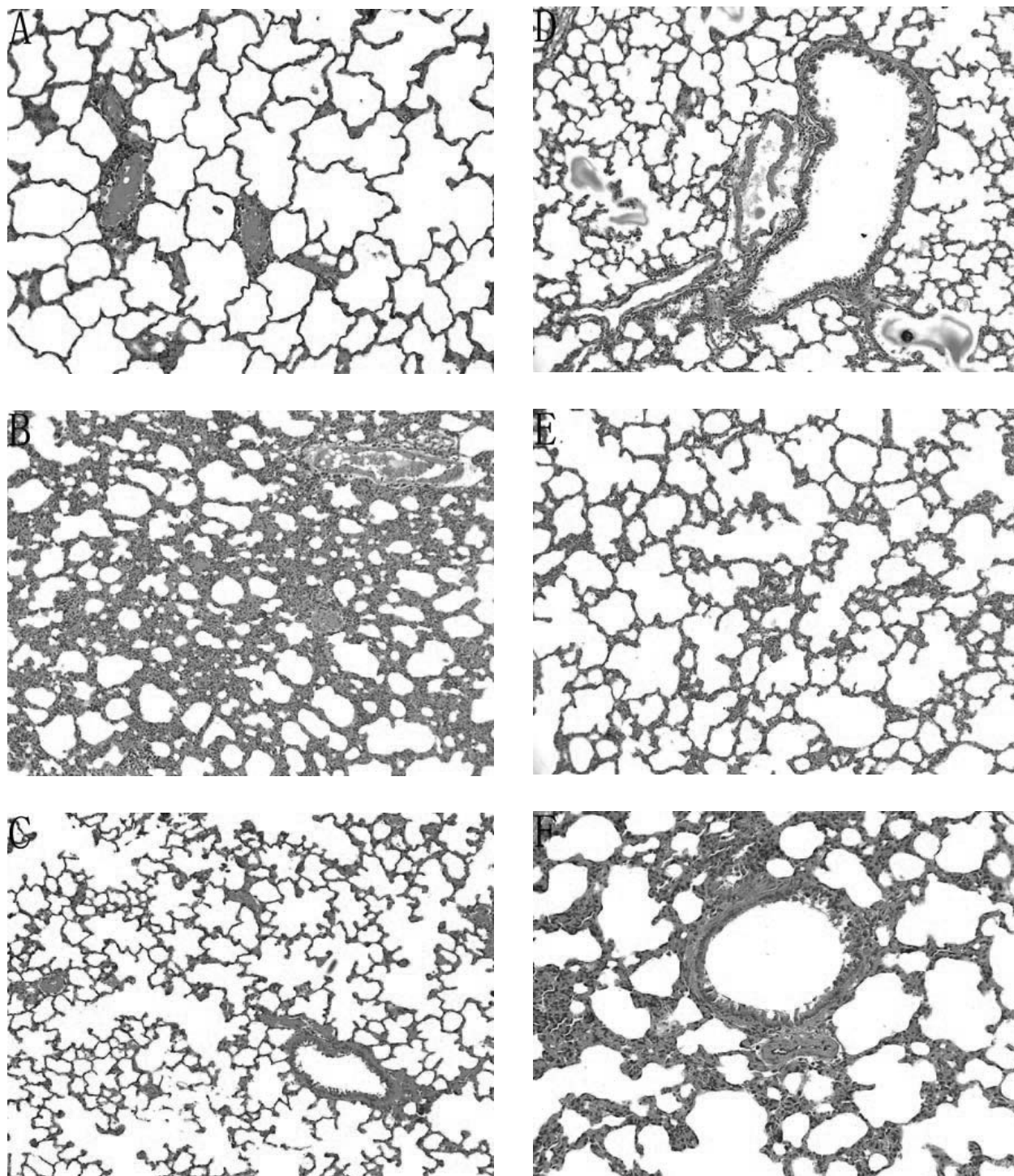


Figure 9 Observation of congestion and edema in lung interstitial tissue of rat under light microscope. (A) Inhalation aerosol of OVA. (B) Inhalation aerosol of 10^{-6} M UCN. (C) Pretreatment with 10^{-5} M astressin 20 min before inhalation aerosol of 10^{-6} M UCN. (D) Pretreatment with 10^{-4} M cromolyn 20 min before inhalation aerosol of 10^{-6} M UCN. (E) Pretreatment with 10^{-4} M azelastine 20 min before inhalation aerosol of 10^{-6} M UCN. (F) Pretreatment with 10^{-4} M montelukast 20 min before inhalation aerosol of 10^{-6} M UCN. HE $\times 100$.

may be important sources of endogenous UCN in lung tissues.

Previous studies reported that acute stress increases skin CRH that can trigger mast cell-dependent vascular permeability and that intradermal injection of UCN can induce skin mast-cell degranulation and increase vascular permeability. The actions of both CRH and UCN can be inhibited by pretreatment with the mast-cell stabilizer disodium cromoglycate (cromolyn) (Singh *et al.* 1999a, 1999b, Michael *et al.* 2003). In the present study, we report for the first time that UCN increases pulmonary vascular permeability significantly in rats. We show that the extravasated EB in lung tissues is markedly increased after inhalation aerosol of 10^{-6} and 10^{-7} M UCN. UCN aerosol inhalation resulted in obvious pulmonary congestion and edema observed under light-microscope HE staining.

To investigate whether the effect of UCN is mediated by specific CRH receptors, we pretreated the rats with astressin before administration of UCN. We found that UCN-induced edema and EB extravasation in rat lung tissues were attenuated significantly by pretreatment with astressin. UCN-induced EB extravasation was reduced by astressin nearly to the level in normal control. The finding that the nonselective peptide CRH receptor antagonist astressin reduces vascular permeability triggered by UCN indicates that the effect of UCN is mediated by CRH receptors.

Our findings also show that the enhanced pulmonary vascular permeability induced by 10^{-6} M UCN is clearly inhibited by pretreatment with the mast-cell membrane stabilizer cromolyn. However, cromolyn at a concentration as high as 10^{-4} M cannot inhibit the effect of UCN on lung vascular permeability entirely. This suggests that enhanced vascular permeability in lung induced by UCN may involve mechanisms other than mast-cell degranulation.

Azelastine is an antihistamine agent and has been shown to inhibit the release of histamine from rat mast cells after immunologic and nonimmunologic stimuli, as well as antagonize the proinflammatory effects of histamine (Chand *et al.* 1985, Mosges & Klimek 1998, Takao *et al.* 1999). Here, we also report that pretreatment with azelastine inhibits vascular permeability induced by inhalation aerosol of 10^{-6} M UCN. It has been shown that rat pleural mast cells secrete about 30% histamine in response to CRH (Boucher *et al.* 1996). Our results suggest that UCN may stimulate histamine release through CRH receptors expressed on lung mast cells, which can increase lung vascular permeability. Histamine appears to be one of the main mediators involved in the *in situ* effect of UCN because the H_1 receptor antagonist azelastine inhibited most of the vasodilatory response. However, even 10^{-4} M azelastine could not reduce the EB extravasation induced by UCN to the normal control level, suggesting that histamine is not the only factor in the enhanced vascular permeability in lung induced by UCN.

Cysteinyl leukotriene (CysLT) is a potent proinflammatory mediator. It has been recognized that leukotriene can increase vascular permeability and mucus secretion. To examine whether the vasodilatory molecule leukotriene mediated the vasodilatory effect of UCN, the cysteinyl leukotriene receptor antagonist montelukast was inhaled by aerosol before treatment with UCN. The results showed that montelukast failed to inhibit the increased pulmonary vascular permeability triggered by UCN, suggesting that leukotriene may not be the main inflammatory factor involved in the *in situ* vasodilatory effect of UCN.

In contrast, Turnbull *et al.* (1996) demonstrated that edema owing to thermal injury in rats is inhibited by systemic UCN treatment, suggesting that UCN may decrease vascular permeability. However, the pharmacologic doses of UCN administered i.v. had probably produced hypotension, decreasing the ability of the animals to mount a full peripheral inflammatory response. Recently, Torpy *et al.* (1999) demonstrated that equivalent levels of hypotension produced by UCN or hydralazine are associated with decreased inflammatory responses to carrageenin, and the lowered blood pressure correlated with the decrease of inflammatory response. Thus, we suggest that UCN administered i.v. acts as vasodilator, leading to hypotension, rather than as immunologically competent UCN that directly decreases vascular permeability. In this study, UCN was locally administered by inhalation aerosol to lung tissues, which may be insufficient to result in hypotension. Therefore, the effect of UCN on elevation of pulmonary vascular permeability appeared. The effects of UCN on pulmonary vascular permeability remain to be further studied.

In conclusion, our present study demonstrated for the first time that UCN is strongly expressed in rat lung and that inhalation aerosol of UCN increases pulmonary vascular permeability through activation of CRH receptors. Mast cells and histamine may also be involved in this process as important effector cells and an inflammatory mediator. Peripherally produced UCN in lung may act as an autocrine or paracrine proinflammatory mediator; may be involved in the pathophysiology of some inflammatory lung diseases accompanied by enhanced vascular permeability, such as allergic asthma and pulmonary edema; and may lead to new forms of treatment. The exact physiologic function and pathophysiologic role of UCN in lung remain to be further investigated.

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