Effect of lipopolysaccharide on expression and characterization of cholecystokinin receptors in rat pulmonary interstitial macrophages[1]

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ABSTRACT

AIM: To investigate the effect of lipopolysaccharide (LPS) on the expression and the binding characteristics of cholecystokinin receptors (CCK-R) in rat pulmonary interstitial macrophages (PIMs). **METHODS:** The PIMs isolated from rat lung tissues were purified by the collagenase digestion method combined with alveolar lavage and pulmonary vessel perfusion. The expression of CCK-R mRNA was detected by RT-PCR and Southern blot analysis and the binding experiments were performed by radioligand binding assay (RBA). **RESULTS:** CCK-A receptor (CCK-AR) and CCK-B receptor (CCK-BR) mRNA were detected in rat PIMs and their RT-PCR amplified products had a size of approximately 1.37 kb and 480 bp, respectively. The relative expression of CCK-BR mRNA was higher than that of CCK-AR mRNA after incubation with LPS for 0.5, 2, and 6 h. The expression of CCK-R mRNA could be upregulated obviously by LPS. Southern blot analysis of RT-PCR amplified CCK-AR and CCK-BR mRNA products using [γ-^{32}P]ATP 5'-end-labelled probe showed specific hybridization bands. The specific binding of [^{3}H] CCK-8S to rat PIM membranes was detected in the rats administered with LPS for 48 h, but not in normal rats. Scatchard analysis of the saturation curves suggested the presence of CCK-R with a high affinity (K_d=0.68±0.28 nmol/L) and a low binding capacity (B_{max}=32.5±2.7 fmol·mg^{-1} protein) in rat PIMs. The specific binding of [^{3}H] CCK-8S to rat PIM membranes was inhibited by unlabelled CCK-8S (IC_{50}=2.3±0.8 nmol/L), CCK-AR specific antagonist CR1409 (IC_{50}=0.19±0.06 μmol/L) and CCK-BR specific antagonist CR2945 (IC_{50}=3.2±1.1 nmol/L). **CONCLUSION:** Two types of functional CCK-AR and CCK-BR existed in rat PIMs and their expression could be upregulated by LPS.

INTRODUCTION

The uncontrolled inflammatory response is considered as the major factor in the pathogenesis of infectious diseases and multiple organ dysfunction syndromes (MODS). It is well known that the activation of monocytes/macrophages plays a critical role in inducing the inflammatory response[1]. Macrophages stimulated by LPS or other inflammatory factors produce and release large quantity of various proinflammatory cytokines including TNF-α, IL-1β, IL-6 and so on. Overproduction of the cytokines can result in systemic inflammatory response syndrome (SIRS) and MODS[2]. The lung is highly sensitive to LPS to be dysfunctional, suggesting that pulmonary macrophages activated by LPS re-

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lease some mediators to produce the tissue injury. Pulmonary macrophages consist of three major subpopulations including alveolar macrophages (AMs) located primarily in the alveolar spaces, pulmonary interstitial macrophages (PIMs) which reside within the lung parenchyma, and pulmonary intravascular macrophages. It was reported that there was a significant increase in production of reactive oxygen intermediates from PIMs, but not from AMs in the rats treated with LPS. The treatment also markedly enhanced phagocytosis only in PIMs and caused a significant increase in chemotaxis of PIMs toward C5a. These data demonstrate that PIMs play an important role in the inflammatory response of the lungs in acute endotoxemia[3].

CCK is discovered initially in the gut as a gastrointestinal hormone with the function of contracting gallbladder, and subsequently localized in the central and peripheral nervous system as a neurotransmitter or neuromodulator to modulate dopamine release and reward[4]. CCK is identified as several different size of the peptide including 4, 8, 33, 39, and 58 amino acid forms. The sulfated carboxyl-terminal octapeptide (sCCK-8) is the biologically predominant active form localized in the small intestine, blood and CNS[5]. Recently, some studies demonstrate that sCCK-8 causes an *in vitro* inhibition of LPS-induced TNF-α production, sCD14 release and mCD14 expression in rat PIMs[6]. Consistently, the production of proinflammatory cytokines including TNF-α, IL-1β, and IL-6 in endotoxin shock (ES) rat was also inhibited by sCCK-8 *in vivo*[7,8]. These results suggested that sCCK-8 had anti-inflammatory effect to some extent, a new field about the biological action of CCK, which was also confirmed by a morphological observation that sCCK-8 clearly lessened the inflammatory lesions in lung, spleen and liver tissues in ES rat[9]. However, the anti-inflammatory mechanism and signal transduction of sCCK-8 remain unclear. CCK exerts a variety of physiological actions through its cell surface receptors, which have been pharmacologically classified into two subtypes CCK-AR and CCK-BR according to their affinity for the peptide agonists sCCK-8 and gastrin[10]. The physiological functions that are associated with the CCK receptor have generated interest in the development of receptor-specific drugs as potential therapeutic options for some diseases. The present study was undertaken to investigate the expression and binding characterization of CCK receptor and the effect of LPS on its expression in rat PIMs.

MATERIALS AND METHODS

Chemicals Collagenase IA, LPS (*E Coli* 0111: B4), CCK-8S, proglumide, CR-1409, CR-2945, aprotinin, sigmacote, and DNase I were from Sigma Company. RPMI-1640 culture medium, phenylmethylsulfonylfluoride (PMSF), and TRIzol reagents were obtained from Gibco BRL (Gercy-Pontoise, France). Avian myeloblastosis virus reverse transcriptase, Taq DNA polymerase, and Gel shift assay system were purchased from Promega Company. [Propionyl-^{3}H]-cholecystokinin octapeptide (sulphated) ([^{3}H]CCK-8S, 2849 GBq/mmol) was from Amersham Company; all other reagents used were of analytical pure grade.

Animals Adult healthy female Sprague-Dawley (SD) rats (180-220 g, Grade II, Certificate No 04057) were obtained from Experimental Animal Center of Hebei Province. For RT-PCR, 10 rats were killed directly to prepare PIMs. For binding experiments, 10 rats divided into two groups randomly, were injected with LPS (LPS group, 5 g/L, 10 mg/kg) or Saline (control group, the volume was equal to LPS) through lingual vein 48 h before killed to prepare PIM membranes.

Preparation of rat PIMs PIMs were isolated from perfused rat lungs with a collagenase digestion technique, modified as Wizemann *et al*[3] described. Alveoli were lavaged 12 to 14 times with 4 ºC phosphate-buffered saline (PBS) containing EDTA 0.6 mmol/L (PBS-EDTA) to remove alveolar macrophages. Lung vessels were perfused with 100 mL PBS-EDTA to remove the monocytes and other blood cells. Then the lung tissues were cut into 500 μm slices followed by digestion in 175 U/mL collagenase IA containing Dnase I 0.1 g/L and fetal bovine serum (FBS) 100 ml/L in a shaking 37 ºC water bath for 60 min. The suspension was then filtered through 30 μm mesh. After washed 2 times (400×*g*, 4 ºC, 10 min) with PBS, the cells were resuspended in RPMI-1640 medium containing FBS 15 ml/L, penicillin 100 U/mL, and streptomycin 100 g/L and incubated for 2 h at the conditions of 37 ºC and 5 % CO_2. Nonadherent cells were removed by gentle washing with warm medium. Remaining adherent cells contained more than 93 % PIMs and the contamination by polymorphonuclear leukocytes and alveolar macrophages was less than 7 %. PIMs viability was greater than 95 %, determined by trypan blue exclusion assay.

Primers and probes primers and probes of CCK receptor were designed according to Mostein's report[11]. The sequences of CCK-AR primers were 5'-CTC GCT CGC CCA GAA CTC TAC CAA GGA ATC AAA TTT GAT GC-3' (sense) and 5'-CTG GTT CGG CCC ATG GAG CAG AGG TGC TCA TGT GGC TGT AG-3' (antisense). The sequences of CCK-BR primers were

5'-CTC GCT CGC CCA GAA CTC TAC CTA GGA CTC CAC TTT GA-3' (sense) and 5'-CTG GTT CGG CCC ACG CAC CAC CCG CTT CTT AGC CAG CA-3' (antisense). The sequences of β-actin primers were 5'-GAG ACC TTC AAC ACC CAG CC-3' (sense) and 5'-TCG GGG CAT CGG AAC CGC TCA-3' (antisense). The sequences of CCK-AR and CCK-BR probes were respectively 5'-CGG GGG CCG GGG ACT TCT GCA AGT AAC AGC CAT CAC TAT CCT CAT A-3' and 5'-AGC TAC GCT GGT TAC AGG CCG GCA GCC CCC GTT-3'. The oligonucleotide probes were labeled with [γ-^{32}P]ATP. All the primers and probes were synthesized in Sangon Corporation (Shanghai).

Semi-quantitative RT-PCR for the detection of CCK receptor mRNA PIMs were prepared as above and plated on culture dishes. After washed 3 times with serum-free medium, cells were stimulated with LPS (10 mg/L) for 0.5-12 h. Total cellular RNA was prepared with TRIzol reagents. The concentration of RNA was determined from absorption at 260 nm. cDNA was synthesized from 2 μg of the total RNA by extension of random primers with avian myeloblastosis virus reverse transcriptase. PCR of the cDNA was performed in a final volume of 25 μL containing $MgCl_2$ 2 mmol/L, *Taq* DNA polymerase 1 units and specific primers 1 μmol/L. The RT-PCR was performed in the following procedures: 42 ºC for 40 min, 1 circle; 94 ºC for 2 min, 1 circle; 94 ºC for 45 s, 50 ºC for 45 s, 72 ºC for 45 s, 28 cycles, finally with further extension at 72 ºC for 10 min. Amplification of β-actin was always involved to serve as control of reaction efficacy. As positive controls, the RNA extracted from rat pancreas (for CCK-AR) and brain tissues (for CCK-BR) were also performed RT-PCR in parallel. The synthesized PCR products were separated by electrophoresis on an agarose gel (18 g/L) and analyzed by Gel-Pro analyzer version 3.1 software (Media Cybernetics). The ratio of arbitrary unit (AU, $D_{area} \cdot D_{density}$) of target genes over β-actin was used for expressing the relative level of mRNA expression.

Southern blot analysis of PCR-amplified products PCR-amplified products of CCK-AR and CCK-BR mRNA were electrophoretically separated on a 1.5 % agarose gel, denatured, and transferred onto Hybond-NA$^+$ membranes. Southern blot filters were hybridized in the buffer (containing SDS 0.5 %, 6×SSC, 5×Denhard's, salmon sperm DNA 100 g/L, formamide 50 %) at 42 ºC for 10 h, using a [γ-^{32}P] 5'-end-labelled rat CCK-AR and CCK-BR probes. After hybridization, filters were washed at 50 ºC: 2×SSC/0.1 % SDS for 2 ×20 min and 0.1×SSC/0.1 % SDS for 20 min, (1×SSC=150 mmol/L risodium citrate, 15 mmol/L sodium chloride). Radiolabelled bands were visualized by exposure to X-ray films at -80 ºC for 3 d.

Preparation of PIMs membranes PIMs were harvested with PBS containing EDTA 0.4 g/L, pelleted by centrifugation (4 ºC, 400×*g*, 10 min) and immediately used for experiments. PIMs membranes were obtained as described previously [12]. Briefly, PIMs were resuspended in ice-cold 50 mmol/L Tris-HCl buffer (PH 7.4) containing EDTA 1 mmol/L, PMSF 0.05 g/L and approtinin 1.0×10^5 KU/L. After 15 min incubation at 4 ºC, cells were disrupted by sonication for two 10 s bursts at maximal power and tune meter separated by 10 s intervals. The homogenate was centrifugated (600×*g*) at 4 ºC for 10 min. The supernatant was centrifugated (60 000×*g*) at 4 ºC for 30 min. The pellet was resuspended in 20 mmol/L HEPES containing $MgCl_2$ 5 mmol/L, NaCl 20 mmol/L, BSA 0.5 g/L and PMSF 50 mg/L and was immediately frozen at -80 ºC until used. Proteins were measured by the method of Bradford using bovine serum albumin as standard.

Binding experiments [^{3}H]CCK-8S was found to attach to plastic and glass surfaces and all experiments were therefore performed in tubes and with pipette tips which were siliconized with Sigmacote. For the equilibrium binding studies, the crude membrane homogenate was incubated with [^{3}H]CCK-8S (0.05-6.4 nmol/L) at 4 ºC for 12 h and free ligands were removed by fast filtration through glass-fiber filter (GF/B, Whatman International Ltd). The filters were washed three times with 3 mL cold incubation buffer, and bound [^{3}H]CCK-8S (trapped on the filter) was counted with a liquid scintillation counter (LS-6500, BECKMAN INSTRUMENTS, INC). The specific binding was defined as the difference between total and nonspecific binding, which was measured in the absence and presence of 30 μmol/L proglumide, respectively[13,14].

Statistical analysis Data were expressed as means±SD and analyzed by ANOVA and least significant difference test using SPSS statistical program. Statistical significance was accepted when $P<0.05$. Binding data were analyzed by the method of Scatchard using the non-liner curve-fitting program LIGAND. Half-maximal inhibition (IC_{50}) was calculated by the method of log-probit.

RESULTS

Effect of LPS on CCK-AR and CCK-BR mRNA expression in rat PIMs CCK-AR and CCK-BR mRNA

were detected in rat PIMs by RT-PCR, and their amplification products had a size of approximately 1.37 kb and 480 bp, respectively. In each cell or tissue sample, all β-actin amplification products were of 420 bp length. The relative expressed quantity (ratio of arbitrary unit over β-actin) of CCK-AR and CCK-BR mRNA in control group was (30±4)% and (35±3)%, respectively, and significant increases in their expression were observed at indicate time after incubating rat PIMs with LPS (P<0.01), but with an exception of which at 0.5 h for CCK-AR mRNA (P>0.05). The relative expressed quantity of CCK-BR mRNA was higher than that of CCK-AR mRNA after incubation with LPS for 0.5, 2, and 6 h (P<0.01, Fig 1).

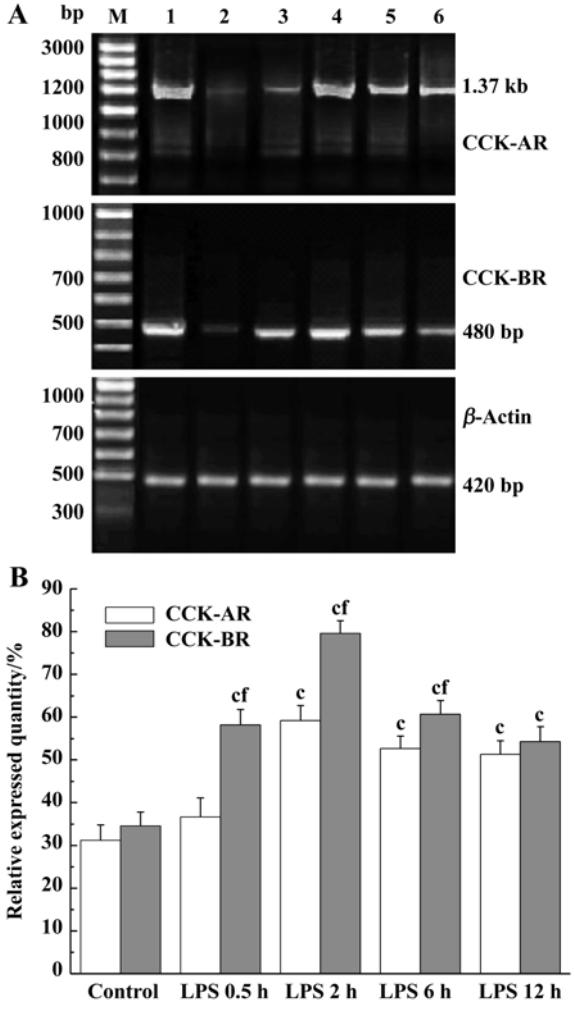

Fig 1. Effects of LPS on CCK-AR and CCK-BR mRNA expression in rat PIMs. A) Analysis of RT-PCR amplified CCK-AR, CCK-BR, and β-actin mRNA products. M: marker; 1: Positive control, products amplified from pancreas (CCK-AR) and brain (CCK-BR); 2: Control; 3-6: LPS 0.5, 2, 6, and 12 h, respectively. B) Alteration of relative expressed quantity of CCK-AR and CCK-BR mRNA. n=3. Mean±SD. ^{c}P<0.01 *vs* control; ^{f}P<0.01 *vs* CCK-AR.

Southern blot analysis Southern blot analysis of PCR amplified CCK-AR and CCK-BR mRNA products using [γ-^{32}P]ATP 5'-end-labelled probes showed specific hybridization bands (Data not shown). It confirmed the specificity of RT-PCR amplified CCK-AR and CCK-BR mRNA products.

Effect of time and temperature on [^{3}H]CCK-8S binding Under our experimental conditions, the specific binding of [^{3}H]CCK-8S to rat PIMs membranes was detected in LPS group, but not in control group, and it was a time- and temperature-dependent process (Fig 2). The rate of binding was rapid at 37 ºC and 21 ºC, became maximal at 30 min and then declined comparative quickly too. Reducing the incubation temperature from 37 ºC and 21 ºC to 4 ºC produced a steady increase in the rate of the binding reaction. The apparent equilibrium state was attained at about 180 min and was maintained until 12 h. These conditions were found to be optimal for [^{3}H]CCK-8S binding to rat PIMs membranes.

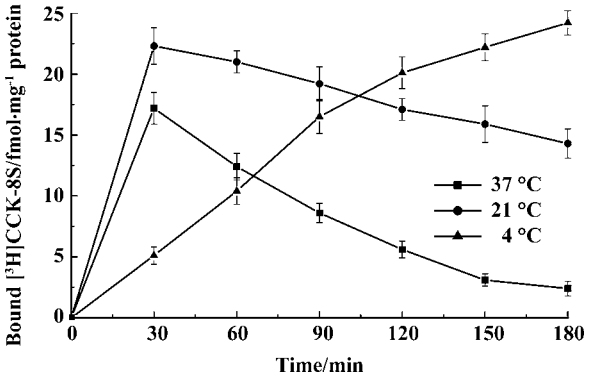

Fig 2. Time-course of specific binding of [^{3}H]CCK-8S to rat PIM membranes as a function of incubation temperature. [^{3}H]CCK-8S (5 nmol/L) was incubated with PIM membranes at 37 °C, 21 °C, or 4 °C for indicated time. n=3. Mean±SD.

Receptor binding of [^{3}H]CCK-8S at steady state The curve of [^{3}H]CCK-8S binding to PIM membranes was saturable, and the straight line obtained by Scatchard analysis indicates the presence of a single class of [^{3}H]CCK-8S binding sites (Fig 3). The dissociation constant (K_d) and maximal binding capacity (B_{max}) were estimated to be 0.68±0.28 nmol/L and 32.5±2.7 fmol·mg^{-1} protein , respectively. The non-specific binding of [^{3}H]CCK-8S was always below 15 % of total at steady state.

Receptor specificity of [^{3}H]CCK-8S binding Specificity of CCK receptors in rat PIMs was investi-

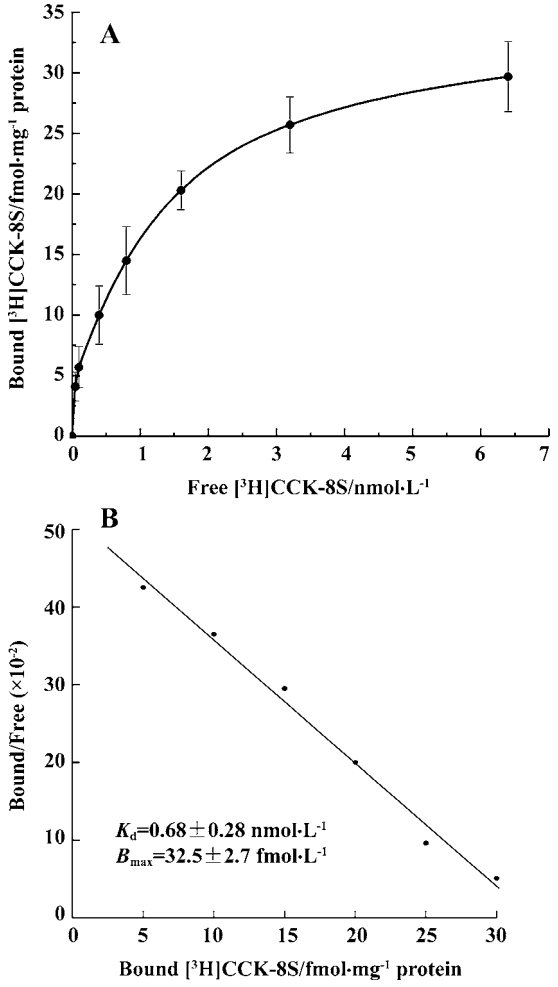

Fig 3. Equilibrium of the specific binding of [^{3}H]CCK-8S to rat PIM membranes induced by LPS (A) and Scatchard analysis of the binding data (B). Rat PIM membranes were incubated with different concentration of [^{3}H]CCK-8S in the absence (total binding) and presence (nonspecific binding) of 30 μmol/L proglumide at 4 ºC for 12 h. *n*=3. Mean±SD.

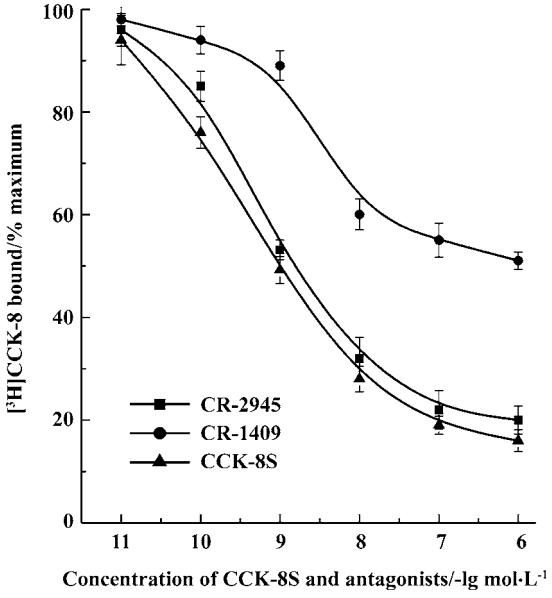

Fig 4. Competitive inhibition of [^{3}H]CCK-8S binding to rat PIM membranes by unlabelled CCK-8S, CCK-AR selective antagonist CR-1409 and CCK-BR selective antagonist CR-2945. Binding is expressed as the percentage of radioactivity specifically bound in the absence of unlabelled CCK-8S and antagonists. *n*=3. Mean±SD.

gated by determining the specific binding of 5 nmol/L [^{3}H]CCK-8S in the presence of unlabelled CCK-8S, CCK-AR selective antagonist CR-1409 and CCK-BR selective antagonist CR-2945. Competition binding studies demonstrated inhibition of specific binding of [^{3}H]CCK-8S in a dose dependent manner by unlabelled CCK-8S, CR-1409 and CR-2945 and the concentrations required to inhibit 50 % of the specific binding (IC_{50}) were 2.3± 0.8 nmol/L, 0.19±0.06 μmol/L, and 3.20±1.13 nmol/L, respectively (Fig 4).

DISCUSSION

CCK is a neuropeptide expressed in the endocrine I-cells of the small intestinal mucosa and in widespread central and peripheral neurons[15]. Whereas intestinal CCK regulates the release of pancreatic enzymes and the contraction of the gall bladder, neuronal CCK is a transmitter or modulator assumed to be involved in a variety of central nervous system (CNS) functions such as feeding behavior, anxiety, analgesia, memory, immunomodulation, and anti-opioid effect[5,16,17]. The CCK receptor is a seven-transmembrane domain (TM) G protein-coupled receptor (GPCR) that has attracted considerable interest as a target for drug-discovery efforts, based on its important physiological role in the gastric mucosa, CNS, and immune cells[18]. Two types of CCK receptor (CCK-AR and CCK-BR) have been identified on the basis of their affinities for a structurally and functionally related family of peptide with identical COOH-terminal pentapeptide sequences[19]. These receptors were cloned and the CCK-BR gene was found to be identical to the gastric receptor gene. CCK-AR is highly selective to sulfated analogues of CCK and the antagonist L-364, 718, whereas CCK-BR has similarly high affinity to both sulfated and nonsulfated peptide analogues of CCK/gastric peptides and the antagonist L-365, 260[10]. CCK-AR is found principally in the gastrointestinal tract and selective areas of the CNS, while CCK-BR is found principally in the CNS and selective areas of the gastrointestinal tract, on pancreatic acinar

cells, lymphocytes and parietal cells[20,21]. These findings suggested that CCK receptors were widely distributed around various kinds of tissues and their presence provided the structural basis for CCK to exert a broad array of physiological action.

Cong *et al*[22] studied the distribution of CCK receptors in lung tissues using highly sensitive method of *in situ* RT-PCR and conventional *in situ* hybridization. The results demonstrated that CCK-AR and CCK-BR genes were present in pulmonary vascular endothelial cells, macrophages, bronchial epithelial cells, and alveolar epithelial cells, which play an important role in mediating the regulatory actions of CCK-8 on these cells. A recent study from our laboratory has shown that CCK-8 inhibited LPS-induced NF-κB activity and IκB degradation in PIMs, which was abrogated by proglumide [23]. These results suggested that CCK-8 might bind to CCK receptors on PIMs to interfere with the activation of macrophages during inflammation, but the expressed subtypes of CCK receptors in PIMs were not fully clarified. In present study, the expression of CCK-AR and CCK-BR mRNA were detected for the first time by RT-PCR and Southern blot analysis in rat PIMs, their amplification products were approximately 1.37 kb and 480 bp, respectively. The relative expressed quantity of CCK-BR mRNA was higher than that of CCK-AR mRNA. The results of radioligand binding assay also provided evidences for the existence of functional CCK receptors in rat PIMS. These data indicated that CCK receptors on PIMs provided the structural basis for CCK-8 to regulate the functions and activation of PIMs, which may be beneficial to the control of pulmonary inflammatory responses.

There are many factors influence the binding of [^{3}H]CCK-8S to rat PIMs membrane, including the incubation time and temperature. In comparison with several other G protein-coupled receptors, the CCK receptors were quite labile, losing a considerable number of binding sites during incubation at 30 ºC or 37 ºC[12]. Our results suggested that the rate of binding was rapid at 37 ºC and 21 ºC, and reached the peak at 30min, and then declined comparative quickly. We also found that the rate of the binding reaction produced a steady increase when the incubation temperature was reduced from 37 ºC and 21 ºC to 4 ºC, and the apparent equilibrium state was attained at about 180 min, and was maintained until 12 h. The stability of CCK receptors in this study was characterized by their ability to bind a specific agonist, which requires the active conformation of whole receptor-G protein complexes. Earlier studies have indicated that receptor-G protein complexes, as well as G protein themselves, are much more labile than receptors[12], therefore, it can be proposed that the loss of [^{3}H]CCK-8S binding activity is not mainly connected to the thermoinactivation of CCK receptors but also by the decomposition of receptor-G protein complexes. In the present study, it was found that the binding of [^{3}H]CCK-8S to rat PIMs membrane was relatively fast, reversible and saturable, whereas optimal experimental conditions for binding isotherms appeared to be an incubation for 12 h at 4 ºC in a buffer containing 20 mmol/L HEPES, 5 mmol/L $MgCl_{2.}$, 20 mmol/L NaCl and 0.5 g/L BSA. These conditions should be considered a compromise between many different factors which affect binding and had to be taken into account during the experiments.

The Scatchard analysis of the binding data suggested the presence of CCK receptors with high affinity (K_d=0.68±0.28 nmol/L) and low binding capacity (B_{max}=32.5±2.7 fmol·mg^{-1} protein) in rat PIMs. In comparison with the CCK receptors in pancreas (K_d=0.27± 0.01 nmol/L, B_{max}=360.18±1.09 fmol·mg^{-1} protein)[24], the CCK receptors had a low density in rat PIMs. With respect to specificity, the unlabelled CCK-8s and selective CCK-R antagonists were investigated by determining the inhibition of [^{3}H]CCK-8S specific binding. As could be seen from the competition binding assay, the binding of [^{3}H]CCK-8S was inhibited by unlabelled CCK-8s, CCK-AR selective antagonist CR-1409 and CCK-BR selective antagonist CR-2945, but their IC_{50} were different, suggesting the binding was specific and the two subtypes CCK receptors were present in rat PIMs and it was accordance with RT-PCR results.

Riepl *et al*[25] reported that plasma CCK increased significantly in the aorta, the portal vein, the superior caval vein, and the internal jugular vein at the end of the 2 h endotoxin infusion, but no changes in plasma CCK were seen in hemorrhagic shock. Our previous data demonstrated that the expression of CCK-AR and CCK-BR mRNA could be upregulated obviously by LPS in rat spleen and myocardium[8,26]. In the present study, up-regulation of CCK-AR and CCK-BR mRNA expression was observed after incubation rat PIMs with LPS for 2 h, and remained high level to 12 h and the specific binding of ^{3}H-CCK-8S was not detected in normal rat PIMs, but was detected in the rats administrated with LPS for 48 h. It is postulated that increase of CCK concentration in plasma and up-regulation of CCK receptor expression in target cells may be a protective mechanism of body during endotoximia. The possible

pathways of CCK-AR and CCK-BR mRNA expression increase by LPS are as follows: (1) Positive or homologous regulation, that is increase of CCK synthesis induced by LPS leads to up-regulation of CCK receptor expression. (2) Heterologous regulation — LPS binds to its receptor CD14 in target cells and then results in increase of CCK receptor mRNA expression; (3) LPS exerts its effect on CCK receptor mRNA expression through enhanced expression of fast reactive gene. It is indicated that LPS leads to injury of body, meanwhile, switches on the protective mechanism of body during endotoximia. Increase of CCK-AR and CCK-BR mRNA expression may be one of the protective mechanisms.

In conclusion, CCK-AR and CCK-BR mRNA expression were detected by RT-PCR and the presence of functional CCK receptors was confirmed by radoligand binding assay in rat PIMs, and their expressions were upregulated obviously by LPS. So it might be postulated that CCK-8 exerts anti-inflammatory effect through its receptors in target cells during endotoximia.

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