Effect of calcitonin gene-related peptide on the expression of triggering receptor expressed on myeloid cells-1 in lipopolysaccharide-induced macrophages

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Abstract: Objective To determine the effect of calcitonin gene-related peptide (CGRP) on triggering receptor expressed on myeloid cells-1 (TREM-1) in the lipopolysaccharide (LPS)-induced macrophages and its signal transduction pathway. Methods The levels of TREM-1 mRNA in the macrophages were observed by reverse transcription-polymerase chain reaction (RT-PCR), and flow cytometry was performed to detect TREM-1 protein expression levels in the macrophages. Results CGRP had no regulating effect on the expression of TREM-1 in the macrophages; LPS could up-regulate macrophages to express TREM-1; CGRP increased TREM-1 mRNA expression in LPS-induced macrophages in dose and time-dependent manner; CGRP increased TREM-1 protein expression in LPS-induced macrophages, which could be partially reversed by H-7 or H-89 (P < 0.05). Conclusion CGRP can regulate the LPS-induced macrophages synthesis and secretion of TREM-1, and the intracellular signal transduction pathway is related to PKA and PKC.

Key words: calcitonin gene-related peptide; triggering receptor expressed on myeloid cells-1; lipopolysaccharide; macrophage

降钙素基因相关肽对脂多糖诱导的巨噬细胞表达髓样细胞触发受体-1 的影响

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【摘要】目的:探讨降钙素基因相关肽（CGRP）对脂多糖（LPS）诱导的小鼠巨噬细胞表达髓样细胞触发受体-1 (TREM-1) 的影响及其信号传导途径。方法:采用反转录-聚合酶链式反应 (RT-PCR) 观察巨噬细胞 TREM-1 mRNA 表达量的变化, 应用流式细胞术检测巨噬细胞表面 TREM-1 蛋白的表达。结果: CGRP 对未受刺激的巨噬细胞表达 TREM-1 无明显影响, LPS 可诱导巨噬细胞表达 TREM-1; CGRP
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预处理呈剂量和时间依赖性上调 LPS 所致的巨噬细胞 TREM-1 mRNA 的表达; 同时, CGRP 上调 LPS 所致的巨噬细胞膜表面 TREM-1 蛋白的表达; 上述作用均可被 PKC 阻断剂 H-7 和 PKA 阻断剂 H-89 部分逆转 (P < 0.05)。结论: CGRP 上调 LPS 诱导的巨噬细胞表达 TREM-1, 其胞内信号传导途径与 PKA 和 PKC 有关。

【关键词】 降钙素基因相关肽； 髓样细胞触发受体-1； 脂多糖； 巨噬细胞
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Calcitonin gene-related peptide (CGRP) was firstly found with recombinant DNA technology and molecular biology techniques in 1983. It was reported that CGRP, expressed on the sensory neurons and many other tissues, performed as a vasodilator peptide with function of relaxing blood vessels, increasing vascular permeability, and promoting mucus secretion as well as other biological effects[1-2]. When airway inflammation happens, the level of CGRP increases significantly and it possibly participates in neurogenic inflammation through regulating local inflammatory cells. Macrophages have functions of getting rid of bacteria, aging cells and stimulating lymphocyte immune response, etc[3]. CGRP receptor anchors on cell membrane of human and mouse monocytes/macrophages with function of immune regulation[4-5]. Triggering receptor expressed on myeloid cells-1 (TREM-1) was found in 2000 by Bouchon, et al.[6] which expresses on cell membrane of neutrophils and monocytes with function of inflammatory amplification[7]. TREM-1 has been regarded as a new target for anti-inflammatory treatment. Recent studies showed that CGRP and TREM-1 expression in the lung tissue would increase under the condition of inflammation, but their relevance and significance remain unclearly. In this study, we aimed to observe the effect of CGRP on TREM-1 expression on lipopolysaccharide (LPS)-induced macrophages, and to explore the signal transduction mechanism. Furthermore, this study revealed the role of neuropeptides in acute inflammation and explored its role in diagnosis and treatment of pulmonary infection diseases.

1 MATERIALS AND METHODS

1.1 Main reagents
DMEM medium, LPS, CGRP, H-7, and H-89 were purchased from Sigma Company, USA. TREM-1 antibody and FITC-labeled anti-mouse TREM-1 antibody were products of Santa Cruz, USA. Calf serum was bought from Hangzhou Sijiqing Company, China. Reverse transcription (RT) kit and Taq DNA polymerase were purchased from MBI Company, Canada. All primers were synthesized by Shanghai Bio-engineering Company, China. TRIzol was the product of Invitrogen Company, USA. DEPC-treated water and normal melting point agarose were purchased from Dingguo corporations, China. While other reagents were all of analytical grade level.

1.2 Cultivation of macrophages

The macrophages cell line (RAW264.7) was purchased from Cell Center, Xiangya School of Medicine, Central South University. The macrophages were cultured in DMEM/high glucose complete medium at 37℃ under 5% CO2. The medium was replaced with serum-free medium for 8 h, when the cells confluence to 80%. Then the cells were treated as follows: (1) Time-effect studies: after CGRP (10^-8 mol/L) pretreatment for 30 min, LPS (1 μg/mL[8]) was added into cultured macrophages for 2, 4, 6, 12, and 24 h, respectively; (2) Dose-effect studies: macrophages were pretreated with different concentrations of CGRP (10^-10 – 10^-6 mol/L) for 30 min, then macrophages were cultured with LPS (1 μg/mL) in combination with CGRP for 6 h; (3) Signal transduction pathway studies: after CGRP (10^-8 mol/L) and H-7 (10^-5 mol/L) or H-89 (10^-5 mol/L) pretreatment for 30 min, macrophages were cultured with LPS (1 μg/mL) in combination with CGRP and H-7 or H-89 for 6 h.

1.3 Expression of TREM-1 in macrophages detected by flow cytometry

Unlabeled first antibody was added into the tubes,
and then 100 μL cell suspension were added into each tube. All samples were incubated for 30 min at 4 °C for staining, and washed by PBS. After centrifuged at 2000 r/min for 5 min, the supernatant was discarded. The macrophages were incubated for 30 min at 4 °C with fluorescent pigment labeled secondary antibody. Each sample was washed by PBS and the supernatant was also abandoned after centrifuged at 2000 r/min for 5 min. The cells were resuspended with 1% paraformaldehyde, and then tested by flow cytometry.

1.4 TREM-1 mRNA expression in macrophages detected by RT-PCR

The total RNA was extracted from macrophages by TRIzol. RT was completed according to kit instructions and all products were saved at −20 °C after an instant centrifuge. PCR primers were designed as TREM-1 sense primer: 5′-CTGCTGTGCGTGTCTTT-3’ , anti-sense primer: 5′-TCATCCGAGGATGTTA-3’, with an amplified fragment of 338 bp. PCR reaction conditions were pre-denaturation at 94 °C for 3 min, 30 thermo-cycles (30 s at 94 °C, 30 s at 53 °C, and 1 min at 72 °C), and 5 min at 72 °C. GAPDH was used as internal control, sense primer: 5′-ACCA- CAGTCCATGCGCATCAG-3’, anti-sense primer: 5′- TCCACCACCTGTGCTGTA-3’, with an amplified fragment of 450 bp. PCR reaction conditions: pre-denaturation at 94 °C for 3 min, 30 thermo-cycles (30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C), and 5 min at 72 °C. Reaction system: 12.5 μL 2 × master PCR mix, 2 μL primer with the concentration of 10 pmol/μL, 8.5 μL DEPC-treated water, 2 μL template. PCR amplified products were saved at 4 °C. PCR products (5 μL) were electrophoresed on 1.5% agarose gel (containing ethidium bromide of 0.5 μg/μL). Software of Quantity One was used for analysis of the gray area, and the radio of gray scale values of TREM-1 and GAPDH was represented TREM-1 mRNA expression.

1.5 Statistical analysis

Data were shown as mean ± standard deviation (x ± s) and analyzed with SPSS 13.0 statistical package. The significance of differences between groups was tested by ANOVA. P < 0.05 was considered as significant difference.

2 RESULTS

2.1 Effect of CGRP on TREM-1 expression in LPS-induced macrophages

The results of RT-PCR showed that CGRP (10−10 − 10−6 mol/L) increased TREM-1 mRNA expression in LPS-induced macrophages in a dose-dependent manner (P < 0.05, Fig. 1). CGRP (10−8 mol/L) increased the TREM-1 mRNA expression in LPS-induced macrophages at 2, 4, 6, 12, and 24 h (P < 0.05, Fig. 2), among which the role of 6 h point had the maximal effect.

![Fig. 1 Effect of different concentrations of CGRP on TREM-1 mRNA expression in LPS-induced macrophages (x ± s, n = 5). A: Electropherogram; B: Polygram. M: Marker; L: LPS (1 μg/mL); C1: CGRP (10−10 mol/L); C2: CGRP (10−9 mol/L); C3: CGRP (10−8 mol/L); C4: CGRP (10−7 mol/L); C5: CGRP (10−6 mol/L). Compared with the L+C1 group, * P < 0.05, ** P < 0.01.](image-url)
Fig. 2 Effect of CGRP (10^{-8} mol/L) on TREM-1 mRNA expression in LPS-induced macrophages at different time points (\( \bar{x} \pm s, n=5 \)). A: Electropherogram; B: Polygram. M: Marker. Compared with the 2 h group, * \( P < 0.05 \).

The results of flow cytometry showed that compared with the control group, there was no difference with expression of TREM-1 protein on macrophages in CGRP treatment 6 h group (Fig. 3), suggesting that CGRP didn’t affect TREM-1 protein expression of macrophages without inducement. The expression of TREM-1 protein in macrophages significantly increased after LPS stimulating for 6 h (\( P < 0.05 \)). TREM-1 protein expression of LPS-induced macrophages increased when the cells cultured with LPS (1 \( \mu \)g/mL) in combination with CGRP (10^{-8} mol/L) for 6 h (\( P < 0.01 \), Fig. 3).

Fig. 3 Effect of CGRP (10^{-8} mol/L) on TREM-1 protein expression in LPS-induced macrophages (\( \bar{x} \pm s, n=3 \)). Compared with the control group, * \( P < 0.05 \), ** \( P < 0.01 \); compared with the LPS group, ### \( P < 0.01 \).
2.2 Effect of H-7 and H-89 on TREM-1 expression in LPS-induced macrophages

The results of RT-PCR showed PKC inhibitor H-7 and the PKA inhibitor H-89 could partially reverse the effect of CGRP on increasing the expression of TREM-1 mRNA of LPS-induced macrophages at 6 h ($P < 0.05$), while there was no significant change of TREM-1 mRNA level when H-7 and H-89 affected alone (Fig. 4).

The results of flow cytometry showed that PKC inhibitor H-7 and the PKA inhibitor H-89 could partially reverse the effect of CGRP on enhancing the expression of TREM-1 protein in LPS-induced macrophages at 6 h ($P < 0.01$), while there was no significant change of TREM-1 protein expression when H-7 and H-89 affected alone (Fig. 5).

![Effect of signal pathway inhibitors on TREM-1 mRNA expression in LPS-induced macrophages](image1)

**Fig. 4** Effect of signal pathway inhibitors on TREM-1 mRNA expression in LPS-induced macrophages ($\bar{x} \pm s$, $n = 5$). A: Electropherogram; B: Histogram. M: Marker; Con: Control; L: LPS 1 μg/mL; L+C: CGRP $10^{-8}$ mol/L; H-7, H-89: 10 $^{-5}$ mol/L. Compared with the control group, * $P < 0.05$, ** * $P < 0.01$; compared with the LPS group, ** ** $P < 0.01$; compared with the L+C group, Δ $P < 0.05$.

![Effect of signal pathway inhibitors on TREM-1 protein expression in LPS-induced macrophages](image2)

**Fig. 5** Effect of signal pathway inhibitors on TREM-1 protein expression in LPS-induced macrophages ($\bar{x} \pm s$, $n = 3$). LPS: 1 μg/mL; CGRP: $10^{-8}$ mol/L; H-7, H-89: 10 $^{-5}$ mol/L. Compared with the LPS + CGRP group, * * $P < 0.01$. 
3 DISCUSSION

CGRP widely distributes in nervous system, cardiovascular system, lungs and many organs, involving in regulation of physiological and pathological processes\(^9\text{-}^\text{10}\). The relationship between CGRP and inflammation is very complex. CGRP has a strong pro-inflammatory property which could promote T lymphocytes to adhere to fibronectin, accelerate the migration of lymphocyte to inflammation sites\(^1\text{1}\), and increase the activity and expression of metalloproteinkinase-9 (MMP-9) in human bronchial cells\(^1\text{2}\). The native immune response is main form of early anti-infection and the immune cells will be activated after Toll-like receptor (TLRs) and other inflammatory receptor binding together to clear pathogens and form adaptive immune response\(^1\text{3}\). It has been reported that TREM-1 could amplify TLR-induced signals to regulate the innate immune response, and play an essential role in inflammatory response\(^1\text{4-}^\text{15}\). Studies have showed that patients with asthma acute episode and animal with asthma has significant increase of CGRP in plasma and bronchoalveolar lavage fluid\(^1\text{6}\), while TREM-1 increases in plasma and bronchoalveolar lavage fluid of pneumococcal pneumonia and lung cancer patients\(^1\text{7-}^\text{18}\). All above suggests that CGRP and TREM-1 expression might be increased in case of lung inflammations.

It is generally believed that soluble TREM-1 (sTREM-1) is secretary subtype of TREM-1, which is lack of transmembrane domain. TREM-1 and sTREM-1 have the same clinical significance. Some studies observed that sTREM-1 concentration in serum increased in patients with chronic obstructive pulmonary, and its level was negatively correlated with the restoration of lung function\(^1\text{9}\). In this study, CGRP was found to increase mRNA transcription and protein expression of TREM-1 in LPS-induced macrophages. These indicated that CGRP may promote the secretion of inflammatory cytokine and chemotactic factor through TREM-1, subsequently recruit polymorphonuclear neutrophils to clear original stimulation and terminate pathogens, which is an important innate immune component in lung. However, the continued expression of TREM-1 would have an effect on inflammatory amplification, extending the repair time or missing it, which will lead to protracted heal after lung injury. Thus, in the early stages of pulmonary infection, CGRP and TREM-1 concentration in the lavage fluid could be used to quickly determine the extent of lung injury. At the same time, both CGRP and sTREM-1 could be treated as targets in actively anti-inflammatory treatment. It may be a new and effective way in diagnosis and treatment of acute lung injury in pulmonary infectious diseases.

Protein kinase C (PKC) and protein kinase A (PKA) play an essential role in intracellular signal transduction. In this experiment, CGRP could promote the expression of TREM-1 in LPS-induced macrophages, while the effect could be partially reversed by PKC inhibitor H-7 or PKA inhibitor H-89, indicating that their intracellular signaling pathway were associated with PKC and PKA. Based on the complex nature of CGRP and TREM-1 in the lungs, further researches need to be done.

REFERENCES:


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