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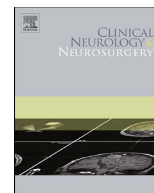

Increased expression of P2X7 receptor in peripheral blood mononuclear cells correlates with clinical severity and serum levels of Th17-related cytokines in patients with myasthenia gravis

Yong Zhang[a,1,*], Yanyan Zhang[a,b,1], Hao Li[a,1], Xiao Jia[a], Xiuying Zhang[a], Yan Xia[a], YuZhong Wang[c], Linlin Fu[d], Chenghua Xiao[a,*], Deqin Geng[a]

[a] Department of Neurology, Affiliated Hospital of Xuzhou Medical university, Xuzhou, Jiangsu, China
[b] Department of Neurology, The Third People's Hospital of Xuzhou, Xuzhou, Jiangsu, China
[c] Department of Neurology, Affiliated Hospital of Jining Medical University, Jining, Shandong, China
[d] Department of Pathogenic Biology and Lab of Infection and Immunology, Xuzhou Medical university, Xuzhou, Jiangsu, China

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ABSTRACT

Objectives: P2X7R is a well-documented activator of innate and adaptive immune responses. We aimed to measure the expression levels of P2X7R in peripheral blood mononuclear cells (PBMCs) from patients with myasthenia gravis (MG) and to investigate whether the expression of P2X7R is associated with pathogenesis of MG.

Patients and methods: A total of 32 patients with MG (12 generalized MG (GMG) and 20 Ocular MG (OMG) and 22 healthy donors were recruited in this study. The quantitative MG score was used to evaluate the clinical severity. Real-time PCR and western blot were used to measure the levels of P2X7R expressed in PBMCs. Serum Th17-related cytokines (IL-1β, IL-6, IL-17 and IL-21) were tested by ELISA. PBMCs from MG patients were purified and challenged by LPS with or without a selective P2X7R inhibitor (BBG).

Results: Our results showed that the expression of P2X7R mRNA and protein in PBMCs was increased in MG patients compared with healthy controls, with higher expression in generalized patients (GMG) than in ocular patients (OMG). In addition, P2X7R expression presents a significantly positive correlation with clinical severity and serum levels of IL-1β, IL-6, IL-17 and IL-21 in MG. In cultured MG PBMC, LPS challenge led to up-regulated P2X7R expression accompanied with increased production of IL-1β, IL-6, IL-17 and IL-21. Importantly, P2X7R blockade with BBG significantly attenuates the LPS-induced production of cytokines.

Conclusion: P2X7R expression was up-regulated in MG and LPS-P2X7R axis may be involved in the pathogenesis of MG by promoting Th17 immune response.

Myasthenia gravis (MG) is an autoimmune-mediated neuromuscular disease and its incidence has been increasing lately [1]. The disorder is caused by antibodies binding to components in the neuromuscular junction, impairing neuromuscular transmission. Over 80% of patients have antibodies against acetylcholine receptor (AChR) [2]. AChR-specific CD4 + T cells i.e. T-help cells, which can be detected in most MG patients, likely have an important role in MG, because they modulate the synthesis of anti-AChR Ab and may be the prime movers in the pathogenesis of MG [3]. Recently, T-helper type 17 (Th17) cells were found to play a critical role in the development of a murine model of human MG [4,5]. In addition, clinical observation from our group as well as other groups have revealed that serum levels of interleukin-17A(IL-17A) and interleukin-21(IL-21), two signature cytokines of Th17 cells, were increased in MG patients compared with healthy controls [6–8].

The P2X7 receptor (P2X7R), an ATP-gated cation channel, is expressed in various immune cells and plays a key role in promoting the release of pro-inflammatory cytokines, including interleukin-1 beta (IL-1β) and interleukin-6(IL-6) [9–11], which are involved in Th17 cell polarization [12,13]. Association of P2X7R gene polymorphisms with systemic lupus erythematosus (SLE), one of the classic autoimmune diseases, was found in a Chinese population [14]. Moreover, expression of P2X7R on peripheral blood mononuclear cells (PBMC) in patients with primary Sjögren's syndrome (pSS) was significantly higher than

* Corresponding author.
[1] Yong Zhang, Yanyan Zhang and Hao Li contributed equally to this work.
E-mail addresses: zyxzmu@163.com (Y. Zhang), chenghuaxiaoxzbj@163.com (C. Xiao).

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healthy controls [15]. Blockade of P2X7 receptors could ameliorates disease severity of several animal models of inflammatory autoimmune diseases [16–18]. Based on these findings, we here put forward the hypothesis that in MG patients this receptor may be over activated, thereby putatively contributing to the enhanced Th17 associated inflammatory cytokines production in the disease.

The aim of this study is to detect the mRNA and protein expression level of P2X7R in PBMC from MG and healthy controls, and analyzed the correlations of P2X7R with clinical severity and serum levels of Th17-related proinflammatory cytokines (IL-1β, IL-6, IL-17 and IL-21) of MG patients. Besides, in vitro studies were performed to explore the effect of P2X7R in LPS-mediated Th17 immune response in patients with MG.

1. Materials and methods

1.1. Materials

The primers of P2X7R and β-actin were synthesized by Sangon Biotech (Shanghai, China). The Quantscript RT Kit were from Tiangen Biochem (Beijing, China). Lipopolysaccharide (LPS) and Brilliant blue G (BBG) was from Sigma-Aldrich (USA). Antibodies: Rabbit anti-human P2X7R was from Boster Biological Technology (Wuhan, China).

1.2. Study population

Thirty-two patients with MG were enrolled in this study from the Department of Neurology at Affiliated Hospital of Xuzhou Medical College. The diagnosis of MG was made based on the following criteria: typical history and signs of fluctuating weakness of voluntary muscles, presence of serum antiacetylcholine receptor antibodies (AChR Ab), definite clinical improvement on injection of the cholinesterase inhibitor, edrophonium, and decremental pattern on repetitive nerve stimulation [19]. The patients were ranked according to the classification of MG (Osserman) [20]. The quantitative MG score (QMG) was used to evaluate the clinical severity of MG patients as previously described [21]. All patients were classified into one of two groups: ocular MG (20 patients) or generalized MG (12 patients). All patients were seropositive for anti- AChR antibodies. The patients did not have other autoimmune diseases, ongoing infection and malignancies. None of the patients had received any immunomodulatory drugs within the past three months. The control group consisted of 22 healthy subjects with no inflammatory diseases. Our study received prior approval by local ethic committee and informed consent was obtained from each subject. The details of the MG patients are summarized in Table 1.

1.3. PBMC isolation

About 20 ml of venous blood from each subject was collected into heparinized vacuum tubes. After centrifugation at 3000 rpm for 15 min, the PBMCs were isolated by Ficoll gradient separation with lymphocyte isolation agent (Beijing Solarbio Science & Technology Co., Ltd.). Serum samples were obtained and stored at −80 °C.

1.4. RT-PCR

The expression of P2X7R mRNA in PBMC was evaluated by RT-PCR. Total RNA from PBMCs of MG patients and healthy controls was extracted using TRIzol (Invitrogen, USA). The concentration of RNA was detected by ultraviolet absorption spectrometry. The same amount of RNA (2 μg) was reversely transcribed to cDNA using the Quantscript RT Kit according to the manufacture's instruction. PCR was performed with P2X7R specific primers (sense 5′-TCCGAGAAACAGGGATAA-3′, and anti-sense 5′- ACTCGCACTTCTTCCTGT -3′) for 30 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s). Human β-actin was amplified as an internal control. For β-actin, we used the flowing primers: the forward 5′-AGCGAGCATCCCCCAAAGTT-3′ and the reverse 5′-GGGCACGAAGGCTCATCATT-3′. The RT-PCR was performed at least twice for each sample.

1.5. Western blot

The expression of P2X7R protein in PBMC was assessed by western blot. Proteins were extracted from PBMC using a modified TRIzol one-step extraction method. Protein concentration was determined by a Bradford kit (Bio-Rad, Hercules, CA, USA). The protein was separated by a verticaldodcyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%), and subsequently transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked in TBS/5% skim milk overnight and then incubated with primary antibody. Membranes were then incubated with a peroxidase labeled secondary antibody and bound antibodies were detected by the ECL western blotting analysis kit (Pierce, Thermo Fisher Scientific, Rockford, IL). The quantification of P2X7R to β-actin levels was done by densitometry analysis.

1.6. PBMCs culture and stimulation

PBMCs from GMG ($n = 8$), OMG patients ($n = 8$) and healthy controls ($n = 8$) were isolated as described above. After being washed twice in phosphate buffered saline (PBS), PBMCs were suspended in RPMI 1640 supplemented with 5% calf bovine serum (CBS, Life Technologies, Inc.) and 1% penicillin–streptomycin (Life Technologies, Inc.). The cell suspension was adjusted to a concentration of 2×10^5 cells/ml, and cultured with or without LPS (1 μg/ml) stimulation for 24 h in a 24-well plastic culture plate in a final volume of 1 ml at 37 °C in 5% CO 2/95% air. In some MG patients, LPS treatment was preceded by a 30 min incubation with the P2X7R antagonist BBG (1 μmol/l). The viability of PBMC cultures was monitored periodically before and after transduction using trypan blue (Sigma-Aldrich) staining. Cells with > 90% viability were continued for additional experiments. After 24 h of culture, the cells were harvested and their mRNA and protein expression of P2X7R was determined by RT-PCR and Western blotting, supernatants were collected for further were analysis of the pro-inflammatory cytokines IL-1β, IL-6, IL-17, and IL-21.

Table 1
General information of study subjects.

Information	MG patients	Ocular MG	Generalized MG	HCs
Number of cases	32	20	12	22
Age (years)	50.56 ± 16.15	47.95 ± 17.79	54.92 ± 12.45	50.36 ± 15.64
Female/male	19/13	12/8	7/5	13/9
Thymoma, thymic hyperplasia	2	1	1	–

MG: myasthenia gravis; HCs: healthy controls
The age between two groups (MG: HCs) had no significant differences ($P > 0.05$) and the group ratios were no significant different ($P > 0.05$).

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