Expression and Significance of protein 4.1R in rat models with diaphragmatic weakness

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Abstract

Abstract Background To explore the significance of protein 4.1R expression in the diaphragmatic weakness animal and cell models and its preliminary mechanism.

Methods Rats were intraperitoneally injected with Lipopolysaccharide (LPS) to construct diaphragmatic weakness models. Histopathology of diaphragmatic tissues was detected by Hematoxylin eosin (HE) staining. L6 cells were induced by LPS to establish the myasthenic cell models and transfected with 4.1R-siRNA to knockdown 4.1R. Immunohistochemistry was performed to detect the expression of acetylcholine receptor (AChR) and 4.1R; The expression of desmin

Results LPS could induce the diaphragmatic weakness of rats. The expression of AChR in diaphragmatic weakness tissues was lower, while that of desmin was that in the control group. 4.1R was up-regulated in the diaphragmatic weakness models, and related to the severity. After knockdown of 4.1R in LPS induced L6 cells, the expression of AChR was up-regulated significantly. But there was not difference of contractile proteins.

Conclusions Protein 4.1R was upregulated in diaphragmatic weakness model in vivo and in vitro and might be involved into the occurrence of myasthenia gravis.

Background

Myasthenia gravis (MG) is a chronic autoimmune disease, which is caused by a lack of acetylcholine receptor (AChR) at the neuromuscular junction resulting in fluctuating weakness of striated muscles. The clinical presentation is characterized by muscle weakness and fatigability [1]. Diaphragmatic weakness is understood to be a typical presentation of MG crisis. Currently, the pathogenesis of MG remains still unclear, while it is commonly believed to be related to infection, drugs, genetics and other environment factors [2].

As one of the cytoskeletal protein, protein 4.1R is composed of 864 amino acids which is encoded by EPB41 gene located in chromosome 1p33-p32. Protein 4.1R is widely expressed in tissues and a variety of subcellular compartments including plasma membrane, cytoplasm and nucleus [3]. Studies have shown that protein 4.1R plays an important role in hereditary anaemias, control of heartbeat, tumors suppression and other diseases [4,5]. Studies found that Protein 4.1R expressed abnormally in various tumors and there is a negative regulation function for 4.1R in the activation, and anti-tumor immunity of T cells [6, 7]. Delhommeau et al. [8] also found that protein 4.1R was colocalized with the expression of dystrophin in muscles of Becker patients, and absent from dystrophin-deficient sarcolemma of patients with Duchenne muscular dystrophy. So it could be speculated that protein 4.1R might be associated with the occurrence of myasthenia related diseases. However there was rarely study on the relationship between 4.1R and myasthenia, so it is lack of evidence. In the present study, the expression and significance of protein 4.1R in LPS-induced diaphragmatic weakness rat or cell models were analyzed and the correlation of 4.1R and the myasthenia was initially explored in *vivo* and in *vitro*, so as to provide a new idea and target for the pathogenesis and treatment of MG.

Methods

Animals

24 male SD rats, weight (180–200)g, were purchased from Shanghai SLAC laboratory animal Co.Ltd. [license number: SCXK(Shanghai) 2017–0005]. After one week of conventional adaptive feeding, rat models of diaphragmatic weakness were established.

Grouping and modeling

24 rats were randomly divided in 4 groups: control group: given normal saline by intraperitoneal injection; low LPS group: 5mg/kg LPS by intraperitoneal injection; middle LPS group: 10mg/kg LPS by intraperitoneal injection, and high LPS group: 15mg/kg LPS by intraperitoneal injection, each group contained 6 rats. The behavioral changes of the rats were observed in 24h. After 24h, the rats in each group were sacrificed by injected 1% pentobarbital sodium. The chest was quickly opened, diaphragmatic tissues were isolated. Part of diaphragmatic tissues was fixed in 4% formaldehyde solution for histopathological experiments, and the other part was stored in liquid nitrogen to extract the mRNA or protein.

Cell culture

L6 cell line (Beijing Solarbio technology co. LTD, China) was maintained in DMEM containing 10% fetal bovine serum, penicillin (50 µg/ml) and streptomycin (50 µg/ml) in an incubator with suitable humidity and 5% CO₂, at 37°C. The cells were digested by trypsin and passage at 80% confluence. And the diaphragmatic muscle weakness cell model in vitro was conducted by 10 µg/ml LPS.

Transfection

The lentiviral vectors containing 4.1R shRNA (4.1R-siRNA) or a control shRNA sequence (NC-siRNA) were purchased from GenePharma (Shanghai, China). The sequence of 4.1R siRNA: GAAGGAGATAGAAGCTTGG and the NC sequence: ACTACCGTTGTATAGGTGT. The L6 cells at logarithmic phase were seeded at 1.5 × 10⁵ cells/well into a 6-well plate and cultured overnight for transfection. According to the instruction, 4.1R-siRNA or NC-siRNA lentiviral vector was mixed with ExFect2000 Transfection Reagent in the proportion 1:1 and transfected into L6 cells, respectively. After transfection for 6h, the cells were cultured in a new medium with 10 µg/ml LPS for 48h for the following experiments.

Quantitative Real-time PCR(qRT-PCR)

Trizol-centrifuge method was used to extract total RNA in diaphragmatic tissues and cells. The concentration and purity of RNA were detected by Meriton SMA4000 and reverse-transcribed to cDNA using HiScript® 1st Strand cDNA Synthesis Kit according to the manufacturer's instructions. And amplification of 4.1R was carried out using the ChamQ™ SYBR Color QPCR Master Mix according to the protocol. Reaction conditions: 95°C for 30s, a total of 45 cycles: 95°C for 10s, 55.7°C for 30s. $2^{-\Delta\Delta Ct}$ method was used to calculate the amount of 4.1R mRNA, with GAPDH as reference. sing

Western blot Analysis

Total proteins in diaphragmatic tissues and cells were extracted using a protein extraction kit and the concentration of protein was detected by bicinchoninic acid (BCA) methods. The proteins were analyzed by SDS-PAGE and electrotransferred on the PVDF membrane. The membrane was blocked by the TBST buffer with 5% skimmed milk. Primary antibody: anti-EPB41, anti-myosin, anti-demosin, anti-F-actin and anti-GAPDH were incubated at 37°C for 2h. HRP labeled secondary antibody was incubated for 1h at room temperature. The membrane developed using an ECL detection kit at ChemiDoc™ Touch Imaging System(Bio-Rad). GAPDH as the internal reference, the relative expression of protein was calculated using Image J.

Hematoxylin eosin(HE) staining

The diaphragmatic tissues were fixed with 4% formaldehyde, dehydrated using different concentration ethanol and embedded in paraffin. A serial sections were cut at a thickness of 4μm and processed for HE staining and immunochemistry.

The paraffin sections were dewaxed and rehydrated, and soaked into hematoxylin solution for 5min. After being washed with water, the paraffin sections were stained by eosin solution for 3–5min. After washing the floating color, the sections were dehydrated and sealed with neutral resin. The pathological of diaphragmatic tissues in each group were observed under a microscope.

Immunohistochemistry

After being deparaffinized at 65°C and rehydrated in a series of ethanol, the paraffin sections were treated with 3% H₂O₂ solution for 15min at 37°C to inhibit endogenous peroxidase activity and treated with 0.01mol/L sodium citrate buffer for retrieve the antigen. After washing with PBS, the transfected cells were fixed with 4% paraformaldehyde for 20min, treated with 0.1% Triton X100 for 20min, and 3% H₂O₂ solution for 15min at 37°C to inhibit endogenous peroxidase activity. Then the sections and cells were blocked by PBS solution with 5% BSA for 1h, and incubated overnight at 4°C in primary antibody. PBS was considered as the negative control. After washing with PBS, the secondary antibody was added and incubated for 1h at room temperature. The 3,3'-diaminobenzidine tetrahydrochloride substrate was added and developed in the dark. Then the sections were observed and photographed under a microscope. Five fields were randomly selected in each section, and the semi-quantitative method was used to evaluate the expression of the proteins. The semi-quantitative evaluation criterion: Staining intensity: 0 represents no obvious staining color, 1 represents mild, 2 represents moderate, 3 represents severe color intensity; Proportion of positive cells: 0 point: ≤5%; 1 point: 6%~25%; 2 point: 26%~50%; 3 point: 51%~75%; 4 point: 76%~100%. The score of positive degree was the sum of staining intensity and proportion of positive cells. 5 views in each sample were selected and the final score was shown by mean±standard deviation.

Results

Rat behaviors and pathological characters of diaphragmatic muscle tissues

The rats in the control group were in good spirits and sensitive reaction. While after being injected with different concentration of LPS, the rat especially in the high LPS group performed to be slow in response, listless in spirits, shortness of breath and eyes secretions increase.

HE staining was performed to observe the pathological characters of diaphragmatic tissues in the rat models with LPS-induced diaphragmatic weakness(Figure 1). Histological findings showed that the muscle fibers of the control group were regularly arranged without morphological changes. In the low LPS group and middle LPS group, a small amount of muscle fibers became atrophy, even breakage. While the muscular fibers were disordered, twisted and broke, and interstitial edema and inflammatory cells infiltration in the high LPS group, which was more severe than that in the control group. The results suggested that LPS could destruct the histological patterns of the severity of diaphragmatic weakness increased with the dose of LPS.

The expression of AchR in diaphragmatic weakness rat models

It is known that a loss of AchRs on the neuromuscular junction impairs neuromuscular transmission and induces muscle weakness. So the expression of AchR in diaphragmatic weakness models was detected by immunohistochemistry (Figure2). The results showed that the AchR mainly expressed in the surface membrane of muscle cells. The expression of AchR in the control group, low LPS group, middle LPS group and high LPS group were significantly different, the expression of AchR in the control group was higher than that in the LPS groups($P<0.05$, Table 1), the expression of AchR was decreased with the concentration of LPS. The results showed that LPS constructed the diaphragmatic weakness models successfully and the severity of the diaphragmatic weakness was related to the LPS dose.

The expression of contractile proteins in diaphragmatic weakness rat models

The expression of contractile proteins myosin and desmin in diaphragmatic tissues were further detected by western blot. The results showed that expression of desmin in LPS groups were higher than that in the control group in concentration-independent manner, while there was no difference of myosin among groups (Figure 3). It was speculated that LPS may induce the expression of contractile protein desmin in diaphragmatic tissues irritability.

Protein 4.1R was upregulated in diaphragmatic weakness rat models

QRT-PCR, western blot and immunohistochemistry were used to detect the expression of 4.1R mRNA and protein in the control rats and diaphragmatic weakness rats. Compared with the control group, the expression of protein 4.1R was significantly increased in the LPS groups both in the mRNA and protein levels (Table 2), which was more obvious with the concentration of LPS. Immunohistochemical results showed that the protein 4.1R was mainly expressed in the cell membrane and cytoplasm, and diffusively expression in few nuclei of muscle cells (Figure 4). The degree of positive expression of protein 4.1R was consistent with the results of western blot and qRT-PCR. The results indicated that the more severe the diaphragmatic weakness was, the higher the protein 4.1R expressed, and protein 4.1R might be related to the occurrence and progress of diaphragmatic weakness.

4.1R regulated the expression of AchR in LPS-induced L6 cells

It is known that a loss of AchRs on the neuromuscular junction impairs neuromuscular transmission and induces muscle weakness, and the above results showed that the expression of AchR was down-regulated in the diaphragmatic weakness models. So, it was speculated that protein 4.1R might regulate the expression of AchR *in vivo* and was involved in the diaphragmatic weakness. In order to prove its possible mechanism, LPS was used to induce the L6 cells to establish the myasthenic cell models and down-regulation of the expression of 4.1R. Then the expression of AchR and protein 4.1R were detected by immunohistochemistry and western blot. The result showed that the AchR mainly expressed in the cell membrane of L6 and the expression level of AchR in LPS induced L6 cells were lower than that in BC group (Figure 5). The results of WB showed that the expression of 4.1R in LPS induced L6 cells was higher than BC group and the protein 4.1R was down-regulated in the 4.1R-siRNA+LPS group, while the expression of AchR in 4.1R-siRNA+LPS group was higher than that in the NC-siRNA+LPS group and BC+LPS group (Figure 6A and B), suggesting that knockdown of protein 4.1R could increase the expression of AchR. The results indicated that protein 4.1R could regulate the expression of AchR *in vitro* directly or indirectly.

4.1R could not affect the expression of contractile proteins in LPS induced L6 cells

The results above showed that in diaphragmatic weakness rat models, the contractile proteins were up-regulated. In order to explore the association of protein 4.1R and contractile proteins in myocyte, the expression of desmin and myosin were detected by western blot in LPS induced L6 cells (Figure 6A and C). There was no difference in the expression of myosin, desmin between the BC groups and LPS induced L6 groups, not consistent with the results *in vivo*. This was probably because that the high expression of contractile proteins was affected by the stress response *in vivo*, while there was no stress response *in vitro*. And there was no significant difference in the expression of myosin and desmin among BC+LPS, NC-siRNA+LPS and 4.1R-siRNA+LPS groups, demonstrated that protein 4.1R could not affect the expression of muscle contractile proteins in LPS induced L6 cells *in vitro*.

Discussion

Protein 4.1R is the earliest isolated and identified member of 4.1R family, which contain five members, 4.1B, 4.1G, 4.1N, 4.1O and 4.1R [9]. There are three highly conservative domains in protein 4.1R: membrane-bound domains in the N terminal, spectrin/actin binding domain (SABD), and C-terminal domain (CTD). Studies have demonstrated that 4.1R is a multifunctional protein—as a membrane cytoskeleton, 4.1R could interact with erythrocyte transmembrane protein, such as spectrin, short actin, band 3 and glycophorin C to maintain the normal erythrocyte shape and functions [10, 11]; 4.1R interacts with the other transmembrane or membrane-associated proteins, regulating the activity of Na^+/K^+ and Ca^{2+} /calmodulin, such as regulating repolarization and calcium handling in the heart [12, 13]; 4.1R is a suppressor in variety of tumors [14]; 4.1R binds to the adaptor protein LAT (linker of activation of T cells) and inhibits the phosphorylation of LAT and the activity of T cells [15]. So abnormal expression of protein 4.1R was associated with hypohemia, heart disease, cancers, autoimmune disease and other diseases. Myasthenia gravis is an autoimmune disease of the nervous system [16], and the previous study has been found that protein 4.1R was abnormal expressed in patients with MG [17]. It was speculated that 4.1R might play an important role in MG disease or other related disease. However, the function of 4.1R in muscle cells, particularly the physiological function of 4.1R *in vivo* remains largely unexplored. In the present study, the LPS-induced diaphragmatic weakness models was used to explore the expression and significance of 4.1R *in vivo*, and preliminarily understand the mechanism of 4.1R *in vitro*, which was of great significance for the pathogenesis and treatment of MG.

It is known that blocked AchR could cause the occurrence of muscle weakness in MG patients [18]. The present study also showed that AchR was mainly expressed in the membrane of myocytes, and downregulated in the diaphragmatic weakness models, which was related to the severity of the diaphragmatic weakness. And in the diaphragmatic weakness models *in vivo*, the expression of protein 4.1R in the control group was significantly lower than that in the LPS groups in the both levels of mRNA and protein, and protein 4.1R was expressed in the cell membrane and cytoplasm, and diffuse expression in few nuclei of muscle cells detected by immunohistochemistry, which suggested that protein 4.1R might be related to the occurrence of myasthenia, consistent with the present study [17]. And *in vitro*, the expression of protein 4.1R was upregulated in the LPS induced L6 cell, the cell experiments also confirmed that the protein 4.1R might be associated with the myasthenia. In addition, the results confirmed that protein 4.1R could not affect the expression of contractile proteins, so the protein 4.1R was not associated with the contractile protein in the myasthenia.

Literature indicated that as a member of protein 4.1 family, 4.1N was a binding partner of the α -7ACh receptor. DCP-LA enhanced signal intensities for the α 7 ACh receptor at the membrane surface in PC-12 cells, which was significantly suppressed by knocking down 4.1N[19]. And there was a high homology of CTD region between 4.1N and 4.1R. So it was speculated that 4.1R might be regulate the AchR, leading to myasthenia. In order to explore the mechanism, the effect of protein 4.1R on the expression of AchR in vitro was detected by immunohistochemistry and it was found that knockdown of protein 4.1R promoted the expression of AchR in LPS induced L6 cells, inferring that protein 4.1R could regulate the expression of AchR, directly or indirectly. It has been shown that 4.1R directly bound to a variety of transmembrane- or membrane-associated proteins. For example, 4.1R could regulate the activity of the housekeeping Na^+/H^+ exchanger(NHE) 1, mediating the regulation of intracellular Ca^{2+} concentration and pH in almost cell types[20]. And 4.1R could bind to C terminus of PMCA1b, which could be activated by Ca^{2+} /calmodulin, regulating the small intestinal calcium absorption and calcium homeostasis[21]. As an ion channel, conformational change of AchR after binding to acetylcholine, the channel was opened to allow Na^+ , K^+ , Ca^{2+} to pass, which could maintaining the the relaxation and contraction of skeletal muscle[22]. So it was possible that the abnormal expression of protein 4.1R could regulated AchR ion channel, inducing the abnormal release of AchR, leading to the abnormal muscle contractions and the occurrence of muscle weakness. However, the mechanism of the protein 4.1R directly or indirectly negatively regulate the expression of AchR still needs further experiment to verify.

Conclusion

Protein 4.1R was upregulated in diaphragmatic tissue and LPS induced myocyte, and involved in the occurrence of myasthenia gravis, which might be associated with the regulation of AchR. In this study, the expression and significance of 4.1R in the MG preliminarily reflected the mechanism of 4.1R in MG, which might provide a new idea for the pathogenesis of MG and a new target for the treatment of MG. However, the role of protein 4.1R in L6 cells, and the mechanism between protein 4.1R and AchR remained to be further explored.

Declarations

Ethics approval

This study was approved by the ethical standards ethics committee of The First Affiliated Hospital of Henan University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors declare that they have no conflicts of interest to this work.

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Author contributions

Authors in this manuscript have contributed the work below:

YH L contributions to the conception or design of the work and the acquisition, analysis, interpretation of data for the work; YQ L and XY J drafted the work and revised it critically for important intellectual content; HM L and DD W contribution to the case collection and experiments operation; WQ W and XC W contribution to the manuscript final revision. All authors have read and approved the manuscript.

Acknowledgement

Not applicable.

Abbreviations

Lipopolysaccharide

LPS

Hematoxylin eosin

HE

acetylcholine receptor

AchR

Myasthenia gravis

MG

Quantitative Real-time PCR

qRT-PCR

bicinchoninic acid

BCA

Na⁺/H⁺ exchanger

NHE

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Tables

Table 1 The expression of AchR in diaphragmatic muscle tissues in each of group(±s)

Groups	AchR expression by IHC
control group	5.31±0.51
low LPS group	4.97±0.35*
middle LPS group	4.25±0.24*#
high LPS group	3.87±0.25*#§
Comparison	F=8.623,P=0.000

IHC: immunohistochemistry. *, P<0.05 vs. control group; #, P<0.05 vs. low LPS group; §, P<0.05 vs. middle LPS group.

Table 2 The expression of protein 4.1R in diaphragmatic muscle tissues in each of group(±s)

Groups	4.1R mRNA	4.1R protein by WB	4.1R protein by IHC
control group	1	0.41±0.02	4.04±0.15
low LPS group	1.52±0.10*#	0.50±0.03*	4.46±0.24*
middle LPS group	2.01±0.12*#	0.58±0.04*#	4.83±0.31*#
high LPS group	3.19±0.13*#§	0.65±0.05*#§	5.43±0.26*#§
Comparison	F=11.124, P=0.000	F=7.324, P=0.002	F=8.623, P=0.000

WB: western blot; IHC: immunohistochemistry. *, P<0.05 vs. control group; #, P<0.05 vs. low LPS group; §, P<0.05 vs. middle LPS group.

Figures

Figure 1

HE staining of diaphragmatic tissues in diaphragmatic weakness models. A: control group, B: low LPS group, C: middle LPS group, D: high LPS group.

Figure 2

Immunohistochemistry of AchR in diaphragmatic weakness models. A: control group, B: low LPS group, C: middle LPS group, D: high LPS group. The brown granules represent positive expression of AchR.

Figure 3

Expression of contractile proteins myosin and desmin in diaphragmatic weakness models. *, $P < 0.05$ vs. control group; #, $P < 0.05$ vs. low LPS group; \$, $P < 0.05$ vs. middle LPS group.

Figure 4

Expression of 4.1R protein in diaphragmatic weakness models by immunohistochemistry. A:control group, B:low LPS group, B: middle LPS group, D:high LPS group. The brown granules represent positive expression of protein 4.1R.

Figure 5
 Expression AchR in L6 cells detected by immunohistochemistry(400×). A: BC group, B: LPS group, C: NC-siRNA+LPS group, D:4.1R-siRNA +LPS group, E:PBS negative control, F: Comparison of expression score of AchR. BC: blank control; *, $P<0.05$ vs. BC group; #, $P<0.05$ vs. LPS group; \$, $P<0.05$ vs. NC-siRNA+LPS group.

Figure 6

Relative expression of protein 4.1R and contractile protein in L6 cells. BC:blank control;*, $P < 0.05$ vs. BC group; #, $P < 0.05$ vs. LPS group; \$, $P < 0.05$

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