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Apelin-13 regulates LPS-induced N9 microglia polarization involving STAT3 signaling pathway

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ABSTRACT

The process of neurodegenerative diseases has always been accompanied by neuroinflammatory response characterized by microglia activation. Two phenotypes of microglial polarization: the classically activated M1 type and the alternative activated M2 type, have been described. Although apelin-13 has been shown to have neuroprotective effects, its specific mechanism of anti-neuritis is still unclear. The aim of this study was to investigate whether apelin-13 can exert anti-neuroinflammatory effects by regulating the polarization of N9 microglia. MTT assay showed that 0.1 μM apelin-13 (24 h) and 2 μg/mL LPS (6 h) treatment had no significant effect on cell viability of N9 microglia. The combined treatment of Apelin-13 and LPS did not affect the viability of N9 microglia. N9 microglia were pretreated with 0.1 μM apelin-13 for 24 h, followed by incubation with LPS for 6 h. Morphological results indicated that apelin-13 (0.1 μM) inhibited LPS-induced N9 microglial activation as observed by smaller soma and slender process compared to LPS-treated group. Western blot confirmed that apelin-13 decreased the level of proinflammatory factor iNOS, IL-6 and up-regulated the level of anti-inflammatory factor arg-1 and IL-10 in N9 microglia. Flow cytometry revealed that apelin-13 inhibited the expression of M1 microglia activation marker CD86 and up-regulated the expression of M2 marker CD206. Furthermore, the data displayed that apelin-13 decreased the expression of p-STAT3 and the radio of p-STAT3/t-STAT3 in M1-type N9 microglia induced by LPS. In conclusion, our results indicated apelin-13 ameliorated neuroinflammation by shifting N9 microglial M1 polarization toward the M2 phenotype, the underlying mechanism of which may be related to STAT3 signals.

1. Introduction

Neurodegenerative disorder is a chronic progressive disease characterized by degeneration of neurons. In the occurrence and development of neurodegenerative diseases, there is always an inflammatory response characterized by microglia activation in the brain (Monzon et al., 2018). Microglia originates from the primitive hematopoietic cells of the embryo yolk sac and is also the main immune cell of the central nervous system. As the brain's first line of defense, microglia can not only recognize the functions of external pathogens to infect and repair tissues (Zhan et al., 2014), but also produce cytokines and chemokines, and secrete factors that promote the development of the nervous system, thereby promoting the development of the central nervous system and maintenance brain homeostasis (Frost and Schafer, 2016). Microglia polarization refers to different functional phenotypes due to their own heterogeneity, differences in microenvironment and responses to stimuli signals. At present, there are two phenotypes for microglial polarization: classical activation state M1 type that is induced by lipopolysaccharide (LPS) (Wu et al., 2018a), and alternative, or anti-inflammatory M2 type that is activated by interleukin-4 (IL-4) (Machado-Pereira et al., 2017). The former participates in the process of central nervous system injury and the latter mediates the process of nerve repair and regeneration of the brain.

Originally, Apelin was a peptide, which extracted and purified by Tatemoto et al. from bovine stomach tissue (O'Dowd et al., 1993). It is an endogenous ligand for the orphan G protein-coupled receptor, the angiotensin receptor-like protein J receptor (Tatemoto et al., 1998; Haghparast et al., 2018). Apelin contains a variety of subtypes, such as apelin-36, apelin-17 and apelin-13. Moreover, apelin-13 can exert neuroprotective effects by acting on APJ receptors on cerebral microglia in rats with cerebral ischemia, suggesting the presence of APJ receptors in microglia. (Xin et al., 2015). Apelin-13 is the most

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biologically active and abundantly expressed in the nervous system. Previous studies have shown that apelin-13 is involved in various pathological and physiological processes of the nervous system including inflammation response, depression, cognitive function and regulation of neurohormones and adenohypophysial hormones (Li et al., 2016; O'Carroll et al., 2013). The potential neuroprotective effect of apelin-13 in improving neuroinflammation has been elucidated: apelin-13 has been shown not only to have a neuroprotective effect against cerebral ischemia-reperfusion injury (Chu et al., 2017), but also to repair the damaged brain barrier and thereby alleviate secondary damage by inhibiting glial activation and pro-inflammatory cytokine secretion (Bao et al., 2016; Chen et al., 2015). In addition, it has been reported that apelin-13 exerts an anxiolytic effect by activating SIRT1 to inhibit NF-KB pathway and improving neuroinflammation (Fan et al., 2018). Furthermore, in a mouse epilepsy model induced by pentylenetetrazole (PTZ), apelin-13 has been reported to protect cortical neurons from PTZ-induced neuroinflammation and apoptosis, thereby exerting anticonvulsant and neuroprotective effects (Kalantaripour et al., 2016). The above results demonstrate that apelin-13 plays a neuroprotective role by inhibiting the neuroinflammation.

Signal transducer and activator of transcription 3 (STAT3) has also been demonstrated to be involved in neuronal differentiation, inflammation and neurodegenerative disease. The relationship between STAT3 and neuroinflammation has been widely recognized. STAT3 is reported to involve in neuroinflammation by microglia activation induced by LPS (Zhu et al., 2018) and regulation of inflammation cytokine secretion (Liu et al., 2018). It also has been demonstrated that upregulation of DYRK1b may regulate astrocyte activation in LPS-induced neuroinflammatory responses through interaction with STAT3 (He et al., n.d.). In addition, STAT3 binding site has been reported to participate in inflammation-induced intestinal apelin expression (Han et al., 2008). However, the role of STAT3 in central apelin remains unclear.

The mechanism by which apelin-13 is resistant to neuroinflammation is unclear, although it is thought to have neuroprotective effects. Microglia are important functional cells that mediate neuroinflammation in the central nervous system. STAT3 acts as an important signal transduction molecule involved in neuroinflammation and microglia activation. Therefore, in the current study we aimed to explore whether apelin-13 can inhibit the inflammatory response from the perspective of microglia polarization via suppressing STAT3 expression, thus providing a potential therapeutic strategy for the development of neuroinflammation.

2. Materials and methods

2.1. Materials

Cell lines Murine N9 microglia cells lines were purchased from the Shanghai Si-xin Biotechnology Ltd. (Shanghai, China).

2.2. Main reagents

Minimum Essential Medium (MEM) was purchased from HyClone (Los Angeles, CA, USA). The fetal bovine serum (FBS) was obtained from Gibco (NY, USA). Trypsin, Penicillin-Streptomycin Liquid and Prussian Blue Iron Stain Kit were purchased from Solarbio (Beijing, China). Lipopolysaccharides was purchased from Sigma-Aldrich (serotype O55:B5). Apelin-13 was purchased from GL. Biochem Ltd. (Shanghai, China). iNOS, Arginase 1 (ARG-1), β-Actin and secondary antibody were obtained from R&D.

3. Methods

3.1. Cell culture and grouping

N9 microglia cells lines were incubated in Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin Liquid at 37 °C incubator containing 5% CO_2. The medium was changed daily (Coelho et al., 2017). The N9 microglia were divided into four groups: cells were incubated with pure medium (control group), cells were incubated in medium containing apelin-13 at concentrations of 0.01、0.1 and 1 μM for 24 h (apelin-13 group), cells were incubated in medium containing 2 μg/mL LPS for 6 h (LPS group) and cells were pre-incubated with apelin-13 for 24 h and then treated with LPS for 6 h (apelin-13 + LPS group). Samples are collected by centrifugation for subsequent experiments.

3.2. Cell viability detection by MTT assay

N9 microglial cells viabilities were evaluated by an MTT assay as described previously (Machado-Pereira and Santos, 2017). Briefly, the cells were cultured in 96-well plates (Corning, Denmark) at a density of 1.0× 104 cells/well and control wells contained with EME medium only. Apelin-13 was added to a half of wells to a final concentration of 0.01, 0.1, 1 μmol/L. MTT solution (20 μL of 5 mg/mL) was added to all the wells and cells were incubated at 37 °C for 4 h. Following, Dimethyl sulfoxide (DMSO) was added to stop the reaction. Optical density (OD) was measured at the 570 nm wavelength by an ELX-800 microplate assay reader (Bio-tek, Winooski, VT, USA), and the results were expressed as a percentage of absorbance measured in the control cells.

3.3. Flow Cytometry measurement of polarization markers

N9 cells surface markers, CD86 and CD206 were detected by flow cytometry (Baksh et al., 2007). Briefly, cells were harvested by trypsin (without EDTA), then centrifuged at 1000 rpm for 10 min, washed twice with PBS and collected cell pellet, incubated with specific target antibody for 30 min at 4 °C in the dark. The antibodies used for N9 microglia polarization markers as follows: PE anti-mouse CD86 (Biolegend, Cat, 105,007) and FITC anti-mouse CD206 (Biolegend, Cat, 141,703). Finally, cells were rewashed twice with PBS, resuspended in 500 μL of PBS and analyzed using flow cytometer.

3.4. Western blot analysis for protein expression levels of cytokines

N9 cells were collected and lysed on ice with radioimmunoprecipitation assay (RIPA) lysis buffer (0.15 M NaCl, 0.05 M Tris, 5 mM ethylene glycol tetra-acetic acid, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulphate, and 10 mM dichlorodiphenyltrichloroethane) and a cocktail of proteinase inhibitors. Protein concentration was quantified by the Bovine serum albumin (BSA) assay. 12 μL of equivalent protein were loaded by 12% SDS polyacrylamide gels and electrophoresed for 90 min at room temperature. After 2 h blocking with 5% skim milk at 37 °C, the blots were incubated with primary antibodies: anti-Arg-1 and anti-iNOS antibodies overnight at 4 °C. Following, the blots were washed several times with TBST and incubated for 2 h at room temperature with secondary antibodies diluted 1:5000. Finally, the blots were washed with TBST in the same way and the level of protein was determined by enhanced chemiluminescence (ECL) exposure (Menassa and Gomez-Nicola, 2018).

3.5. Optical microscope for observing cell morphology

The original medium was discarded after the treatment was finished, N9 microglia were washed with pre-cooled PBS for 2–3 times, then fixed with 4% cell fixative for 30 min and finally observed under optical microscope.

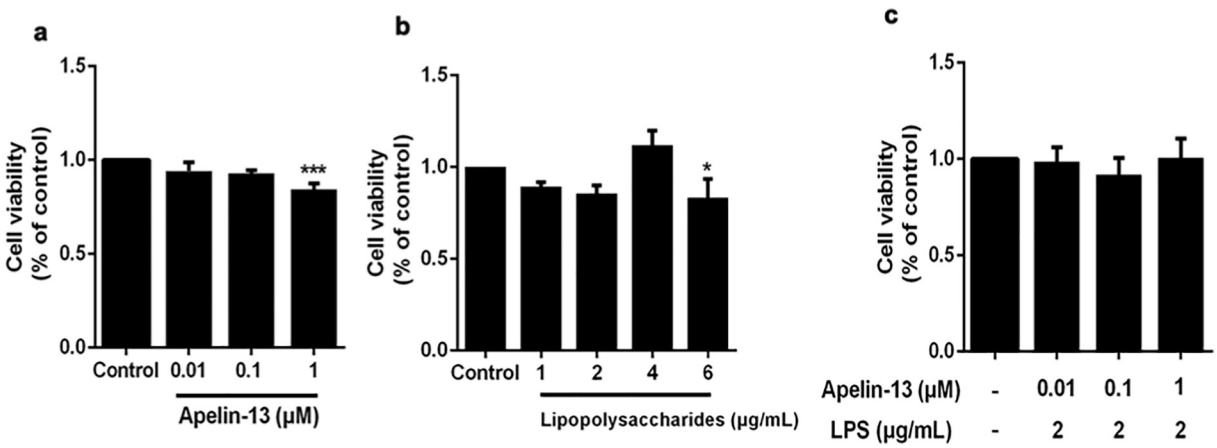

Fig. 1. Cell viability was measured by MTT assay. N9 microglia cells were incubated with 0.01, 0.1 or 1 μM of apelin-13 for 24 h and stimulated with 1, 2, 4 or 6 μg/mL of LPS for another 6 h. Data were presented as mean ± SEM (n = 3). *P < 0.05; ***P < 0.001 vs. the control.

3.6. Statistical analysis

All results were analyzed by SPSS 18.0 software. Data were shown as the mean ± SEM. Significant differences between groups were determined using one-way ANOVA. P-value < .05 are regarded as statistically significant.

4. Results

4.1. Effect of different Apelin-13 and LPS on viability of N9 microglia

We firstly detected whether the concentration of the apelin-13 and LPS had effect on cell viability by using MTT assay. The results showed that the cell viability was significantly decreased in apelin-13 (1 μM) group compared with the control group (P < 0.001). Whereas, treatment of apelin-13 (0.01 and 0.1 μM) alone had no effect on the cell viability of N9 microglial (Fig. 1a). Compared with the control group, LPS at concentrations of 1, 2, and 4 μM had no significant effect on N9 microglia activity, but 6 μg/mL LPS significantly reduced cell viability. In addition, different concentrations of apelin-13 (0.01, 0.1and1 μM) and LPS (2 μg/mL) combined stimulation did not significantly affect the viability of N9 microglia. Therefore, 0.1 μM of apelin-13 and 2 μg/mL of LPS were selected as the dose for subsequent experiments.

4.2. Effect of LPS and Apelin-13 on morphology of N9 microglia

In general, the microglia are in a resting state and the cell branches are slender. Apelin-13 alone had no significant effect on the morphology of N9 microglia. N9 microglial cells were activated after LPS treatment for 6 h. Activated microglial cells had characteristics of enlarged cell body and synaptic retraction, exogenous pre-incubated of apelin-13 significantly inhibited LPS-induced morphology changes of N9 microglia as observed by smaller soma and slender process compared to LPS-treated group, indicating pretreatment of apelin-13 could inhibit LPS-induced morphological polarization of N9 microglia to some extent (Fig. 2).

4.3. Effect of apelin-13 on secretion of pro-inflammatory factors in N9 microglial cells induced by LPS

Next, we would investigate whether exogenously administered apelin-13 can antagonize LPS-induced inflammatory responses. After pretreatment with apelin-13 (0.1 μM) for 24 h, N9-microglial cells were incubated with LPS (2 μg/mL) for 6 h. iNOS expression in the cells treated with LPS was significantly up-regulated compared with the control group (P < 0.001). Apelin-13 could effectively reverse the

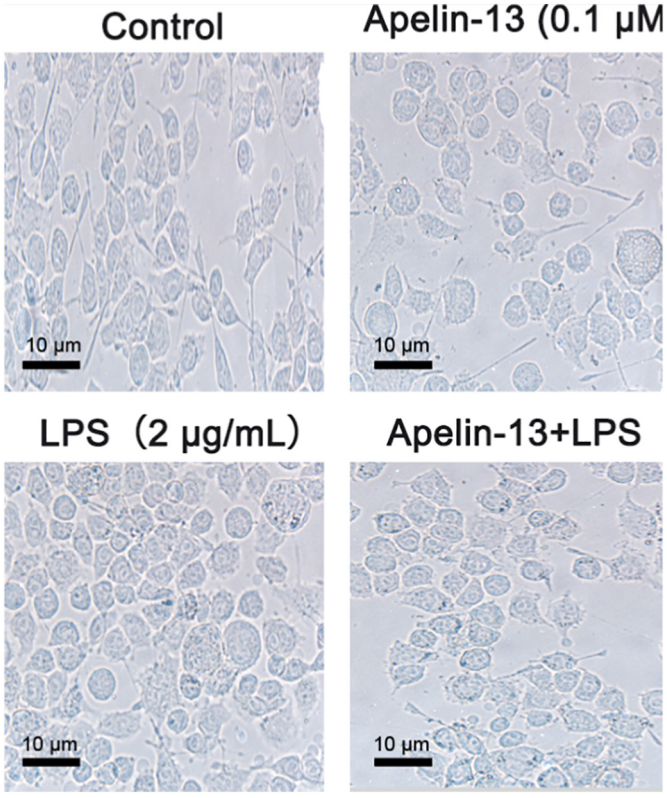

Fig. 2. Effect on LPS and apelin-13 on morphology of N9 microglia. After N9 microglial cells were incubated with basal medium (24 h), apelin-13 (0.1 μM, 24 h) or LPS (2 μg/mL, 6 h). Cell morphology observed with a microscope (20×).

increase of LPS-induced iNOS expression (P < 0.001) (Fig. 3). Similarly, the protein level of IL-6 of N9 microglia in the LPS-treated group was also significantly higher than that in the control group (P < 0.001). Pretreatment with apelin-13 significantly inhibited LPS-induced increase in IL-6 expression in microglial cells (P < 0. 05) (Fig. 3).

4.4. Effect of apelin-13 on secretion of anti-inflammatory factors in N9 microglial cells induced by LPS

In addition to pro-inflammatory factors, we further analyzed the ability of N9 microglia to secrete anti-inflammatory factors under

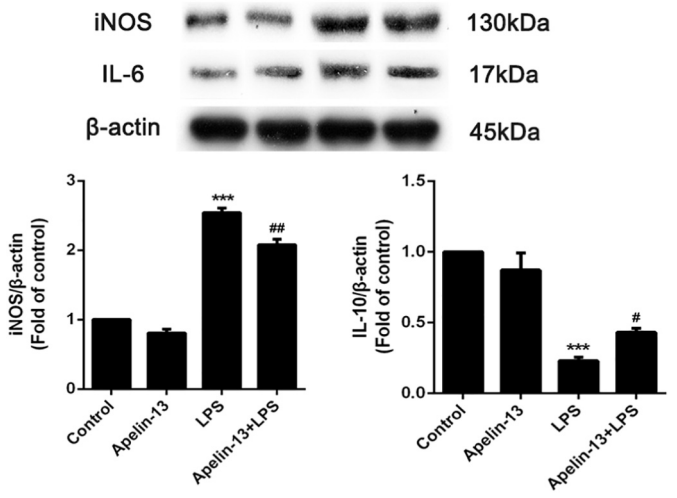

Fig. 3. Effect of apelin-13 on secretion of pro-inflammatory factors in N9 microglial cells induced by LPS. N9 microglia cells were pre-treated with LPS (2 μg/mL) or emem (Control) for 6 h followed by apelin-13 (0.1 μM) or emem (Control) for another 24 h. Western blot analysis of protein expression of iNOS and IL-6. Data were presented as mean ± SEM (n = 3). ***P < 0.001 vs. the control; #P < 0.05; ##P < 0.01 vs. the LPS-treated.

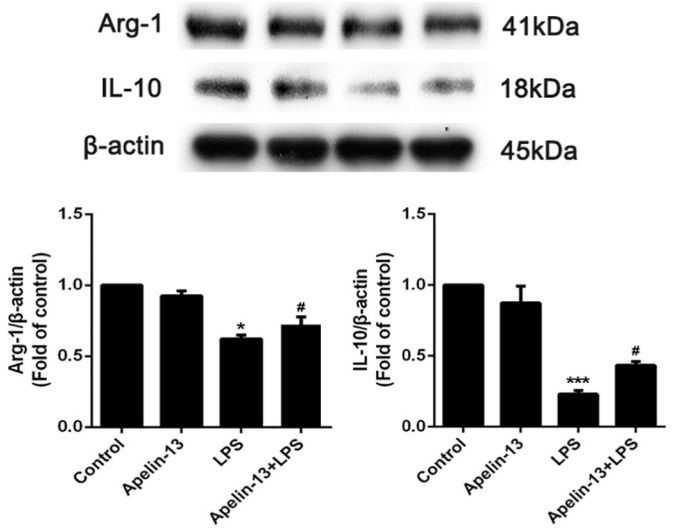

Fig. 4. Effect of apelin-13 on secretion of anti-inflammatory factors in N9 microglial cells induced by LPS. N9 microglia cells were pre-treated with LPS (2 μg/mL) or emem (Control) for 6 h followed by apelin-13 (0.1 μM) or emem (Control) for another 24 h. Western blot analysis of protein expression of arg-1 and IL-10. Data were presented as mean ± SEM (n = 3). *P < 0.05; ***P < 0.001 vs. the control; #P < 0.05 vs. the LPS-treated.

different treatment conditions. Compared with the control group, the expression of arg-1 (P < 0. 05)and IL-10 (P < 0.001) of N9 microglia in the LPS group was significantly decreased; Apelin-13 pretreatment significantly up-regulated the protein levels of the two anti-inflammatory factors compared with the LPS group (Fig. 4).

4.5. Effect of apelin-13 on activation markers of N9 microglial cells induced by LPS

To investigate the expression of N9 microglial activation markers, we detected the average fluorescence intensity in the cells by flow cytometry. The results showed that LPS induced a significant increase in the relative mean fluorescence intensity of CD86 in microglia (P < 0.01). In contrast, the relative fluorescence intensity of CD206 was significantly reduced compared to the control group (P < 0.05). Apelin-13 up-regulated CD206 expression on N9 microglia compared to LPS-treated group (P < 0.05). Expression of CD86 was significantly inhibited by apelin-13 treatment compared with LPS-treated group (P < 0.05) (Fig. 5 a and b).

4.6. STAT3 is involved in the process of apelin-13 regulating polarization of N9 microglia induced by LPS

Based on the role of STAT3 signaling in neuroinflammation, microglia activation, and apelin expression, we therefore examined whether this signal is involved in the process that apelin-13 regulated polarization of N9 microglia induced by LPS by measuring STAT3 and p-STAT3 protein levels. LPS up-regulated the expression of p-STAT3 (Fig. 6b) (P < 0.01) and p-STAT3/t-STAT3 (Fig. 6c) (P < 0.01) ratio in N9 microglia compared to control group, although the protein level of total-STAT3 was not affected by LPS (Fig. 6a). However, apelin-13 pretreatment effectively decreased the protein level of p-STAT3 (Fig. 5b) (P < 0.001) and the ratio of p-STAT3/t-STAT3(Fig. 6c) (P < 0.001) in N9 microglia compared with LPS group, indicating STAT3 signaling may involve in the process of apelin-13 regulating polarization in N9 microglia.

5. Discussion

In the current experiment, we found that pretreatment with apelin-13 inhibited LPS-induced N9 microglia activation in terms of morphology. We were particularly interested in the role of apelin-13 in LPS-induced polarization of N9 microglia. On the one hand, the protein level of the iNOS, IL-6 and mean fluorescence intensity (MFI) of CD86 were significantly higher than that of the control group. Apelin-13 administration suppressed the level of iNOS, IL-6 and CD86. On the other hand, apelin-13 upregulated the LPS-induced decrease in protein levels of anti-inflammatory cytokine arg-1, IL-10 and M2-type marker CD206 in N9 microglial cells. The above results shown that apelin-13 may regulate LPS-induced N9 microglia cell polarization by affecting the secretion of inflammatory cytokines and the expression of microglia activation markers.

Morphological changes occur when microglia are activated. The resting microglial cells grow a round or ramified morphology processes and acquire an amoeboid morphology with synaptic retraction when they are stimulated by foreign bodies (Menassa and Gomez-Nicola, 2018; Wu et al., 2018b). In the present study, N9 microglia activated by LPS tended to became amoebic from branching. Apelin-13 pretreatment changed the morphology of N9 microglia, as observed by smaller somatic cells and slender synapses, indicating the repressive role of apelin-13 in the N9 activity induced by LPS from a morphological point of view.

Arginase metabolism is particularly important for balancing microglial immune responses. Arginine participates in systemic inflammatory responses through two mechanisms: iNOS induced by interleukin-1 (IL-1) or Tumor Necrosis Factor-γ (TNF-γ) is a pro-inflammatory factor of M1-type polarized microglia; Arg-1 stimulated by IL-4 or IL-10 is an anti-inflammatory marker of M2-type polarized microglia. The former is highly expressed in inflammatory-responsive diseases (Mansuy and Boucher, 2004), the latter can generate ornithine, which is beneficial for cell regeneration and wound healing and repair (Toedebusch et al., 2018). Here, it was reported that iNOS released form M1-polarazied N9 microglia induced by LPS produced a large amount of hydroxy-L-arginine, which is a natural and effective inhibitor of arginase. Therefore, we believe that the anti-inflammatory effects of apelin-13 in several neurodegenerative disease models may be caused by shifting M1-type microglia to M2 phenotype (Lisi et al., 2017).

As for two different marker proteins, namely CD86 and CD206, were detected by flow cytometry. The protein CD86 (M1 marker) is a molecule expressed on antigen-presenting cells that provide

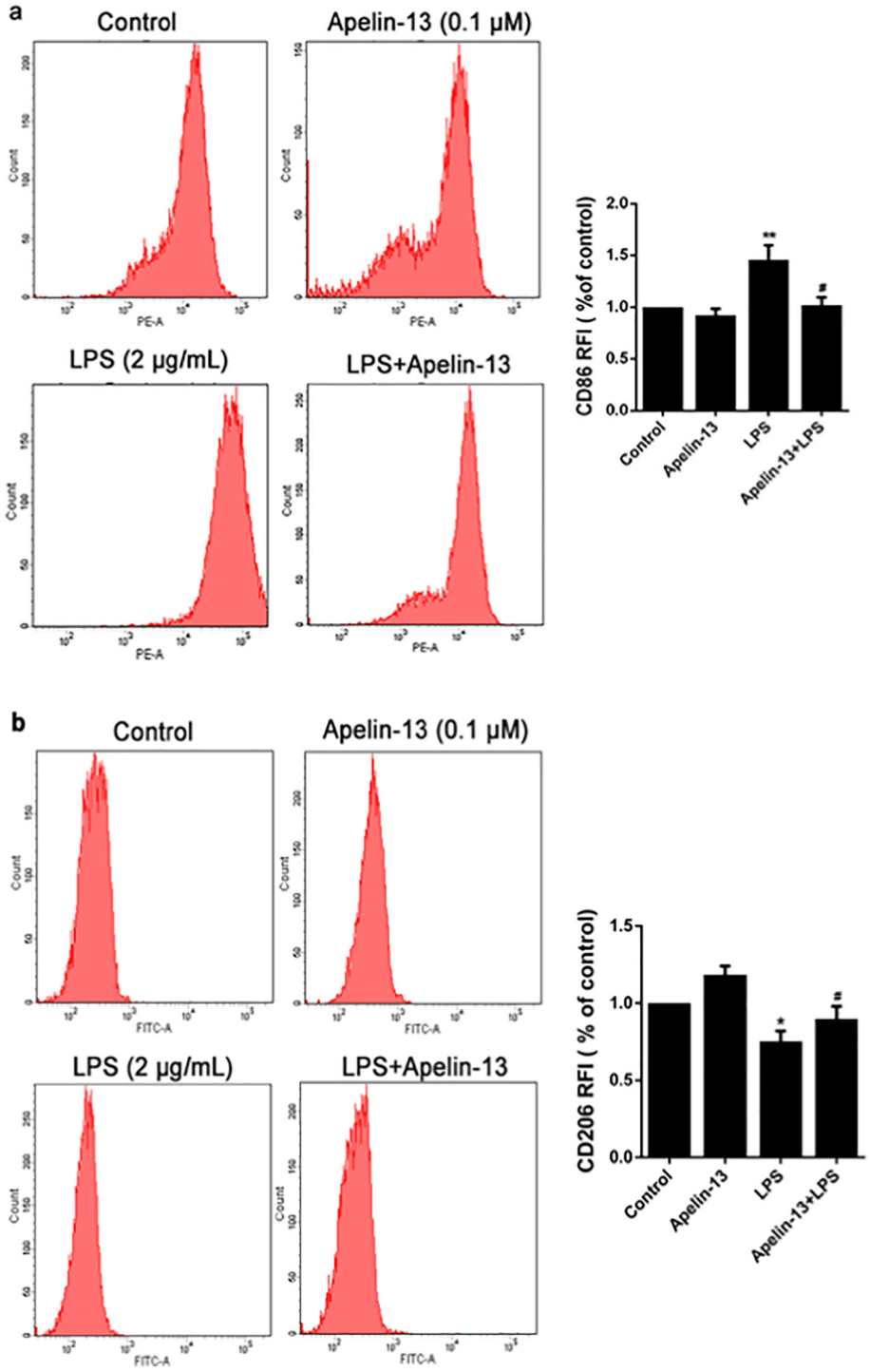

Fig. 5. Effect of apelin-13 on activation markers of N9 microglial cells induced by LPS. N9 microglia cells were pre-treated with LPS (2 μg/mL) or emem (Control) for 6 h followed by apelin-13 (0.1 μM) or emem (Control) for another 24 h. Microglia activation markers CD86(a) and CD206(b) measured by Flow Cytometry. Data were presented as mean ± SEM (n = 3). **P < 0.01 vs. the control; #P < 0.05 vs. the LPS-treated.

costimulatory signals necessary for T cell activation and survival. Consistent with previous studies, there was high expression of CD86 in N9 microglia treated with LPS. Furthermore, apelin-13 suppressed LPS-induced elevated levels of CD86 in N9 microglia. The M2-type marker protein CD206 is expressed only in the perivascular and choroid-associated macrophages in the brain but is less expressed in parenchymal microglia (Galea et al., 2005). From our point of view the results, CD206 was indeed lowly expressed on microglia, which further exacerbated the effect by LPS. Apelin-13 could alleviate the continuous decrease of CD206 expression induced by LPS in N9 microglial cells to a certain extent.

The occurrence of inflammatory reactions is an unavoidable pathological process in various forms of central nervous system injury and insults (Xanthos and Sandkuhler, 2014). Microglia respond to various microenvironmental signals with two functional polarization states to mediate immune and inflammatory responses of central nervous systems. The M1 phenotype of microglia releases a variety of pro-inflammatory mediators and free radicals that impair brain repair (Jha

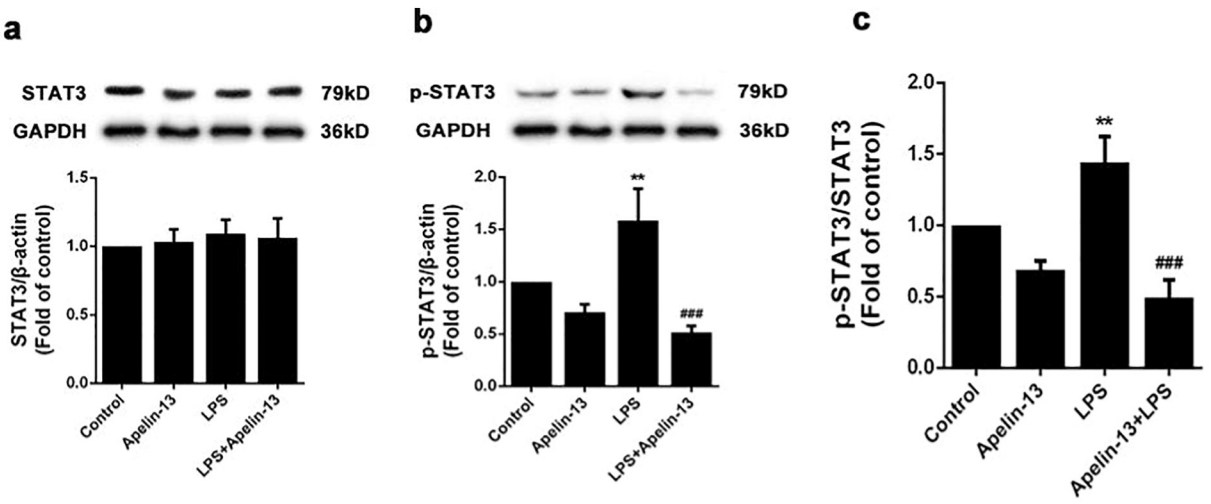

Fig. 6. STAT3 is involved in the process of apelin-13 regulating polarization of N9 microglia induced by LPS. N9 microglia cells were pre-treated with apelin-13 (0.1 μM) or emem (Control) for 24 h followed by LPS (2 μg/mL) or emem (Control) for another 6 h. Western blot analysis of protein expression of STAT3 (a) and p-STAT3 (b). Data were presented as mean ± SEM (n = 3). $^{**}P < 0.01$ vs. Control; $^{\#\#\#}P < 0.05$ vs. the LPS-treated.

et al., 2016). In contrast, the M2 phenotype of microglia phagocytose damaged nerve cell debris through scavenger receptors and matrix degrading enzymes, inhibiting excessive inflammatory responses, promoting tissue repair and neuronal regeneration and avoiding secondary inflammatory damage.

The phenotypic changes of microglia are inseparable from neurodegenerative diseases caused by neuroinflammation. In the early stage of intracerebral hemorrhage, microglia are activated into M1 type and release inflammatory factors IL-1β、IL-6、TNF-α and chemokine CXCL2 to promote neuroinflammation injury. Repeated brain trauma can cause severe cortical lesions, and the M1 phenotype can last from several months to several years (Zhao et al., 2015). In the final stage of cerebral hemorrhage, the microglia M2 phenotype is more commonly seen as a major repair function. Therefore, the transformation of microglia phenotype from M1 to M2 is of great significance for the treatment of subsequent brain parenchymal injury. It has previously been reported that both M1 and M2-type microglia are present in the brains of both normal mice and Parkinson's mice. The difference is that the expression level of M1 microglial cells in PD mouse brain is more than that of M2 type. It has been documented that rosiglitazone regulates microglia polarization for neuroprotection with a target of M2 phenotype over M1 proinflammatory phenotype (Pisanu et al., 2014). Thus, microglia activation and M1/M2 phenotype switching may be involved in the pathological process of PD.

STAT3 signaling has been implicated in microglia cell activation in neuroinflammatory responses induced by LPS (Bode et al., 2012). IL-6/STAT3 is a classical inflammatory signaling pathway. IL-6 binds to its receptor and activates JAKs, JAKs phosphorylate the gp130-specific tyrosine residues in the cytoplasmic region, which provides a binding site for the STAT3 protein SH2 region. STAT3 is then activated by phosphorylation and the activated STAT3 protein forms a dimer to the nucleus, which regulates gene expression after binding to DNA (Che et al., 2019). Numerous studies have demonstrated that transduction of STAT3 signaling regulates microglia activation (Li et al., 2018). Consistent with the above studies, our study also demonstrated that LPS induced the release of IL-6 by M1-type polarized N9 microglia to activate downstream STAT3 signaling, whereas apelin-13 blocked LPS-induced phosphorylation of STAT3 in polarized M1 N9 microglia. We therefore reasoned that the effect of apelin-13 on polarization in microglial cells challenged with LPS might be partly depended on a STAT3-associated mechanism.

In conclusion, apelin-13 down-regulated the expression of pro-inflammatory factor iNOS and the marker CD86 in M1-type microglia induced by LPS and increased the level of anti-inflammatory factor arg-1 and the expression of marker CD206 in M2-type microglia, it means that apelin-13 may down-regulate LPS-induced inflammatory response by regulating N9 microglial polarization. At the same time, apelin-13 pretreatment inhibited the increase of STAT3 expression in M1-type polarized N9 microglia induced by LPS. That is to say, we proposed a possible role of apelin-13 in LPS induced N9 microglia polarization. Therapies by targeting apelin-13 though STAT3 signaling in microglia polarization and central nervous system might be a new way of treating neurological inflammatory diseases.

Declaration of Competing Interests

The authors declare no conflict of interest.

Acknowledgments

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