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LxA4 Inhibits LPS Induced Proinflammatory Response and Microglia Polarization through Notch Signaling Pathway

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Abstract

Objective: to analyze the inhibitory effect of lipoxin A4 (LXA4) on lipopolysaccharide (LPS) - induced proinflammatory response and microglial polarization through Notch signaling pathway.

Methods: mouse microglial BV2 cells were cultured in vitro and divided into control group (serum-free medium), LXA4 group (100nmol /L LXA4), LPS induced group (200ng/ml LPS), LXA4 + LPS group (100nmol / L LXA4 + 200ng / ml LPS), DAPT + LPS group (100nmol/L LXA4+200ng/ml LPS), DAPT + LXA4 + LPS group (10μM DAPT+200ng/ml LPS). RT-PCR, Western blot and ELISA were used to detect the effects of LxA4 on LPS induced inflammatory response, polarization and downstream molecular activity of Notch signaling pathway.

Results: the expression of IL-1 β and TNF - α mRNA in LXA4 + LPS group was significantly lower than that in LPS induced group, while IL-10 mRNA expression was significantly higher than that in LPS induced group ($P < 0.05$). The expression of iNOS mRNA and protein in LXA4 + LPS group was significantly lower than that in LPS induced group, and Argl mRNA and protein expression was higher than that in LPS induced group ($P < 0.05$). Notch1, Hes1 mRNA and protein expressions in LXA4 + LPS group were significantly lower than those in LPS induced group, and the expression of Hes5mRNA and protein in LXA4 + LPS group was significantly higher than that in LPS induced group ($P < 0.05$). Compared with DAPT + LPS group and LXA4 + LPS group, iNOSmRNA in DAPT+LXA4+LPS group was significantly increased, and ArglmRNA was significantly decreased ($P < 0.05$). Compared with DAPT + LPS group and LXA4 + LPS group, the levels of IL-1 β and TNF - α in DAPT+LXA4+LPS group were significantly increased and IL-10 was decreased obviously ($P < 0.05$).

Conclusion: LxA4 can inhibit LPS induced proinflammatory response and cell polarization of microglia, and its mechanism may be related to the regulation of Notch signaling pathway.

Keywords: LXA4; Notch signaling pathway; LPS; Microglia; Proinflammatory response; Polarization

Introduction

Microglia is the main effector cells involved in the immune response of the central nervous system, accounting for about 10%~20% of the glial cells, and play a dual role in neuroprotection and nerve injury in the pathological process [1]. In addition, microglia act as the first line of defense against the central nervous system. When a foreign pathogen invades or a cell dies, ischemia and inflammatory stimulation render the microglial cells to react and be activated, and release inflammatory factors to mediate the inflammatory response, which is manifested as proliferation, directional migration to the injured site, phagocytosis of metabolites, etc. [2] According to the relevant data, moderate microglial activation plays a protective role in the process of central nervous system injury, but over-activated microglial cells may accelerate the progress of some central nervous system diseases. The release of cytotoxic factors, nitric oxide and other inflammatory factors will also cause further damage to nerve cells, thus participating in the pathological development of

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nervous dysfunction [3-4]. Therefore, inhibiting microglia activity may be an effective way to treat central nervous system diseases. LipoxinA4 (LXA4), as an important inflammatory regulator, can inhibit the activation of neutrophils, restrain the production of pro-inflammatory factors, play the role of anti-inflammatory and pro-inflammatory regression [5]. In this study, lipopolysaccharide (LPS) was used to induce microglia inflammation model and analyzed whether it can inhibit microglial proinflammatory response and cell polarization through Notch signaling pathway with LXA4 as drug intervention means.

1. Materials and Methods

1.1 Experimental reagent and instrument

Mouse microglial BV2 cells (Shanghai Aoyin Biological Technology Co., Ltd.); DAPT (TargetMol China); LPS (Shanghai GOYBIO Industrial Co., Ltd.); Cell lysis solution (Shanghai Xin Fan Biological Technology Co., Ltd.); BCA protein quantitative kit (Wanleibio); Nucleoprotein extracted kit (Shanghai ACMEC Biochemical Technology Co., Ltd.); Trigol (Shanghai Yan Mu Industrial Co., Ltd.); iNOS, Argl, Notch1, Hes1, Hes5 antibodies (American Abcam Company).

Desktop cryogenic high-speed centrifuge (Shanghai Hengfei Biotechnology Co., Ltd.); Inverted microscope (Beijing Jiayuan Industrial Technology Co., Ltd.); Micropipettor (Shanghai Feze Trade Co., Ltd.); PCR instrument (Shanghai VEDENG Medical Co., Ltd.); Thermostat water bath (Hangzhou Noted Scientific Equipment Co., Ltd.); Thermostatic drying oven (Beijing Qinye Yongwei Technology Co., Ltd.); Electrophoresis apparatus (Jiangsu BMD Labservice Co., Ltd.); Transfer membrane tank (Beijing Neobio Science&Technology Co., Ltd.).

1.2 Cell culture and grouping

BV2 cells were cultured in MEM/EBSS medium with 10% fetal bovine serum, penicillin and streptomycin 100 u/ml, the cells were placed in an incubator at 37℃ and 5%CO2. When growing to about 80%, the trypsin was used to digest cells and transfer and store through DMSO. It was transferred once every 2~3d. The logarithmic growth period cells were taken for subsequent experiments. The cells were divided into control group (serum-free medium), LXA4 group (100nmol /L LXA4), LPS induced group (200ng/ml LPS), LXA4 + LPS group (100nmol / L LXA4 + 200ng / ml LPS), DAPT + LPS group (100nmol/L LXA4+200ng/ml LPS), DAPT + LXA4 + LPS group (10μM DAPT+200ng/ml LPS).

1.3 Test method

①Detection of gene expression with RT-PCR method: The total RNA was extracted from microglia using Trizol method, RNA concentration and purity were detected. According to the instructions of the reverse transcription kit, cDNA was synthesized by reverse transcription. CDNA product was cooled on ice and diluted, and stored in-20℃ refrigerator. With cDNA as the template and β-actin as the internal control, 40 cycles were extended at 95℃ for 3 min, 95℃ for 12 seconds and 62℃ for 55 seconds. 2-△△CT formula was used to analyze interleukins 1β, 10 (IL-1β, IL-10), tumor necrosis factor -α (TNF- α), Nitric oxide synthase (iNOS), Arginase-1 (Arg1), Notch1, Hes1 and Hes5mRNA.

② Detection on the expression of proteins by Western blot: Cell Protein Concentration was detected with BCA method. Appropriate amount of sample was taken for SDS-PAGE and 10% separation gel and 5% concentration gel were configured for gel electrophoresis. The protein in the gel was transferred to PVDF membrane after electrophoresis, and PVDF membrane was immersed in 5% skim milk powder for 1 h. Inos, Argl, Notch1, Hes1 and Hes5 antibody were added to incubate PVDF membrane in 4℃ refrigerator for overnight, and the corresponding second antibody was incubated at room temperature for 1 h, and ECL chemiluminescence reagent was used to expose and develop, and then stored.

③ Detection of inflammatory factor levels with ELISA method: After centrifugation, the supernatant was placed in an EP tube. After rewarming, ELISA kit was used to dilute concentrated biogenic antibodies and avidin antibodies at 1:100. Cell supernatant was balanced at room temperature before the use. 3 parallel holes were set for each sample according to the kit instructions. The absorbance was measured at 450 nm wavelength and IL-1β, IL-10 and TNF-α content were calculated.

1.4 Statistical methods

All the data were analyzed by SPSS21.0 software package. The measurement data are represented by () and comparison of data between two groups was performed by t test. Variance was used to analyze comparison of data between groups. $P<0.05$ was regarded as a statistical difference. $\bar{x} \pm s$

2. Result

2.1 Effect of LXA4 on LPS- induced microglial inflammatory factors

The expression of IL-1βand TNF-α mRNA in LPS induced group was significantly higher than that in the control group, and the difference was statistically significant (P<0.05). The expression of IL-1β and TNF-α mRNA in LXA4 + LPS group was obviously lower than LPS induced group, while the expression of IL-10 mRNA was obviously higher than LPS induced group, and the difference was statistically significant (P<0.05). See Table 1

Table 1. **Effect of LXA4 on LPS- induced microglial inflammatory factors** $\bar{x} \pm s$

Group	Sample size	IL-1β mRNA	IL-10 mRNA	TNF-α mRNA
Control group	10	4.25±1.33	1.36±0.47	5.45±1.36
LXA4 group	10	2.41±0.58	1.89±0.55	6.20±1.47
LPS induced group	10	20.47±7.16*	6.47±1.63*	70.23±15.49*
LXA4 + LPS group	10	8.56±2.42#	15.37±6.74#	28.64±8.89#

Notes: Compared with the control group, *P<0.05; Compared with LPS induced group, #P<0.05;

2.2 Effects of LXA4 on LPS-induced microglia M1 and M2 biomarker

The expression of iNOS mRNA and protein in LPS induced group was significantly higher than that in the control group, and the difference was statistically significant (P<0.05). LPS induced group was not significantly different from the control group in Argl mRNA and protein expression (P>0.05). The expression of iNOS mRNA and protein in LXA4 + LPS group was significantly lower than that in LPS induced group, and Argl mRNA and protein expression was higher than that in LPS induced group (P < 0.05). See Table 2, Figure 1.

Table 2. **Effects of LXA4 on LPS-induced microglia iNOS, Argl mRNA and protein** $\bar{x} \pm s$

Group	Sample size	iNOS		Argl	
		mRNA	Protein	mRNA	Protein
Control group	10	1.00±0.05	1.02±0.41	1.00±0.03	0.56±0.06
LXA4 group	10	1.56±0.12	0.97±0.12	4.55±1.32	0.58±0.07
LPS induced group	10	25.68±6.30*	1.74±0.36*	1.22±0.42	0.53±0.04
LXA4 + LPS group	10	10.45±5.23#	1.03±0.25#	9.54±1.47#	1.48±0.17#

Notes: Compared with the control group, *P<0.05; Compared with LPS induced group, #P<0.05;

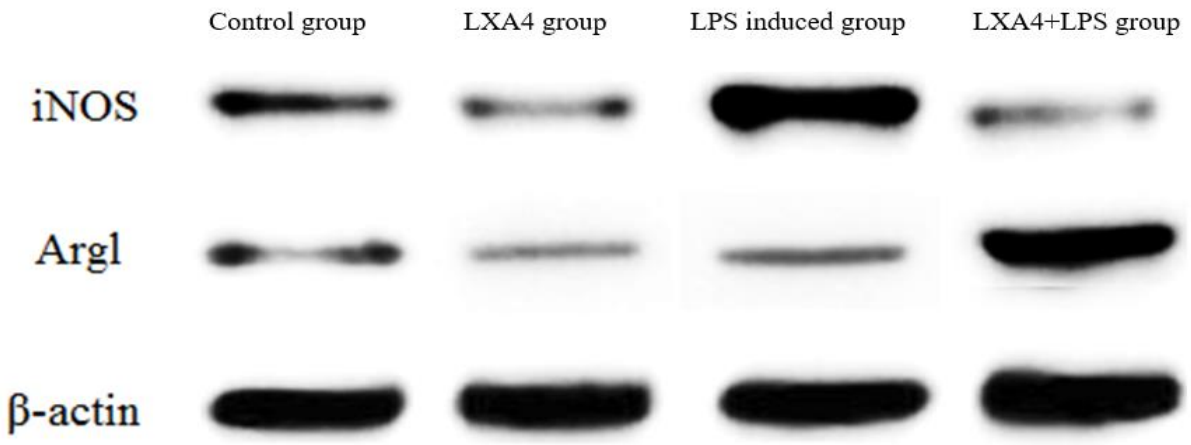

Figure 1. **Detection of expression of iNOS and Argl protein using Western blot method**

2.3 Effects of LXA4 on downstream molecules of Notch signaling pathways in microglia

The expression of Notch1, Hes1 mRNA and protein in LPS induced group was significantly higher than that in control group, and the difference was statistically significant (P<0.05). Notch1, Hes1 mRNA and protein expressions in LXA4 + LPS group were significantly lower than those in LPS induced group, and the expression of Hes5mRNA and protein in LXA4 + LPS group was significantly higher than that in LPS induced group, and the difference was statistically significant (P < 0.05). See Table 3 and Figure 2

Table 3. **Effects of LXA4 on Notch1, Hes1, Hes5mRNA and protein in microglia ()** $\bar{x} \pm s$

Group	Notch1		Hes1		Hes5	
	mRNA	Protein	mRNA	Protein	mRNA	Protein
Control group	1.00±0.02	0.47±0.12	1.00±0.03	0.88±0.05	20.00±1.12	1.00±0.02
LXA4 group	5.46±1.18	0.40±0.11	1.15±0.12	0.82±0.12	21.36±5.32	1.15±0.14
LPS induced group	62.33±15.96*	1.56±0.13*	68.32±14.20*	2.56±0.38*	23.33±12.03	1.17±0.13
LXA4 + LPS group	10.23±3.21#	0.58±0.16##	16.38±6.20	1.20±0.11#	352.36±47.25	2.58±1.20#

Notes: Compared with the control group, *P<0.05; Compared with LPS induced group, #P<0.05;

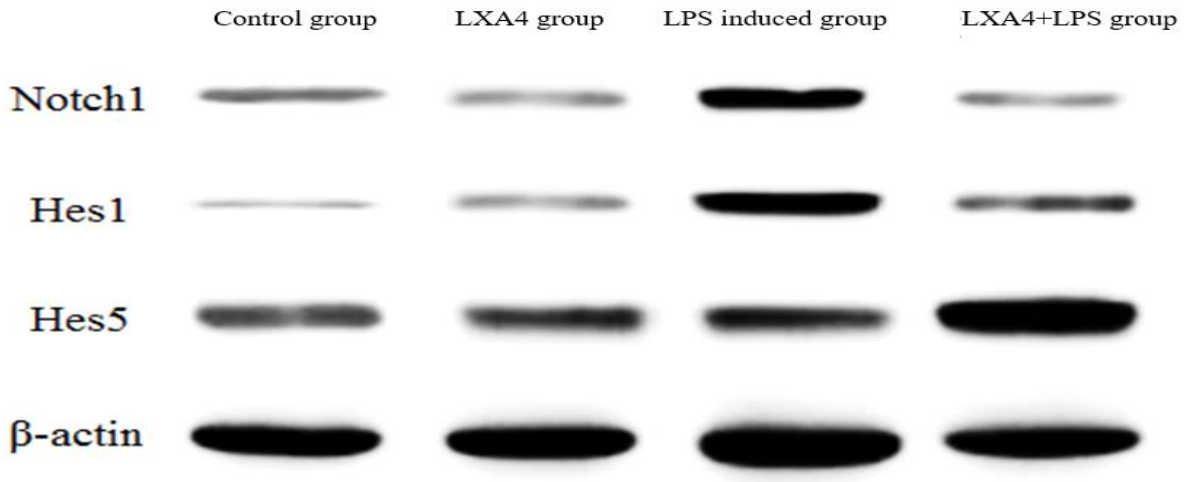

Figure 2. **Detection of expression of Notch1, Hes1 and Hes5 protein using Western blot method**

2.4 Effects of LXA4 on microglia M1 and M2 biomarkers after regulating Notch signaling pathway

The expression of iNOS and ArglmRNA in LPS induced group was obviously higher than the control group (P<0.05). The expression of iNOSmRNA in DAPT + LPS group and LXA4 + LPS group was obviously lower than LPS induced group (P<0.05). The expression of ArglmRNA in LXA4 + LPS group was obviously higher than that in LPS induced group (P<0.05). Compared with DAPT + LPS group and LXA4 + LPS group, iNOSmRNA in DAPT+LXA4+LPS group was significantly increased, and ArglmRNA was significantly decreased (P < 0.05). See Table 4

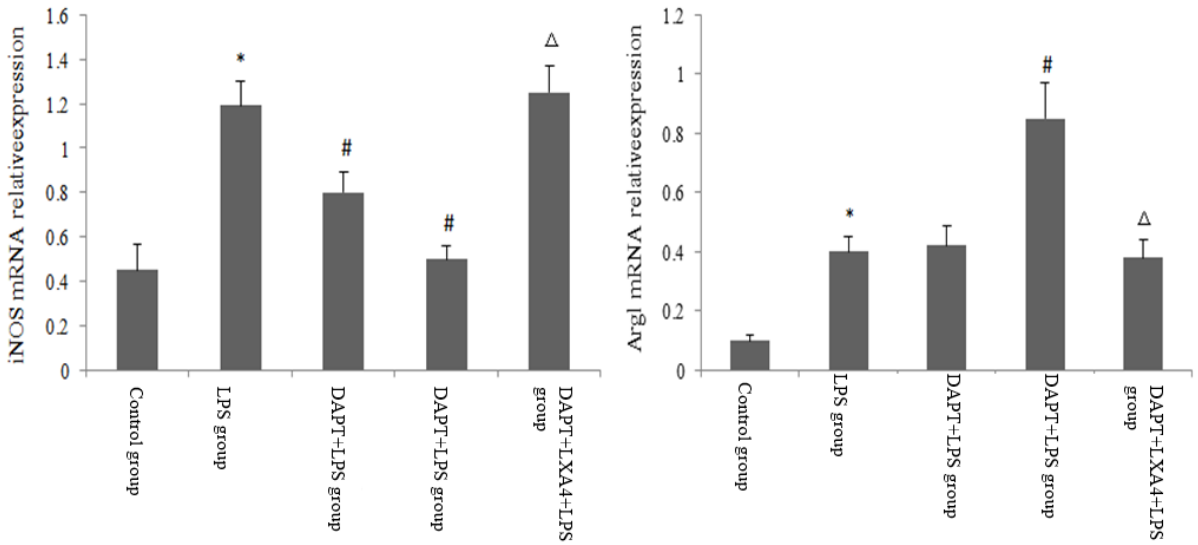

Figure 3. **Effects of LXA4 on microglia NOS and Argl mRNA after regulating Notch signaling pathway**

Notes: Compared with the control group, *P<0.05; Compared with LPS induced group, #P<0.05; Compared with DAPT + LPS group and LXA4 + LPS group, △P<0.05

2.5 Effects of LXA4 on inflammatory factors following microglia polarization after regulating Notch signaling pathway

The IL-1β and TNF-α levels of LPS induced group were significantly higher than that of the control group, and the difference was statistically significant (P<0.05). The IL-1β and TNF-α levels of DAPT + LPS group and LXA4 + LPS group were significantly higher than that of LPS induced group (P<0.05); IL-10 level of LXA4 + LPS group was higher than LPS induced group (P<0.05). Compared with DAPT + LPS group and LXA4 + LPS group, the levels of IL-1 β and TNF - α in DAPT+LXA4+LPS group were significantly increased and IL-10 was decreased obviously (P < 0.05).

Table 5. **Effects of LXA4 on inflammatory factors following microglia polarization after regulating Notch signaling pathway ($\overline{x} \pm s$)**

Group	Sample size	IL-1β（pg/ml）	IL-10（pg/ml）	TNF-α（ng/l）
Control group	10	7.25±1.33	4.78±1.24	1.00±0.02
LPS induced group	10	20.32±5.67*	4.80±1.36*	2.85±0.41*
DAPT + LPS group	10	15.12±4.25#	3.25±1.02	1.52±0.13#
LXA4 + LPS group	10	10.22±3.48#	7.68±2.44#	1.53±0.11#
DAPT+ LXA4 + LPS group	10	19.47±5.33△	4.79±1.33△	1.84±0.23△

Notes: Compared with the control group, *P<0.05; Compared with LPS induced group, #P<0.05; Compared with DAPT + LPS group and LXA4 + LPS group, △P<0.05

3. Discussion

Central nervous system diseases such as ischemic stroke and Parkinson's disease are often accompanied by a large number of neuronal necrosis and apoptosis. Microglia often gathers around these necrotic lesions, releases inflammatory cytokines such as NO, TNF-αand IL-6 in large quantities and further exacerbates central nervous system cell and tissue damage [6]. Therefore, the microglial mediated inflammatory response in the pathogenesis of these diseases has gradually attracted clinical attention. Microglial cells have different morphology and function in the pathological state of nervous system injury and disease. After exogenous stimuli or changes in the brain microenvironment, microglia can be rapidly activated. The activated microglia is M1 and M2 types [7]. iNOS is a biological molecular marker of M1 type. The increased level can produce many NO. It can exert toxic effects through a variety of mechanisms such as mitochondrial damage, peroxidation and DNA damage. Argl, as a biological molecular marker of M2 type, can compete with iNOS for arginine substrates to reduce no release and promote tissue damage repair [8]. As a result, the regulation of microglia M1 and M2 balance is of great significance for the improvement of various inflammatory related nervous system diseases.

LXA4 is a kind of lipid anti-inflammatory transmitter produced by the body's own acute inflammatory reaction, which can regulate the expression of various inflammatory reactions extensively. By binding to its receptor, it can inhibit the signal transduction pathway and reduce the production and release of inflammatory factors [9]. According to the relevant data, LXA4 can inhibit the recruitment of neutrophils, regulate the balance of pro-inflammatory and anti-inflammatory factors, play an anti-inflammatory and anti-regression role, and can limit inflammatory damage and regulate tissue repair [10]. Some researchers have discovered in animal experiments that LXA4 can significantly reduce the expression level of proinflammatory mediators TNF-α and IL-1β in rats with macrophages induced by bone cement particles, and has a significant negative regulatory effect on inflammatory response [11]. Other researchers have found that LXA4 receptors exist on the surface of microglia, suggesting that microglia may be a target for LXA4 in the central nervous system [12]. The results showed that LXA4 intervention could significantly reduce the expression of IL-1β and TNF-α inflammatory factors and iNOS M1 related factors, increase the expression of IL-10 and Argl M2 related factors, suggesting that LXA4 intervention can significantly regulate the M1 to M2 transformation of microglia.

Notch signaling pathway is an important signaling pathway to regulate embryonic development, which is involved in the dynamic changes from cell structure to nervous system function. It can regulate cell activation by activating and regulating related transcription factors. It also plays an important role in the activation and polarization of microglia [13]. Some foreign scholars have found that Notch-1 receptor intracellular gene can activate Notch signaling pathway and maintain cell proliferation of mouse or human neural stem cells. Human neural stem cells will be differentiated after DAPT blocking Notch

signaling pathway. Thus it can be seen that Notch signaling pathway plays an important role in the activation and proliferation of glial cells, and can maintain the normal development of glial cells [14-15]. The study results concluded that LXA4 can affect the expression of downstream molecules of Notch signaling pathway at both gene and protein levels, and iNOSmRNA was obviously increased in DAPT+ LXA4 + LPS group and ArglmRNA was obviously reduced (P<0.05), suggesting that LXA4 may regulate microglia polarization through Notch signaling pathway. In addition, it was found that IL-1β and TNF-αlevels were obviously increased and IL-10 was obviously reduced in DAPT+ LXA4 + LPS group (P<0.05), suggesting LXA4 regulates microglia polarization and influences inflammatory factor levels through Notch signaling pathway.

To sum up, LxA4 can inhibit LPS induced proinflammatory response and cell polarization of microglia, and its mechanism may be related to the regulation of Notch signaling pathway.

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