The Regulatory Effect of Lycium Barbarum Polysaccharide on I κ B Phosphorylation and NF - κ B Nuclear Translocation in RAW264.7 Cells

Hong Yin

Yangzhou University

Lili Bai

Ningxia Medical University

Shuying Deng

Ningxia Medical University

Lihua Li

lianshui county peoples hosp

Tingting Liu

Ningxia Medical University

Huizhen Cai (✉ 18169010183@163.com)

<https://orcid.org/0000-0002-2507-3631>

Research

Keywords: Lycium barbarum polysaccharide, Diabetes mellitus, Inflammatory factor, MyD88-dependent pathway

Posted Date: June 4th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-30556/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Scope: RAW264.7 cells was used to study the regulatory effect of LBP on I κ B phosphorylation and NF - κ B nuclear translocation.

Methods: (1)After 5, 10, 20 and 30 minutes of LBP intervention, Western blotting was used to detect the expression of I κ B and P-I κ B in each group, (2)After 5 minutes of intervention with different doses of LBP,Western blotting was used to detect the expression of P-I κ B and I κ B in the cells of each group, (3)After 30 minutes of LBP intervention, the distribution of NF - κ B nuclear translocation was observed by immunofluorescence, and the number of NF - κ B nuclear translocation was detected by Western blotting.

Results : The results of the time-dependent regulation of I κ B phosphorylation by LBP showed that when stimulated by LPS, I κ B phosphorylated rapidly, the level of P-I κ B increased and I κ B decreased, and the level of I κ B increased at each time point in the combined treatment group of LPS (1 μ g / ml) + LBP (25 μ g / ml).

After LPS stimulation, the expression of I κ B decreased and the expression of phosphorylated I κ B increased ($P < 0.05$); after LPS (L μ g / ml) + LBP (25,50 μ g / ml), the level of I κ B increased ($P < 0.05$).

After LPS stimulation, the green fluorescence was mainly distributed in the nuclear area, and after LBP stimulation, the distribution of green fluorescence was slightly increased; in LPS + LBP group, the distribution of green fluorescence in the cytoplasm was increased, and the distribution of green fluorescence in the nucleus was decreased. After LPS stimulation, NF - κ B level in cytoplasm decreased ($P < 0.05$), and NF - κ B level in nucleus increased significantly ($P < 0.05$). After LPS (L μ g / ml) + LBP (25, 50, 100 μ g / ml), NF - κ B level in cytoplasm increased ($P < 0.05$), and NF - κ B level in nucleus decreased ($P < 0.05$).

Conclusion: Collectively, these results suggested that LBP's anti-inflammatory effects were relevant to the interruption of NF-kappa B activation.

1. Introduction

NF - κ B is a key regulatory factor in the inflammatory mechanism of T2DM. Both NF - κ B and its inhibitor I κ B are key molecules of TLR4 / MyD88 dependent pathway, and play an important role in mediating the activation of inflammatory cytokines transcription^[1]. Under normal conditions, NF - κ B and inhibitor I κ B are stably bound in the cytoplasm and exist in the inactive form. When the cells are stimulated to produce stress, phosphorylation of I κ B can release the inhibition of NF - κ B in the cytoplasm, and make NF - κ B shift to the nucleus to regulate the transcription and expression of a variety of inflammatory factors, such as TNF- α , IL-1 β , IL-6, etc. In turn, the high expression of inflammatory factors will further increase the activation of this signaling pathway, up regulate the activity of NF - κ B, and aggravate the inflammatory cascade reaction^[2].

RAW264.7 cells are derived from mouse peritoneal macrophages. It has been confirmed that RAW264.7 cells stimulated by lipopolysaccharide (LPS) are one of the classical cell models to study inflammation [1]. LPS can induce the formation of M1 macrophages, activate NF - κ B signaling pathway, induce IL-1 β , TNF- α , IL-6, IL-8, nitric oxide (no) and inducible nitric oxide synthetase (inducible nitric oxide) Synthase, iNOS and so on [1], and the activation of NF - κ B signaling pathway and the generation of a large number of inflammatory factors have been confirmed to be closely related to the pathogenesis and inflammatory response of T2DM.

The fruit of *Lycium barbarum* (Goji berry) has been commonly used as traditional Chinese medicine and herbal food for health promotion in countries. *Lycium barbarum* polysaccharides (LBP) has been most widely researched and considered to be the main bioactive substance. The monosaccharide composition of LBP contained rhamnose, arabinose, xylose, mannose, glucose, galactose, galacturonic acid [2].

In this study, LPS stimulated RAW264.7 macrophages were used to study the regulatory effect of LBP on subsequent I κ B phosphorylation and NF - κ B nuclear translocation in MyD88 dependent pathway. The effects of LBP on the phosphorylation of I κ B in dose-response, time-dependent and nuclear translocation of NF - κ B were studied.

2. Materials And Methods

Preparation of LBP

LBP was prepared as described previously [2]. Dried *L. barbarum* was made into a powder and decocted with water (60 °C) by a traditional method used for Chinese medicinal herbs after degreasing. Then it was filtered by regenerated cellulose membranes of 300 kDa, 100 kDa, 80 kDa, 50 kDa and 30 kDa (0.2 MPa, 60 °C) after centrifuging. The resulting fraction was retained and vacuum-dried at 40 °C. Neutral sugars were determined by phenol-H₂SO₄, acidic sugars by carbazole and proteins by the Coomassie Brilliant Blue G-250 method .

LBP we prepared was a brown powder composed of neutral sugars (78.23%) and acidic sugars (14.83%). The protein content was < 6.92%.

Cell lines and reagents

The mouse macrophage RAW264.7 cells were purchased from China Center for Type Culture Collection (Shanghai, China).

Rabbit anti- β -Actin monoclonal antibody, rabbit monoclonal antibody against NF- κ B p65, I κ B etc. were purchased from Abcam (Cambridge, MA). Secondary antibodies were obtained from Boster Co.(Wuhan, China). Goat anti rabbit IgG labeled fluorescent antibody was purchased from Santa cruz (Shanghai, China). DMEM medium, fetal bovine serum (FBS), Penicillin and Streptomycin Solution were purchased

from HyClone (Logan, UT, USA). LPS were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade.

Cell culture

RAW 264.7 cells were cultured in DMEM medium, supplemented with 10% FBS, 100 U/mL Penicillin, 100 µg/mL Streptomycin Solution and maintained at 37 °C in a humidified atmosphere with 5% CO₂.

Time dependent regulation of I κ B phosphorylation by LBP

RAW264.7 cells were grown in six-well plates (1×10^6 cells/well) for 24 h. In brief, 1×10^6 RAW264.7 cells were pre-treated with control, LPS(1 µg/mL), LBP 25 µg/mL, LPS + LBP 1 µg/mL + 25 µg/mL, respectively, for 5, 10, 20, 30 min. At the end of culture, the protein was extracted and the expression of I κ B and P-I κ B was detected by Western blot.

Dose effect regulation of I κ B phosphorylation by LBP

RAW264.7 cells were grown in six-well plates (1×10^6 cells/well) for 24 h. 1×10^6 RAW264.7 cells were pre-treated with control, LPS(1 µg/mL), LBP 25, 50, 100 µg/mL, LPS 1 µg/mL + LBP 25 50 100 µg/mL for 5 min. At the end of culture, the protein was extracted and the expression of I κ B and P-I κ B was detected by Western blot.

Total and nuclear protein extraction

For I κ B, P-I κ B, NF - κ B etc. analysis, protein expression by western blotting, total and nuclear protein extracts were prepared from pure macrophage using commercial kits (Biosynthesis Biotechnology Co., LTD, Beijing, China). The protein concentration was determined by bicinchoninic acid (BCA) assay and stored at - 80 °C until analyzed.

Western blotting

Sixty (60) µg of cell extract were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was blocked for 1 h with 5% non fat milk in TBST and then incubated with a rabbit monoclonal antibody against I κ B (Abcam Company, UK) at 4 °C over night. After washing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibodies (1:5000; Boster Co., Wuhan, China) for 60 min at room temperature. After additional washing, bound conjugates were detected by ECL superSignal™ West Pico substrate (Pierce, Rockford,

IL, USA). Proteins were visualized by exposing the blot to X-ray film, photographed with a digital camera, and then the net intensities of the individual bands were measured using Bandscan 5.0 software. Rabbit anti- β -Actin monoclonal antibody (AbcamCompany, UK) was used as the loading control, and I κ B protein expression was normalized to Actin.

The WB process of P-I κ B, NF - κ B are the same as that of I κ B.

Immunofluorescence staining

RAW264.7 cells on glass coverslips were incubated with control, LPS(1 μ g/mL), LBP 25 μ g/mL, LPS + LBP 1 μ g/mL + 25 μ g/mL, respectively, for 30 min. After 30 min fixation with 4% paraformaldehyde and 30 min permeabilization with 0.1% Triton X-100, cells were blocked for 1 h with 5% bovine serum albumin (BSA) at room temperature. Then, cells were first incubated with the primary rabbit anti-NF- κ B p65 antibody at 4 °C overnight, followed by incubation with the secondary Alexa Fluor-conjugated secondary antibody for 1 h at room temperature. Nuclear of cells were stained with DAPI. A fluorescence microscope was employed for observation of the images.

RAW264.7 cells were grown in six-well plates (1×10^6 cells/well) for 24 h. 1×10^6 RAW264.7 cells were pre-treated with control, LPS(1 μ g/mL), LBP 25, 50, 100 μ g/mL, LPS 1 μ g/mL+LBP 25 50 100 μ g/mL for 30 min. At the end of culture, cytoplasmic protein and nucleoprotein were extracted and the number of nuclear translocation of NF - κ B was detected by Western blot.

Statistical analysis

All data are expressed as means $\pm$ standard error (SEM) and represented at least three independent experiments. ANOVA and Student's *t*-test were used to perform multiple comparisons for statistical significance, and $p < 0.05$ was considered statistically significant.

3. Results And Discussion

3.1 Time dependent regulation of I κ B phosphorylation by LBP

When the cells were stimulated by LPS, I κ B phosphorylated rapidly, the level of P-I κ B increased and I κ B decreased (see Fig. 1). The phosphorylated I κ B is then ubiquitinated and finally recognized and degraded by proteasome. After adding LBP 5 min alone, I κ B in the cells increased, I κ B level decreased after 10 min, and I κ B increased after 20 min to 30 min, indicating that LBP can also activate I κ B, but its effect is more moderate compared with LPS model. After LPS (1 μ g / ml) combined with LBP (25 μ g / ml), the level of I κ B increased at each time point compared with LPS model, which indicated that LBP inhibited the phosphorylation of I κ B caused by LPS stimulation.

3.2 Dose effect regulation of I κ B phosphorylation by LBP

Figure 2 After LPS stimulation, compared with the blank group, the expression of I κ B decreased and the expression of phosphorylated I κ B increased ($P < 0.05$), indicating that LPS has a certain stimulating effect on I κ B. This is consistent with the results of Peng Qiang et al.

The level of I κ B in RAW264.7 cells stimulated by LBP alone (25, 50, 100 μ g / ml) decreased ($P < 0.05$), but there was no significant difference compared with LPS model group ($P > 0.05$), indicating that the stimulation of I κ B by LBP alone was similar to LPS. Previous studies have shown that LBP can increase the number of peritoneal macrophages and pseudopods in mice, enhance the phagocytic ability, and improve the immune ability of the body^[1]. In this study, compared with the blank group, the I κ B decreased, the NF - κ B distribution and expression in the cytoplasm decreased, and the NF - κ B distribution in the nucleus increased after the addition of LBP alone, indicating that LBP also has the activation effect on the NF - κ B pathway in RAW264.7 cells, which may be related to the fact that both LBP and LPS belong to polysaccharide. LPS can activate macrophages because there are receptors of LPS on the surface of macrophages^[2]. Although LBP and LPS belong to different polysaccharides, they still have similarities in structure. Therefore, LBP may affect the function of macrophages by binding with receptors on the surface of macrophages.

When LBP (25, 50, 100 μ g / ml) was used alone, the level of P-I κ B in the cells was lower than that in LPS group ($P < 0.05$), suggesting that the stimulation effect of LBP on I κ B was weaker than that of LPS. After LPS (L μ g / ml) + LBP (25,50 μ g / ml) treatment, the level of I κ B in the cells increased ($P < 0.05$), which indicated that LBP could weaken the phosphorylation of I κ B by LPS.

Zhang Xiaorui et al.^[3] showed that lbp4-ol purified from LBP could significantly up regulate TLR4 / MD2 expression in peritoneal macrophages and RAW264.7 cells. Combined with the results of this study, LBP could activate TLR4 / NF - κ B pathway in macrophages. For the cells only added with LBP, compared with the blank group, the expression level of P-I κ B did not increase, it is possible that ubiquitination of I κ B occurs rapidly after phosphorylation, and is finally recognized and degraded by proteasome.

3.3 Regulation of nuclear translocation of NF - κ B by LBP

In Fig. 3, green fluorescence represents NF - κ B. It can be seen that there are a lot of green fluorescence in the cytoplasm area of normal cells, and the color of the nuclear area is relatively dark. After stimulated by LPS, NF - κ B was activated into the nucleus, so there was a lot of green fluorescence in the nucleus. When macrophages were treated with 25 μ g / ml LBP alone for 30 minutes, the green fluorescence was distributed in both nucleus and cytoplasm. This indicated that the activation of NF - κ B by LBP was milder than that of LPS. When the cells were treated with LPS (L μ g / ml) + LBP (25 μ g / ml), compared with the LPS model group, the distribution of green fluorescence in the cytoplasm increased, which indicated that LBP had a certain inhibitory effect on LPS induced NF - κ B intense nuclear translocation.

As can be seen from Fig. 4, there is a large amount of NF - κ B in the normal growth cell cytoplasm, and the expression level of NF - κ B in the nucleus is low. When LPS stimulated the cells, compared with the blank group, the relative expression of NF - κ B in the cytoplasm decreased ($P < 0.05$), and the level of NF - κ B in the nucleus increased significantly ($P < 0.05$), indicating that LPS can activate NF - κ B.

When LBP (25, 50, 100 μ g / ml) alone was used to stimulate the cells, the level of NF - κ B in the cytoplasm decreased compared with that of the normal control group ($P < 0.05$), but the decrease was significantly smaller than that of LPS model, which indicated that LBP alone could stimulate NF - κ B activation, but it was milder than LPS.

After LPS (1 μ g / ml) + LBP (25, 50, 100 μ g / ml) was used, the NF - κ B level in the cytoplasm was significantly higher than that in the LPS model group ($P < 0.05$), while the NF - κ B level in the nucleus was significantly lower than that in the LPS model group ($P < 0.05$), which indicated that LBP could effectively inhibit the inflammatory stimulation of LPS on cell.

Wang Yalin et al.^[1] found that Dictyophora polysaccharide can inhibit LPS induced NF - κ B inflammatory signal pathway activation by regulating TLR4 expression, I κ B α phosphorylation and NF - κ B nuclear translocation,

Peng Qiang et al.^[2] used L. rutenicum polysaccharide (LRGP3) to interfere with LPS stimulated RAW264.7 cells, the results showed that LRGP3 significantly inhibited the production of TNF - α , IL-6, NO, the expression of iNOS, TLR4 and the degradation of I κ B α , and reduced the expression of p65nf - κ B protein. The above studies showed that plant polysaccharides had a certain inhibitory effect on the inflammatory activation of RAW264.7 cells induced by LPS.

In this study, compared with LPS group, the level of I κ B in RAW264.7 cells treated with LBP & LPS increased, the distribution and expression of NF - κ B in the cytoplasm increased, and the distribution and expression of NF - κ B in the nucleus decreased, indicating that LBP has a certain inhibitory effect on I κ B phosphorylation and NF - κ B nuclear translocation downstream of TLR4 / MyD88 dependent pathway in LPS stimulated macrophages.

Conclusion

LBP has a certain inhibitory effect on I κ B phosphorylation and NF - κ B nuclear translocation downstream of TLR4 / MyD88 dependent pathway in LPS stimulated macrophages.

Abbreviations

LBP

Lycium barbarum polysaccharide ;

IL-6

Interleukin-6;

IL-1 β
Interleukin – 1 β ;
TGF- β 1
Transforming growth factor- β 1;
IL-10
Interleukin – 10;
IL-8
Interleukin – 8;
TNF- α
Tumor necrosis factor – α ;
TLR4
Toll-like receptors 4;
MyD88
Myeloid differentiation factor 88;
I κ K β
Inhibitive κ K β ;
I κ B
Inhibitive κ B;
P-I κ B
Phosphorylated Inhibitive κ B;
NF - κ B
Nuclear factor- κ B;
BCA
Bicinchoninic acid ;
SDS-PAGE
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis;
PVDF
Polyvinylidene difluoride ;
T2DM
Type 2 diabetes mellitus;

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Design of the study was done by HY and HC. Experiments were performed by HY, SD, LB and LL. Data analyses were performed by SD, LB and TL. The manuscript was written by HY and was approved by HC. All authors read and approved the final manuscript.

Funds

Science and technology research project of ningxia higher education institutions (NGY2018-66), The National Natural Science Fund (81460494, 81803235), 2017 youth backbone talent cultivation plan of Ningxia Medical University (202/30200102) and Top Discipline of Public Health and Prevent Medicine, Education Department of Ningxia (NXYLXK2017B08) supported this research.

Availability of data and materials

All data generated or analysed during this study are included in this published article or are available from the corresponding author on reasonable request.

Ethics approval

The study protocol was reviewed and approved by the Animal Care and Use Committee at Ningxia Med Univ (Yinchuan, China).

Consent for publication

All authors support the submission to this journal.

Competing interests

The authors declare that they have no competing interests.

References

1. Karin M , Delhase M . The I κ B kinase (IKK) and NF- κ B: Key elements of proinflammatory signaling. *Seminars in Immunology* 2000; 12(1):85-98.
2. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004;4(7):499-511.
3. Liu F, Zhang X, Ling P, Liao JS, Zhao MS, Mei L, et al. Immunomodulatory effects of xanthan gum in LPS-stimulated RAW 264.7 macrophages. *Carbohydr Polym* 2017;169(4): 65-74.
4. Han B, Dai Y, Wu H, Zhang YY, Wan LH, Zhao JL, et al. Cimifugin Inhibits Inflammatory Responses of RAW264.7 Cells Induced by Lipopolysaccharide. *Med Sci Monit* 2019;25(1): 409-17.

5. Safavian D, Leung C H, Kapus A, Ailenberg M ,Szaszi K ,Shani R , et al. Hemorrhagic Shock/Resuscitation Reduces the M2 Phenotype of Alveolar Macrophages. *SHOCK* 2018;51(2): 213-20.
6. Xie C, Li X, Zhu J. Magnesium isoglycyrrhizinate suppresses LPS-induced inflammation and oxidative stress through inhibiting NF- κ B and MAPK pathways in RAW264.7 cells. *Bioorgan Med Chem* 2019;27(3):516-24.
7. Potterat, O. Goji (*Lycium barbarum* and *L-chinense*): Phytochemistry, Pharmacology and Safety in the Perspective of Traditional Uses and recent Popularity. *Planta Medica* 2010; 76(1): 7–19.
8. Cai H, Yang X, Cai Q, Reb BB, Qiu HY, Yao ZQ. *Lycium barbarum* L. Polysaccharide (LBP) Reduces Glucose Uptake via Down-Regulation of SGLT-1 in Caco2 Cell. *Molecules* 2017;22(2):1-12.
9. Yakun L , Yan C . The Influence Of *Lycium Barbarum* Polysaccharide To The Function Of Intracellular Immunization. *forestry science and technology information* 2013;2:10-1.
10. Ayala A , Elphick G F , Kim Y S , Huang X , Chung C S . Sepsis-induced potentiation of peritoneal macrophage migration is mitigated by programmed cell death receptor-1 gene deficiency. *Journal of Innate Immunity* 2013;6(3): 325-38.
11. Sato-Nishiwaki, Michiko, Aida, ShibataY, Kimura T, Yamauchi K, et al . Reduced Number and Morphofunctional Change of Alveolar Macrophages in MafB Gene-Targeted Mice. *Plos One* 2013;8(9):e73963.
12. Kim T,Wjoh EH, Kim B, Kim DH. Ginsenoside Rg5 ameliorates lung inflammation in mice by inhibiting the binding of LPS to toll-like receptor-4 on macrophages. *International Immunopharmacology* 2012;12(1):110-6.
13. Zhang X, Qi C, Cheng J, LiuG, Huang LJ, Wang ZF, et al. *Lycium barbarum* polysaccharide LBPF4-OL may be a new Toll-like receptor 4/MD2-MAPK signaling pathway activator and inducer. *Int Immunopharmacol* 2014;19(1):132-41.
14. Shen T , Wang G , You L , Zhang L, RenHW, Hu WC, et al. Polysaccharide from wheat bran induces cytokine expression via the toll-like receptor 4-mediated p38 MAPK signaling pathway and prevents cyclophosphamide-induced immunosuppression in mice. *Food & Nutrition Research* 2017: 61(1):1344523.
15. Wang Y, Lai L, Teng L,Li YH, Cheng JQ, Chen JH, et al. Mechanism of the anti-inflammatory activity by a polysaccharide from *Dictyophora indusiata* in lipopolysaccharide-stimulated macrophages. *Int J Biol Macromol* 2019;126:1158-66.
16. Wang WQ, Hu XY, Shen P. Sodium houttuynfonate inhibits LPS-induced inflammatory response via suppressing TLR4/NF- κ B signaling pathway in bovine mammary epithelial cells. *Microbial Pathogenesis* 2017;107:12-6.
17. Peng Q, Liu H, Shi S. *Lycium ruthenicum* polysaccharide attenuates inflammation through inhibiting TLR4/NF- κ B signaling pathway. *Int J Biol Macromol* 2014;67(3): 330-5.
18. Fan HY , Qi D , Yu C, Zhao F , Du Y. Paeonol protects endotoxin-induced acute kidney injury: potential mechanism of inhibiting tlr4-nf-kb signal pathway. *Oncotarget* 2014;7(26):39497-510.

19. YeHY, Jin J, Jin LW, Chen Y, Zhou ZH, Li ZY. Chlorogenic Acid Attenuates Lipopolysaccharide-Induced Acute Kidney Injury by Inhibiting TLR4/NF-kappa B Signal Pathway. *Inflammation* 2017;40(2):523-9.

Figures

FIG.1 Time dependent regulation of IκB phosphorylation by LBP

Figure 1

Note: compared with LPS group, Pa<0.05.

Figure 2

Dose effect regulation of I κ B phosphorylation by LBP Note: compared with control, Pa<0.05; compared with LPS group, Pb<0.05 .

Figure 3
Immunofluorescence analysis: Regulation of nuclear translocation of NF - κ B by LBP

Figure 4

Regulation of nuclear translocation of NF - κ B by LBP Note: compared with control, Pa<0.05; compared with LPS group, Pb<0.05 .