The Effect of Blocking TLR4 Receptor on the Pathogenicity of Escherichia Coli after it infected the Endometrium of Dairy Cow

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Abstract: Based on TLR4 in the innate immune response, it is found that blocking the downstream signal receptor subunit of TLR4 after Escherichia coli infection could significantly reduce the phosphorylation of NF-κB protein as well as the dissociation of I-κB and NF-κB protein, and prevent NF-κB from entering the nucleus and activating related genes factors. Thus, inflammatory cytokines such as TNF-α, IL-1β and IL-6 could not excessively express. After infection, blocking the secretion of inflammatory cytokines initiated by TLR4 showed an outbreak-like response effect, reducing the inflammatory damage caused by excessive secretion of inflammatory cytokines, and had a good protective effect on cells.

1. Introduction

Endometritis is a major disease affecting the reproductive capacity of dairy cows. The incidence of endometritis in the United States is as high as 53%, [1] while in China it is 20% to 50%. The disease is caused by the invasive infection of pathogenic microorganisms. [2] The transcription-related factors in the downstream pathway of TLR4 are NF-kB, AP-1 and IRF-5. The oligomerization of TLR4 receptors promotes the formation of junction complexes containing TIRAP, MyD88 and TIR regions after the pathogenic bacterial infection. TRAF6 decorated with ubiquitin chains can combine two continuous acting kinase complexes to ensure the phosphorylation and activation of IkBa. Phosphorylated IkBa can be detected by the receptor component of E3-ligase complex (b-TrCP), and finally IKKa is activated and plays a role in the initiation of non-classical pathways. Once IkBa is destroyed, the dimer of NF-kB (composed of p65 and P50 proteins) can be recognized into the nucleolus by a- and b-input proteins with the nuclear pore. Activated NF-kB pathway will promote the transcription of related genes so that inflammatory factors will be secreted.

In this study, structural domain peptide inhibitors were used to disturb this process, trying to understand the role of TLR4 in E. coli infection more accurately.

2. Research Materials and Research Methods

2.1 Experimental animals

Healthy dairy cows were selected and the endometrial samples were collected by endometrial biopsy samplers. [3] Firstly, the collected endometrial tissues were put into the sterilized vials from the sterilized samplers through the rectal grasp method for test detection.

2.2 Main reagents and strains

Biological reagents included DNA bacterial genome extraction kit, DNA gel recovery kit, DNA Marker (15000, 2000) and Plasmid Extraction kit purchased from the Tiangen Company, as well as PCR-related reagents, the PGMT cloning kit, the DGGE denaturant, DH 5α competent cells and the pMD-18T vector purchased from TaKaRa Bioengineering (Dalian) Co., Ltd.

Chemical reagents included deionized water (Millpore purification column), acrylamide, bisacrylamide, urea, Tris and EDTA (Promega), glacial acetic acid (purchased from Shanghai Chemical Reagent Company), formamide, TEMED, Ammonium Persulfate, Triton and EB (Sigma),

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anhydrous ethanol (purchased from Tianjin Chemical Reagent Company), NaOH (purchased from Shanghai Chemical Reagent Company) and formaldehyde (purchased from Beijing Chemical Reagent Company).

Standard E. coli ATCC8739 was purchased from Shanghai Jianglai Biological Technology Co., Ltd.

2.3 The design, synthesis and detection of TIR domain blocking peptide

According to the protein sequence of TLR4 found in NCBI database and the homology analysis carried out though software, the TIR domain blocking peptide was designed and synthesized according to the conservative domain of the sequence connected with TIR. This is mainly used to study the regulation of TLR4 downstream pathway in subsequent inflammation after E. coli infection.

2.4 Isolation and culture of cells

The endometrial tissues were cut in a small amount of digestive juice. The shredded tissue was transplanted into a 50 mL cell centrifugal tube, and the volume of tissue was less than 1/3 of the cell tube volume. Then digestive juice by the wet weight of 10 mL/g was added. The digestion time was 3 hours at 37℃. After digestion, the 500 g tissues were centrifuged at 4℃ for 10 minutes. The fat suspended on the surface was carefully absorbed and discarded. Then small amount of sediment was taken out and diluted with the culture medium. The digestive degree of tissues was observed under the microscope. Completely digested tissues were clusters of cells. The sediments were washed four times with culture medium and transferred to a new culture tube. The fresh culture medium was added for suspension culture at 37℃ for 4 hours. Fibroblasts adhere faster than epithelial cells, so they remain in the cell tube. Mixtures containing epithelial cells were transferred to cell culture flasks. On the second day, fresh medium was added to the old culture medium with dead cells and other debris without adherence. After 3 days, many epithelial cell clusters were observed. If there is still residual fibroblast contamination, 0.25% trypsin should be digested at room temperature for 5 minutes, and then the fibroblasts can be removed by differential digestion. On the second day, fluid exchange and passage should be carried out depended on cell growth.

2.5 Cell processing and grouping

Tissues were treated with a homogenizer. The cell type was primary endometrial cells with purity of 95%. Cell viability was detected by the kit. Then cells were separated by a cell sieve. The isolated cells were inoculated into 6 empty culture plates, and after growing to the appropriate density, the E. coli liquid was added into the culture medium as the control group (group A) without treatment. The standard E. coli ATCC8739 was added (group B). The blocking peptide was added after the standard E. coli ATCC8739 liquid was added (group B+). The E. coli liquid isolated from diseased cattle was added (group C). After adding E. coli culture liquid isolated from diseased cattle, blocking peptide was added (group C +).

2.6 Cell damage

After 3 hours of treatment, LIVE/DEAD cell detection kit was used to detect cells in each culture hole. The dead cells were stained red while the living cells were green. Then cells were observed under an immunofluorescence microscope.

2.7 Detection of inflammatory cytokines

TNF-α, IL-1β and IL-6 were detected as typical inflammatory cytokines. Cell culture supernatants were extracted and centrifuged in a centrifugal tube for 12 000 r/min; the precipitation was discarded. The liquid supernatant was collected and detected. ELISA was performed according to the instructions. The value of $OD_{450\ nm}$ was measured by enzyme labeling instrument; the concentration was calculated according to the standard curve already drawn.

2.8 Real-time fluorescence quantitative PCR analysis

Total RNA was extracted through cell RNA extraction kit. The concentration of RNA was obtained by measuring the value of OD_{260}; and the integrity of RNA was detected by agarose gel electrophoresis. The first chain of DNA was synthesized according to instructions of the reverse transcription kit produced by TaKaRa.

Primers were synthesized through Premier 5.0. SYBR Premix Ex Tag kit was used to carry out real-time fluorescence quantitative PCR in a 25 μL system. 1 mu-L of upstream and downstream primers (10 pmol/μL) were take out; 2.5 μL of template DNA, 1.7 μL of Rox Reference Dye II, 11 μL of buffer solution, and 7.8 μL of sterilized double-steamed water were used. The reaction condition was 35s at 95℃, 5s at 95℃, 40s at 60℃, and 40s at 60℃ for 40 cycles. Relative quantitative method was used to analyze the results of three repetitive holes in each sample.

2.9 Western blot analysis

Firstly, cell lysate was added. After 15 minutes of cell lysis, 12 000 r/min centrifugation was carried out and the precipitation was discarded. The supernatant liquid was collected and detected. Pre-cracking liquid was added, then the sample was placed in a shaker for slow shaking for 5 minutes to ensure complete cracking. Then the supernatant was centrifuged at 4℃ and the speed of 10 000 r/min for 20 min. SDS-PAGE was used to analyze the supernatant liquid. The membrane was transferred by a semi-dry membrane converter. 50 g/L BSA was sealed for 2 h, then incubated at 4℃ for overnight. TBST was rinsed 4 times for 5 minutes each time, and the second antibody was incubated. Chemiluminescent ECL coloration, the chemiluminescent imaging system was used for photographic observation.

3. Experiment Results

3.1 Cell damage

Through LIVE/DEAD detection and analysis of cell damage, the survival of cells can be intuitively seen. No dead cells were found in the blank group; cell death was obvious in the ATCC8739 strain and isolation group ($P < 0.05$). Comparing the ATCC8739 strain and isolation groups with and without the blocking peptide, the number of cell death in the blocking peptide groups were significantly lowered ($P < 0.01$). Compared with ATCC8739 strain, the cell damage ability of the isolated strain was significantly greater than that of the ATCC8739 strain.

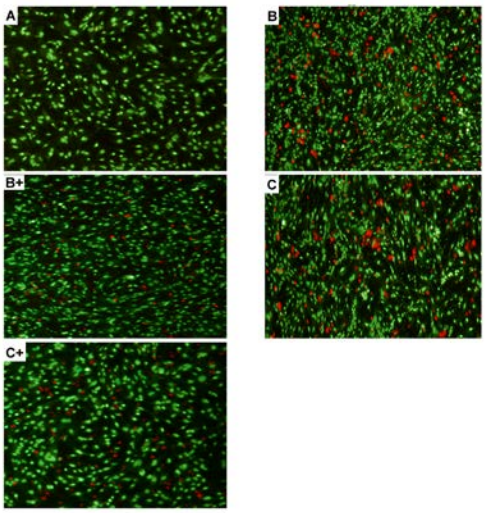
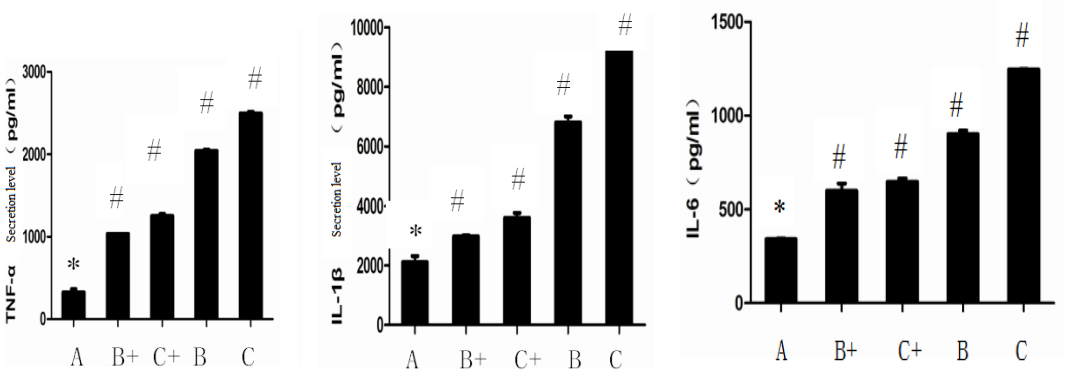


Figure 1. Cell damage detection

3.2 Secretion of inflammatory cytokines

Instructions on the TNF-α, IL-1β and IL-6 ELISA kits were followed in the determination. The values of $D_{450\,nm}$ were measured by enzyme labeling instrument, and the concentrations of TNF-α,

IL-1β and IL-6 were calculated according to the standard curve. The results are shown in Figure 2. In the stimulation groups of E. coli isolation, inflammatory factors increased significantly. The changes of TNF-α and IL-1β were more obvious; the secretion increased ($P < 0.05$). IL-6 also increased, but the change was not significant. The ATCC strain stimulation group also showed certain inflammatory reaction, but the secretion of inflammatory factors was lower than that of the isolate stimulation group. It was especially obvious in the changes of TNF-α and IL-1β, in which the secretion increased ($P < 0.05$). IL-6 also increased, but the change was not significant. In the control group, TNF-α, IL-1β and IL-6 also increased slightly, which was probably caused by the environment, since the differences have no significant meaning in satistics. The cytokine secretions of the ATCC strain group and the isolated treatment group with blocked peptide were significantly lower than those of non-treated groups ($P < 0.05$). There was no significant difference in the blank group.



Note: Compared with group C, * means significant difference ($P < 0.05$); compared with group A, # means significant difference ($P < 0.05$). Same below

Figure 2. Secretion levels of inflammatory cytokines

3.3 Changes of TLR4 gene and protein expressions

TLR4 is a major receptor protein for Gram-negative bacteria. The results showed that both ATCC strains and isolates could significantly increase the expression of TLR4 mRNA in endometrial cells (as shown in Figure 3). The addition of blocking peptide had no effect on the expression level of TLR4 mRNA. The expression of TLR4 protein was further analyzed by the Western blotting. It was found that both ATCC strains and isolates could significantly increase the expression of TLR4 protein in endometrial tissue ($P < 0.05$), but blocking peptide had no effect on the expression of TLR4 protein (as shown in Figure 3). The results showed that blocking peptide did not affect the expression of TLR4, but only influence the function of protein.

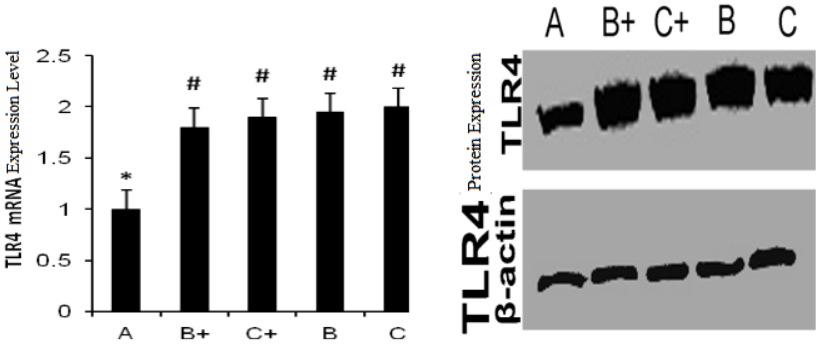


Figure 3. TLR4 gene and protein expression levels

3.4 Effects on the NF-κB signaling path

NF-κB signaling pathway was detected. The results showed that the expression of NF-κB mRNA in endometrial tissues of the the ATCC strain and the isolate stimulation groups was significantly increased, and the expression of inhibitory protein I-κB was also significantly increased (as shown in Figure 4). When blocking peptide was added, the stimulating effect decreased significantly.

Further analysis of NF-κB protein by immunoblotting showed that the stimulation of ATCC strains and isolates had no effect on the expression of NF-κ B protein, but could promot the phosphorylation of NF-κB. Correspondingly, the expression of I-κB phosphorylated protein increased ($P < 0.05$). After blocking peptide was added, that function was significantly decreased, showing that blocking TLR4 after infection could decrease the expression of NF-κB in endometrial cells.

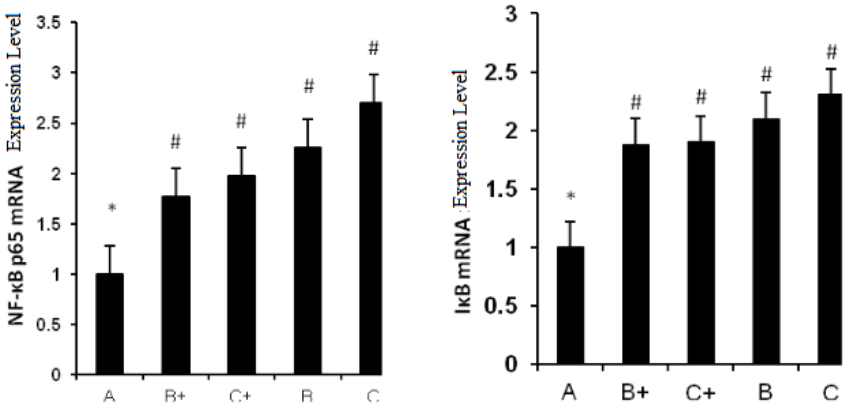


Figure 4. Gene expression levels of NF-κB and I-κB

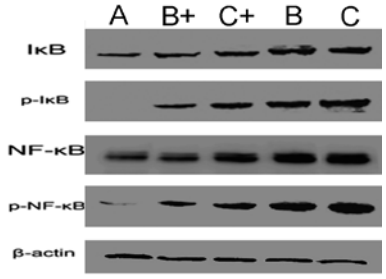


Figure 5. Protein expression levels of the NF-κB signal path

4. Discussions

TLR4 controls the transcription factors of the NF-KB/Rel family, which has the Rel homologous region with DNA linkage and subunit dimerization. Members of this family are inhibited by the IkB inhibitor protein family. IkB proteins are linked to NF-kB/Rel to prevent their nuclear localization signals and recognition on DNA. The hydrolysis of these proteins marked the elimination of their inhibition funciton and the activation of NF-kB. Phosphorylated/activated IKKb phosphorylates IKB in the two serine regions at the N-terminal, allowing the recognition of SCFB ubiquitinated ligase complexes. Then IkBa is labeled by proteasomes, releasing RelA and NF-KB and exposing their nuclear site signals. [4, 5] Then they enter the nucleus and connect DNA at the kB component, driving the expression of inflammatory factors.

The results showed that after blocking the downstream domain of TLR4 after infection, a typical pheonmenon is the desreasing of inflammatory factor secretion, but the secretion level is still higher than the normal. Blocking TLR4 after infection can increase cell defense and reduce cell damage. After blocking TLR4, the expression of NF-κB in endometrial cells is significantly inhibited and the phosphorylation is also decreased, which indicats that blocking TLR4 from activiting downstream signaling pathways could effectively reduce the secretion of inflammatory factors.

TLR4 plays a regulating role in downstream signaling pathways after pathogenic bacteria infection. It is the key to the timely defensive response of cells and the protection of cells. It is also essential in the effective regulation of signaling pathways. It is suggested that some drugs or biological agents can be used to partially inhibit the function of TLR4 in the treatment of endometritis in dairy cows, so as to reduce the excessive secretion of inflammatory factors which

may cause unnecessary inflammatory damage to the body after the pathogen infection.

It has been found that blocking the downstream signal receptor subunit of TLR4 after E. coli infection can significantly reduce the phosphorylation of NF-kappa B protein as well as the dissociation of I-κB and NF-κB protein, prevent the gene of NF-κB from entering the nuclear and acting related factors, and prevent the overexpression of inflammatory cytokines such as TNF-α, IL-1βt and IL-6. It has a good protective effect on cells.

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