Lactobacillus acidophilus Strain Suppresses the Transcription of Proinflammatory-Related Factors in Human HT-29 Cells

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Previous studies have shown that lactic acid bacteria can inhibit inflammatory responses, but the mechanisms are very little known. In this study, transcription and expression of three proinflammatory factors, iNOS, PTGS-2, and IL8, which are closely related to the inflammatory response, were investigated by luciferase reporter assay and RT-PCR in HT-29 cells treated by Lactobacillus acidophilus. The results showed that the live L. acidophilus sharply down-regulated the transcription of these three genes. Because there was a NF-κB binding site located at -265 bp, -225 bp, and -95 bp upstream of the iNOS, PTGS-2, and IL8 promoters, respectively, we further addressed the effects of NF-κB on transaction of the three promoters by cotransfection. As was expected, NF-κBs remarkably up-regulated the activity of the reporter gene and, no effect of NF-κB binding site mutation of the IL8 promoter in HT-29 cells. In conclusion, the live L. acidophilus decreased the transcriptional activity of NF-κB and, in turn, inhibited the transaction of NF-κB on the three proinflammatory factors mentioned above.

Key words: Lactobacillus acidophilus, NF-κB, proinflammatory-related factors

Inflammation is a beneficial host response to external challenge or cellular injury that leads to the release of a complex array of inflammatory mediators, finalizing the restoration of tissue structure and function. However, prolonged inflammation can be harmful, contributing to the pathogenesis of many diseases.

A number of cytokines are involved in the inflammation response. iNOS (inducible nitric oxide synthase), PTGS-2 (prostaglandin endoperoxide synthase 2, also called cyclooxygenase-2), and IL-8 (interleukin 8) are among the most important proinflammatory-related factors. iNOS, which catalyzes the oxidative deamination of l-arginine to produce NO (nitric oxide), is responsible for a prolonged and massive production of NO [30]. NO in a large quantity by iNOS can provoke deleterious consequences such as septic shock and inflammatory diseases [28, 31]. Prostaglandins also play a major role as mediators of the inflammatory responses. The rate-limiting enzyme in the synthesis of prostaglandins is prostaglandin endoperoxide synthase. Two isoforms of PTGS have been found: PTGS-1 and PTGS-2. PTGS-1 is constitutively expressed in the gastrointestinal tract, like in most tissues, whereas PTGS-2 expression is associated with inflammatory events [19, 29]. PTGS-2 is induced by several stimuli, and is responsible for the production of large amounts of pro-inflammatory prostaglandins at the inflammatory site [11]. IL-8 belongs to the family of chemokines. It mediates the activation and migration of neutrophils from peripheral blood into tissue and hereby plays a pivotal role in the initiation of inflammation. Thus it is important in inflammatory lung diseases like bronchial asthma or severe infections by RSV (Respiratory syncytial virus). More recently, it has been shown that IL-8 also exhibits potent angiogenic activities both in vitro and in vivo [10].

Based on these observations, it has been hypothesized that inhibition of NO, prostaglandins, and IL-8 production in macrophages, by blocking iNOS, PTGS-2, and IL-8 expressions or their activities, could serve as the basis for the potential development of anti-inflammatory drugs.

NF-κB (nuclear factor-κB) is an important transcription factor complex that regulates the expression of many genes involved in immune and inflammatory responses [14]. In unstimulated cells, NF-κB is constitutively localized in the cytosol as a heterodimer by physical association with an
inhibitory protein called IκB (inhibitor κB) [2]. Many stimuli, including LPS (lipopolysaccharide), cytokines, activators of protein kinase C, oxidants, and viruses, activate NF-κB via several signal transduction pathways that all lead to phosphorylation of IκB. Following activation, NF-κB heterodimer is rapidly translocated to the nucleus, where it activates the transcription of target genes, including the genes encoding the proinflammatory cytokines, adhesion molecules, chemokines, and inductive enzymes such as PTGS-2 and iNOS. In turn, the products regulated by NF-κB, such as TNF-α (tumor necrosis factor-α) and IL-1β (interleukin-1β), also lead to the activation of NF-κB. This means that there is a complex regulatory loop that amplifies and perpetuates inflammatory responses. Because NF-κB plays such a pivotal role in the amplifying loop of the inflammatory response, it has become a logical target for new types of anti-inflammatory treatment.

LAB (lactic acid bacteria) are safe microorganisms that reduce disturbance caused by intestinal microorganisms, ameliorate the development of microflora [3], have anti-diabetic and anti-hyperlipidemic effects [7, 27], inhibit carcinogenesis, and induce nonspecific activation of the host’s immune system [16]. Some experiments and human studies have been carried out in order to clarify how LAB regulate inflammatory responses. Lactobacillus casei inhibits the expression of inflammatory cytokines in DSS (dextran sulfate sodium)-induced colitic mice [6]. Lactobacillus acidophilus and Bifidobacterium lactis show intestinal anti-inflammatory activity in the TNBS (trinitro-benzene-sulfonic acid) model of rat colitis [23]. However, the mechanisms underlying the effects of probiotics on the inflammatory response and transcriptional regulation of inflammation factors are still being outlined.

In this paper, the effects of a L. acidophilus strain on the regulation of proinflammatory factors in human HT-29 cells were investigated. These results indicate that live L. acidophilus decreased the transcriptional activity of NF-κB and, in turn, inhibited the transaction of NF-κB on the three proinflammatory factors above.

**Materials and Methods**

**Microorganism and Related Preparations**

*L. acidophilus* (TCCC 11036) was cultured in MRS (Merck, Germany) broth anaerobically at 37°C for 24 h and checked by Gram staining and resuspended in PBS at different concentrations.

(i) **Live L. acidophilus (LAL).** The bacteria were centrifuged at 4,000 rpm for 15 min, resuspended at the designated concentration in HT-29 medium, and used immediately.

(ii) **Heat-killed L. acidophilus (LAD).** Heat-killed *L. acidophilus* was prepared by heating bacteria at 80°C for 10 min; bacteria were then centrifuged and resuspended as described above.

(iii) **L. acidophilus broth culture medium (LAS).** *L. acidophilus* was cultured in MRS broth for 24 h. The supernatant was separated by centrifugation at 4,000 rpm for 15 min. LAS was saved at −20°C until use.

(iv) **Staphylococcus aureus** (TCCC11048) (SAL) and heat-killed *S. aureus* (SAD). The Gram-positive bacterium *S. aureus* was cultured in LB broth. The bacteria were centrifuged at 4,000 rpm for 15 min, resuspended at the designated concentration in HT-29 medium, and used immediately. Heat-killed *S. aureus* was prepared by heating bacteria at 80°C for 10 min; bacteria were then centrifuged and resuspended as described above.

**Cell Culture**

The human HT-29 cells were obtained from the American Type Culture Collection and grown in DMEM (Dulbecco’s modified Eagle’s medium; Sigma, USA) supplemented with 10% (v/v) fetal bovine serum at 37°C in a 5% CO2 humidified incubator. For each experiment, HT-29 cells were collected by dissociation of a confluent stock culture with 0.25% trypsin and 1 mmol/L EDTA. The cells used for all experiments were cultured in triplicate at a density of 5×105 cells/ml in 24-well tissue culture plates (Corning) with various components from Lactobacillus strains. LPS (100 ng/ml) (*E. coli* O55:B5, Sigma, USA) was used as a positive control.

**Plasmids, Transient Transfections, and Luciferase Reporter Assays**

The human iNOS promoter reporter plasmid (iNOS-Luc) and PTGS-2 promoter reporter plasmid (PTGS-2-Luc) were constructed. The IL-8 promoter reporter construct IL-8-Luc wild type (162/44) and NF-κB site mutation construct (IL-8mutNFκB-luc) were gifts from Brasier (University of Texas Medical Branch, Galveston, TX, USA). pNFKB-Luc (Clontech) was a commercial vector. pCMV2 constructs containing cDNAs for human NF-κB1 (p50) and RelA (p65) were generous gifts from Dr. Marty Mayo of the University of North Carolina [18]. All constructs were verified by DNA sequencing (Invitrogen, Shanghai, China). DNA transfections of cells were carried out in 24-well plates by using TurboFect (**Invitrogen**, Shanghai, China). DNA transfections were carried out in 24-well plates by using TurboFect in vitro Transfection Reagent (Fermentas) according to the manufacturer’s instructions. To control for transfection efficiency between groups, 0.1 µg of a plasmid containing a promoter-driven green fluorescent protein gene (pEGFP-N1) was added to each well. Cells were lysed with Reporter lysis buffer (Promega). Luciferase activity was assayed with a Biotek Gen5 Microplate Reader using a commercially available kit (Promega).

**RT-PCR Analysis**

Five microgram of total RNA was reverse transcribed with moloney murine leukemia virus-reverse transcriptase (Promega) by oligo(dT) primers for 90 min at 42°C in 20 µl reaction mixtures, and then further diluted to 100 µl with water for the subsequent procedures. The resulting cDNA was amplified by RT-PCR using the following: human GAPDH primers (sense, 5'-ATTCACCGCAGCACTCAAAGG-3' and anti-sense, 5'-GCCGAGGCGGCCGAGATGA-3'); human iNOS primers (sense, 5'-AGAGTGGGAGAGTCCAGACC-3' and anti-sense, 5'-AGGACAGCAGCAATGATGG-3'); human PTGS-2 primers (sense, 5'-TCCAAATGAGATTGTGGGAAAATTGTGCT-3' and anti-sense, 5'-AGATCATCTCCTGGCTGAGATCTT-3'); human IL-8 primers (sense, 5'-CTTGGCACGCTTCTGGATTTTC-3' and anti-sense, 5'-GCCTTTTCAAAATTTCCCAGG-3'). The protocol consisted of 30 cycles of incubation at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by extension for 5 min at 72°C. The amplified products were analyzed by 2% agarose gel electrophoresis and visualized by EB staining under UV light.
RESULTS

Effects of Different Bacterial Preparations on the Transcription of Proinflammatory-Related Gene

L. acidophilus and S. aureus (as a negative control) were examined for their abilities to influence proinflammatory-related gene promoter construct in human HT-29 cells. The human iNOS promoter reporter plasmid (iNOS-Luc) and PTGS-2 promoter reporter plasmid (PTGS-2-Luc) were constructed as illustrated in Fig. 1A.

To identify various proinflammatory-related gene promoter constructs in response to L. acidophilus, HT-29 cells transfected with iNOS-Luc, PTGS-2-Luc, IL-8-Luc, or IL-8mutNFκB-Luc were treated with LAL, LAD, LAS, and SAL or SAD, respectively. LPS was used as a positive control in this experiment [26]. As can be observed (Fig. 1B), in HT-29 cells transfected with iNOS-Luc, PTGS-2-Luc, or IL-8-Luc, the expression of luciferase gene was dramatically suppressed by live L. acidophilus but induced by boiled strains and supernatant of L. acidophilus fermented broth. Considering that the NF-κB binding site was located on the promoter region of iNOS, PTGS-2, and IL8, we deduced that NF-κBs might be involved in the regulation of proinflammatory-related genes by L. acidophilus or its related preparations.

To test the effects of L. acidophilus on the endogenous expression of iNOS, PTGS2, and IL-8, we used a semi-quantitative RT-PCR to evaluate the levels of these proinflammatory-related gene mRNAs after the L. acidophilus treatment. In agreement with the transfection results (Fig. 1C), the mRNA levels of iNOS, PTGS-2, and IL-8 were also down-regulated by live L. acidophilus, and increased by the boiled strains and fermented broth of L. acidophilus.

NF-κBs Were Responsible for the Regulation of Proinflammatory-Related Gene

To test the role of NF-κBs in the transcription of the proinflammatory-related gene, HT-29 cells were transfected with or without p65, and p50, followed by stimulation with PDTC (10 µM). Cells were harvested 24 h post-transfection, and (A) luciferase activity was measured or (B) RT-PCR was performed. The blank bar stands for cells transfected with iNOS, PTGS-2, or IL-8 promoter-luc construct alone.

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with iNOS-Luc, PTGS-2-Luc, or IL-8-Luc vector, respectively, along with p65 and p50 expression plasmids. From the luciferase activity measured (Fig. 2A), the heterodimer p65-p50 remarkably up-regulated the expression of reporter genes, much higher than p65 or p50 homodimer. In contrast, in HT-29 cells transfected with IL-8mutNFκB-Luc, the expression of the luciferase gene almost had no response to the various forms of NF-κB dimers. PDTC (pyrrolidine dithiocarbamate), a widely-used NF-κB inhibitor, significantly attenuated the expression of the reporter gene [15].

Furthermore, the effects of NF-κB on the endogenous transcription of iNOS, PTGS-2, and IL-8 were detected using the method of RT-PCR, and the results also showed that iNOS, PTGS-2, and IL-8 were all up-regulated by NF-κBs (Fig. 2B). Taken together, these results indicated that NF-κBs might play a vital role in the regulation of expression of iNOS, PTGS2, and IL-8.

**Effect of L. acidophilus on pNF-κB -Luc**

To investigate the role of the L. acidophilus in NF-κBs regulation, we tested the effects of L. acidophilus on NF-κB transcription with the methods of luciferase reporter assay and RT-PCR analysis. As shown in Fig. 3, the expression of the reporter gene was markedly suppressed by treatment with the live L. acidophilus cells. In contrast, S. aureus had little effect on the luciferase level. Strikingly, supernatant of L. acidophilus fermented broth (LAS) remarkably increased the expression of the reporter gene.

**DISCUSSION**

Lactobacilli are commensal bacteria that can be found in the gut. Some strains of lactobacilli have been isolated and are being used in clinical and experimental studies for their immunomodulatory properties [1, 12]. Based upon some animal experiments and human studies [16, 24], there are a potential therapeutic role for probiotics and prebiotics in patients with inflammatory bowel disease. However, large, double-blinded controlled trials are needed to confirm the efficacy and to document dosage and treatment parameters [17]. Whether these positive results were in part due to patient selection or use of probiotics in liquid rather than dried form remains to be determined in a larger patient study. Suffice it to say, the role of probiotics in treating or preventing inflammation has not yet been clearly proven [20].

In this study, we investigated the involvement of L. acidophilus strain versus the commensal pathogenic S. aureus on the induction of genes involved in typical proinflammatory factors, including iNOS, PTGS-2, and IL-8.

In this study, we demonstrated that live L. acidophilus down-regulates the transcription of proinflammatory genes. As indicated in this paper, the live L. acidophilus could sharply decrease the expression of iNOS, PTGS-2, and IL-8 in HT-29 cells. Genetic studies have revealed an important role for NF-κBs in immune and inflammatory responses [4]. NF-κB p50 protein has been shown *in vitro* to play a role in repression of proinflammatory gene transcription, as recently shown for TNF-α [22, 25]. Dysregulation of NF-κB can lead to the constitutive overproduction of pro-inflammatory cytokines, which is associated with a number of chronic inflammatory disorders, including rheumatoid arthritis and Crohn’s disease [8, 9]. Although NF-κBs appear to play a critical role in cytokine-mediated inflammation by up-regulating the transcription of a specific set of cytokine genes in response to inflammatory stimuli, it is uncertain whether NF-κBs have a significant role in the differential production of NF-κB-dependent cytokines or in coordinating the production of these cytokines. Conversely, the heat-killed L. acidophilus (LAD) and fermented supernatant broth (LAS) stimulated the expression of iNOS, PTGS-2, and IL-8 in HT-29 cells. It was deduced that some components of the cells were resolved when were heated or fermented, such as LPS, cell wall-associated polysaccharides, proteins, and lipoteichoic acids [11, 13]. These substances can stimulate the cell signal pathway-related genes.

In the present study, the mechanisms of L. acidophilus regulation of proinflammatory genes iNOS, PTGS-2, and IL-8 in HT-29 cells were discussed. In the 5' upstream region of these genes, we found the unique κB-binding site 5'-GGGGRNWYYCC-3', through which NF-κBs can drive their transcription. A variety of stimuli or inhibitors, such as microbial and viral products, cytokines, DNA damage, and noxious chemicals [5], have been showed to have abilities to regulate the activity of NF-κB’s signal pathway. Here, we found that L. acidophilus could also act as an inhibitor of NF-κB-dependent transcription and down-regulate iNOS, PTGS-2, and IL-8 *via* this cell signal pathway.
References


