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Effects of Tricin on Viability and Inflammation-Related Gene Expression in Lipopolysaccharide-Stimulated Bovine Mammary Epithelial Cells in Vitro: A Postprint

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Abstract

This study aimed to investigate the effects of tricin on the activity and expression of inflammation-related genes in dairy cow mammary epithelial cells cultured in vitro and stimulated by lipopolysaccharide (LPS). The in vitro cultured dairy cow mammary epithelial cells were divided into six groups: the control group was treated with basal medium, while the experimental groups were treated with basal medium supplemented with 1 g/mL LPS (Group L), 1 g/mL LPS and 2.5 g/mL tricin (Group L+2.5), 1 g/mL LPS and 5.0 g/mL tricin (Group L+5), 1 g/mL LPS and 10.0 g/mL tricin (Group L+10), and 1 g/mL LPS and 15.0 g/mL tricin (Group L+15), respectively, and cultured for 24 h. The results showed that: 1) Compared with the control group, the relative proliferation rate of dairy cow mammary epithelial cells was extremely significantly decreased in Group L (P<0.01), but extremely significantly increased in Groups L+2.5 and L+10 (P<0.01); 2) Compared with Group L, the superoxide dismutase activity in Groups L+10 and L+15 was significantly increased (P<0.05), while the lactate dehydrogenase activity and nitric oxide concentration were significantly decreased (P<0.05); 3) Compared with Group L, the relative expression levels of interleukin-8, interleukin-1 , tumor necrosis factor- , Toll-like receptor 2, and Toll-like receptor 4 in Groups L+5 and L+10 were all extremely significantly decreased (P<0.01), and the relative expression level of myeloid differentiation factor 88 in Group L+5 was significantly decreased (P<0.05). These results suggest that under LPS stimulation, the addition of tricin can improve the activity and antioxidant capacity of dairy cow mammary epithelial cells, inhibit the expression of inflammation-related genes, and exert anti-inflammatory effects.

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Full Text

Effects of Tricin on Viability and Expressions of Genes Related to Inflammation of Bovine Mammary Epithelial Cells Stimulated by Lipopolysaccharide in Vitro

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Abstract

This experiment aimed to investigate the effects of tricin on viability and expressions of genes related to inflammation of bovine mammary epithelial cells (BMECs) stimulated by lipopolysaccharide (LPS) in vitro. The BMECs cultured in vitro were divided into 6 groups and cultured for 24 h. The control group was cultured in basal culture medium, while the treatment groups were cultured in basal culture medium supplemented with 1 g/mL LPS (L group), 1 g/mL LPS + 2.5 g/mL tricin (L+2.5 group), 1 g/mL LPS + 5.0 g/mL tricin (L+5 group), 1 g/mL LPS + 10.0 g/mL tricin (L+10 group), and 1 g/mL LPS + 15.0 g/mL tricin (L+15 group), respectively. The results showed that: 1) Compared with the control group, the relative proliferation rate of BMECs in the L group was significantly decreased ($P<0.01$), whereas those in the L+2.5 and L+10 groups were significantly increased ($P<0.01$). 2) Compared with the L group, the superoxide dismutase activity of cells in the L+10 and L+15 groups was significantly increased ($P<0.05$), while lactate dehydrogenase activity and nitric oxide concentration were significantly decreased ($P<0.05$). 3) Compared with the L group, the relative expression levels of interleukin-8, interleukin-1 , tumor necrosis factor- , Toll-like receptor 2, and Toll-like receptor 4 in the L+5 and L+10 groups were significantly decreased ($P<0.01$), and the relative expression level of myeloid differentiation primary response 88 in the L+5 group was significantly decreased ($P<0.05$). These results indicated that tricin supplementation could enhance the viability and antioxidant capacity of BMECs under LPS stimulation and exert anti-inflammatory effects by inhibiting the expression of inflammation-related genes.

Keywords: tricin; anti-inflammatory; bovine mammary epithelial cell; lipopolysaccharide; cell proliferation

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Introduction

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria such as Escherichia coli. When bacteria infect mammary tissue, released LPS can cause tissue damage and subsequent inflammation. Previ-

ous studies have demonstrated that LPS can increase the expression of inflammatory cytokines such as tumor necrosis factor- (TNF-) and interleukin-1 (IL-1) in bovine mammary epithelial cells, reduce antioxidant enzyme activities, increase malondialdehyde (MDA) concentration and cell membrane permeability, and ultimately promote cell apoptosis.

Tricin, also known as 4 ,5,7-trihydroxy-3 ,5 -dimethoxyflavone, is a flavonoid compound first discovered in wheat leaves infected with rust disease and subsequently found in alfalfa, rice, sorghum, and barley. Research has shown that tricin can inhibit the proliferation of bacteria, fungi, and influenza viruses, exerting antimicrobial and antiviral effects. It also possesses mild estrogen-like activity and antioxidant properties. Additionally, tricin can inhibit the proliferation of human rectal cancer cells, induce apoptosis, and suppress the proliferation of mouse T and B lymphocytes, indicating immunosuppressive effects.

Under LPS stimulation, flavonoids such as astragalin, morin, and puerarin can inhibit the expression of TNF- and interleukin-6 (IL-6) in mammary epithelial cells, reduce nuclear factor- B (NF- B) activity, and decrease the synthesis of phosphorylated NF- B inhibitor protein (I B), p38, extracellular signal-regulated kinase (ERK), and p65. These findings suggest that flavonoids can exert anti-inflammatory effects by inhibiting NF- B signaling pathway activation and reducing downstream inflammatory cytokine expression. However, the effects of tricin on bovine mammary epithelial cells under LPS stimulation have not been reported. Therefore, this study investigated the impact of tricin on BMECs under LPS stimulation to explore its potential anti-inflammatory effects and provide a scientific basis for its application in animal production.

1.1 Experimental Materials

Tricin (purity 98% by HPLC analysis) derived from oats was provided by Shanghai Yuanye Biological Technology Co., Ltd. LPS (serotype O55:B5) was purchased from Sigma-Aldrich (USA). The bovine mammary epithelial cells used in this study were obtained through tissue block culture method in our laboratory, with culture and identification methods previously described by Zhan et al.

1.2.1 Cytotoxicity Detection

The cytotoxicity of tricin was assessed using the CCK-8 assay. Bovine mammary epithelial cells were divided into 8 groups and cultured in basal culture medium supplemented with 0 (control), 2.5, 5.0, 7.5, 10.0, 15.0, 25.0, and 40.0 g/mL tricin, respectively, with 5 replicates per group. Tricin was dissolved in dimethyl sulfoxide (DMSO) (Beijing Solarbio Science & Technology Co., Ltd.), with the final DMSO concentration in the medium below 2‰. Mammary epithelial cells were seeded in 96-well plates at a density of 1×10 cells/mL and incubated at 37°C in a 5% CO incubator. After cell adhesion, the medium was removed, cells were washed with phosphate-buffered saline (PBS), and the various treatment

media were added. Following 24 h of culture, 10 L of CCK-8 solution was added to each well, and after 3 h of incubation, absorbance at 450 nm was measured using a microplate reader to calculate the relative proliferation rate and evaluate tricin cytotoxicity. The relative proliferation rate was calculated as: Relative proliferation rate (%) = (OD nm of treatment group / OD nm of control group) × 100.

1.2.2 Cell Viability Detection

Cell viability was also assessed using the CCK-8 method. Bovine mammary epithelial cells were divided into 6 groups: a control group cultured in basal culture medium, and treatment groups cultured in basal medium supplemented with 1 g/mL LPS (L group), 1 g/mL LPS + 2.5 g/mL tricin (L+2.5 group), 1 g/mL LPS + 5.0 g/mL tricin (L+5 group), 1 g/mL LPS + 10.0 g/mL tricin (L+10 group), and 1 g/mL LPS + 15.0 g/mL tricin (L+15 group). The experimental procedures were the same as described in section 1.2.1. Cell viability was determined based on the relative proliferation rate.

1.2.3 Antioxidant Capacity Measurement

Bovine mammary epithelial cells were divided into 6 groups using the same grouping method as in section 1.2.2. Cells were seeded in 6-well plates at a density of 5×10 cells/mL. After 24 h of culture, cell culture medium and cells were collected to detect nitric oxide (NO) and malondialdehyde (MDA) concentrations, as well as lactate dehydrogenase (LDH) and superoxide dismutase (SOD) activities. Detection methods followed the instructions provided with the assay kits (Nanjing Jiancheng Bioengineering Institute).

1.2.4 Relative Expression of Inflammation-Related Genes

Bovine mammary epithelial cells were divided into 6 groups using the same grouping method as in section 1.2.2 and seeded in 6-well plates at a density of 5×10 cells/mL. After 24 h of culture, the cell culture medium was removed, 1 mL of Trizol was added, and the mixture was allowed to stand for 10 min. Total RNA extraction was performed according to the kit instructions provided by Tiangen Biotech Co., Ltd. The extracted total RNA was assessed for concentration and purity using a One Drop instrument, and cDNA synthesis was performed using a reverse transcription kit from Roche (Switzerland), with operational procedures, reaction systems, and reagent preparation as previously described by Zhan et al. Real-time quantitative PCR kits were provided by Roche (Switzerland), with PCR reaction preparation and conditions as previously described by Zhan et al.

Primers were designed using Primer 5.0 software based on gene sequences from GenBank and synthesized by Invitrogen (USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal reference gene. Primer sequences

are listed in Table 1 . Relative gene expression levels were calculated using the Ct method.

1.3 Data Processing

Experimental data were first organized using Excel 2007 and analyzed using SPSS 21.0 software. ANOVA was performed, and multiple comparisons were conducted using the LSD method. Data are presented as mean ± standard error. Differences were considered extremely significant at $P<0.01$ and significant at $P<0.05$.

2.1 Cytotoxicity of Tricin

As shown in Table 2 , when tricin concentration ranged from 2.5 to 15.0 g/mL, the relative proliferation rate of BMECs increased with increasing tricin concentration. When tricin concentration ranged from 15.0 to 40.0 g/mL, the relative proliferation rate decreased with increasing concentration. These results indicate that tricin enhances cell viability within a certain concentration range, while excessive concentrations produce cytotoxic effects.

2.2 Effects of Tricin on Cell Viability Under LPS Stimulation

As shown in Table 3 , the relative proliferation rate of BMECs in the L group was significantly lower than in all other groups ($P<0.01$). The cell viability in the L+2.5 and L+10 groups was significantly higher than in both the control and L+15 groups ($P<0.01$). These results demonstrate that LPS stimulation reduces BMEC viability, while tricin supplementation can enhance cell viability.

2.3 Effects of Tricin on Antioxidant Capacity Under LPS Stimulation

As shown in Table 4 , SOD activity in the L+2.5, L+5, L+10, and L+15 groups was significantly higher than in other groups ($P<0.05$), with the L group showing the lowest activity. MDA and NO concentrations in the L+10 group were significantly lower than in the L group ($P<0.05$). LDH activity was highest in the L group, while the L+2.5, L+5, L+10, and L+15 groups showed significantly reduced LDH activity compared to the L group ($P<0.05$). These results indicate that tricin supplementation can enhance the antioxidant capacity of cells under LPS stimulation.

2.4 Effects of Tricin on Expression of Inflammation-Related Genes Under LPS Stimulation

As shown in Table 5 , compared with the control group, all other groups showed significantly increased relative expression levels of interleukin-8 (IL-8), IL-1 , IL-6, and TNF- ($P<0.01$). Compared with the L group, the L+5 and L+10 groups showed significantly decreased relative expression levels of IL-8, IL-1 , and TNF- ($P<0.01$). The relative expression levels of Toll-like receptor 2 (TLR2) and

Toll-like receptor 4 (TLR4) in the L group were significantly higher than in other groups (P<0.01). The relative expression level of myeloid differentiation primary response 88 (MyD88) in the L group was significantly or extremely significantly higher than in the control and L+5 groups (P<0.05 or P<0.01). No significant differences were observed in the relative expression levels of transforming growth factor- 1 (TGF- 1) and nucleotide-binding oligomerization domain 1 (NOD1) among groups (P>0.05). These results indicate that LPS increases the expression of inflammation-related genes in BMECs, while tricin supplementation can suppress their expression.

3.1 Effects of Tricin on Cell Viability Under LPS Stimulation

Previous in vitro studies have demonstrated that supplementation with 100 ng/mL daidzein and genistein, 10-1000 mg/L soybean isoflavones, and 50-100 g/mL alfalfa flavonoids can significantly promote BMEC proliferation. In the present study, supplementation with 2.5-15.0 g/mL tricin increased the relative proliferation rate of BMECs, consistent with these previous findings. However, when tricin concentration exceeded 15.0 g/mL, the relative proliferation rate decreased, suggesting that high concentrations of tricin may be cytotoxic. Therefore, to avoid cytotoxic effects, subsequent experiments were limited to tricin concentrations below 15.0 g/mL.

Under LPS stimulation, the relative proliferation rate of BMECs decreases with increasing LPS concentration. This study also found that LPS stimulation significantly reduced BMEC proliferation, demonstrating its inhibitory effect on cell growth. Liu et al. reported that LPS stimulation significantly decreased the relative proliferation rate of mammary epithelial cells, while supplementation with different concentrations of alfalfa total flavonoids under LPS stimulation significantly enhanced cell viability in a dose-dependent manner. Our findings are consistent with these results, indicating that appropriate concentrations of tricin can promote BMEC proliferation under LPS stimulation.

3.2 Effects of Tricin on Antioxidant Capacity Under LPS Stimulation

Under normal physiological conditions, free radicals are maintained in a dynamic balance. Excessive free radicals can cause lipid peroxidation of unsaturated fatty acids, producing malondialdehyde that leads to cell disintegration and death. Under LPS stimulation, SOD and catalase (CAT) activities decrease while MDA concentration increases in porcine intestinal epithelial cells and BMECs. LDH is an oxidoreductase involved in glycolysis that is released when cells are damaged. LPS can increase LDH release from BMECs, with LDH activity in cell culture supernatant increasing with LPS concentration. NO is an important signaling molecule that plays a crucial role in inflammatory responses. LPS induction increases cellular NO concentration, which activates cyclooxygenase-2 (COX-2) and induces apoptosis. These findings demonstrate that LPS reduces antioxidant enzyme activity, increases intracellular free radical concentration, causes cell damage, and inhibits cell proliferation.

Under LPS stimulation, naringin can increase SOD activity and reduce MDA concentration in mouse hearts, while tea polyphenols can increase SOD activity and decrease LDH activity and MDA concentration in human bronchial epithelial cells. Additionally, tricin, isoliquiritigenin, liquiritigenin, and naringenin can inhibit NO release in LPS-induced RAW264.7 cells. Our results are consistent with these studies, suggesting that appropriate tricin supplementation can protect BMECs from oxidative damage and enhance cell viability by improving antioxidant capacity.

3.3 Effects of Tricin on Expression of Inflammation-Related Genes Under LPS Stimulation

Transforming growth factor- (TGF-) plays an important role in regulating tissue inflammation and repair, with LPS significantly increasing TGF- 1 secretion in rat peritoneal mesothelial cells. NOD1 is distributed in various tissues and cells throughout the body and plays a crucial role in innate immunity and inflammatory responses. Under LPS stimulation, NOD1 expression is significantly increased in piglet liver and hypothalamic-pituitary-adrenal axis. Our results showed that LPS stimulation increased the relative expression levels of TGF- 1 and NOD1 in BMECs, while tricin supplementation had no significant effect, suggesting that tricin does not exert anti-inflammatory effects through modulation of TGF- and NOD signaling pathways.

LPS is an important inflammatory factor that can induce cytokines such as TNF- , IL-1 , and IL-6 to participate in inflammatory responses. Studies have found that tricin can significantly downregulate TNF- and IL-6 production in human peripheral blood mononuclear cells under LPS stimulation, inhibit TLR4, MyD88, and TIR domain-containing adaptor-inducing interferon- (TRIF) activity, and enhance anti-inflammatory effects in human umbilical vein endothelial cells by modulating MAPK and PI3K/Akt pathways, inhibiting NF- B signaling, and reducing COX-2 and TNF- concentrations. Furthermore, tricin can significantly reduce TNF- and IL-1 concentrations in bronchoalveolar lavage fluid of asthmatic mice and decrease the relative expression levels and protein concentrations of TLR4, MyD88, and NF- B p65 in alveolar macrophages. Our study also found that under LPS stimulation, tricin could inhibit the expression of TNF- , IL-8, IL-6, IL-1 , TLR4, TLR2, and MyD88 in BMECs. These findings suggest that the anti-inflammatory effect of tricin in BMECs may be related to its regulation of the TLR/MyD88/NF- B pathway.

4 Conclusion

1. Tricin can enhance the antioxidant capacity and relative proliferation rate of BMECs under LPS stimulation.
2. Tricin may exert anti-inflammatory effects by regulating the TLR/MyD88/NF- B pathway, reducing inflammatory cytokine expression and NO production.

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