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# The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide

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## ABSTRACT

Caffeic acid possesses multiple biological effects, such as antibacterial, antioxidant, antiinflammatory, and anticancer growth; however, what effects it has on bovine mastitis have not been investigated. The aim of this study was to verify the antiinflammatory properties of caffeic acid on the inflammatory response of primary bovine mammary epithelial cells (bMEC) induced by lipopolysaccharide (LPS), and to clarify the possible underlying mechanism. Bovine mammary epithelial cells were treated with various concentrations (10, 50, 100, and 200 μg/mL) of LPS for 3, 6, 12, and 18 h; the results showed that LPS significantly inhibited cell viability in a time- and dose-dependent manner. When cells were treated with LPS (50 μg/mL) for 12 h, the cell membrane permeability significantly increased, which promoted cell apoptosis. Various concentrations (10, 25, and 50 μg/mL) of caffeic acid could weaken the inflammation injury of bMEC induced by LPS without cytotoxicity. Proinflammatory cytokines (IL-8, IL-1β, IL-6, and tumor necrosis factor α) from bMEC were decreased. Nuclear transcription factor κB activity was weakened via blocking κB inhibitor α degradation and p65 phosphorylation. All these showed that the protective effect of caffeic acid on LPS-induced inflammation injury in bMEC was at least partly achieved by the decreased production of proinflammatory cytokines mediated by the effect of reducing the κB inhibitor α degradation and p65 phosphorylation in the nuclear transcription factor κB pathway. The use of caffeic acid would be beneficial in dairy cows during *Escherichia coli* mastitis as a safe and natural antiinflammatory drug.

**Key words:** bovine mammary epithelial cell, lipopolysaccharide, inflammation, caffeic acid

## INTRODUCTION

Bovine mastitis, which causes serious economic losses, is one of the most common cow diseases throughout the world. Bacterial infections of the bovine mammary gland can result in a spectrum of clinical outcomes, ranging from acute and life threatening to chronic and subclinical, which affect mammary tissue integrity and reduce the production performance of cows (Seegers et al., 2003). Therefore, it is of prime importance to determine how to detect and eradicate bacteria earlier and control the inflammation (Menzies et al., 1995; Porcherie et al., 2012; Quesnell et al., 2012). Currently, antibiotics are still an effective treatment of bovine mastitis, but are limited because of the growing problem of drug resistance and food safety concerns over their use in recent years (Boehmer, 2011; Kerro Dego et al., 2012). Thus, safe and effective treatments of bovine mastitis continue to garner attention in veterinary research.

Coliform mastitis is often characterized by severe inflammation and impaired milk production, which can even lead to death of the animal. *Escherichia coli* is one of the major mastitis pathogens responsible for clinical mastitis in cows (Burvenich et al., 2003; Bradley et al., 2007; Botrel et al., 2010). Lipopolysaccharide, a major integral structural component of the outer membrane of *E. coli* and one of the best-studied microbial products, is a potent initiator of inflammation and endotoxin shock. Toll-like receptor 4 (**TLR4**) is indispensable for LPS signaling (Shimazu et al., 1999; Miyake, 2004). The TLR4-LPS interaction leads to the rapid and coordinated activation of various intracellular signaling pathways, including the mitogen-activated protein kinase (**MAPK**) cascades, translocation of nuclear factor κB (**NF-κB**), and so on (Risco et al., 2012; Hines et al., 2013). Nuclear factor κB regulates the expression of cytokines, chemokines, antiapoptotic, and cell growth factors, which are essential mediators of immune and inflammatory responses. Thus, inhibition of NF-κB activation has attracted attention as a therapeutic approach for intervention in immune and inflammatory events (Li and Verma, 2002; Calzado et al., 2007).

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In the cow udder, the expression of TLR2 and TLR4 has frequently been demonstrated (Petzl et al., 2008; Bougarn et al., 2010). The mammary immune response to LPS has been studied in vitro (Wellnitz and Kerr, 2004; Strandberg et al., 2005; Günther et al., 2012) and in vivo (Schmitz et al., 2004; Wellnitz et al., 2010; Morimoto et al., 2012), and the initial response is similar to that for whole bacteria (Hoeben et al., 2000; Wellnitz and Bruckmaier, 2012). Those studies clearly demonstrate that mammary epithelial cells have a strong innate immune defense capability and capacity to attract circulating immune effector cells, such as neutrophils (Strandberg et al., 2005; Ibeagha-Awemu et al., 2008), but fewer studies have focused on the excessive inflammatory response of epithelial cells affecting themselves, or reduced inflammatory injury of breast tissue by weakening the immune response.

Caffeic acid, a natural phenolic compound, is abundant in medicinal plants. Caffeic acid and its synthetic derivatives possess multiple biological effects such as antibacterial, antioxidant, antiinflammatory, and anticancer growth (Shin et al., 2004; Jung et al., 2007; Búfalo et al., 2013). Caffeic acid could act on multiple signaling pathways associated with inflammation in various cell lines. It could inhibit LPS-induced inflammation response of mouse leukemic monocyte macrophage (Raw 264.7) cells through the inhibition of NF-κB, p38 MAPK, and c-Jun $NH_2$-terminal kinase 1/2 activation (Puangpraphant et al., 2011; Búfalo et al., 2013). However, the effect of caffeic acid on mastitis has not been investigated. The aim of this study was to investigate the protective effect of caffeic acid against LPS-induced inflammation injury of bovine mammary epithelial cells (**bMEC**) and to clarify the possible mechanisms.

## MATERIALS AND METHODS

### bMEC Isolation, Cell Culture, and Treatment

Bovine mammary epithelial cells were isolated from 5 lactating cows as previously described and cryopreserved in liquid nitrogen (Lahouassa et al., 2007; Porcherie et al., 2012). Cells were incubated at 37°C in 5% $CO_2$. Cells cultured in medium consisting of Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (**DMEM/F12**) supplemented with 10% fetal bovine serum (Gibco, Grand island, NY), 100 U/mL of antibiotic (penicillin and streptomycin; Sigma-Aldrich, St. Louis, MO), 5 μg/mL of insulin, 1 μg/mL of hydrocortisone, 5 μg/mL of transferrin, and 1 μg/mL of progesterone (Sigma-Aldrich) were used at passage 3 or 4 for further research.

Caffeic acid (>98% purity; National Institutes for Food and Drug Control, Beijing, China) and LPS (*E. coli* serotype O55:B5, Sigma-Aldrich) were diluted in DMEM/F12 medium (1 mg/mL). All cells were washed with serum-free medium and serum starved for 2 h before incubation with caffeic acid or LPS.

### Cell Viability Assay and Flow Cytometric Analysis

To measure cell viability, equivalent numbers of bMEC were plated on 96-well multiplies and cultured in DMEM/F12 medium containing 5% fetal bovine serum at a density of $1.2 \times 10^5$ cells/mL. After cells were grown to 90% confluence, all cells were washed twice with PBS and serum starved for 2 h before incubation with caffeic acid or LPS for different times, and then 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL; Amresco LLC, Solon, OH) was added to each well and incubated at 37°C for 4 h, and well media was aspirated and the formazan product dissolved using dimethyl sulfoxide (Amresco LLC). The remaining formazan product was analyzed using a microplate reader (Bio-Rad Laboratories Inc., Foster City, CA) at a fixed absorption wavelength of 490 nm and reference wavelength of 630 nm.

To analyze apoptosis, cells were treated with LPS or caffeic acid according to the experimental requirements. Following each specific treatment, all cells were trypsinized (Gibco), washed twice with PBS, and then stained with annexin V/propidium iodide (Invitrogen Inc., Carlsbad, CA), and flow cytometric analysis was performed according to the manufacturer's instructions (Beckman Coulter Inc., Fullerton, CA).

### RNA Extraction and Gene mRNA Expression Analysis

Total RNA was isolated from bMEC using a phenol and guanidine isothiocyanate-based TRIzol reagent (Invitrogen Inc.) according to the manufacturer's instructions. The RNA quality assessment and reverse transcription (**RT**) were performed as previously described (Yu et al., 2010a). Reverse transcription-generated cDNA encoding β-actin, IL-1β, tumor necrosis factor α (**TNF-α**), IL-6, and IL-8 were amplified by real-time PCR using selective primers listed in Table 1. Quantitative PCR analysis was carried out as previously described (Yu et al., 2010b). The PCR reaction system (25 μL in total) contained 12.5 μL of SYBR Green PCR mix (Stratagene, La Jolla, CA), 0.375 μL of reference dye, 1 μL of each primer (both 10 μmol/L), 1 μL of cDNA template, and 9.125 μL of diethylpyrocarbonate-treated water.