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Stevioside inhibits inflammation and apoptosis by regulating TLR2 and TLR2-related proteins in *S. aureus*-infected mouse mammary epithelial cells

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ABSTRACT

Stevioside is a natural sweetener that is commonly used in traditional medicine and as a food additive. The object of this study was to investigate the anti-inflammatory and anti-apoptosis function of stevioside and the possible molecular mechanisms for such activity in *Staphylococcus aureus (S. aureus)*-infected mouse mammary epithelial cells (MMECs). The cells were treated with varying doses of stevioside before infection with *S. aureus*. The live/dead cells were detected by immunofluorescence microscopy. The pro-inflammatory cytokines were determined by ELISA. The mRNA of TLR2 and proteins related to NF-κB, MAPK and apoptosis were analyzed by q-PCR. The relative protein expression levels were determined by Western blot. The results indicated that stevioside inhibited the mRNA and protein expression of TNF-α, IL-6 and IL-1β dose-dependently in *S. aureus*-stimulated MMECs. Stevioside suppressed the *S. aureus*-induced expression of TLR2 and proteins of the NF-κB and MAPK pathways as well as apoptosis. The mRNA levels of IκBα, p38, ERK, JNK, p65, caspase-3 and Bax were not influenced by the stevioside treatment. Stevioside exerts anti-inflammatory and anti-apoptotic properties by inhibiting the release of cytokines and the activation of TLR2 and proteins of the NF-κB and MAPK signaling pathways, as well as caspase-3 and Bax.

1. Introduction

Mastitis is a serious threat to human and dairy cows. Mastitis can lead to the human discontinuation of breastfeeding, which provides optimal infant nutrition [1]. Dairy cows suffering from mastitis deliver drastically reduced milk yield and product quality. This disease results in great economic losses in the dairy cow industry world-wide [2,3].

Staphylococcus aureus (*S. aureus*) is one of the most common gram-positive bacterial pathogens and causes mammary infections in humans and dairy ruminants [4,5]. *S. aureus* infection leads to the inflammation and apoptosis of host cells [6,7]. Studies have demonstrated that mammary epithelial cells (MECs) respond rapidly and sensitively to bacterial intrusion by activating several pattern recognition receptors (PRRs) through microbe-associated molecular patterns (MAMPs). Toll-like receptors (TLRs) are involved in innate immunity, and the interaction between different TLRs can determine the magnitude and type of immune response [8]. TLR2 was identified as a key immune receptor in the TLR family. Many classes of microorganisms such as *S. aureus* and the bacterial cell wall components peptidoglycan and lipoteichoic acid have been found to activate TLR2 [9,10]. MECs possess the ability to react by producing mediators of inflammation and local defense [5,11] due to the presence of several pathogens sensors.

Some studies do not support the extensive use of antibiotics such as cephalexin and penicillin for mastitis during lactation. All communities should avoid the imprudent use of antibiotics due to the spread of drug-resistant *S. aureus* strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) and other multi-resistant pathogens [12]. Moreover, the use of antibiotics could result in an increased risk of antibiotic residues in milk [13]. Thus far, the issues of antibiotic residues and treatment resistance have practically no effective solutions.

Stevioside is obtained from leaves of *Stevia rebaudiana*, which is commonly used in traditional medicine and in food additives as a natural sweetener. Stevioside is reported to possess some therapeutic benefits such as anti-hyperglycemic [14], anti-hypertensive [15], anti-diarrheal [16], immunomodulatory [17,18], anti-inflammatory [19], and anti-apoptosis effects [20]. However, whether stevioside exhibits an analogous anti-inflammatory and anti-apoptotic effect in *S. aureus*-infected MMECs remains unclear. In the present study, we sought to examine the anti-inflammatory and anti-apoptosis effects of stevioside in *S. aureus*-infected MMECs and to investigate the potential mechanisms for its activity.

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2. Materials and methods

2.1. Experimental animals and strains

In total, 15 day-gravidity BALB/c mice (6–8 weeks old, weighing 40–45 g, purchased from Norman Bethune University of Medical Science (NBUMS) of Jilin University) were used in the present study. All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals established by the US National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Jilin University.

The *Staphylococcus aureus* SA113 (ATCC35556) (obtained from Mikrobielle Genetik, University of Tubingen, Germany) was cultured in Mueller-Hinton II cation-adjusted broth (BD Biosciences, Sparks, MD, USA) at 37 °C for 18 h with constant shaking.

2.2. Experimental groups

S. aureus was added to 6 well plates (1×10^7 CFU/ml). Stevioside was dissolved in DMEM: -F12/-1:1 at concentrations of 300 μg/ml, 100 μg/ml, or 30 μg/ml. The *S. aureus* suspension was add to stimulate the MMECs for 3 h after incubation with stevioside for 1 h.

Mouse mammary epithelial cells isolated from BALB/c mice were divided into three experimental groups as follows:

1) Microbionation (*S. aureus*) group (MG): The cell model of *S. aureus*-stimulated MMECs was established, and the cells were treated with PBS.
2) Stevioside administration groups (SG): The cell model of *S. aureus*-stimulated MMECs was established, and the cells were treated with stevioside at 300, 100, or 30 μg/ml. The dosage of stevioside was determined based on the results of a previous study.
3) Control group (CG): The cells were treated with PBS (as a vehicle control) at the same volume and time points as the stevioside treatment.

2.3. Chemicals and reagents

Stevioside (HPLC ≥98%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) (Fig. 1). Rat tail collagen was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM: F12/1:1), Foetal Calf Serum (FCS) and trypsin /EDTA were purchased from Hyclone (Logan, UT, USA). Collagenase I and Collagenase II were purchased from Invitrogen (CA, USA). EGF Epidermal Growth Factor, Transferrin and T3 were purchased from PeproTech (LA, USA). Mouse TNF-α, IL-6, and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biolegend (San Diego, CA, USA). Live/Dead Cell Staining Kit (ab65470) and Anti-TLR2 antibody (ab108998) was purchased from Abcam (UC, UK). Cleaved Caspase-3 (Asp175) Rabbit mAb, Caspase-3 (8G10) Rabbit mAb, Bax Antibody, Bcl-2 (D17C4) Rabbit mAb, MAPK Family Antibody Sampler Kit, Phospho-MAPK Family Antibody Sampler Kit and NF-κB Pathway Sampler Kit were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). β-actin (KM9001) and HRP-conjugated goat–mouse antibodies were provided by Sungene Biotech Co. Ltd (Tianjin, China). Horseradish peroxidase conjugated goat anti-rabbit and goat-mouse antibodies were purchased from GE Healthcare (Buckinghamshire, UK). All other chemicals were of reagent grade.

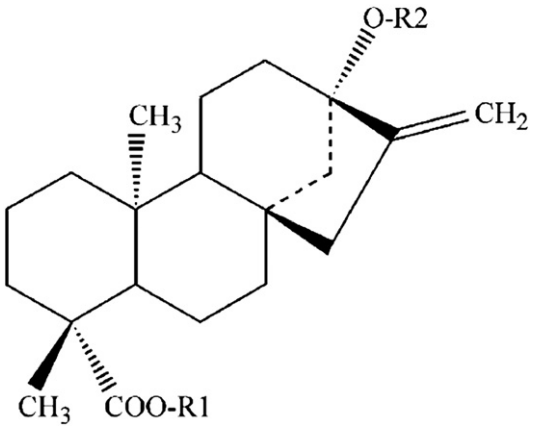

Fig. 1. Chemical structure of stevioside.

2.4. Cell separation and biological detection

The primary mouse mammary epithelial cells (MMECs) were isolated from BALB/c mice that had been pregnant for 15 days. The methods were referenced from previous studies [21,22]. The mammary gland tissue were collected, minced to paste, and incubated with a collagenase I/II/trypsin digestion mixture at 37 °C with shaking for 1 h. Then, the mixture was centrifuged at 250 (×*g*) for 5 min, and the supernatant was then removed. The tissue pellet was mixed together with DMEM/F12 and centrifuged three times at 250 (×*g*) for 5 min each. After treatment with EDTA and trypsin/EDTA, the sample was filtered with a 40-μm cell strainer to remove any remaining cell clumps and debris. The cells were suspended and transferred to a tissue culture flask and incubated three times for 1 h at 37 °C and 5 % CO_2.

The primary cells were cultured in DMEM:F12/1:1 containing 10% FCS, 0.5% transferrin, 0.1% T3 and 0.5% EGF at 37 °C with 5 % CO_2. The cell media were changed every 24 h. In all experiments, the MMECs were cultured in 6-well plates until the cells achieved a proper density of 1×10^6 cells/ml and had spread over the bottom of the well.

The cells cultured on coverslips were stained to identify cytokeratin-18 after achieved confluency [23], which is specifically expressed as a skeleton protein. The cells were incubated with cytokeratin-18 antibody and FITC-labeled goat anti-rabbit IgG (H + L). The cell nuclei were stained with Hoechst and observed under confocal laser scanning.

Table 1
Sequence of primers used in current investigation in FqRT-PCR.

Gene	Primer	Sequence 5′ > 3′	Product Size(bp)
TNF-α	Sense	CCTATGTCTCAGCCTCTTCTCAT	214
	Anti-sense	CACTTGGTGGTTTGCTACGA	
IL-1β	Sense	TGAAATGCCACCTTTTGACAG	185
	Anti-sense	CCACAGCCACAATGAGTGATAC	
IL-6	Sense	GGAGAGGAGACTTCACAGAGGA	103
	Anti-sense	ATTTCCACGATTTCCCAGAGA	
ERK	Sense	GCTCTGCCCTATTTCATCTTGT	162
	Anti-sense	ATCCAATCACCCACACACAG	
JNK	Sense	CTCCAGCACCCATACATCAAC	121
	Anti-sense	TCAGTTCTTTCCACTCCTCTATTG	
	Sense	TGAAATGCCACCTTTTGACAG	
	Anti-sense	AGACCGTTTCAGTCCATCATTC	
P38	Sense	ACACATCCAACAGACCAATCAC	100
	Anti-sense	ATTTCCACGATTTCCCAGAGA	
P65	Sense	CCAGAAGAGGAGAGGAGGGTAT	111
	Anti-sense	GGGATTTAGAGAAAAGGGGACTA	
IκB	Sense	TACCCCTCTACATCTTGCCTGT	238
	Anti-sense	GTGTCATAGCTCTCCTCATCCTC	
Caspase-3	Sense	ACTGGAAAGCCGAAACTCTTC	133
	Anti-sense	CATACAGGAAGTCAGCCTCCA	
Bax	Sense	GAGACACCTGAGCTGACCTTG	195
	Anti-sense	GAAGTTGCCATCAGCAAACAT	
Bcl-2	Sense	ATGTGTGTGGAGAGCGTCAAC	177
	Anti-sense	CAGCCAGGAGAAATCAAACAG	
β-actin	Sense	TAAAACGCAGCTCAGTAACAGTCGG	182
	Anti-sense	TGCAATCCTGTGGCATCCATGAAAC	