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Activation of liver X receptors inhibit LPS-induced inflammatory response in primary bovine mammary epithelial cells

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ABSTRACT

Liver X Receptors (LXRs) belong to the nuclear receptor superfamily, have been reported that activation of LXRs with synthetic ligands has anti-inflammatory effects in various inflammatory diseases. This study aims at investigating the effects of T0901317 (T0), a synthetic LXRs ligand, on lipopolysaccharide (LPS)-stimulated primary bovine mammary epithelial cells (bMECs). BMECs were stimulated by LPS in the presence or absence of T0. The results showed that treatment with T0 significantly inhibited LPS-induced tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) expression. LPS-induced NF-κB activation was also suppressed by T0. Furthermore, T0 was found to inhibit the translocation of TLR4 to lipid rafts. T0 could activate ATP-binding cassette transporter A1 (ABCA1) dependent pathway which induced cholesterol efflux from cells and disrupted the formation of lipid rafts. Thus, based on those findings we proposed that LXRs agonist might become a novel therapeutic target for inflammation.

1. Introduction

The innate immune system participated in the first line of defense of host to invading pathogens, particularly during the initiation of infection (Hoffmann et al., 1999). Lipopolysaccharide (LPS) is the cell wall component of the Gram-negative bacterium *E. coli*, which triggers strong immune responses in the mammary gland and bovine mammary epithelial cells. LPS initiates intracellular signaling by binding to a Toll-like receptor, TLR4, which requires formation of a complex, including LPS, LPS-binding protein, CD14 (LPS correceptor), TLR4, and adaptor protein MD-2. Initial studies demonstrated that activation of TLR4 engaged a set of TLR signaling cascades, which eventually mediated activation of the transcriptional factor NF-κB, thereby resulting in cytokines production (Lu et al., 2008; Palsson-McDermott and O'Neill, 2004; Triantafilou and Triantafilou, 2005).

Translocation of TLR4 into lipid rafts attributes to the initiation of LPS intracellular signaling. Lipid rafts are liquid-ordered (l_o) phase microdomains on biological membranes that enriched in cholesterol, sphingomyelin, saturatedfatty acids and specific patterns of receptors and signaling proteins (Eggeling et al., 2009; Lingwood and Simons, 2010; Sonnino and Prinetti, 2013). ATP-binding cassette transporter A1 (ABCA 1) is an integral transmembrane lipid transporter. ABCA1 functions in mediating cellular cholesterol efflux via the reverse cholesterol transport pathway (Li et al., 2013; Yvan-Charvet et al., 2010). Recruitment of TLR4 into the plasma membrane lipid rafts has been observed upon LPS stimulation (Olsson and Sundler, 2006; Triantafilou et al., 2002). Increasing evidence indicates that disruption of lipid rafts could inhibit LPS-induced inflammatory responses (Nakahira et al., 2006; Olsson and Sundler, 2006). Liver X receptors (LXRs) are ligand-dependent nuclear transcription factor, which regulate the expression of a number of genes involved in cholesterol metabolism. Two isoforms of LXR α and β have been identified, while LXRβ is expressed ubiquitously, LXRα is expressed predominantly in spleen, liver, intestine, adipose and lung (Rebe et al., 2012). The LXRs are activated by natural ligands (e.g., oxysterols) (Janowski et al., 1999; Janowski et al., 1996) and by synthetic ligands (e.g.T0) (Shi et al., 2014; Wang et al., 2011). Once activated, the nuclear receptor forms a heterodimer with Retinoid X Receptor (RXR), which binds to the LXR response element (LXRE) on DNA, leading to transcription of various genes important in cholesterol metabolism.

In the past, most studies were focused on the role of LXRs on the regulation of cholesterol and lipid metabolism. Studies performed during the recent decade have showed that LXRs modulate the development of inflammatory responses (Korf et al., 2009; Zelcer and Tontonoz, 2006). Activation of LXR pathway in inflammatory cells leads to the decreases of pro-inflammatory cytokines (TNFα, iNOS, IL-6, COX-2, IL-1β) production (Joseph et al., 2003; Myhre et al., 2008). In addition to these *in vitro* anti-inflammatory effects, LXRs strongly exert anti-inflammatory effects in the mouse models of inflammation, such as contact dermatitis (Joseph et al., 2003), atherosclerosis (Joseph et al.,

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2002) and lung injury (Birrell et al., 2007; Wang et al., 2011). Furthermore, controlling LXR activation may prove useful in regulating milk fat production in lactating dairy cows. T0901317 is one of the most commonly used in experimental studies which activate both LXR isoforms with potency. Our previous study has been investigated that LXRs agonist T0901317 had a protective effect on LPS induced mastitis in mice (Fu et al., 2014a). In the study, we aimed to explore further the possible role of T0 on the modulation of LPS-stimulated inflammation in bovine mammary epithelial cells (bMECs) and elucidate the potential mechanism.

2. Materials and methods

2.1. Materials

T0901317, Dimethyl sulfoxide (DMSO), Lipopolysaccharide (LPS, *Escherichia coli* 055: B5), and 3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide (MTT) were all provided by Sigma Chemical Co. (St. Louis, MO, USA). DMEM medium/nutrient mixture F-12 Ham (DMEM/F–12 K) and fetal bovine serum (FBS) were obtained from Hyclone. For Western blot assays, the following Abs were used: LXRα (Abcam; ab176323), ABCA1 (Abcam; ab18180), GM1 (Abcam; ab23943), TLR4 (Cell Signaling Technology; 14358), NF-κB (Cell Signaling Technology; 3033), IκBα (Cell Signaling Technology; 2859), HRP-conjugated goat anti-rabbit antibodies (GE Healthcare; RPN4301). All other chemicals were of reagent grade.

2.2. Primary bovine mammary epithelial cell culture

Epithelial cells from the mammary tissues of healthy Holstein cows in mild- and late-lactation were prepared in accordance with the established methods described previously (Pantschenko et al., 2000). The experiment was approved by Jilin Animals Protection Association. Briefly, mammary tissues were collected from five lactating cows. Isolated cells were plated in plastic culture dishes in DMEM/F–12 K supplemented with 10% FBS, 100 U/mL penicillin and streptomycin, 5 mM sodium acetate, 5 μg/mL hydrocortisone, 10 μg/mL insulin and 1 μg/mL amphotericin B. At 72 h of culture, primary mammary epithelial cells formed monolayer island aggregates free of fibroblasts, which were detached by selective trypsinization. Cells from passages 2–5 were used for further research. The cells were cultured in DMEM/F–12 K containing 10% FBS and 1% of penicillin–streptomycin at 37 °C in an incubator with 5% CO_2.

2.3. Cell viability

To measure cell viability, bMECs were plated at a density of 2×10^5 cells/mL in 96-well plates in a 37 °C, 5% CO_2 incubator for 4 h, then the cells were pretreated with T0 (2.5, 5, 10 μM) for 18 h in the absence or presence LPS (1 μg/mL). The cells were washed twice with PBS, and then 10 μL MTT (5 mg/mL) was added to each well and further incubated at 37 °C for 4 h. The supernatant was removed and the formazan product dissolved with 150 μL/well of DMSO. The optical density was measured at 570 nm on a microplate reader (TECAN, Austria).

2.4. RNA extraction and real-time quantitative polymerase chain reaction

BMECs were treated with various concentrations of T0 (2.5, 5, 10 μM) for 12 h followed by incubation by LPS (1 μg/mL) for 3 h. Total RNA was extracted from bMECs using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's protocol. Total RNA concentration and purity were measured with a spectrophotometer, and 5 μg of RNA was reverse-transcribed into cDNA with the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Primers were designed based on sequences from the National Center for Biotechnology Information Database. As shown in Table 1. QRT-PCR was performed using FastStart Universal SYBR® Green Master (Roche) on a Real-time PCR System (Applied Biosystems, Carlsbad, California, USA) in 25 μL reactions. The PCR reactions were carried out as follows: activation at 50 °C for 2 min and 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. Data were expressed as relative gene expression = $2^{-\Delta\Delta Ct}$.

Table 1
Primers used in this study.

Gene	Primer	Sequence 5′ > 3′	Product size (bp)
TNF-α	Sense	GCCTCCCTCTCATCAGTTCTA	246
	Anti-sense	GGCAGCCTTGTCCCTTG	
IL-1β	Sense	ACCTGTGTCTTTCCCGTGG	162
	Anti-sense	TCATCTCGGAGCCTGTAGTG	
IL-6	Sense	AGTTGTGCAATGGCAATTCTGA	223
	Anti-sense	CCCCAGCATCGAAGGTAGA	
GAPDH	Sense	TGCTGTCCCTGTATGCCTCT	224
	Anti-sense	TTTGATGTCACGCACGATTT	
β-actin	Sense	TCACCAACTGGGACGACA	206
	Anti-sense	GCATACAGGGACAGCACA	

2.5. Western blotting

BMECs were seeded into six-well plates and incubated for 12 h, then pretreated with various concentrations of T0 (2.5, 5, 10 μM) for 12 h followed by stimulation with 1 μg/mL LPS for 3 h. The cells were harvested and washed twice with cold PBS. Protein lysates from the cells were prepared with mammalian protein extraction reagent (Thermo Fisher Scientific). Lysates were precipitated at 12000 rpm for 8 min at 4 °C. Total protein content was determined using BCA method. The supernatants were mixed with one quarter volume of 4 × SDS sample buffer, boiled for 10 min and stored at −20 °C. Next, Equal amounts of protein extracts (50 μg) were separated by 10% SDS-polyacrylamide gel and transferred onto the PVDF membrane, then blocked with 5% non-fat milk in 0.05% TBST. Membranes were incubated with primary antibodies (1:1000 dilution) at 4 °C overnight, and then washed 3 times with TBST for 10 min before incubation with a 1: 2000 dilution of HRP-conjugate goat anti-rabbit antibodies. Finally, blots were generated with the ECL plus Western Blotting Detection System (Amersham Life Science, UK).

2.6. Isolation of lipid rafts and quantification of cholesterol levels in lipid rafts

The lipid raft domains were isolated from bMECs as previously described (Kim et al., 2004). Briefly, bMECs were lysed in ice-cold MBS buffer (25 mM MES, pH 6.5, 150 mM NaCl, 1 mM Na3VO4, 1% Triton X-100), and then incubated on ice for 30 min. Lysates were mixed with 4 mL of 40% sucrose by mixing with 2 mL of 80% sucrose, and then this was overlaid with 4 mL of 35% sucrose solution in MBS buffer, followed by 4 mL of 5% sucrose in MBS buffer. Samples were centrifuged at 100,000*g* for 18 h and fractionated into 12 subfractions. The levels of cholesterol in lipid rafts were assayed by gas–liquid chromatography as previously described (Fu et al., 2014b).

2.7. Cholesterol replenishment experiment

BMECs were treated with culture medium alone or medium containing T0 (2.5, 5, 10 μM) at 37 °C for 12 h. BMECs then were washed with PBS and incubated in the absence or presence of cholesterol (84 μg/mL) for 1 h. The cells were exposed to LPS (1 μg/mL) for 3 h. The translocation of TLR4 to lipid rafts was analyzed by western blotting as mentioned above.