International Immunopharmacology 11 (2011) 1740–1746

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

The effect of taurine on the toll-like receptors/nuclear factor kappa B (TLRs/NF-κB) signaling pathway in *Streptococcus uberis*-induced mastitis in rats

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ARTICLE INFO

Article history:
Received 5 May 2011
Received in revised form 16 June 2011
Accepted 17 June 2011
Available online 13 July 2011

Keywords:
Taurine
Streptococcus uberis
Mastitis
Rat
TLRs/NF-κB signaling pathway

ABSTRACT

To investigate whether taurine ameliorates mammary damage in a rat model of *S. uberis* mastitis by suppressing inflammation related to the toll-like receptors/nuclear factor kappa B (TLRs/NF-κB) signaling pathway. Starting on gestation day 14 and continuing until parturition, 100 mg/kg of taurine (group TS) or an equal volume of physiological saline (group CS) was administered daily to rats. Seventy-two hours after parturition, rats were infused with 100 cfu of *S. uberis* into each of 2 mammary glands. The resultant inflammation, evidenced by swelling, degeneration of secretory epithelium, increased tissue loss and neutrophil (PMN) infiltration was observed. Pretreatment with taurine attenuated inflammatory changes and significantly decreased mRNA expression of TLR-2 (8 h post *S. uberis*-injection, PI), NF-κB p65 (16 h and 24 h PI), and NF-κB DNA binding activity (16 h PI). Tumor necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS) levels were also decreased. Significant differences ($P<0.05$) were present at 24 h and 48 h PI for TNF-α and at 16 h PI for iNOS. TLR-4 mRNA expression was increased by taurine administration and significant differences were observed at 8 h, 16 h and 24 h PI. These results suggest that the in vivo relationship of immunomodulatory reagents with TLRs is complex. Taurine may modulate inflammatory injury induced by *S. uberis* in mammary glands though TLR-2 and TLR-4. Suppression of inflammation may be related to TLRs/NF-κB and may be one mechanism of taurine action in controlling *S. uberis* mastitis.

1. Introduction

Streptococcus uberis (*S. uberis*) is an environmental pathogen of emerging importance as a mastitis causing organism in dairy animals. Conventional measures are ineffective in controlling this pathogen and *S. uberis* accounts for approximately 33% of the isolates obtained from clinical cases of bovine mastitis [1,2]. Therefore, new and innovative approaches for *S. uberis* mastitis control are needed. In recent years, research has focused on regulating the natural defense mechanisms of the mammary gland during periods of heightened disease susceptibility through the application of a variety of immunomodulatory compounds [3–7].

Taurine (2-aminoethane sulfonic acid) is the most abundant free amino acid in most animal tissues and plays an important role in several essential biological processes [8]. It is a membrane stabilizer and regulates calcium flux. Taurine possesses antioxidant properties and regulates the release of proinflammatory cytokines in animals and humans [9–11]. A large number of reports suggests a key role of taurine and its derivatives in the innate immune response and suggests its use in the prevention and treatment of various topical infections and chronic inflammatory diseases [12–14].

Toll like receptors (TLRs) play a central role in driving host inflammatory responses and promoting adaptive immunity following infection. They accomplish these functions by serving as host sensors of conserved structural components of viruses, bacteria, fungi, and protozoa [15–17]. The structure of TLRs defines the exquisite sensitivity of receptors toward microbial structures. TLRs have an ectodomain that contains leucine-rich repeats, a transmembrane region and a cytoplasmic Toll/IL-1-Receptor (TIR) domain. The TIR domain interacts with myeloid differentiation primary response protein 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) to initiate signaling which ultimately induces the expression of numerous immune response genes through activation of activator protein (AP)-1, nuclear factor kappa B (NF-κB) and other transcription factors [18,19]. Taurine has the ability to regulate the inflammatory process in vivo by inhibiting the activation of NF-κB and thus inhibiting the generation of proinflammatory cytokines [20]. To our knowledge, the relationship between taurine regulation of the inflammatory process and the changes in TLRs has not been defined. The study described herein was conducted to determine the effect of taurine on the TLRs/NF-κB signaling pathway in *S. uberis*-induced mastitis in rats.

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doi:10.1016/j.intimp.2011.06.008

2. Materials and methods

2.1. Animals

Seventy-two, healthy, pregnant, SD rats (five months old and weighing 300–350 g) were purchased from the Experimental Animal Center of Southeast University (Nanjing, China). They were housed in individual cages and provided water and food ad libitum. Following acclimatization, rats were randomly divided into two groups (each n = 36). The experiments followed the guidelines of the regional Animal Ethics Committee.

2.2. Treatment

Starting on gestation day 14, taurine 100 mg/kg (dissolved in sterile pyrogen-free physiological saline, group TS) or an equal volume of physiological saline (group CS) was administered to each rat by gavage each morning until parturition. Seventy-two hours after parturition, 30 rats from each group were infused with 100 cfu of *S. uberis* (*S. uberis* 0140J, capsular strain, ATCC) in 100 μL physiological saline each into the left 4 (L4) and right 4 (R4) teats. Six rats from each group served as controls. The offspring were weaned 2 h prior to *S. uberis* inoculation. Following administration of ether anesthesia, the teat area of L4 and R4 was moistened with 75% ethanol, a 33-gage needle fitted to a 1-mL syringe was gently inserted into the mammary duct, and 100 μL of *S. uberis* was slowly infused. Just prior to inoculation (control group defined as 0 h) and at 8, 16, 24, 48 and 72 h post *S. uberis*-injection (PI), six rats at each time point were euthanized, and mammary tissues were aseptically collected and stored at −70 °C until analyzed.

2.3. Preparation of mammary tissue

Mammary tissues were weighed placed on ice and homogenized (Kinematica AG Switzerland) with sterile physiological saline (1:4, W/V) and then centrifuged at 2000×*g* for 40 min at 4 °C. Fat was removed and the supernatants were collected and centrifuged again at 2000×*g* for 20 min at 4 °C to remove any remaining fat. The resultant supernatants below the fat were collected and stored at −20 °C for later analysis. Protein concentration was determined using the Bradford method.

2.4. Histologic examination

Tissue specimens were fixed in 10% formalin for 24 h. Standard dehydration and paraffin-wax embedding procedures were used to produce tissue blocks. Hematoxylin and eosin stained slides were prepared using standard methods. The numbers of PMNs in alveoli and histologic changes in mammary tissue were observed by light microscopic (Olympus BH2 Japan) analyses at a magnification of 100×. Four sections of rat mammary tissue were scored for each animal. Ten fields were selected randomly per tissue section and assigned a score of 1, 2 or 3 according to the number of PMNs in the microscopic field, where 1 = none or few PMNs present, 2 = moderate PMN infiltration, and 3 = marked PMN infiltration. Areas of tissue loss were semi-quantified using a scoring system where 1 = less than 20% adipose tissue present per 100× field, 2 = 21% to 50% adipose tissue, and 3 = more than 50% adipose tissue. Hemorrhage and degeneration in the mammary alveoli were scored as 1 = no or minimal hemorrhage and degeneration, 2 = mild hemorrhage and degeneration, and 3 = severe hemorrhage and degeneration [21]. Evaluation of each section was conducted in a blinded fashion by two of the authors.

2.5. RNA extraction and RT-PCR

2.5.1. RNA extraction

Total RNA was extracted from mammary tissue using TRIZOL reagent (TaKaRa, Dalian, China) according to the manufacturer's protocols. The concentration was quantified by measuring absorbance at 260 nm (Eppendorf Biophotometer). The ratios of absorption (260/280 nm) of all samples were between 1.8 and 2.0. Aliquots of the RNA samples were subjected to electrophoresis through a 1.4% agarose formaldehyde gel to verify their identity.

2.5.2. Real-time quantitative RT-PCR

Synthesis of first strand complementary DNA (cDNA) was performed with reverse transcriptase and Oligo$(dT)_{18}$ primer (TaKaRa, Dalian, China), according to the manufacturer's instructions. The final volume of 20 μl contained 10 units of AMV reverse transcriptase, 1 mM dNTP mixture (TaKaRa, Dalian, China), 20 units of recombinant RNasin ribonuclease inhibitor (TaKaRa, Dalian, China), and 50 pmol of Oligo$(dT)_{18}$ primer. After incubation (42 °C, 60 min), the mixture was heated (95 °C, 5 min). An aliquot of the cDNA samples was mixed with 25 μl SYBR® Green PCR Master Mix (TaKaRa, Dalian, China) in the presence of 10 pmol of each forward and reverse primer for TLR-2, TLR-4 and NF-κB p65 (Table 1) and then subjected to PCR under standard conditions (43 cycles). As an internal control, the same RT products were subjected to PCR in the presence of a second pair of primers specific to rat β-actin. All primer sequences were synthesized by Invitrogen Biological Company (Shanghai, China). Mixtures were incubated in an ABI Prism 7300 Sequence Detection System (Applied Biosystems) programmed to conduct one cycle (95 °C for 10 min) and 43 cycles (95 °C for 15 s and 62 °C for 1 min). Results (fold changes) were expressed as $2^{-\Delta\Delta Ct}$ with $\Delta\Delta Ct = (Ct\ ij - Ct\ \beta\text{-actin}\ j) - (Ct\ i1 - Ct\ \beta\text{-actin}1)$, where Ct ij and Ct β-actin j are the Ct for gene i and for β-actin in a sample (named j), and where Ct i1 and Ct β-actin1 are the Ct in sample 1, expressed as the standard. In this study, 0 h for the CS group is determined as standard, thus leading to a relative expression of $1 = 2^0$ at this time point.

2.6. Detection of tumor necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS)

The levels of TNF-α were measured by radioimmunoassay. Commercial kits were purchased from the Institute of Radiation of Science and Technology Development Center of the General Hospital of People's Liberation Army (Beijing, China). The assay was conducted following the protocol of the manufacturer. All samples and standards were assayed in triplicate. The inter- and intra-assay coefficients of variation at the concentrations obtained in these experiments were 6.4 and 2.4% and the detection limit was 0.3 ng/g protein.

The activity of iNOS was determined using commercial kits purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). iNOS activity in homogenates of mammary tissue and serum were evaluated following the manufacturer's protocols.

Table 1
Sequences of oligonucleotides used for PCR.

Target gene	Accession number	Orientation	Primers sequence (5′-3′)
β-actin	NM_031144	Forward	CCCTGTGCTGCTCACCGA
		Reverse	ACAGTGTGGGTGACCCCGTC
TLR-2	NM_198769.2	Forward	CGCTTCCTGAACTTGTCC
		Reverse	GGTTGTCACCTGCTTCCA
TLR-4	NM_019178	Forward	GATTGCTCAGACATGGCAGTTTC
		Reverse	CACTCGAGGTAGGTGTTTCTGCTAA
NF-κB p65	AF079314	Forward	CTTCTGGGCCATATGTGGAGAT
		Reverse	TCGCACTTGTAACGGAAACG