International Immunopharmacology 16 (2013) 429–435

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

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

The role of NADPH oxidase in taurine attenuation of *Streptococcus uberis*-induced mastitis in rats

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ARTICLE INFO

Article history:
Received 26 October 2012
Received in revised form 4 May 2013
Accepted 6 May 2013
Available online 26 May 2013

Keywords:
Streptococcus uberis
Mastitis
Taurine
p47phox
p22phox

ABSTRACT

In order to evaluate the role of taurine on the oxidative stress mediated by NADPH oxidase in *Streptococcus uberis*-induced (*S. uberis*) mastitis, rats were administered daily (per os) 100 mg/kg of taurine (group TS) or an equal volume of physiological saline (group CS) from gestation day 14 until parturition. Seventy-two hours after parturition, approximately 100 cfu of *S. uberis* was infused into each of 2 mammary glands. Pretreatment with taurine significantly decreased mRNA and protein expression of p47phox and p22phox in mammary tissues. The total anti-oxidation capability (T-AOC) levels and superoxide dismutase (SOD) activities decreased, while malondialdehyde (MDA) levels increased both in mammary tissues and serum of rats with intramammary *S. uberis* infusion. Gavage administration of taurine moderated this change. Concentrations of interleukin-1β (IL-1β) and IL-6 in mammary glands decreased as a result of taurine administration. Significant differences ($P < 0.05$) were present at 48 and 72 h post *S. uberis*-infusion (PI) for IL-1β and 72 h PI for IL-6. Our data indicate that, in *S. uberis*-induced mastitis, taurine has the ability of regulating redox conditions which leads to the suppression of oxidative stress and secretion of proinflammatory cytokines. This phenomenon may be ascribed to taurines's ability to inhibit the expression of NADPH oxidase.

1. Introduction

Streptococcus uberis (*S. uberis*) is an environmental pathogen of emerging importance as a mastitis causing agent in dairy animals. It accounts for approximately 33% of the isolates obtained from clinical cases of bovine mastitis [1,2]. A better understanding of the pathogenesis of *S. uberis* infection is requisite in controlling this disease. Thomas et. al. in 1994 observed that the acute inflammatory response was accompanied by large numbers of neutrophils (PMNs) in the secretory acini of bovine mammary glands following *S. uberis* infusion [3]. However, phagocytosis and killing of this pathogen by PMNs has been shown to be ineffective in preventing infection. Conversely, phagocytosis of *Escherichia coli* and *Staphylococcus aureus* by bovine PMNs has been well documented [4–6]. At the infection site, the accumulated PMNs activate the release of antibacterial peptides, proteases and reactive oxygen species (ROS) (i.e., superoxide anion, hydrogen peroxide, hydroxyl radicals and hypochlorous acid) which cause tissue injury if overproduced [7,8]. ROS play crucial roles in various physiological processes, including innate immunity, modulation of redox-dependent signaling cascades, and as cofactors in the production of hormones and pro-inflammatory cytokines [7]. NADPH oxidases, which are expressed in most mammalian cell types, catalyze the generation of ROS. Increasing evidence suggests that NADPH oxidases and ROS are involved in inflammatory diseases such as rheumatoid arthritis, diabetic nephropathy and inflammatory bowel disease, among others [9–11]. To our knowledge, the role of NADPH oxidase in *S. uberis* mastitis has not been defined.

Taurine is the most abundant free amino acid in most animal tissues and plays an important role in several essential biologic processes. A large number of reports have demonstrated the key role of taurine and its derivatives in the innate immune response and suggest its use in the treatment of various topical infections and chronic inflammatory diseases [12–14]. Our previous studies revealed that taurine has the ability to increase T regulatory cells, down-regulate inflammatory signaling pathways and protect the mammary gland from damage from the *S. uberis* challenge [15,16]. The protection by taurine and its derivatives on inflammatory injury may be related to NADPH oxidase. For example, Choi HS et. al. in 2006 established that taurine chloramine inhibits PMA-stimulated superoxide production in human neutrophils by inhibiting phosphorylation and translocation of p47phox, a requirement for the translocation of cytosolic NADPH oxidase to the plasma membrane, eventually blocking the assembly of NADPH oxidase complex [17]. Das et. al. in 2012 found that taurine administration decreased elevated blood glucose and proinflammatory cytokine levels, reduced renal oxidative stress through inhibiting the activity of NADPH oxidase and was beneficial in regulating diabetes-associated renal complications [11]. These findings led to our interest in the role of taurine

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http://dx.doi.org/10.1016/j.intimp.2013.05.007

on oxidative stress mediated by NADPH oxidase in *S. uberis*-induced mastitis. Herein, we report the role of NADPH oxidase in a rat model of taurine attenuation of *S uberis*-induced mastitis.

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 containing 36 animals. 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 daily to each rat by gavage and continued until parturition. Seventy-two hours after parturition, 30 rats from each group were infused with 100 cfu in 100 μL of *S. uberis* (*S. uberis* 0140J, capsular strain, ATCC) into the left 4th (L4) and right 4th (R4) teats. Six rats from each group served as controls. The offspring were weaned 2 h prior to experimental infusion. Following administration of ether anesthesia, the teat area of L4 and R4 were moistened with 75% ethanol, a 33-gauge needle fitted to a 1-mL syringe was gently inserted into the mammary duct, and 100 μL of *S. uberis* were slowly infused. Just prior to inoculation (control group defined as 0 h) and at 8, 16, 24, 48 and 72 h post *S. uberis*-infusion (PI), six rats at each time point were euthanized and serum and mammary tissues aseptically collected. Serum was stored at −20 °C and mammary tissues at −70 °C until analyzed.

2.3. Preparation of mammary tissue and serum

Mammary tissues were weighed and homogenized (Kinematica AG, Switzerland) with sterile physiological saline (1:4, W/V) on ice and then centrifuged at 2000 ×*g* for 40 min at 4 °C. Fat was removed, and the supernatant was collected and centrifuged again at 2000 ×*g* for 20 min at 4 °C to remove any remaining lipid. The supernatant was collected and stored at −20 °C for later analysis. Protein concentration was determined using the Bradford method. Serum was separated by centrifugation (2000 ×*g*, 15 min) and stored at −20 °C until analyzed.

2.4. RNA extraction and RT-PCR

2.4.1. RNA extraction

Total RNA was extracted from mammary tissue using TRIzol reagent (TaKaRa Biotechnology (Dalian) Co., Ltd., 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 identities.

2.4.2. Real-time quantitative RT-PCR

Synthesis of first strand complementary DNA (cDNA) was performed with reverse transcriptase and Oligo$(dT)_{18}$ primer (TaKaRa Biotechnology (Dalian) Co., Ltd., 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 Biotechnology (Dalian) Co., Ltd., Dalian, China), 20 units of recombinant RNasin ribonuclease inhibitor (TaKaRa Biotechnology (Dalian) Co., Ltd., 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 were mixed with 25 μL of SYBR® Green PCR Master Mix (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China) in the presence of 10 pmol of each forward and reverse primer for p47phox and p22phox (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 1 cycle at 95 °C for 10 min, 43 cycles at 95 °C for 15 s and 62 °C for 1 min. The 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.

2.5. Total protein extraction and Western blotting

Total protein extracts were prepared from frozen mammary samples using commercial kits (Pierce Biotechnology, USA). Protein concentrations were determined by bicinchoninic acid (BCA) assay and samples were stored at −70 °C until analyzed.

Tissue extract (60 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, MA, USA). The membrane was blocked for 1 h with 5% non fat milk in TBST and then incubated with a rabbit polyclonal antibody against p47phox or p22phox (Santa Cruz Biotechnology, Inc., CA, USA) at 4 °C overnight. After washing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (1:5000; Wuhan Boster Biological Technology Ltd., Wuhan, China) for 60 min at room temperature. After additional washing, bound conjugates were detected by ECL superSignalTM West Pico substrate (Pierce Biotechnology, USA). The PVDF membrane was quantified by scanning densitometry (Genomic Solutions Inc., Ann Arbor, MI). Mouse anti-GAPDH monoclonal antibody (Bioworld Technology, Inc.) was used as the loading control and p47phox or p22phox protein expression was normalized to GAPDH. The data from each time point from each group were expressed as relative integrated intensity compared with 0 h of the CS group (determined as standard).

2.6. Analyses of total anti-oxidation capability (T-AOC) of superoxide dismutase (SOD) and malondialdehyde (MDA)

SOD, T-AOC and MDA levels in mammary gland homogenate were determined using commercial kits purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The analyses were carried out according to the manufacturer's protocols with a Unic-2100 spectrophotometer. Briefly, the ferric reducing antioxidant

Table 1
Sequences of oligonucleotides used for PCR.

Target gene	Accession number	Orientation	Primer sequence (5′–3′)
β-Actin	NM_031144	Forward	CCCTGTGCTGCTCACCGA
		Reverse	ACAGTGTGGGTGACCCCGTC
p47phox	AY029167.1	Forward	CCTTCATTCGCCACATCG
		Reverse	ATCATACCACCTGGGAGC
p22phox	AJ295951.1	Forward	CCTCCACTTACTGCTGTCCG
		Reverse	GCCTCACTTGGCTTCTTGC