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Immunomodulatory effects of thionin Thi2.1 from *Arabidopsis thaliana* on bovine mammary epithelial cells

Marisol Báez-Magaña, Violeta Díaz-Murillo, Joel E. López-Meza, Alejandra Ochoa-Zarzosa*

Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo, Km 9.5 Carr. Morelia-Zinapécuaro, Posta Veterinaria, C.P. 58893 Morelia, Michoacán, Mexico

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ABSTRACT

Antimicrobial peptides (AMPs) are key elements of plant defense mechanisms, resembling conserved protection strategies also present in mammals. Among the AMPs, plant thionins are particularly interesting due that display antibacterial and antifungal activities. In *Arabidopsis thaliana* have been described four thionins: Thi2.1, Thi2.2, Thi2.3 and Thi2.4. Work from our group shows that Thi2.1 expressed by bovine endothelial cells has direct antibacterial activity against *Staphylococcus aureus* mastitis isolates, bacteria able to persist inside bovine mammary epithelial cells (bMECs). Thus, the objective of this work was to analyze the immunomodulatory effects of the AMP thionin Thi2.1 from *A. thaliana* on bMECs during *S. aureus* infection. According to the results, *S. aureus* internalization into bMECs was reduced in cells pre-treated with Thi2.1 at 5 and 10 μg/mL during 24 h, effect related to the participation of TLR2. In addition, bMECs pre-treated with Thi2.1 (24 h) significantly increased TNF-α (~2-fold) and IL-6 (~7-fold), whereas decreased IL-10 gene expression (~0.5-fold). Interestingly, Thi2.1 inhibits the up-regulation induced by *S. aureus* of TNF-α and IL-10 gene expression, as well as NO production. In addition, Thi2.1 (10 μg/mL) up-regulates the expression of the chemokine IL-8 (~3-fold) in infected bMECs. Some of these effects are related to TLR2 activation. In this sense, Thi2.1 also reduces *S. aureus*-induced TLR2 gene expression and membrane abundance. In conclusion, Thi2.1 from *A. thaliana* modulates bMEC innate immune response by inducing the production of pro- and anti-inflammatory molecules while inhibits *S. aureus* internalization. Some of these effects are mediated by TLR2.

1. Introduction

Arabidopsis thaliana is a prototypical organism for plants. Some of its relevant characteristics include: a small size that favors its growth, short generation time, and facilities for seed production [1]. In addition, *A. thaliana* research has provided useful information in the host-pathogens interactions study, being the key instrument in the understanding of the molecular details of the plant immune system [2].

Antimicrobial peptides (AMPs) are key elements of plant defense mechanisms, which resemble conserved defense mechanisms also present in mammals. AMPs are widespread throughout the plant kingdom, including defensins, lipid transfer proteins, knottins, heveins, snakins and thionins, among others [3]. Each of these AMPs are cationic secreted peptides, which possess a characteristic number of cysteine pairs. These form disulfide bridges in specific three-dimensional folds [4]. Members belonging to these classes are active *in vitro* against fungi and bacteria, oomycetes and herbivorous insects, and their activity has been also demonstrated in transgenic plants [5].

Among the AMPs, plant thionins are particularly important. These peptides have been identified in plants such as barley and wheat. Thionins have antibacterial and antifungal activities, and contain a conserved cysteine-rich domain that shows antimicrobial and cytotoxic properties [6]. Thionins are small basic peptides (44–47 amino acids), characterized by a three-dimensional structure stabilized by six to eight disulfide-linked cysteines. Thionins are classified into two groups, α/β-thionins and γ-thionins (which should more appropriately be called defensins), in relation to their 3-D structure. α/β-Thionins possess two α-helixes, double-stranded β-sheets and a C-terminal coil region, whereas γ-thionins are composed by two amphipathic layers (α/β sandwich), each containing one α-helix and three anti-parallel β-sheets [6]. In *A. thaliana* have been described the thionins: Thi2.1, Thi2.2, Thi2.3 and Thi2.4 [7].

Work from our group and other authors, shows that Thi2.1 has antibacterial, antifungal and cytotoxic activity [8,9]. In addition, in a previous work we reported that thionin Thi2.1 expressed in bovine endothelial cells has direct antibacterial activity against *Staphylococcus aureus* mastitis isolates [10]. We have also reported that bovine mammary epithelial cells (bMECs) treated with conditioned medium

* Corresponding author.
E-mail address: ochoaz@umich.mx (A. Ochoa-Zarzosa).

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containing Thi2.1 reduce the internalization rate of *S. aureus* [11]. However, we do not know if this effect is achieved through the modulation of the innate immune response of bMECs. Recently, we demonstrated that a plant defensin (erroneously named γ-thionin) from *Capsicum chinense* shows immunomodulatory activity in bMECs infected with *S. aureus* [12]. Thus, the aim of this work was to evaluate the immunomodulatory effects of the thionin Thi2.1 from *A. thaliana* in the aforementioned model.

2. Materials and methods

2.1. Peptide

The thionin Thi2.1 used in this work corresponds to the mature region (NH_2-KI**CC**PSNQARNGYSV**C**RIRFSKGR**C**MQVSG**C**QNSDT**C**PRG WVNA-COOH) (Genbank L41244.1). This peptide was chemically synthesized and obtained from Invitrogen (Fig. S1). The formation of disulfide bonds was accomplished by air oxidation using 5% (v/v) aqueous dimethyl sulfoxide, which dissolves 5 mg/mL of Thi2.1 for 24 h at room temperature. The bond formation of Thi2.1 was confirmed by TOF-mass spectrometry (Fig. S1). For all of the experiments, the final concentration of vehicle DMSO was 0.02%, which was considered as the control. Additionally, Thi2.1 biological activity was confirmed by evaluating its antifungal effect against *Candida albicans* ATCC 10231 at 200 μg/mL (data not showed). In this study, Thi2.1 was employed at a range of concentrations of 0.1 to 10 μg/mL, in agreement with reports from our group describing the immunomodulatory effects of these concentrations for other plant AMPs [12].

2.2. Staphylococcus aureus strain

The American Type Culture Collection (ATCC) *S. aureus* subsp. *aureus* 27543 strain isolated from a case of clinical mastitis was used, which has the capacity to invade bMECs [13]. The bacteria were grown at 37 °C overnight in Luria-Bertani broth (LB, Bioxon). The colony forming units (CFU) were adjusted by optical density determination (O.D. 600 nm).

2.3. Primary culture of bMECs

Isolation of bMECs was performed from the alveolar tissue of udders from lactating cows as described [13]. For this study, we used cells from passages 2 to 8 at 80%–90% confluence, which were cultured (in 5% CO_2 at 37 °C) in Petri dishes (Corning-Costar) using growth medium (GM) composed of: DMEM medium/nutrient mixture F-12 Ham (DMEM/F-12 , Sigma) supplemented with 10% fetal calf serum (Equitech-Bio), 10 μg/mL insulin (Sigma), 5 μg/mL hydrocortisone (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco) and 1 μg/mL amphotericin B (Invitrogen).

2.4. Effects of thionin Thi2.1 on S. aureus 27543 growth and bMEC viability

To analyze the effect of thionin Thi2.1 on *S. aureus* growth, 9×10^7 CFU/mL were cultured at 37 °C in LB broth (O.D. 0.2), 1 mL of this culture was incubated with different concentrations of thionin for 24 h. A 1×10^6-fold dilution from this culture was plated on LB-Agar dishes and incubated for 18 h at 37 °C, in order to determine the number of CFUs. For all of the experiments we used controls of gentamicin-treated *S. aureus* (50 μg/mL) and bacteria grown in LB broth with vehicle. To analyze the effect of Thi2.1 on bMEC viability, 5×10^3 cells were incubated with the AMP (0.1–10 μg/mL) for 24 h at 37 °C in a 96-well flat-bottom plate in DMEM/F-12 medium without antibiotics and serum. Next, viability was determined by a trypan blue exclusion assay (Sigma). Controls of bMECs cultured in incomplete medium or Triton 0.1% (Sigma) were included for all of the experiments. Viability of bacteria and bMECs was also monitored by MTT assays (data not shown).

2.5. Effect of thionin Thi2.1 on S. aureus 27543 internalization into bMECs

The effect of Thi2.1 on *S. aureus* internalization into bMECs was evaluated by the gentamicin protection assay as described [13], using a MOI 30:1 bacteria per cell. To this, bMECs were inoculated with bacterial suspensions (9.2×10^7 CFU/mL) and incubated for 2 h in 5% CO_2 at 37 °C. Then, bMECs were washed three times with PBS (pH 7.4) and incubated in DMEM/F-12 K medium without serum supplemented with 50 μg/mL gentamicin for 1 h at 37 °C to kill extracellular bacteria. bMEC monolayers were detached with trypsin (0.05%)-EDTA (0.02%) (Sigma) and lysed with 250 μl of sterile distilled water. bMECs lysates were plated on LB agar using a 100-fold dilution and incubated overnight at 37 °C. The CFU total was calculated using the standard colony counting technique.

For the invasion assays in the presence of the TLR2 blocking antibody, 1 h previous to the addition of *S. aureus* to the bMECs, the anti-TLR2 monoclonal antibody (Abcam, TLR2.1) was added (5 μg/mL) to triplicate wells. Mouse IgGs (purified from normal mouse serum and acquired from Pierce) were used as the negative controls (data not showed). The invasion assays were performed using gentamicin protection assays as described. Data are presented as the percentage of internalization, considering the internalization of control (bMECs treated with vehicle) as 100%.

2.6. Expression analysis of inflammatory response genes

We used bMEC monolayers (~2×10^5 cells cultured in 24-well dishes with 6–10 μg/cm^2 rat-tail type I collagen) incubated with different Thi2.1 concentrations. For the analysis of gene expression, we selected two concentrations of this peptide based on the results from internalization experiments: 5 and 10 μg/mL of Thi2.1 for 24 h. Thereafter, cells were infected with *S. aureus* as described above. bMEC total RNA (5 μg) was extracted from all conditions with Trizol (Invitrogen) according to the manufacturer's instructions, which was used to synthesize cDNA as described [14]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primers (Invitrogen) and PCR conditions used to amplify the bovine mRNAs (TNF-α, IL-1β, IL-6, IL-8, IL-10, TAP, BNBD5 and TLR2) were previously described [15]. Expression analysis of genes related to the inflammatory response was performed by qPCR using the comparative Ct method (ΔΔCt) and the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) in a StepOne Plus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. For the analyses of gene expression of the pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in the presence of the anti-TLR2 blocking antibody, bMECs were treated 6, 12 or 24 h with 5 and 10 μg/mL of Thi2.1 and the antibody was added (5 μg/mL) 1 h previous to the addition of *S. aureus*. Then, RNA extraction and qPCR was performed as described.

2.7. Flow cytometry analysis

bMECs were cultured to 80% confluence on 24-well plates (Corning) in DMEM/F-12 K medium and treated with Thi2.1 and/or *S. aureus*, as described above. After the treatment, the bMECs were detached with mechanical dissociation using a rubber blade in PBS, centrifuged and washed with DMEM/F-12 K medium. The cell pellet was obtained by centrifugation at 3000*g* at 4 °C for 10 min, and was washed twice with PBS. The bMECs were incubated with normal goat serum (5% in PBS, Pierce) for 30 min at 4 °C with shaking; cell pellet was obtained by centrifugation and was incubated with the primary antibody anti-TLR2 (TL2.1, Abcam) at a dilution of 1:150 (PBS containing 0.1% BSA) for 1 h at 4 °C with shaking to analyze the TLR2 membrane abundance (MA). The cells were then washed three times with PBS and incubated with