Molecular Immunology 68 (2015) 445–455

Contents lists available at ScienceDirect

Molecular Immunology

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

The activation of the TLR2/p38 pathway by sodium butyrate in bovine mammary epithelial cells is involved in the reduction of *Staphylococcus aureus* internalization

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ARTICLE INFO

Article history:
Received 8 August 2015
Received in revised form
26 September 2015
Accepted 29 September 2015
Available online 21 October 2015

Keywords:
Staphylococcus aureus
Butyrate
Internalization
TLR2
p38
Epithelial cells

ABSTRACT

Staphylococcus aureus is an etiological agent of human and animal diseases, and it is able to internalize into non-professional phagocytic cells (i.e. bovine mammary epithelial cells, bMECs), which is an event that is related to chronic and recurrent infections. bMECs contribute to host innate immune responses (IIR) through TLR pathogen recognition, whereby TLR2 is the most relevant for *S. aureus*. In a previous report, we showed that sodium butyrate (NaB, 0.5 mM), which is a short chain fatty acid (SCFA), reduced *S. aureus* internalization into bMECs by modulating their IIR. However, the molecular mechanism of this process has not been described, which was the aim of this study. The results showed that the TLR2 membrane abundance (MA) and mRNA expression were induced by 0.5 mM NaB ~1.6-fold and ~1.7-fold, respectively. Additionally, 0.5 mM NaB induced p38 phosphorylation, but not JNK1/2 or ERK1/2 phosphorylation in bMECs, which reached the baseline when the bMECs were *S. aureus*-challenged. Additionally, bMECs that were treated with 0.5 mM NaB (24 h) showed activation of 8 transcriptional factors (AP-1, E2F-1, FAST-1, MEF-1, EGR, PPAR, ER and CBF), which were partially reverted when the bMECs were *S. aureus*-challenged. Additionally, 0.5 mM NaB (24 h) up-regulated mRNA expression of the antimicrobial peptides, TAP (~4.8-fold), BNBD5 (~3.2-fold) and BNBD10 (~2.6-fold). Notably, NaB-treated and *S. aureus*-challenged bMECs increased the mRNA expression of all of the antimicrobial peptides that were evaluated, and this was evident for LAP and BNBD5. In the NaB-treated bMECs, we did not detect significant expression changes for IL-1β and IL-6 and only TNF-α, IL-10 and IL-8 were induced. Interestingly, the NaB-treated and *S. aureus*-challenged bMECs maintained the anti-inflammatory response that was induced by this SCFA. In conclusion, our results suggest that 0.5 mM NaB activates bMECs via TLR2/p38, which leads to improved antimicrobial defense before/after pathogen invasion, and NaB may exert anti-inflammatory effects during infection.

1. Introduction

Staphylococcus aureus is the etiological agent in a wide range of human and animal diseases (Lowy, 1998). Chronic and recurrent infections related to this bacterium have been associated with its ability to internalize and survive within professional and nonprofessional phagocytic cells (NPPCs) (Almeida et al., 1996; Garzoni and Kelley, 2009; Fraunholz and Sinha 2012).

S. aureus internalization occurs mainly by a zipper-like process, which is mediated by α5β1 integrin in the membrane of the host cell (Hauck et al., 2012). However, others host cell proteins are involved in this process, such as TLR2 and CD36 (Alva-Murillo et al., 2014a). Reports have described the participation of TLR2 in *S. aureus* internalization into phagocytic cells (Watanabe et al., 2007; Fournier, 2012; Yimin et al., 2013; Fang et al., 2014), but little is known about the participation of TLR2 during *S. aureus* internalization into NPPCs. In this sense, it is known that the blockage of TLR2 with neutralizing antibodies in human mast cells and bovine mammary epithelial cells (bMECs) decreases the number of internalized *S. aureus* (Rocha-de-Souza et al., 2008; Medina-Estrada et al., 2015). Additionally, TLR2 is located in phagosomes and co-

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http://dx.doi.org/10.1016/j.molimm.2015.09.025
0161-5890/

localizes with different *S. aureus* pathogen-associated molecular patterns (PAMPs) (Müller et al., 2010). Although the role of TLR2 during *S. aureus* internalization is unclear, it has been previously suggested that TLR2 activation is a pre-requisite for this process.

On the other hand, CD36, a membrane glycoprotein that belongs to the class B scavenger receptor family, interacts with TLR2 during *S. aureus* recognition as a coreceptor for diacylglyceride detection through the TLR2/6 complex, which mediates bacterial invasion primarily in phagocytic cells (Hoebe et al., 2005). In HEK 293 and HeLa cells, CD36 overexpression increases *S. aureus* or *Escherichia coli* internalization (Stuart et al., 2005; Baranova et al., 2008). Nevertheless, the role of CD36 in *S. aureus* internalization into bMECs is unknown.

Mammary epithelial cells can display a relevant innate immune response (IIR) in mammary glands (Rainard and Riollet, 2006). In cattle, the bMECs contribution to udder defense is mediated by pattern recognition receptors (PRRs), with TLR2 being the most relevant receptor for *S. aureus* recognition. Additionally, this stimulation leads to the activation of MAPK family members (JNK, ERK and p38) and transcription factors, such as NF-κB, AP-1 or IRF1/2, which in turn stimulates antimicrobial and inflammatory gene expression (Akira et al., 2006; Chiu et al., 2009; Stack et al., 2014). It is well known that p38 activation is triggered by *S. aureus* stimulation (live or killed bacteria or PAMPs) in phagocytic cells (McLeish et al., 1998; Fang et al., 2014; Song et al., 2014; Chekabab et al., 2015). Also, the role of p38 in *S. aureus* internalization has been demonstrated in phagocytic cells (e.g. macrophages and monocytes) by pharmacological inhibition (Kapetanovic et al., 2011). However, in NPPCs (ca. osteoblasts) *S. aureus* did not modify the phosphorylation of p38 (Ellington et al., 2001). In addition, the TLR2/p38 interaction has been related with *S. aureus* stimulation of immune system (antimicrobial peptide expression) but no with bacteria internalization (Menzies and Kenoyer, 2006). An attractive alternative to avoiding bacterial internalization in bMECs is the modulation of their innate immune response to facilitate the elimination of the invading pathogen.

One of the most relevant immunomodulatory molecules is butyrate, which is a short chain fatty acid (SCFA) that is produced in the colonic lumen by the bacterial fermentation of dietary fiber. Additionally, bovine milk is the only mammalian milk that contains this SCFA (2–5% wt) (Jensen, 2002). Butyrate has been associated with several effects at the intestinal and extraintestinal levels, such as cell growth, differentiation, apoptosis induction in transformed epithelial cells, satiety, oxidative stress in the intestine, colonic barrier defense, intestinal epithelial permeability and immune modulation (Hamer et al., 2008; Canani et al., 2011). Moreover, butyrate modulates several signaling pathways in intestinal epithelial cells, including that of the transcription factor AP-1 (Mandal et al., 2001; Hamer et al., 2008; Canani et al., 2011). Additionally, this SCFA possesses anti-inflammatory properties that interfere with NF-κB activation or inhibit interferon-γ signaling, peroxisome proliferator-activated receptor γ (PPARγ) up-regulation, and histone deacetylase (HDAC) (Inatomi et al., 2005; Schwab et al., 2007a; Hamer et al., 2008; Canani et al., 2011). Furthermore, butyrate is a strong inducer of antimicrobial peptides in human and animal cells (Schauber et al., 2003; Zeng et al., 2013; Sunkara et al., 2014).

In previous reports, we demonstrated that short and medium fatty acids reduce *S. aureus* internalization into bMECs (Ochoa-Zarzosa et al., 2009; Alva-Murillo et al., 2012, 2013). In particular, 0.5 mM sodium butyrate (NaB) reduces ~50% *S. aureus* internalization into bMECs and up-regulates tracheal antimicrobial peptide (TAP) gene expression (Ochoa-Zarzosa et al., 2009). However, the molecular mechanisms that are regulated by butyrate regarding bacterial internalization reduction and the modulation of the bMEC innate immune response are unknown. In this study, we showed that 0.5 mM NaB activates bMECs via TLR2/p38, which leads to the improvement of the antimicrobial defense against pathogen invasion and may exert anti-inflammatory effects during infection.

2. Materials and methods

2.1. Staphylococcus aureus *strain*

The *S. aureus* subsp. *aureus* (ATCC 27543) strain used in this study was isolated from a case of bovine clinical mastitis. This strain has a recognized capacity to be internalized into bMECs (Gutiérrez-Barroso et al., 2008). Bacteria were grown overnight in Luria-Bertani broth (LB, Bioxon, México), and the colony forming units (CFU) were adjusted by measuring their optical density at 600 nm (OD 0.2 = 9.2×10^7 CFU/ml).

2.2. Reagents and antibodies

LTA (from *S. aureus*), LPS (from *E. coli* 0111:B4) and sodium butyrate (NaB) were acquired from Sigma–Aldrich (St. Louis, MO, USA). In this study, we used 0.5 mM NaB, which inhibits *S. aureus* internalization into bMECs (Ochoa-Zarzosa et al., 2009). The monoclonal blocking antibodies used were anti-TLR2 (TL2.1, Abcam) and anti-CD36 (FA6-152, Abcam). The MAPK inhibitors, SB20358 (p38), SP600125 (JNK) and U0126 (ERK1/2) were acquired from Cell Signaling Technology^R (Boston, MA). The working solutions were dissolved in dimethyl sulfoxide (DMSO), which was employed as vehicle in the corresponding experiments.

2.3. Primary bovine mammary epithelial cell (bMEC) culture

bMEC isolation was performed on udder alveolar tissue from healthy lactating cows as described previously (Anaya-López et al., 2006). Cells from passages 2–8 were used in all of the experiments. The cells were cultured in petri dishes (Corning-Costar) in growth medium (GM) that was composed of a DMEM medium/nutrient mixture F-12Ham (DMEM/F-12K, Sigma), which was 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, and 1 μg/ml amphotericin B (Invitrogen). The bMECs were grown in 5% CO_2 atmosphere at 37 °C. To perform the *S. aureus* challenge, the bMECs were cultured in serum-free growth medium without antibiotics for 24 h, and then, they were infected.

2.4. Invasion assays

Polarized bMEC monolayers were cultivated on 96-well flat-bottom plates that were coated (Corning-Costar) with 6–10 μg/cm^2 rat-tail type I collagen (Sigma). Prior to the invasion assays, the bMECs (~10×10^3 cells/well) were incubated with 0.5 mM NaB in DMEM/F12K (Sigma) without antibiotics and serum for 24 h. Then, the cells were treated separately with different blocking antibodies, including anti-TLR2 (5 μg/ml, 1 h) and anti-CD36 (0.25 μg/ml, 45 min). Mouse IgG (purified from normal mouse serum that was purchased from Pierce) was used as the negative control. The blocking of the invasion assays was performed using gentamicin protection assays as described (Gutiérrez-Barroso et al., 2008). Briefly, the bMECs that were used in the antibody blockage experiments were infected with *S. aureus* (MOI 30:1 bacteria per cell). For this, the bMECs were inoculated with 3.5 μl of bacterial suspensions to 9.2×10^7 CFU/ml and incubated for 2 h in 5% CO_2 at 37 °C. Then, the bMECs were washed three times with PBS (pH 7.4) and incubated in GM without serum and penicillin and streptomycin, and they were supplemented with 50 μg/ml gentamicin for 1 h at 37 °C to eliminate extracellular bacteria. Finally, the bMEC monolayers were detached with trypsin-EDTA (Sigma) and lysed with 250 μl