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Selenium inhibits *Staphylococcus aureus*-induced inflammation by suppressing the activation of the NF-κB and MAPK signalling pathways in RAW264.7 macrophages

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l-glutamine (PubChem CID: 5961)
dimethyl sulfoxide (PubChem CID: 679)
diethyl pyrocarbonate (PubChem CID: 3051)
sodium dodecyl sulphate (PubChem CID: 3423265)
tween 20 (PubChem CID: 443314)

ABSTRACT

Inflammation is the hallmark of *Staphylococcus aureus* (*S. aureus*)-induced mastitis. Given the interesting relationship between selenium levels and inflammation, this study aimed to demonstrate that selenium modulated the inflammation reaction by suppressing the nuclear factor kappa B (NF-κB) and mitogen activated protein kinase (MAPK) signalling pathways. RAW264.7 macrophages were treated with three different concentrations (1 μmol/l, 1.5 μmol/l, and 2 μmol/l) of Na_2SeO_3 for 12 h before infection with *S. aureus* for 6 h, 8 h, and 10 h. The results showed that selenium significantly reduced the mRNA expression levels of tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6). Furthermore, the release of TNF-α, IL-1β, and IL-6 was decreased significantly with selenium supplementation. In addition, selenium influenced the NF-κB signalling pathway by suppressing the activation of NF-κB *p65* and degradation of inhibitory kappa-B (*IκB*). Selenium also suppressed extracellular regulated protein kinase (*Erk*), c-Jun N-terminal kinase (*Jnk*), and *p38* phosphorylation through the MAPK signalling pathway. In conclusion, selenium played an anti-inflammation role in RAW264.7 macrophages infected with *S. aureus* by suppressing the activation of the NF-κB and MAPK signalling pathways.

1. Introduction

Staphylococcus aureus (*S. aureus*) is an important Gram-positive bacterium that causes a wide spectrum of diseases, such as skin and soft tissue infections (SSTIs), pneumonia, bacteremia, and mastitis (Sowash and Uhlemann, 2013; Ferrer et al., 2015). Macrophages are important immune effector cells that act as the first defence line against pathogens, including *S. aureus* (Brüne et al., 2013). When infection occurs, macrophages are activated by recognizing pathogen-associated molecular patterns (PAMPs) via a series of pattern-recognition receptors (PRRs) (Newton and Dixit, 2012). Activated PRRs eventually result in activation of the nuclear factor kappa B (NF-κB) signalling pathway. The NF-κB-inhibitory kappa-B alpha (*IκBα*) complex is located in the cytoplasm of normal cells (Duntas, 2009). When *IκBα* is phosphorylated and degraded, the subunits (*p65*/*p50*) of the NF-κB heterodimer are released (Asehnoune et al., 2005). As a result, NF-κB *p65*, which is believed to play a central role in inflammation, enters the nucleus, and the genes encoding various cytokines and chemokines, such as tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), are transcribed (Takeda and Akira, 2005). The mitogen activated protein kinase (MAPK) signalling pathway, including *p*38, *Erk*, and *Jnk*, also plays a critical role during inflammation and in some autoimmune diseases (Li et al., 2012; Mavropoulos et al., 2013). Similar to NF-κB, activation of the MAPK signalling pathway can recruit a series of cytokines and chemokines, including TNF-α, IL-1β, and IL-6. These molecules have the

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potential to protect against inflammation and also cause tissue damage or organ failure (Seeley et al., 2012).

There are several forms of selenium, including selenomethionine, sodium selenite, and selenium methylselenocysteine (Fritz et al., 2011). Selenium and its various forms have been studied as well as the biological effects that work through selenoproteins. The majority of selenoproteins contain a selenocysteine residue (Novoselov et al., 2012). Thus far, studies have shown that selenoproteins play modulatory effects on the cellular antioxidant defence system (Duntas, 2009). Selenium can also regulate the inflammatory process and gene transcription (Kaushal et al., 2014). In addition, some studies have shown that small molecular weight selenocompounds, such as ebselen, can influence macrophage functions (Shimohashi et al., 2000). Studies have shown that *in vitro* supplementation of macrophages with selenium enhanced cellular immunity following stimulation with *S. aureus* (Ndiweni and Finch, 1995). However, the mechanisms involved in the effects of selenium on inflammatory reactions in macrophages stimulated by *S. aureus* remain unclear. Thus, the aim of this study was to explore the effects of selenium on *S. aureus*-induced inflammation injury in RAW264.7 macrophages and to confirm the possible mechanisms.

2. Materials and methods

2.1. Preparation of RAW264.7 macrophages and S. aureus

RAW264.7 macrophages were cultured in Dulbecco's Modification of Eagle's Medium (DMEM; Gibco, US) supplemented with 10% heat-inactivated foetal bovine serum (Gibco, US) and L-glutamine (Sigma, US) as described (Bjur et al., 2006). For the infection of nonactivated RAW264.7 macrophages, *S. aureus* cells (29213, ATCC) were cultured in 10 ml of Luria-Bertani (LB) at 37 °C and harvested at the log phase. The number of *S. aureus* cells was determined by serial dilution and the plate count method. Then, the bacteria were diluted to achieve a multiplicity of infection (MOI) of 1:1 (bacteria: cell) in DMEM.

2.2. Analysis of cell viability with a MTT assay

A MTT assay was used to determine cell viability. Briefly, 1×10^5 cells were seeded in 96-well plates and incubated at 37 °C with 5% CO_2. When cells were cultured to more than 80% confluence, cells in each group were separately treated with different concentrations of Na_2SeO_3 (0 μM, 2 μM, 4 μM, 8 μM, 16 μM, 32 μM, 64 μM, and 128 μM) for 12 h. Then, 20 μl of MTT (Amresco, US) was added to each well to incubate with the cells for another 4 h. The medium was removed and formazan formation was dissolved with 150 μl of dimethyl sulfoxide (Amresco, US). The absorbance was measured at 570 nm with a microplate reader (Tecan, Austria).

2.3. RNA extraction and real-time quantitative reverse transcription PCR

Cells were cultured in 6-well plates (5×10^5 cells/well) at 37 °C with 5% CO_2. The experimental groups were treated with Na_2SeO_3 (1 μM, 1.5 μM, and 2 μM) for 12 h. Then, all of the cells were stimulated with *S. aureus* (MOI=1:1). Cells of each group were collected separately at 6 h, 8 h, and 10 h postinoculation (pi). The total RNA of cells was extracted according to the manufacturer's instructions using Trizol reagent (Invitrogen, US). The quantity and quality of the extracted RNA were evaluated with a Nanodrop 2000 spectrophotometer (Thermo, USA). The ratio of absorption (OD 260 nm/OD 280 nm) was between 1.8 and 2.2, and the RNA integrity was verified with electrophoresis using a 1% agarose gel. Then, RNA was converted to cDNA using a reverse transcriptase synthesis kit (Takara, Japan).

Table 1
Sequences, product size, and GenBank accession number of genes.

Gene	Primer sequence (5′–3′)	Product size (bp)	Accession number
IL-6	F: AGTTGTGCAATGGCAATTCTGA R: AGGACTCTGGCTTTGTCTTTCT	223	NM_031168
TNF-α	F: GCCTCCCTCTCATCAGTTCTA R: GGCAGCCTTGTCCCTTG	246	NM_013693
IL-1β	F: ACCTGTGTCTTTCCCGTGG R: TCATCTCGGAGCCTGTAGTG	162	NM_008361
β-actin	F: TGCTGTCCCTGTATGCCTCT R: TTTGATGTCACGCACGATTT	224	NM_007393

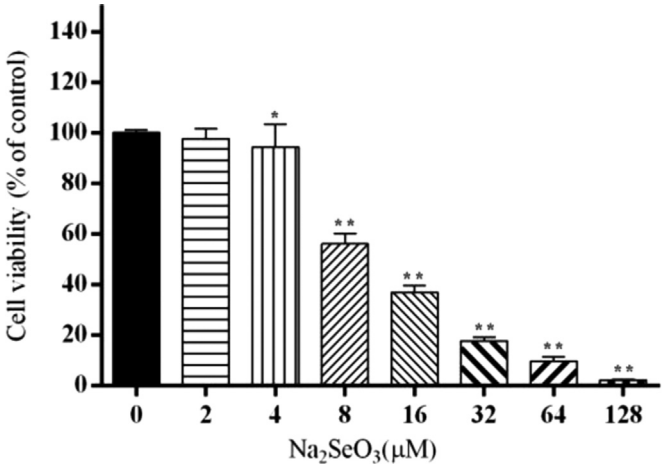

Fig. 1. Effects of selenium on the cell viability of RAW264.7 macrophages at 12 h. The data are presented as the mean ± S.E.M ($n=6$). Asterisks represent a significant change compared with 0 μM selenium. $^{*}P<0.05$ and $^{**}P<0.01$.

The PCR reaction system contained 10 μl of SYBR Green PCR mix, 0.8 μl of each primer (both 10 μmol/l), and 2 μl of cDNA template in a final volume of 20 μl per reaction (Takara, Japan). The sequences of the primers are listed in Table 1. The cycling parameters for real-time PCR were 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s using a CFX connect real-time PCR system (BIO-RAD, US). The real-time quantitative PCR reaction was performed in triplicate for each sample, and the mean value was used to calculate the mRNA expression levels. The fold change (n-fold) for gene expression was calculated using the relative quantification method as previously described (Shao et al., 2012).

2.4. Measurement of TNF-α, IL-1β, and IL-6 in culture supernatants

Cell-free media of each group were collected separately at 6 h, 8 h, and 10 h pi during the experiment as described above. The concentration of the TNF-α, IL-1β, and IL-6 proteins were detected with commercial ELISA kits according to the manufacturer's instructions (Cusabio Biotech, China).

2.5. Western blot analysis of the levels of activation of the NF-κB and MAPK signalling pathways

Cells were seeded in 6-well plates (5×10^5 cells/well) to incubate for at least 12 h to allow cells to adhere to the plates. After washing three times with medium, cells were pretreated with Na_2SeO_3 (1 μM, 1.5 μM, and 2 μM) or left untreated for 12 h and then stimulated with *S. aureus* (MOI=1:1) for 0.5 h. The total proteins were extracted and the protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit