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

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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 kappaB (NF- κ B) and mitogen activated protein kinase (MAPK) signalling pathways. RAW264.7 macrophages were treated with three different concentrations (1 μ mol/l, 1.5 μ mol/l, and 2 μ mol/l) of Na₂SeO₃ 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- α (TNF- α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6). Furthermore, the release of TNF- α , IL-1 β , and IL-6 was decreased significantly with selenium supplementation. In addition, selenium influenced the NF- κ B signalling pathway by suppressing the activation of NF- κ B p65 and degradation of inhibitory kappa-B (*I κ B*). 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- κ B and MAPK signalling pathways.

Keywords

Selenium; *Staphylococcus aureus*; inflammation; NF- κ B; MAPK

Chemical compounds included in this study

The chemical compounds that were used in this study were: selenium (PubChem CID: 6326970); ebselen (PubChem CID: 3194); L-glutamine (PubChem CID: 5961); dimethyl sulfoxide (PubChem CID: 679); diethyl pyrocarbonate (PubChem CID: 3051); sodium dodecyl sulphate (PubChem CID: 3423265); and tween 20 (PubChem CID: 443314).

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 (SowashandUhlemann, 2014; 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 kappaB (NF- κ B) signalling pathway. The NF- κ B-inhibitory kappa-B alpha (*I κ B α*) complex is located in the cytoplasm of normal cells (Duntas, 2009). When *I κ B α* is phosphorylated and degraded, the subunits (*p65/p50*) of the NF- κ B heterodimer are released (Asehnoune et al., 2005). As a result, NF- κ B *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-1 β), and

interleukin-6 (IL-6), are transcribed (Takeda and Akira, 2003). The mitogen activated protein kinase (MAPK) signalling pathway, including *p38*, *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- κ B, activation of the MAPK signalling pathway can recruit a series of cytokines and chemokines, including TNF- α , IL-1 β , and IL-6. These molecules have the 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 seleno compounds, 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% CO₂. When cells were cultured to more than 80% confluence, cells in each group were separately treated with different concentrations of Na₂SeO₃ (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 dimethylsulfoxide (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% CO₂. The experimental groups were treated with Na₂SeO₃ (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 post inoculation (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).

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).

Table 1

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

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

2.4. Measurement of TNF- α , IL-1 β , 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-1 β , 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- κ B 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 Na₂SeO₃ (1 μ M, 1.5 μ M, and 2 μ M) 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 (BioChain, US). The proteins were loaded onto a 10% sodium dodecyl sulphate polyacrylamide gel for electrophoresis and transferred to PVDF membranes (Millipore, Germany). The membranes were blocked in 5% nonfat milk for 1 h to 2 h and incubated overnight with the primary antibodies, including *p65*, *P-p65*, *I κ B α* , *P-I κ B α* , *p38*, *P-p38*, *Erk*, *P-Erk*, *Jnk*, *P-Jnk*, and β -actin (CST, US). Then, all of the membranes were subsequently incubated with HRP-conjugated goat anti-rabbit antibodies (CST, US). The blots were washed with PBS-T and processed with supersignal west pico chemiluminescent substrate.

2.6. Statistical analysis

All data are shown as the mean $\pm$ S.E.M, and experiments were independently repeated at least 3 times. One-way ANOVA and Dunnett's test were used for statistical analyses. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Cell viability influenced by selenium

A MTT assay was used to determine the potential cytotoxicity of selenium. As shown in Figure 1, cell

viability was not affected when cells were exposed to 2 μM of Na_2SeO_3 . However, cell viability declined significantly at 4 μM , 8 μM , 16 μM , 32 μM , 64 μM , and 128 μM ($P < 0.05$ or $P < 0.01$).

3.2. Selenium contributed to the depression of inflammatory cytokine expression in

*RAW264.7 macrophages induced by *S. aureus**

The effects of selenium were analysed by qRT-PCR in RAW264.7 macrophages stimulated by *S. aureus*. As shown in Figure 2, the mRNA expression of TNF- α was increased or significantly increased in the model group and experimental groups ($P < 0.05$, $P < 0.01$, or $P < 0.001$) compared with the control group at 6 h, 8 h, and 10 h pi. However, the mRNA expression of TNF- α in the experimental groups was down-regulated or significantly down-regulated by selenium at 1.5 μM and/or 2 μM compared with the model group ($P < 0.05$ or $P < 0.001$).

The IL-6 mRNA levels were significantly up-regulated by *S. aureus* in the model group and experimental groups at 6 h pi ($P < 0.001$), but the mRNA expression of IL-6 was down-regulated by selenium at 2 μM ($P < 0.05$) compared with the model group. At 8 h and 10 h pi, the mRNA expression of IL-6 was significantly increased in the model group and experimental groups ($P < 0.01$ or $P < 0.001$) compared with the control group. The IL-6 mRNA levels were significantly down-regulated by selenium at 1.5 μM and 2 μM ($P < 0.001$) compared with the model group.

At 6 h, 8 h, and 10 h pi, the mRNA expression of IL-1 β was significantly up-regulated by *S. aureus* in the model group and experimental groups ($P < 0.01$ or $P < 0.001$). However, the mRNA expression of IL-1 β was significantly down-regulated at 6 h and 8 h with selenium concentrations of 1 μM , 1.5 μM , and 2 μM ($P < 0.01$ or $P < 0.001$) and at 10 h with a selenium concentration of 2 μM ($P < 0.001$) compared with the model group.

*3.3. Selenium contributed to suppressing the release of cytokines TNF- α , IL-1 β , and IL-6 induced by *S.**

aureus

As seen in Figure 3, the concentration of TNF- α increased significantly in the model group and experimental groups ($P<0.05$, $P<0.01$, or $P<0.001$) at 6 h, 8 h, and 12 h pi. However, compared with the model group, the concentration of TNF- α decreased differently at 6 h, 8 h, and 10 h with selenium concentrations of 1.5 μ M and 2 μ M ($P<0.05$ or $P<0.01$).

A significant increase in IL-1 β concentration was observed at 6 h, 8 h, and 10 h compared with the control group. However, the increase of the IL-1 β concentration was suppressed at 6 h, 8 h, and 10 h by different concentrations of selenium, 1 μ M, 1.5 μ M, and 2 μ M ($P<0.05$, $P<0.01$, or $P<0.001$), compared with the model group.

The IL-6 concentration increased significantly when cells were inoculated with *S. aureus* in the model group and experimental group ($P<0.01$ or $P<0.001$). The IL-6 concentration in the experimental groups was significantly lower than the model group at 6 h and 8 h ($P<0.01$ or $P<0.001$).

*3.4. Selenium inhibited activation of the NF- κ B signalling pathway in RAW264.7 macrophages induced by *S. aureus**

To determine whether the NF- κ B signalling pathway was involved in the alleviation of inflammatory responses with selenium supplementation or not, key proteins in the signalling pathway were detected. The results showed that *S. aureus* increased phosphorylation of *I κ B α* and *p65* significantly in the model group ($P<0.001$). The phosphorylation levels of *I κ B α* and *p65* were alleviated when RAW264.7 macrophages were pretreated with selenium ($P<0.001$) compared with the model group.

*3.5. Selenium inhibited activation of the MAPK signalling pathway in RAW264.7 macrophages induced by *S. aureus**

S. aureus increased phosphorylation of *p38*, *Erk*, and *Jnk* significantly in the model group

($P < 0.001$), and phosphorylation of *p38*, *Erk*, and *Jnk* was also detected obviously in the experimental groups ($P < 0.05$ or $P < 0.01$). However, the phosphorylation levels of *p38*, *Erk*, and *Jnk* were suppressed significantly when selenium was added to the culture medium ($P < 0.001$) compared with the model group.

4. Discussion

Inflammatory reactions are characterized by redness, pain, swelling, and dysfunction of organs (Conese and Assael, 2001). Although the occurrence of drug resistance increased drastically, antibiotics are still an effective treatment for infection (Liu et al., 2014), and other effective therapies are being developed as well. Selenium is an essential micronutrient that plays an important role in regulating the immune response to infection (Hoffmann and Berry, 2008). When RAW264.7 macrophages were inoculated with *S. aureus*, the gene expression and protein levels of cytokines were inhibited with selenium supplementation in this study. In addition, selenium inhibited activation of the NF- κ B and MAPK signalling pathways. Therefore, selenium can alleviate the inflammatory reaction by suppressing activation of the NF- κ B and MAPK signalling pathways.

It was confirmed that a rapid inflammatory response occurred when RAW264.7 macrophages were stimulated with *S. aureus*, characterized by releasing a large number of proinflammatory cytokines. Such cytokines are beneficial for immune effector cells to eliminate infection during the first stage (Aribi et al., 2015; Porcherie et al., 2012). TNF- α , IL-1 β , and IL-6 are important pro-inflammatory cytokines that regulate the inflammatory response during *S. aureus* infection in a sophisticated manner. The results showed that gene expression and the production of TNF- α , IL-1 β , and IL-6 were significantly increased in RAW264.7 macrophages induced by *S. aureus*, which indicated a drastic inflammatory response. TNF- α is involved in the activation of neutrophils, impairment of endothelial

cells and aggravation of the inflammatory cascade (Murdaca et al., 2015; Koca et al., 2008). IL-1 β and IL-6 also play important roles in the regulation of the host immune response to *S. aureus* (Miller et al., 2007; Ahmed et al., 2011). However, overreaction of proinflammatory cytokines also injures cells and tissues. Therefore, the expression and release of these proinflammatory cytokines must be tightly regulated (Scheibel et al., 2010). Selenium inhibited *S. aureus*-induced gene expression and production of TNF- α and IL-1 β in a dose-dependent manner at all time points. Although selenium did not inhibit *S. aureus*-induced IL-6 expression at 1 μ M, the inhibitory effect of selenium on IL-6 expression was significant at 1.5 μ M and 2 μ M, which indicated that a low concentration of selenium might not be enough to inhibit *S. aureus*-induced IL-6 expression. All of the results further demonstrated that selenium suppressed the inflammation of *S. aureus* infection, with decreased TNF- α , IL-1 β and IL-6 expression and production.

Activation of the signalling pathways of inflammation have been confirmed to play an important role in regulating immune responses (Vunta et al., 2007). Experiments have verified that *S. aureus* led to the activation of multiple signalling pathways in RAW264.7 macrophages, including the MAPK and NF- κ B signalling pathways, which regulate the expression of many inflammatory genes (Fang et al., 2014; Paul et al., 1997). Recently, the phosphorylation levels of NF- κ B *p65* and *p38* MAPK were increased in selenium-deficient mice after infection with *S. aureus*, which verified that selenium deficiency could strengthen the inflammatory reaction (Gao et al., 2015). The NF- κ B complex is mainly regulated by *I κ B α* and *p65* in the cytoplasm. In the present study, when infected with *S. aureus* NF- κ B *I κ B α* and *p65* were phosphorylated. Then, NF- κ B *p65* translocated into the nucleus, where it accelerated the transcription of target genes, such as TNF- α , IL-1 β , and IL-6. The findings of this study are consistent with those of a previous report (Youn et al., 2008). Once selenium was added to the

culture medium, the phosphorylation of *IκBα* and *p65* was inhibited, which demonstrated that selenium suppressed inflammatory reactions in *S. aureus* infected RAW264.7 macrophages through the NF-κB signalling pathway. *Erk*, *Jnk*, and *p38* are three important members of the MAPK family (Song et al., 2003). Accumulative evidence has shown that the MAPK family is mainly involved in inflammation (Kim et al., 2004). When mouse mammary epithelial cells were stimulated with LPS, a selenium supplement could inhibit the LPS-induced inflammatory cytokines through the MAPK signalling pathways (Zhang et al., 2014). In our study, the phosphorylation of *Erk*, *Jnk*, and *p38* were increased after infection with *S. aureus*, indicating that *S. aureus* activated the MAPK signalling pathway in RAW264.7 macrophages. Selenium attenuated the inflammatory responses by suppressing the phosphorylation of *Erk*, *Jnk*, and *p38* through the MAPK signalling pathway.

In summary, selenium can decrease TNF-α, IL-1β, and IL-6 expression and alleviate the inflammatory reaction in RAW264.7 macrophages stimulated by *S. aureus*. The NF-κB and MAPK signalling pathways are involved in the protection of selenium during inflammation. Therefore, selenium supplements may be an effective method for the prevention or treatment of *S. aureus* infectious diseases.

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

Fig. 2. The effects of selenium on TNF- α (A), IL-1 β (B), and IL-6 (C) mRNA expression of RAW264.7

macrophages stimulated with *S. aureus*. Con: control group; Mod: cells treated with *S. aureus* (MOI=1:1) only; Low: Na₂SeO₃ (1 μM) + *S. aureus* (MOI=1:1); Mid: Na₂SeO₃ (1.5 μM) + *S. aureus* (MOI=1:1); Hig: Na₂SeO₃ (2 μM) + *S. aureus* (MOI=1:1). The data are presented as the mean ± S.E.M(n=3).^{###}*P*<0.001 vs. Con; **P*< 0.05 vs. Mod; ***P*< 0.01 vs. Mod; ****P*< 0.001 vs. Mod.

Fig. 3. The effects of selenium on TNF-α (A), IL-1β (B), and IL-6 (C) levels after RAW264.7 macrophages were stimulated by *S. aureus*. The data are presented as the mean ± S.E.M(n=3).[#]*P*<0.05 vs. Con; ^{##}*P*<0.01 vs. Con; ^{###}*P*<0.001 vs. Con; **P*< 0.05 vs. Mod; ***P*< 0.01 vs. Mod; ****P*< 0.001 vs. Mod.

Fig.4. Effects of selenium on the phosphorylation of *IκBα* and *p65* in *S. aureus*-stimulated RAW264.7 macrophages. Con: control group without any processing; Mod: cells treated with *S. aureus* (MOI=1:1) only; Low: Na₂SeO₃ (1 μM) + *S. aureus* (MOI=1:1); Mid: Na₂SeO₃ (1.5 μM) + *S. aureus* (MOI=1:1); Hig: Na₂SeO₃ (2 μM) + *S. aureus* (MOI=1:1). The data are presented as the mean ± S.E.M(n=3).^{###}*P*<0.001 vs. Con; ****P*< 0.001 vs. Mod.

Fig.5. Effects of selenium on the phosphorylation of *p38*, *Jnk* and *Erkin* in *S. aureus* stimulated RAW264.7 macrophages. The data are presented as the mean ± S.E.M(n=3). Groups are the same as Figure 4.

Fig.1.

Fig.2.

Fig.3.

Fig.4.

Fig.5.