Zinc L-Carnosine Inhibits The Secretion of Pro-Inflammatory HMGB1 Protein From Lipopolysaccharide-Induced RAW 264.7 Murine Macrophage

Theng Choon Ooi
Universiti Kebangsaan Malaysia
Kok Meng Chan
Universiti Kebangsaan Malaysia
Razinah Sharif (razinah@ukm.edu.my)
Universiti Kebangsaan Malaysia https://orcid.org/0000-0001-7174-7353

Research Article

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Abstract

Dysregulation in the secretion of high mobility group box 1(HMGB1) protein has been shown to modulate the progression of sepsis. Hence, the primary objective of this study is to explore the potential of zinc L-carnosine (ZnC) in inhibiting the secretion of HMGB1 from RAW 264.7 murine macrophages after induced with lipopolysaccharide (LPS). Generally, RAW 264.7 cells were pretreated with ZnC (0–100 μM) for 2 hours before challenged with LPS (1 μg/mL). After 22 hours of LPS induction, RAW 264.7 cells pretreated with ZnC showed significantly higher intracellular HMGB1 protein levels in a dose-dependent manner, indicating that ZnC was capable to suppress the secretion of HMGB1 protein into the extracellular compartments. Besides, significant increment in intracellular free thiol level was also detected in ZnC pretreated cells. In addition, ZnC was demonstrated to inhibit the late phase NF-κB activation, but not the early phase NF-κB activation. Moreover, after induced with LPS for 30 minutes, pretreatment with ZnC was also demonstrated to increase the phosphorylated-Akt/Akt ratio in RAW 264.7 cells, indicating that the immunomodulatory effects of ZnC may associated with the Akt signaling pathway. In summary, ZnC can prevent the secretion of HMGB1 from RAW 264.7 cells and suppress the NF-κB activation after induction with LPS. Results from this present study propose that ZnC possesses good potential to be used in the management and treatment of sepsis by inhibiting the secretion of HMGB1 from immune cells.

1. Introduction

Sepsis is a life-threatening medical condition that defines by the dysregulation in systemic inflammatory and immune responses toward severe infection that causes organ dysfunction [1]. Innate immune cells can recognize the pathogen-associated molecular pattern (PAMP) at the site of infection or damage-associated molecular pattern (DAMP) that released from injury tissues through its pathogen-recognition receptor (PRR), hence initiating intracellular signaling cascades that activate several inflammation-related transcription factors, including the NF-κB [2]. Consequently, the production of various pro-inflammatory mediators is greatly enhanced, which may further activate more immune cells and induce the production of other acute phase reactants, including the coagulation factors. In an ideal condition, the immune responses should resolve once the invading pathogens are eliminated [2]. However, if the body failed to return to homeostasis and the immune responses are persisted, sepsis may occur because of the excessive production of inflammatory mediators.

Being a DNA bound protein, high mobility group box 1 (HMGB1) is crucial to the maintenance of chromatin structure [3]. HMGB1 usually resides in the nucleus of various cell types, including white blood cells [4]. Accumulating evidence demonstrated that HMGB1 protein also involves in the inflammatory reactions [5]. During infection, the HMGB1 protein located inside the nucleus of activated macrophages can be released actively to the extracellular compartment [3]. Besides, HMGB1 also involves in the sterile inflammation by acting as a DAMP during tissue injuries. HMGB1 that leaks out passively from death or injured cells can initiate the immune responses in the body which subsequently leads to the development of inflammation [3]. The released HMGB1, in turn, can bind to various cell membrane receptors presenting

on other cells such as the toll-like receptor (TLR) and receptor for advanced glycation end-products (RAGE) to trigger the pro-inflammatory responses [3]. As a consequence, the HMGB1 further stimulates the production of pro-inflammatory mediators in immune cells, resulting in the formation of a positive loop that potentiates the inflammatory responses. Moreover, excessive release of HMGB1 protein into the extracellular compartment, especially the systemic circulation, has been linked to the progression of sepsis [6].

Zinc L-carnosine (ZnC), or better known as polaprezinc, is commonly used to treat gastric ulcers in Japan [7]. Besides, ZnC has been reported to have therapeutic effects against pressure ulcer and *Helicobacter pylori*-associated gastritis [8, 9]. Recently, the efficacy of ZnC in the treatment of oral mucositis, esophagitis, proctitis, and taste alterations resulting from chemotherapy or radiotherapy is widely studied as well [10, 11]. The potent antioxidant and anti-inflammatory properties of ZnC has been proposed as the mechanisms of action of underlying such therapeutics effects of ZnC [11, 12]. Previously, ZnC was reported to protect mice against lipopolysaccharide (LPS)-induced endotoxin shock [13]. However, the potential of ZnC in the prevention of sepsis progression via the inhibition of HMGB1 secretion remains unclear. Therefore, this study aims to examine the inhibitory effects of ZnC against the secretion of HMGB1 from RAW 264.7 murine macrophages cell line after LPS induction.

2. Material And Methods

2.1 Cell culture and treatment

The RAW 264.7 murine macrophages cell line (ATCC, USA) was cultured following the protocol provided by the supplier. ZnC powder was purchased from Yonezawa Hamari Chemicals Ltd (Japan) and was dissolved in 0.2 N HCl to prepare a 100 mM ZnC stock solution. Meanwhile, the LPS stock solution (1 mg/ml LPS; Sigma, USA) was prepared by reconstituted the LPS in sterile phosphate-buffered solution. The stock solutions were further diluted to the desired concentration by using cell culture medium before treatment. In general, RAW 264.7 cells were plated at the initial density of 1 x 10^5 cells/ml for 18 hours. Cells were then pretreated with different concentrations of ZnC (0–100 μM) for 2 hours and followed by LPS induction (1 μg/ml) for different periods.

2.2 Determination of protein concentration

The protein concentration of the cell lysates was determined by using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, USA) following the manufacturer's guideline. The bovine serum albumin (BSA) solution was used as the standard solution for this assay. In brief, 10 μl of sample or standard solution was mixed with 200 ul of the reagent in a 96 well plate. The optical density of the mixture at 595 nm wavelength was measured by using the I-Mark™ microplate reader (Bio-Rad Laboratories, USA). The protein concentration of each sample was extrapolated from the BSA standard curve.

2.3 Quantification of intracellular free thiol level

The intracellular free thiol level was quantified as described in the previous study [14]. Prior to the experiment, the pH6.5 reaction buffer and pH8 reaction buffer consisted of 0.1M Na_2HPO_4 and1mM EDTA was prepared in equal volume and adjusted to pH6.5 and pH8 respectively. Following treatment, cells were lysed with 100 µl of cell lysis buffer (50 mM K_2HPO_4; 1mM EDTA, pH 6.5; 0.1% v/v Triton X-100) for 15 minutes in chilled condition before centrifugation at 4000 X g and 4°C for another 15 minutes. On the other hand, the GSH standard solution was prepared freshly by dissolving the GSH in the pH6.5 reaction buffer and further diluted to different concentrations by using the serial dilution technique. Then, 50 µl of standard solution or cell lysate was mixed with 40 µl of pH8 reaction buffer in a 96 well plate before the addition of 10 µl of DTNB solution [4 mg DTNB (Sigma, USA) dissolved in 1 ml pH8 reaction buffer]. After 15 minutes of incubation, the plate was analyzed at 405 nm wavelength was measured by using the I-Mark™ microplate reader (Bio-Rad Laboratories, USA). The intracellular free thiol level of each sample was then extrapolated from the GSH standard curve and was expressed as nmol/mg protein.

2.4 Immunoblotting analysis

Immunoblotting analysis was carried out as previously described [15]. The harvested cells were lysed in the cell lysis buffer and the protein concentration of each sample was adjusted to 1 mg/ml. Then, samples were subjected to protein separation by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were electrotransfer to a PVDF membrane. After that, SNAP i.d. 2.0 protein detection system (Millipore, USA) was used to facilitate the antibodies incubation step. The antibodies (Cell Signaling Technology, USA) used in this study were primary antibodies against Akt, phosphorylated-Akt, glutamate-cysteine ligase catalytic subunit (GCLC), HMGB1, p65, phosphorylated-p65 (Ser536) and β-Actin and the horseradish peroxidase (HRP)-conjugated secondary antibodies. The targeted protein bands were then visualized by using the Luminata Forte Western HRP substrate (Millipore, USA) and the Fusion FX7 Imaging System (Vilber Lourmat, Germany). The β-actin bands were used as the loading control for this study.

2.5 Statistical analysis

In this present study, three independent trials (n = 3) were performed for each experiment. Then, statistical analysis was performed by using the Prism 7.0 software (GraphPad Inc., San Diego, CA). One-way analysis of variance (ANOVA) followed by Tukey's Post-Hoc test was used to analyze the data obtained from experiments. A p-value < 0.05 was considered as statistically significant. All results are expressed as mean ± standard error (SE).

3. Results

3.1 Effects of ZnC on intracellular HMGB1 level

Results showed that pretreatment with 100 µM of ZnC alone did not have any effects on the HMGB1 level in RAW 264.7 cells (Fig. 1). Following LPS induction, the HMGB1 level dropped from 100 % in the negative

control group (cells without ZnC pretreatment and LPS induction) to 51.55 ± 3.55 % ($p < 0.05$) in the positive control group (cells induced with LPS only), and cells pretreated with ZnC showed a concentration-dependent increment in HMGB1 level, suggesting that pretreatment with ZnC can prevent the secretion of HMGB1 from the cells into the extracellular compartment. However, significant increment in HMGB1 level was only detected in cells pretreated with 100 μM of ZnC (78.51 ± 2.09 %) in comparison with the positive control.

3.2 Effects of ZnC on intracellular free thiol level

As shown in Fig. 2a, after 22 hours of incubation, the free thiol level was 163.09 ± 0.58 nmol/mg protein and 166.68 ± 6.04 nmol/mg protein in the negative and positive control group respectively. No significant difference in free thiol level was observed between these two groups. On the other hand, in cells pretreated with 100 μM ZnC, the intracellular free thiol level reduced from 419.78 ± 13.51 nmol/mg protein at the beginning of LPS induction to 159.12 ± 3.65 nmol/mg protein after 16 hours of LPS induction. The free thiol level then bounced back to 239.28 ± 10.88 nmol/mg protein after 24 hours of LPS induction. However, only cells at the beginning and after 4 hours, 8 hours, and 22 hours of LPS induction showed significantly higher free thiol level as compared to the negative control and positive control. On the other hand, after 22 hours of LPS induction, results showed that the intracellular free thiol level of RAW 264.7 cells increased with increasing concentration of ZnC pretreatment (Fig. 2b). However, only cells pretreated with 50 μM (219.12 ± 8.03 nmol/mg protein) and 100 μM (238.01 ± 7.82 nmol/mg protein) of ZnC showed significant increment in free thiol level if compared to the positive control group (176.71 ± 0.55 nmol/mg protein).

3.3 Effects of ZnC on the activation of NF-κB signaling pathway

As shown in Fig. 3a, the phosphorylated-p65/p65 ratio was relatively low in the negative control group (0.47 ± 0.06) and cells pretreated with 100 μM ZnC only (0.50 ± 0.06) without LPS induction. After LPS induction, the phosphorylated-p65/p65 ratio increased gradually with increasing ZnC concentration, indicating that pretreatment with ZnC can enhance the early phase NF-κB activation. Significant increment in the phosphorylated-p65/p65 ratio was detected in cells pretreated with 50 μM (1.32 ± 0.11) and 100 μM (1.51 ± 0.05) ZnC as compared to positive control group (1.00). In contrast, ZnC was able to reduce the phosphorylated-p65/p65 ratio in a concentration-dependent manner ($p < 0.05$) after 22 hours of LPS induction, indicating that ZnC can attenuate the late phase NF-κB activation (Fig. 3b).

3.4 Effects of ZnC on the activation of the Akt signaling pathway

Results showed that the phosphorylated-Akt/Akt ratio in RAW 264.7 cells was significantly lower without LPS induction (Fig. 4). The phosphorylated-Akt/Akt ratio in the negative control group and cells pretreated with 100 μm ZnC only was 0.08 ± 0.01 and 0.11 ± 0.03 respectively. Induction with LPS was showed to activate the Akt signaling pathway, and pretreatment with ZnC can further enhance such activation. The

phosphorylated-Akt/Akt ratio was observed in cells pretreated with 50 μM (1.24 ± 0.02) and 100 μM (1.35 ± 0.06) ZnC was significantly higher than the positive control (1.00).

4. Discussion

It has been reported that HMGB1 can be secreted actively by macrophages induced by various PAMP, including the LPS [4]. The active secretion of HMGB1 into the extracellular compartment during inflammation involves two essential steps. The first step involves the hyperacetylation of the lysine residues at the nuclear localizing sequence (NLS) of HMGB1 via the JAK-STAT signaling pathway, which promotes the shuttling of HMGB1 protein out from the nucleus into the cytoplasm [16]. Then, the second step involves the secretion of the cytoplasmic HMGB1 into the extracellular compartment either via the pyroptosis or exocytosis of secretory lysosomes [16]. Our current findings demonstrated that pretreatment with ZnC was able to inhibit the secretion of HMGB1 into the extracellular compartments. However, the exact mechanisms that underlying such observation remain unclear. Hence, future study is needed to determine how ZnC inhibit the secretion of HMGB1 during inflammation.

During inflammation, reactive oxygen species (ROS) are generated by the immune cells, leading to oxidative burst [17]. The ROS produced may alter the functionality and biological activities of various proteins and enzymes, including the HMGB1. It has been reported that the pro-inflammatory properties of HMGB1 are greatly depending on the redox state of two redox-sensitive cysteine moieties (C23 and C45) [16]. When these two cysteine moieties are in reduced condition, HMGB1 is immunologically inactive. However, excessive production of ROS during inflammation can oxidize the C23 and C45 residues to form a disulfide bond. The resulting conformation changes enable HMGB1 to bind to the TLR and act as a pro-inflammatory mediator. It has been reported that the GSH and thioredoxin can help to maintain HMGB1 in its reduced form after LPS induction in human umbilical vein endothelial cells (HUVEC) and HK-2 human kidney proximal tubule cells, hence limiting the pro-inflammatory activities of HMGB1 [18]. Since GSH and thioredoxin are part of the non-enzymatic antioxidant defense mechanisms in the cells, these thiol-based antioxidants can prevent the HMGB1 protein from being oxidized by scavenging the ROS that being produced during inflammation [19]. Our current results showed that ZnC was able to increase the free thiol level of RAW 264.7 cells after LPS induction, indicating that ZnC may help to limit the pro-inflammatory activities of HMGB1 by keeping it in the reduced state.

It has been reported that the binding of HMGB1 to the TLR can cause the activation of NF-κB signaling [3]. NF-κB is a transcription factor that involves in the regulation of immune response, inflammation, and cell survival [20]. The activation of the NF-κB can induce the expression of various inflammatory mediators, such as the tumor necrosis factor-alpha (TNF-α), interleukin-1, and interleukin-6. Hence, the HMGB1 protein released into the extracellular compartments may cause overproduction of such mediators. Besides, we demonstrated that pretreatment with ZnC was able to attenuate the late phase NF-κB activation, but not the early activation of NF-κB. Similar findings were reported in previous studies as well [13, 21]. This is reasonable since HMGB1 is a late mediator of sepsis pathogenesis due to its delayed released manner [4]. Previously, it has been reported that the secretion of HMGB1 can only be

detected in mice after 8 hours of LPS administration [22]. Hence, it is possible that the suppression of NF-κB activation after 22 hours of LPS induction was partly due to the inhibition of HMGB1 secretion by ZnC pretreatment.

Interestingly, our current findings demonstrated that after 30 minutes of LPS induction, pretreatment with ZnC can enhance the early phase NF-κB activation instead of suppressing its activation. Following LPS stimulation, the TLR4 signals to the activation of the NF-κB via the MyD88 dependent or TRIF dependent signaling pathway [23]. The MyD88 dependent pathway leads to the early phase NF-κB activation while the TRIF dependent pathway signals to the late phase NF-κB activation. Previously, it has been demonstrated that zinc, which is one of the subcomponents of ZnC, can regulate these two pathways differentially [24]. Intracellular free zinc ions were demonstrated to enhance MyD88 dependent NF-κB activation while suppressing the TRIF dependent NF-κB activation in cells. Results from this present study are in agreement with previous findings where ZnC was found to enhance the early phase NF-κB activation while suppressing the late phase NF-κB activation. It is noted that the activation of NF-κB not only can induce the expression of pro-inflammatory mediators, but it may also induce the expression of anti-inflammatory mediators, including the A20 protein [25]. A20 protein is a deubiquitinase that involved in the termination of the NF-κB signaling pathway [26]. Previous study has demonstrated that zinc can downregulate the NF-κB signaling pathway and inhibit the expression of pro-inflammatory mediators via the induction of A20 protein [27]. Hence, further study is needed to determine whether the induction of early NF-κB activation by ZnC pretreatment can lead to the expression of A20 protein and other anti-inflammatory mediators or not.

Furthermore, pretreatment with ZnC was showed to activate the Akt signaling pathway after 30 minutes of LPS induction. Previously, the activation of the Akt signaling pathway was demonstrated to limit the pro-inflammatory responses in LPS-induced macrophages by downregulating the TLR signaling cascade [28, 29]. Besides, the inhibition of activation of the Akt signaling pathway by using inhibitors greatly enhanced the cytokine production and reduced the survival time in LPS-induced endotoxemic mice [30]. The Akt signaling pathway was also reported to be crucial in the polarization of macrophages toward the M2 phenotype [31]. Hence, ZnC may exert its anti-inflammatory effects by activating the Akt signaling pathway.

In conclusion, pretreatment with ZnC can inhibit the secretion of HMGB1 protein from RAW 264.7 cells and suppress the activation of the late phase NF-κB signaling pathway after induction with LPS. Moreover, present findings also demonstrated that ZnC was able to enhance the activation of the Akt signaling pathway, suggesting that Akt may play a crucial role underlying the anti-inflammatory effects of ZnC. Taken together, we propose that ZnC is capable to prevent the occurrence of sepsis by inhibiting the secretion of HMGB1 from immune cells.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

RS and KMC involved in the conception and study design. TCO conducted the experiments and contributed to data analysis and interpretation. TCO drafted the original manuscript. All authors read and approved the final manuscript.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Figures

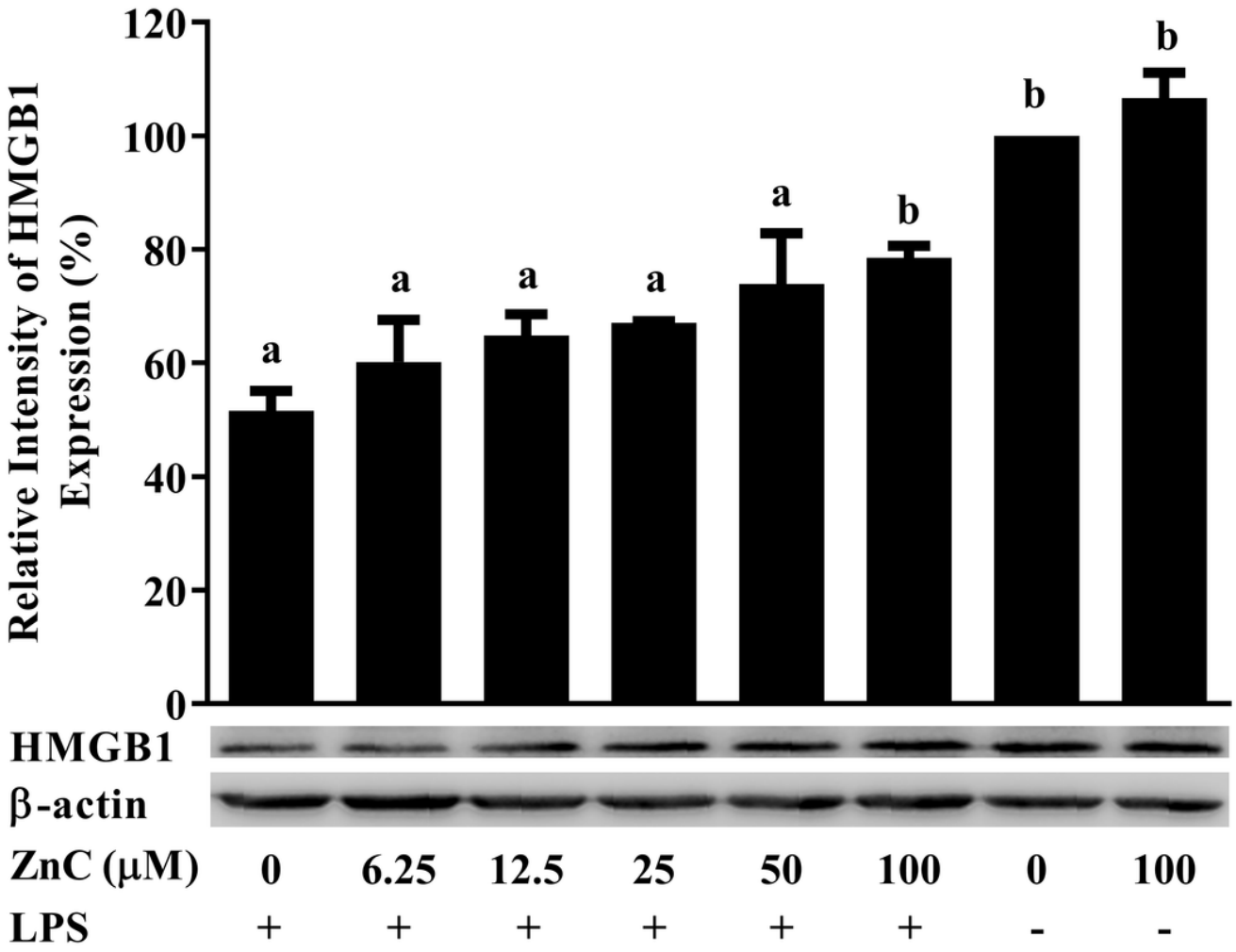

Figure 1

Inhibitory effects of ZnC on the secretion of HMGB1 from RAW 264.7 cells. The lower the intracellular HMGB1 protein level, the more it had been secreted into the extracellular compartment. aSignificant difference ($p<0.05$) as compared to the negative control. bSignificant difference ($p<0.05$) as compared to the positive control. All results are expressed as mean ± SE (n=3)

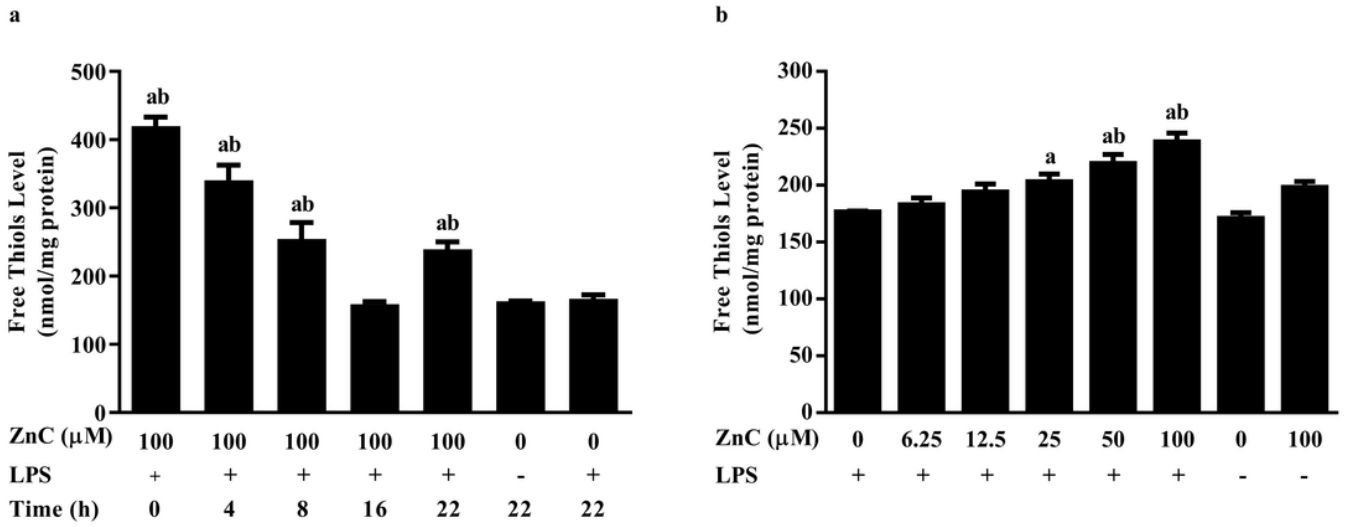

Figure 2

Effects of ZnC on the free thiol level in LPS-induced RAW 264.7 cells in (a) time-dependent manner or (b) concentration-dependent manner. aSignificant difference ($p<0.05$) as compared to the negative control. bSignificant difference ($p<0.05$) as compared to the positive control. All results are expressed as mean ± SE (n=3).

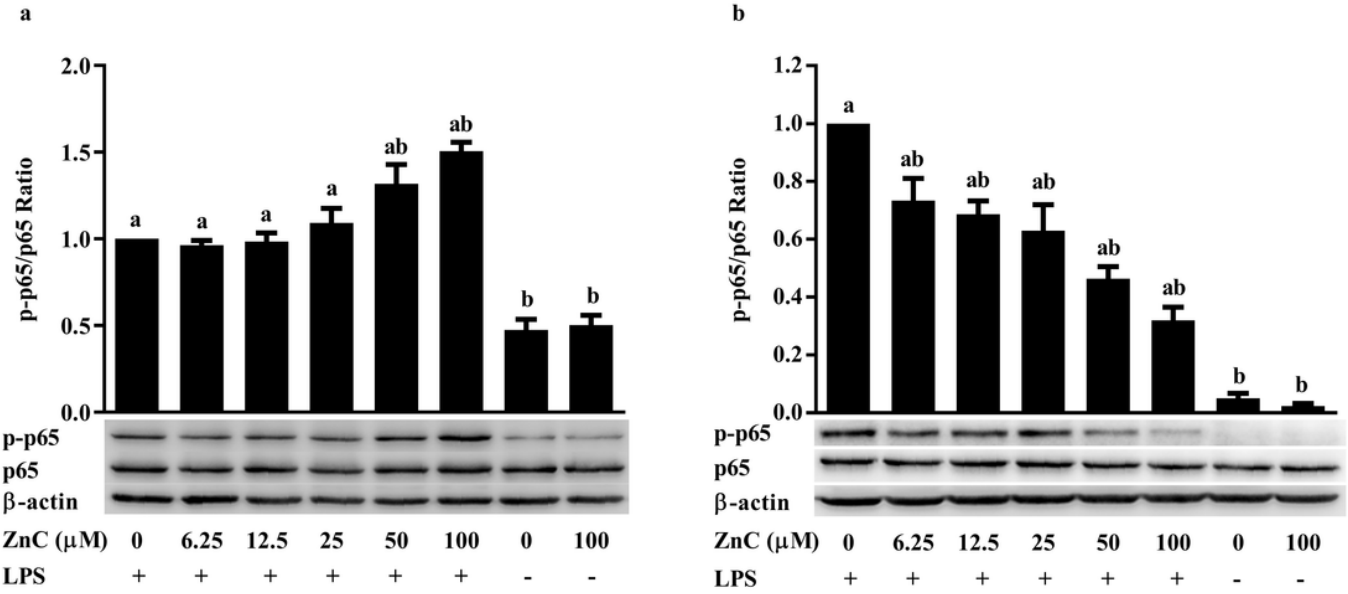

Figure 3

Effects of ZnC on the phosphorylated-p65/p65 ratio in RAW 264.7 cells after induction with LPS (1 μg/ml) for (a) 30 minutes or (b) 22 hours. aSignificant difference ($p<0.05$) as compared to the negative

control. bSignificant difference ($p<0.05$) as compared to the positive control. All results are expressed as mean ± SE (n=3).

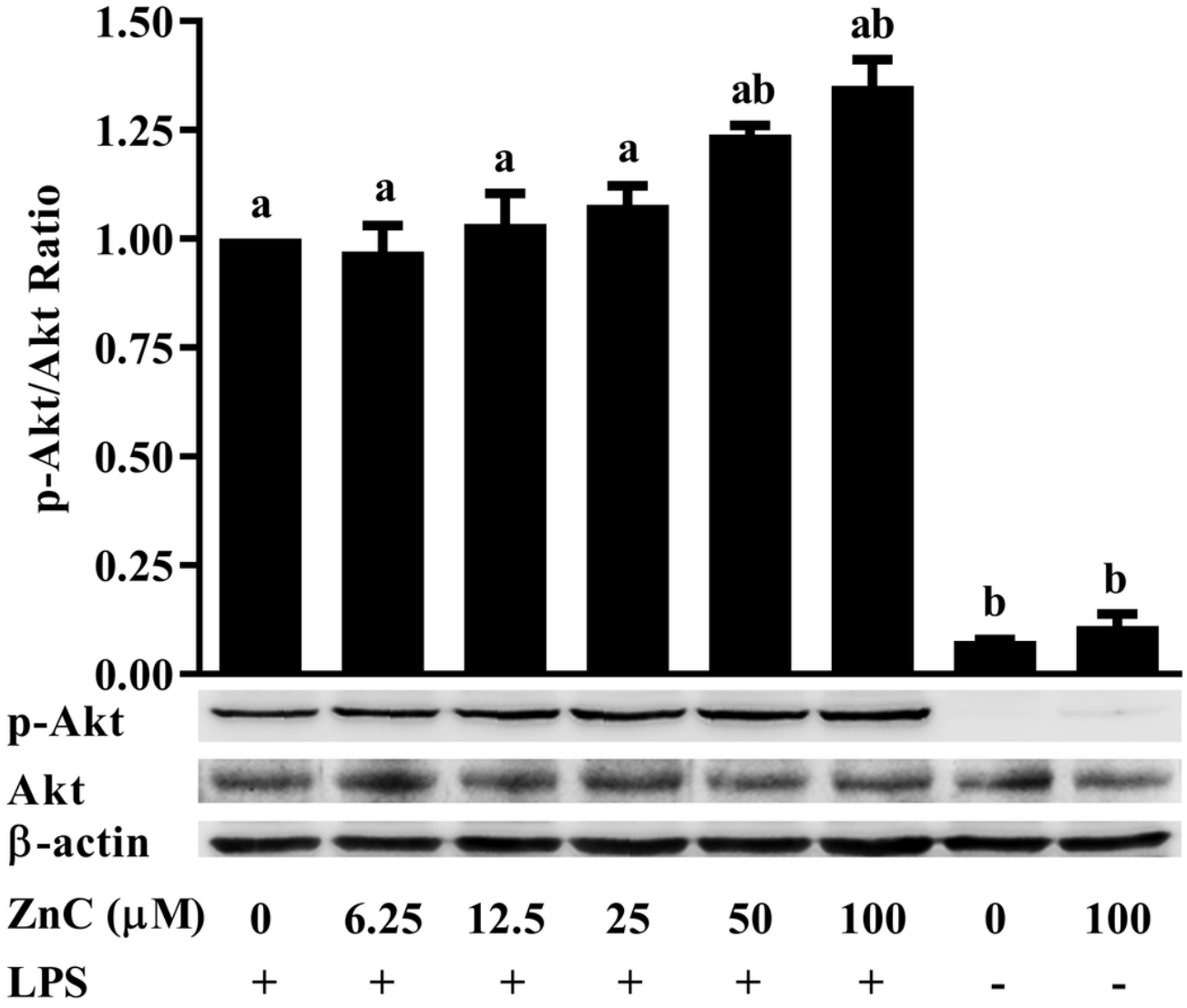

Figure 4

Effects of ZnC on the phosphorylated-Akt/Akt ratio in RAW 264.7 cells after induction with LPS (1 μg/ml) for 30 minutes. aSignificant difference ($p<0.05$) as compared to the negative control. bSignificant difference ($p<0.05$) as compared to the positive control. All results are expressed as mean ± SE (n=3).