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Zinc Carnosine Inhibits Lipopolysaccharide-Induced Inflammatory Mediators by Suppressing NF-κb Activation in Raw 264.7 Macrophages, Independent of the MAPKs Signaling Pathway

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Abstract This study aimed to investigate the role of the mitogen-activated protein kinases (MAPKs) signaling pathway in the anti-inflammatory effects of zinc carnosine (ZnC) in lipopolysaccharide (LPS)-induced RAW 264.7 cells. Cells were pretreated with ZnC (0–100 μM) for 2 h prior to the addition of LPS (1 μg/ml). Following 24 h of treatment, ZnC was found not to be cytotoxic to RAW 264.7 cells up to the concentration of 100 μM. Our current findings showed that ZnC did not protect RAW 264.7 cells from LPS-induced "respiratory burst". Significant increment in intracellular glutathione (GSH) level and reduction in thiobarbituric acid reactive substances (TBARS) concentration can only be observed in cell pretreated with high doses of ZnC only (50 and 100 μM for GSH and 100 μM only for TBARS). On the other hand, pretreatment of cells with ZnC was able to inhibit LPS-induced inducible nitric oxide synthase and cyclooxygenase-2 expression significantly. Furthermore, results from immunoblotting showed that ZnC was able to suppress nuclear factor-kappaB (NF-κB) activation, and highest suppression can be observed at 100 μM of ZnC pretreatment. However, pretreatment of ZnC did not inhibit the early activation of MAPKs. In conclusion, pretreatment with ZnC was able to inhibit the expression of inflammatory mediators in LPS-induced RAW 264.7 cells, mainly via suppression of NF-κB activation, and is independent of the MAPKs signaling pathway.

Keywords Zinc carnosine · Anti-inflammatory · RAW 264.7 cells · NF-κB · MAPK

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Introduction

Lipopolysaccharide (LPS) is a heat-stable endotoxin that can be found in the outer membrane of Gram-negative bacteria [1]. It is well known that induction of macrophages with LPS will cause the activation of toll-like receptor 4 (TLR4), thus recruiting several adaptor proteins to bind to it [2]. Binding of adaptor proteins to TLR4 will trigger the downstream signaling pathways, leading to the activation of several pro-inflammatory transcription factors such as nuclear factor-kappaB (NF-κB) and activator protein 1 (AP-1). Activation of NF-κB requires the phosphorylation and subsequent degradation of inhibitors of NF-κB (IκB) while activation of AP-1 mainly depends on the mitogen-activated protein kinases (MAPKs) signaling [2, 3]. Activation of such transcription factors is essential for the production of pro-inflammatory mediators to mediate the inflammatory process. However, prolonged or uncontrolled inflammatory process will cause detrimental effects to living organisms, such as endotoxin shock [4].

Zinc carnosine (ZnC) is the chelated form of zinc and L-carnosine and is one of the zinc dietary supplements that currently available in the market [5]. Zinc is one of the essential trace elements involved in various biological functions [6]

while L-carnosine (β-alanyl-L-histidine) is a dipeptide that possesses antioxidant, anti-ageing and metal-chelating properties [7]. Previous studies demonstrated that ZnC possesses strong anti-oxidant properties and can help in the maintenance of genomic stability [8, 9]. Besides that, several studies also reported that ZnC can prevent gastric mucosal injury caused by a number of stimuli such as non-steroidal anti-inflammatory drugs (NSAIDs) and Helicobacter pylori [10, 11]. Accumulating evidence revealed that ZnC can help in reducing inflammatory responses in gastric epithelial cells and prevent mice from endotoxin shock [12, 13]. Both studies demonstrated that ZnC is able to suppress NF-κB activation following the induction of inflammation, suggesting that the anti-inflammatory effect of ZnC was due to the inhibition of NF-κB signaling pathway. However, the role of the MAPKs signaling pathway in the anti-inflammatory effect of ZnC is not yet fully understood. Thus, this present study aimed to investigate the role of the MAPKs signaling pathway in the anti-inflammatory effects of zinc carnosine (ZnC) in lipopolysaccharide (LPS)-induced RAW 264.7 cells.

Experimental Procedures

Cell Culture and Treatment

RAW 264.7 macrophages cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10 % fetal bovine serum (FBS; Sigma, USA) and 1 % penicillin-streptomycin (PAA Laboratories GmbH, Austria) at 37 °C with 5 % CO_2 and humidified atmosphere. The initial concentration of zinc was around 5 μM in DMEM supplemented with 10 % FBS. A 100 mM stock solution of ZnC (Yonezawa Hamari Chemicals Ltd, Japan) was prepared by dissolving ZnC powder in 0.2 N HCl and was further diluted in the culture medium to 6.25, 12.5, 25, 50, and 100 μM prior to treatment. This range of ZnC concentration enables this present study to investigate the anti-inflammatory effects of ZnC that falls within the normal physiological range of plasma zinc concentration, as well as in excessive/supplemented conditions. LPS (Sigma, USA) was dissolved by using sterile phosphate-buffered solution (PBS) to become a stock solution with the concentration of 1 mg/ml. LPS was then further diluted in treatment medium to reach the final concentration of 1 μg/ml. Briefly, RAW 264.7 cells at the concentration of 1×10^5 cells/ml were seeded into different well plates or cell culture dishes for 18 h. Cells were then pretreated with ZnC for 2 h prior to the addition of LPS (1 μg/ml) for different time point to induce the inflammation process.

MTT Cell Viability Assay

MTT assay was carried out with slight modification from Mosmann (1983) method [14]. Two hundred microliters of RAW 264.7 cells at the concentration of 1×10^5 cells/ml were seeded into 96-well plates. After treatment, 20 μl of 0.5 % (*w*/*v*) MTT solution was added into each well and incubated for 4 h at 37 °C. The medium was carefully discarded from each well, and 200 μl of dimethyl sulfoxide was then added to dissolve the formazan. After 30 min of incubation at 37 °C, absorbance was read with an I-Mark™ microplate reader (Bio-Rad Laboratories, USA) at 570 nm. The viability of cells was expressed as the percentage relative to untreated cells.

Measurement of Intracellular Reactive Oxygen Species Level

Intracellular reactive oxygen species (ROS) level was detected by using dichloro-dihydro-fluorescein diacetate (DCFH-DA) labeling assay [15]. Treated cells were harvested and transferred into a microcentrifuge tube. The supernatant was discarded after centrifugation, and the pellet was resuspended in 1 ml of fresh pre-warmed FBS-free DMEM with the addition of 1 μl of 10 mM DCFH-DA (Life Technologies, USA). The cell suspension was incubated for 15 min at 37 °C in the dark and followed by centrifugation at 220×*g* for 5 min. The cells were washed with 1 ml ice-cold PBS, and the supernatant was discarded. Then, 500 μl of ice-cold PBS was used to resuspend the pellets. The stained cell suspension was transferred to flow tubes and analyzed using BD FACSCanto II Flow Cytometer (BD Bioscience, USA) on 10,000 cells.

Quantification of Intracellular Glutathione Level

The glutathione (GSH) assay was carried out with a slight modification from Rahman et al.'s method [16]. Treated cells were collected by centrifugation and washed twice by using ice-cold PBS. Cells were then lysed with 100 μl of cell lysis buffer (50 mM K_2HPO_4; 1 mM EDTA, pH 6.5; 0.1 % (*v*/*v*) Triton X-100) in ice-cold condition for 15 min with gently tapping from time to time. After incubation, the mixture was centrifuged at 4000×*g* at 4 °C for 15 min. Then, 50 μl of GSH standard (1.25 to 0 mM GSH [Sigma, USA] dissolved in reaction buffer [0.1 M Na_2HPO_4, 1 mM EDTA, pH 6.5]) and 50 μl of lysate were added to the designated well in a 96-well plate. 40 μl of reaction buffer (0.1 M Na_2HPO_4, 1 mM EDTA, pH 8) was then added into each well and followed by 10 μl of DTNB solution (4 mg DTNB [Sigma, USA] in 1 ml pH 8 reaction buffer). The plate was then incubated for 15 min at 37 °C and then read under I-Mark™ microplate reader (Bio-Rad Laboratories, USA) with the wavelength of 405 nm. GSH level was then determined from

the standard curve and the GSH concentration was express as nmol/mg protein.

Estimation of Lipid Peroxidation

The intracellular thiobarbituric acid reactive substances (TBARS) assay was carried out with a slight modification from Ohkawa et al.'s method [17]. After treatment, cells were harvested and washed with ice-cold PBS for twice. Cells were then lysed with 100 μl of 0.5 % Triton X-100 solution, and whole cell homogenates were used for TBARS assay. One hundred microliters of cell homogenates were mixed with 400 μl of 0.67 % thiobarbituric acid (dissolved in 0.05 N NaOH) and 500 μl of 20 % trichloroacetic acid (dissolved in 0.6 N HCl) in a microcentrifuge tube. The mixtures were then heated in boiling water for 1 h. After cooled down to room temperature, the mixtures were then centrifuged at 4000×*g* for 15 min. The absorbance of the supernatant was measured at 532 nm by using Multiskan™ GO microplate spectrophotometer (Thermo Scientific, USA). The concentration of TBARS in the samples was determined by using tetraethoxypropane as a reference standard and was normalized with protein concentration respectively.

Immunoblotting Analysis

Following treatment, RAW 264.7 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma, USA) containing 1 mM dithiothreitol (Sigma, USA) protease inhibitor (Roche, Germany) and phosphatase inhibitor cocktail (Roche, Germany). Twenty micrograms of proteins were resolved by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride (PVDF) membrane. Immunodetection was performed by using SNAP i.d. 2.0 protein detection system (Millipore, USA), together with primary antibodies against inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), c-Jun N-terminal kinase (JNK), phospho-JNK, extracellular signal-regulated kinase (ERK), phosphor-ERK, p38, phospho-p38, p65, phospho-p65, and β-Actin (Cell Signaling Technology, USA) and horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology, USA). Detection was conducted on each of the membranes by using Amersham enhanced chemiluminescence (GE Health Care, UK) and Fusion FX7 documentation system (Vilber Lourmat, Germany).

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 21.0. For each treatment, three independent experiments were performed and data are expressed as mean ± standard error. Differences between treatment and control values were determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, with $p < 0.05$ considered statistically significant.

Results

Cell Viability

The cytotoxic effect of ZnC was examined using an MTT assay to determine the effective concentration required for treatment. Upon treated with ZnC for 24 h, RAW 264.7 cells did not show any evidence of cytotoxicity at any treatment concentrations, as shown in Fig. 1.

Intracellular ROS, GSH and TBARS Level

RAW 264.7 cells did not show any significant increment in relative fluorescence units (RFU) after treated with 100 μM of ZnC, as shown in Fig. 2a. Upon induction with LPS, RFU increased significantly from 194.0 ± 8.3 in untreated cells to 583.7 ± 34.5 in cells induced by LPS only ($p < 0.001$). However, cells pretreated with all concentrations of ZnC demonstrated higher RFU as compared to LPS-induced untreated cells, with significant increment can be detected at 50 and 100 μM ZnC pretreatment ($p < 0.05$).

Figure 2b showed the intracellular GSH level in LPS-induced RAW 264.7 cells with or without pretreatment with ZnC. Upon pretreatment with ZnC, intracellular GSH level increase slightly from 164.3 ± 3.6 nmol/mg protein in untreated cells to 193.1 ± 9.7 nmol/mg protein ($p = 0.074$). Upon induction with LPS, GSH level did not show any significant changes as compared to untreated cells ($p = 1.000$). Pretreatment of cells with ZnC increased intracellular GSH level in a concentration-dependent manner, with significant increment can be detected at 50 μM (207.5 ± 5.0 nmol/mg protein) and 100 μM (207.6 ± 9.9 nmol/mg protein) of ZnC pretreatment, as compared to cells induced by LPS only (165.8 ± 5.2 nmol/mg protein; $p < 0.01$).

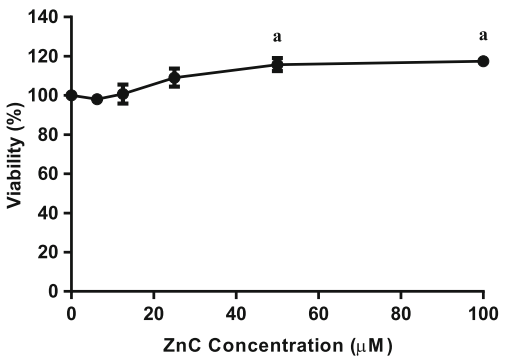

Fig. 1 Cytotoxicity of ZnC on RAW 264.7 cells after 24 h of treatment. [a]Significant difference ($p < 0.05$) as compared to untreated cells. All data are shown as mean ± standard error ($n = 3$)

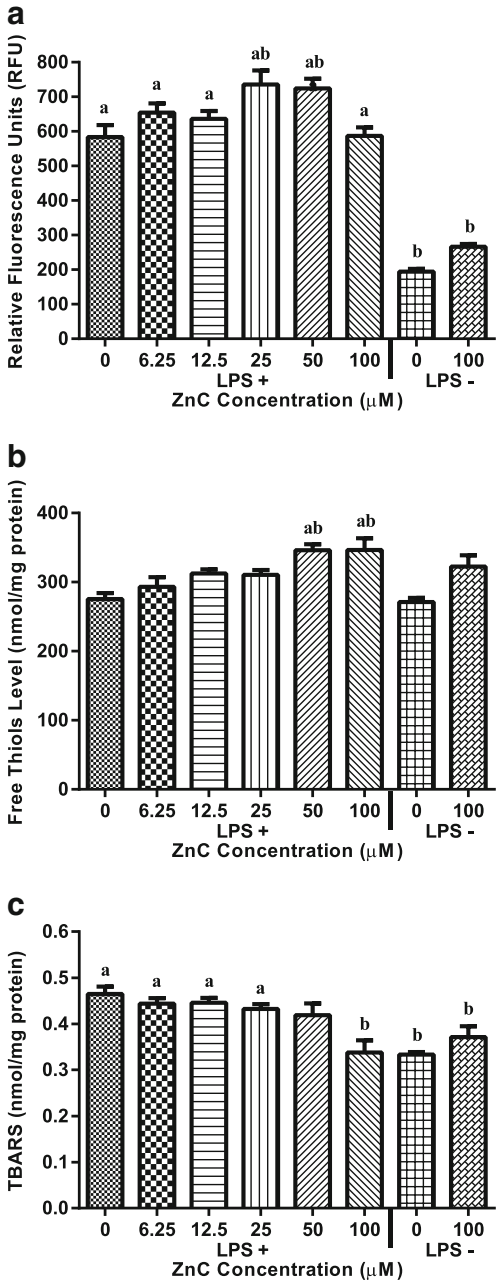

Fig. 2 Intracellular ROS level, GSH level and TBARS concentration in LPS-induced RAW 264.7 cells pretreated with or without ZnC. RAW 264.7 cells were pretreated with various concentration of ZnC (0–100 μM) for 2 h and followed with 22 h of LPS induction. Cells were then harvested and intracellular ROS level (**a**), free thiols level (**b**), and TBARS concentration (**c**) of RAW 264.7 cells were analyzed. [a]Significant difference ($p < 0.05$) as compared to untreated cells. [b]Significant difference ($p < 0.05$) as compared to cells induced by LPS only. All data are shown as mean ± standard error ($n = 3$)

On the other hand, upon induction with LPS, TBARS concentration increased significantly from 333.4 ± 5.2 pmol/mg protein in untreated cells to 464.8 ± 23.4 pmol/mg protein in cells induced by LPS only ($p < 0.01$). Cells treated with 100 μM of ZnC only did not show a significant increment in TBARS concentration (371.5 pmol/mg protein; $p = 0.785$) as compared to untreated cells. Pretreatment of cells with ZnC reduced TBARS concentration in a concentration-dependent manner, with the highest reduction was observed in cells pretreated with 100 μM of ZnC (338.0 ± 26.1 pmol/mg protein; $p < 0.01$) as compared to cells induced by LPS only (Fig. 2c).

Expression Level of iNOS and COX-2

Upon induction with LPS, iNOS and COX-2 expression level increased significantly as compared to those without LPS induction ($p < 0.001$). RAW 264.7 cells did not express iNOS and COX-2 in the absent of LPS, as shown in Fig. 3a, b. Pretreatment of cells with ZnC was able to inhibit iNOS and COX-2 expression at all concentrations, with the highest

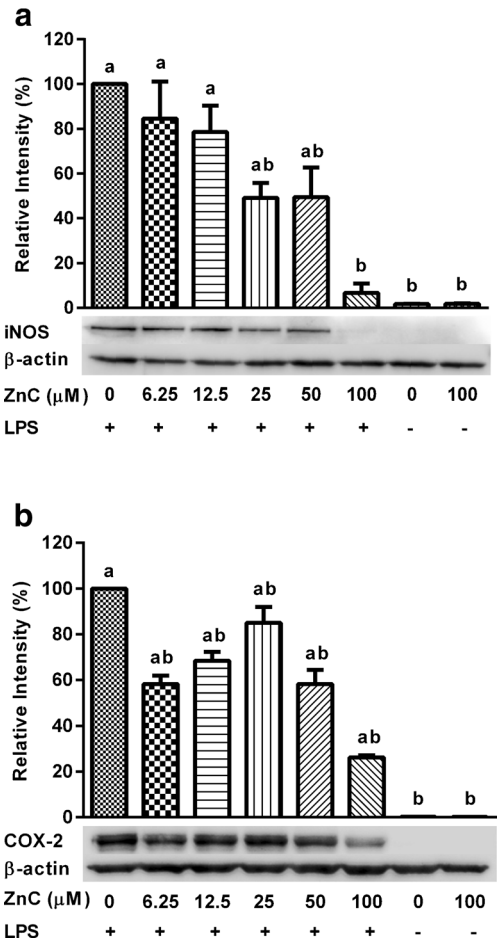

Fig. 3 Effects of ZnC on iNOS and COX-2 expression in LPS-induced RAW 264.7 cells. RAW 264.7 cells were pretreated with various concentration of ZnC (0–100 μM) for 2 h prior to addition of LPS for 22 h. Cells were then lysed and the lysates were used for immunoblotting analysis to determine iNOS (**a**) and COX-2 (**b**) expression levels in RAW 264.7 cells. [a]Significant difference ($p < 0.05$) as compared to untreated cells. [b]Significant difference ($p < 0.05$) as compared to cells induced by LPS only. All data are shown as mean ± standard error ($n = 3$)

inhibition was observed at 100 μM of ZnC treatment (3.7 ± 1.2 % and 26.3 ± 0.5 %, respectively; $p < 0.001$). ZnC caused a dose-dependent inhibition of iNOS expression, with significant inhibition was detected starting from 25 μM of ZnC treatment (56.8 ± 1.3 %; $p < 0.001$). In contrast to iNOS, a significant reduction in COX-2 expression was detected starting from 6.25 μM of ZnC treatment (58.2 ± 3.7 %; $p < 0.001$), and the inhibition was not dose-dependently.

NF-κB Activation

Figure 4 showed phospho-p65 (Ser 536) to p65 ratio in LPS-induced RAW 264.7 cells with or without pretreatment with ZnC. p65 expression was consistent in all treatment conditions, with no significant changes ($p = 0.849$) in expression level was observed. On the other hand, phosphorylation of p65 was not detected in the absence of LPS. However, upon induction with LPS, p65 was phosphorylated, and the phosphorylation can be inhibited by pretreatment of ZnC. ZnC at the concentration of 100 μM demonstrated lowest phospho-p65 to p65 ratio, which was 0.276 ± 0.071.

MAPKs Activities

The basal activities of MAPKs (ERK, JNK and p38) were low in untreated cells, and pretreatment of ZnC did not alter the activities of MAPKs significantly. Induction with LPS for 30 min increased MAPKs activities as compared to untreated cells. However, pretreatment of ZnC did not inhibit the early activation of MAPKs, as shown in Fig. 5.

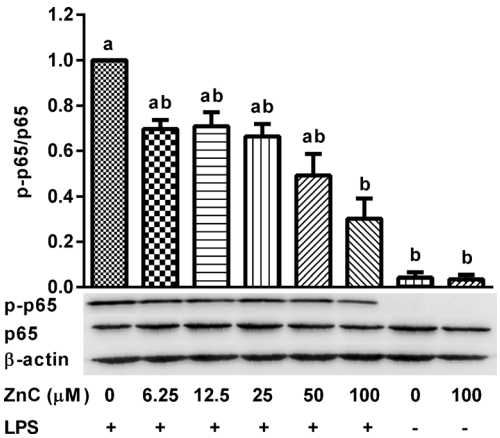

Fig. 4 Effects of ZnC on the activation of NF-κB in LPS-induced RAW 264.7 cells. RAW 264.7 cells were pretreated with various concentration of ZnC (0–100 μM) for 2 h prior to induction of LPS for 22 h. Cells were then lysed and the lysates were used for immunoblotting analysis to determine the p-p65 to p65 ratio. [a]Significant difference ($p < 0.05$) as compared to untreated cells. [b]Significant difference ($p < 0.05$) as compared to cells induced by LPS only. All data are shown as mean ± standard error ($n = 3$)

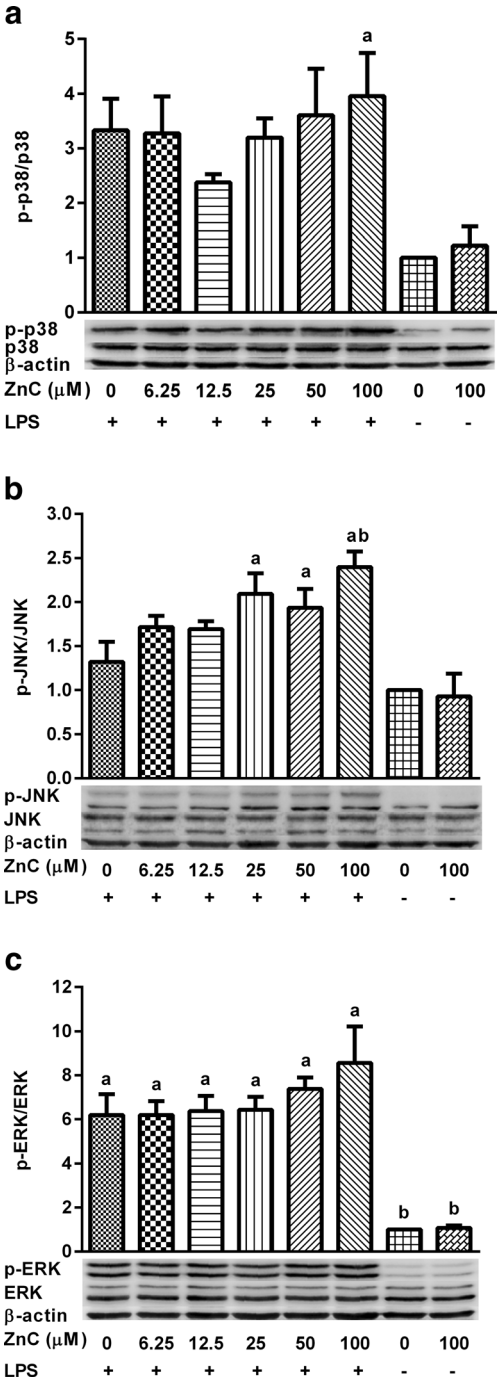

Fig. 5 Effects of ZnC on the activation of MAPKs in LPS-induced RAW 264.7 cells. RAW 264.7 cells were pretreated with various concentration of ZnC (0–100 μM) for 2 h prior to induction of LPS for 30 min. Cells were then lysed and the lysates were used for immunoblotting analysis to determine the activities of p38 (**a**), JNK (**b**), and ERK (**c**) in RAW 264.7 cells. [a]Significant difference ($p < 0.05$) as compared to untreated cells. [b]Significant difference ($p < 0.05$) as compared to cells induced by LPS only. All data are shown as mean ± standard error ($n = 3$)

Discussion

Treatment of cells with LPS has been shown to induce "respiratory burst" through the assembly and activation of NADPH

oxidase, thus inducing ROS production and consequent lipid peroxidation [18]. In this present study, pretreatment of ZnC did not help to reduce the ROS production in LPS-induced RAW 264.7 cells, and the induction of intracellular GSH expression and reduction in TBARS concentration can only be observed in cells pretreated with high doses of ZnC only. Significant reduction of TBARS concentration in cells pretreated with 100 μM of ZnC would probably due to the increment in intracellular GSH synthesis, which relieve the oxidative condition of the cells. Although ZnC has been reported to possess antioxidant properties and was able to quench malondialdehyde (MDA) in several studies [8, 9, 19], however, the effect as observed in this study is only marginal. Different in experimental designs (cell type or in vivo model) and compounds being used to induce the oxidative condition could partly explain the variation in our observation as compared with previous studies [8, 9, 19].

Generally, induction of cells with LPS will activate TLR4 signaling pathway, leading to the activation of transforming growth factor-β activated kinase 1 (TAK1) [2, 3]. TAK1, in turn, will phosphorylate and activate the IκB kinase (IKK) complex and MAPKs. Activation of IKK complex and MAPKs will trigger the activation of NF-κB and AP-1 respectively, thus leading to the expression of several inflammatory mediators such as iNOS and COX-2 [2, 3]. Results from this present study showed that ZnC was able to inhibit NF-κB activation in a dose-dependent manner after induction of RAW 264.7 cells with LPS. Ohata et al. demonstrated in 2010 that 100 μM of ZnC was able to suppress NF-κB activation in LPS-induced RAW 264.6 cells; thus, this is in agreement with our current findings [13]. Moreover, ZnC was also reported to suppress the activation of NF-κB was also reported in gastric epithelial cells and rat colonic mucosa as well [12, 20]. Hence, the inhibition of iNOS and COX-2 expression as observed in this study was due to the suppression of NF-κB activation by ZnC.

Apart from NF-κB, induction of macrophages with LPS also activates the MAPKs signaling pathway, which lead to the expression of pro-inflammatory mediators [2, 3]. MAPKs can be grouped into three major subfamilies, which are ERK, JNK and p38. It is noted that maximal activation of MAPKs occurs around 30 min after LPS induction [21]. Hence, we further examined whether ZnC inhibits early MAPKs activation in LPS-induced RAW 264.7 cells. Pretreatment of cells with ZnC did not inhibit the activation of ERK, JNK and p38 after 30 min of LPS induction. Thus, our present findings suggest that MAPKs is not involved in the inhibitory effect of ZnC on the expression of pro-inflammatory mediators, and the inhibitory effect is solely based on the suppression of NF-κB activation.

In summary, pretreatment of ZnC was able to inhibit the expression of inflammatory mediators such as iNOS and COX-2 in LPS-induced RAW 264.7 cells, mainly via suppression of NF-κB activation, and is independent of the MAPKs signaling pathway. Current findings give a better understanding of the mechanism underlying the anti-inflammatory effects of zinc in macrophages. This can help in promoting the usage of zinc as one of the preventative measures for inflammation-related diseases.

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