Effect of zinc oxide nanoparticles on the expression of pro-inflammatory proteins in murine macrophages RAW 264.7 cells

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Abstract

Zinc is a microelement essential for the body. It has a great impact on the proper development and renewal of tissues, reproductive system, skin condition, or immune processes. Zincis involved practically in all aspects of the immune system and the production and activation of white blood cells.

This work aimed to determine the effect of zinc oxide nanoparticles (ZnONP) on the expression of pro-inflammatory proteins in murine macrophages RAW 264.7, activated with lipopolysaccharide (LPS). Using the immunodetection technique the expression of cyclooxygenase 2 (COX-2), prostaglandin E2 synthase (cPGES), prostaglandin F2α receptor (FP receptor) and nuclear factor Nrf2 was determined. Statistically the highest expression of COX-2, cPGES, and FP receptor was observed in LPS-activated macrophages. RAW 264.7 cells supplementation with ZnONP 100 nmol and 500 nmol and LPS activation resulted in repression of COX-2 and cPGES, and an increased expression of Nrf2 protein when compared to control.

The results suggest an anti-inflammatory effect and activation of the antioxidant system by ZnONP in RAW 264.7 macrophages. It seems appropriate to conduct further research on the molecular mechanism of action of ZnONP in eukaryotic cells.

Keywords

inflammation, zinc oxide nanoparticles, COX-2, Nrf2

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Abbreviations

COX-2, prostaglandin endoperoxide synthase 2 (cyclooxygenase 2); cPGES; cytosolic prostaglandine E2 synthase; FP, prostaglandin F2α receptor; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; ZnONP, zinc oxide nanoparticles

Introduction

Inflammation is a response of the organism for the harmful factors such as bacteria, injury, viruses etc. Main role in response of the organism for the inflammatory factors is triggered by the immunological system. As a result of the effects of the aforementioned factors, the repair process is initiated, aimed to neutralizing the damaging factor and restoring the homeostasis of the organism [1-3]. Fundamental role in this response play immunological cells and mediators of inflammations like cytokines, chemokines, eicosanoids and other factors. One of them is zinc, a trace element essential for the proper functioning of cells, its development and function. Macrophages, neutrophils, natural killer (NK) cells and lymphocytes needs zinc for its proper function [1, 3]. Zinc also influences on NF-κB signaling pathway during inflammation. That pathway is activated by the pro-inflammatory stimuli. Zinc is one of the inhibitors of NF-κB pathway and is also responsible for its regulation. It is important during resolution of inflammation and prevents tissue damage by the immunological cells [2,3].

The anti-inflammatory effect of zinc is proved and was widely studied. Research were carried out on man, rodents and on cell lines. The effect of many zinc compounds were investigated. The experiments on rodents prove the anti-inflammatory effect of zinc, and its positive influence

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of the anti-inflammatory activity of non steroidal anti-inflammatory drugs [4]. Experiments also involved zinc oxide nanoparticles (ZnONP). Experiments on Wistar rats showed the anti-inflammatory activity of ZnONP in the dose 14 mg/kg intraperitoneally (i.p.) [5]. The research carried out on cell lines also shoved promising results. Zinc oxide nanoparticles shoved antioxidative and anti-inflammatory activity [6]. On the other hand it was shown, that ZnONP may induce oxidative stress in cells and acts as a pro-inflammatory agent [7]. Depending on nanoparticles features, such as method of synthesis, which determines their surface properties, pro-inflammatory or anti-inflammatory effect were obtained.

The aim of the study was to determine the effect of ZnONP on the pro-inflammatory proteins level in murine macrophages RAW 264.7, activated with lipopolysaccharide (LPS).

Materials and methods

Cell cultures

Mus musculus monocytes/macrophages (RAW 264.7, TIB-71, ATCC, USA) were cultured in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics: penicillin (100 IU/mL) and streptomycin (100 μg/mL) in Falcon cell culture vessels (75cm^2). Cell culture was performed in an air atmosphere containing 5% carbon dioxide, in an incubator, at 37°C until reaching 90% confluence. The cultures were washed with phoshate buffer solution (PBS without Ca and Mg ions). Microscopic observations were performed to demonstrate normal cell morphology. Cell viability was determined by trypan blue exclusion test. Experiments were carried out on 6-well culture plates (Sarstedt, Germany), on which a 264.7 RAW cells were seeded at a density of 1x105 cells/mL. After 48 hours cells were incubated for 24 hours with a suspension of ZnONP (Sigma-Aldrich, USA; average φ of nanoparticles below 35 nm) at a concentration 100 and 500 nmol. After this period, 10 ng/mL of LPS was added and macrophages were incubated for another 24 hours. Cells were then collected and prepared for further analysis.

Western blot analysis

Proteins from cell lysates were isolated using M-PER buffer (Thermo Fisher Scientific Inc., USA) with protease and phosphatase inhibitors (Merck, Germany). After centrifugation at 10,000 × g of cell lysates, the supernatant was taken. Bradford method was used to determine total protein concentration. Forty μg of protein was solubilized in the Laemmli buffer with 2% 2-mercaptoethanol. Proteins were separated using 10% polyacrylamide gel electrophoresis with addition of sodium dodecyl sulfate (SDS) and were transferred to PVDF membranes by transblot (BioRad, USA). After transfer, the membranes were blocked in the presence of a solution of 1% casein in TBST (Tris Buffer Saline with 1% Tween) and incubated with anti-COX-2 (GeneTex, USA), anti-cPGES (GeneTex) anti-Nrf2 (GeneTex) and anti-FP receptor (Cayman Chemical, USA) diluted 1:1000. In addition, endogenous control was the anti-β-actin antibody (GeneTex). After membrane rinsing in TBST buffer, the membranes were incubated with horseradish peroxidase conjugated antibody (Thermo Fisher Scientific Inc.). Signal intensities were determined by chemiluminescence using Chemi Doc Camera and Image Lab software (BioRad).

Statistical analysis

Results are presented as means ± SD of at least six independent experiments. A one-way ANOVA along with the Scheffe *post hoc test* were performed to compare the differences between groups,. Calculations were made using the STATISTICA 12 software (StatSoft, USA) and statistical significance was established as $p<0.05$.

Results

Statistically the highest expression of pro-inflammatory proteins: cyclooxygenase 2, prostaglandin E2 synthase and prostaglandin F2α receptor was reported in LPS-activated macrophages ($p=0.000$). In each case with respect to the control group, the increase in protein expression in LPS-activated 264.7 LPS cells was more than double (Fig. 1A, B, C). The addition of 100 nmol of ZnONP did not affect the expression of Nrf2 (Fig. 1D) and cPGES, but decreased the expression level of COX-2 (Fig. 1A). Increasing the amount of zinc oxide nanoparticles to 500 nmol did not result in significant changes in protein expression as compared to RAW cells 264.7 supplemented with 100 nmol ZnONP. In macrophages after adding 100 and 500 nmol zinc oxide nanoparticles and activated LPS, COX2, cPGES and FP receptor expression were lowered compared to LPS-activated macrophages ($p=0.000$).

Discussion

After incubation with different ZnONP concentrations (100 and 500 nmol) and after

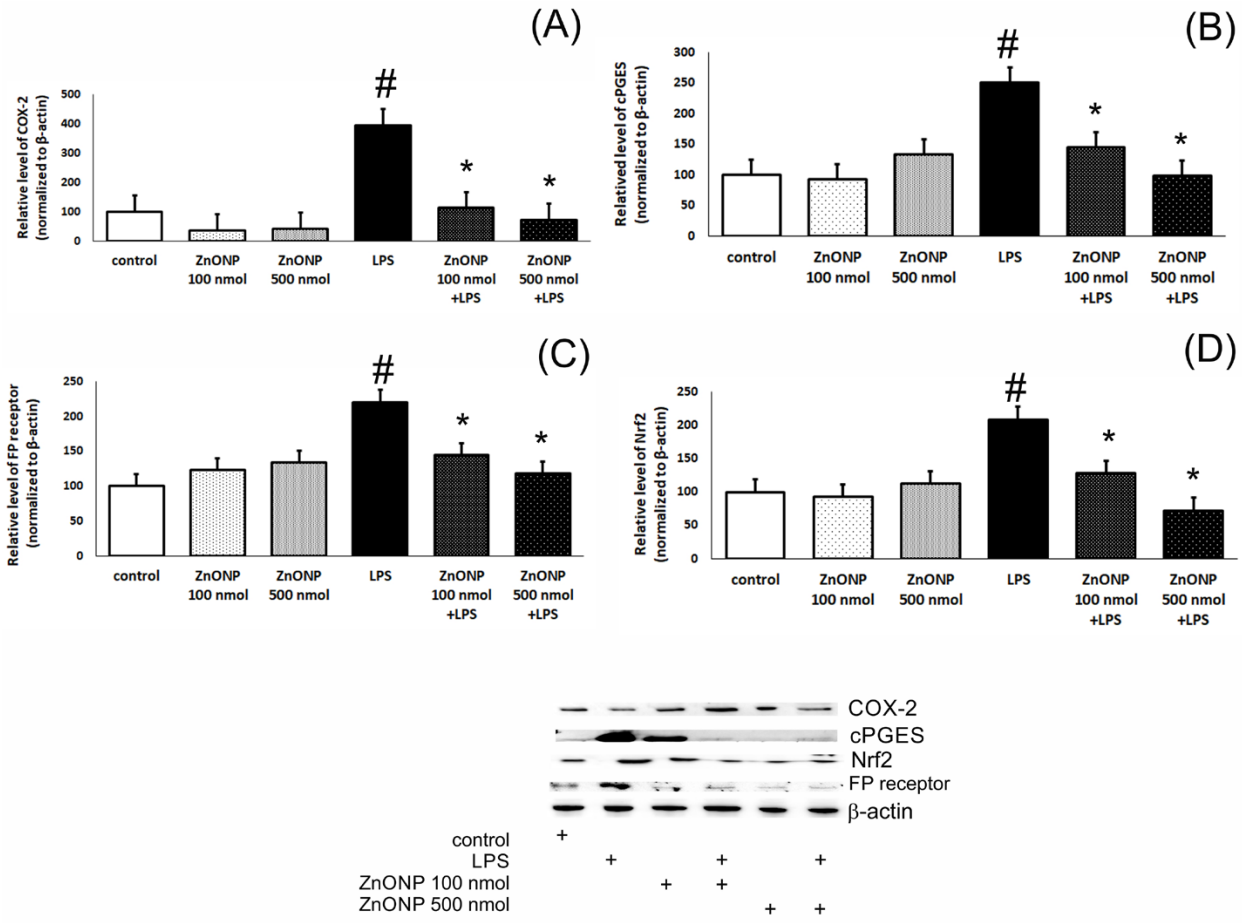

Figure 1. Relative levels of proteins: COX-2 (A), cPGES (B), FP receptor (C) and Nrf2 (D) in RAW 264.7 cells activated with LPS. # vs. control, p<0.001; * vs. LPS, p<0.001.

activation with LPS, the level of pro-inflammatory proteins was decreased in RAW 264.7 cells, in comparison to macrophages incubated only with LPS. This may lead to the hypothesis that zinc oxide nanoparticles acts as anti-inflammatory agent in the murine mouse macrophages. Presented results are consistent with previous study concerning ZnONP and its anti-inflammatory and immunomodulating activity [5]. Our results are consistent with the work of Nagayiothi *et al* [6]. In mentioned research the anti-inflammatory activity of zinc oxide nanoparticles was observed. ZnOnP repressed the COX-2 level in activated with LPS RAW 264.7 cells [6].

Zinc is a trace element, essential for the proper anti-inflammatory response of the organism. It is necessary for the proper functioning of the immunological system. Zinc is necessary for the maturation and proper functioning of the neutrophils, natural killer (NK) cells and macrophages [7,8]. It is necessary for the proper humoral and cellular response. Thymulin (thymus hormone) secretion is also zinc dependent. Thymulin directly influence the functioning of the Th1 cells. Zinc deficiency may lead to the insufficient thymulin secretion, which may cause impaired immunological response of the organism [9]. In case of zinc deficiency the level of T lymphocytes (regulative and helper) is disrupted, the activity of NK cells is decreased. The level of interleukins and interferons is also decreased [8,9].

It is proved that zinc deficiency leads to the increased sucteptibility for the infections. Zinc supplementation may positively influence the duration of the viral and bacterial infections [10].

Diet rich in Zn is one of the factors that strengthen the natural immunological response of the body. The natural sources of zinc, rich in that trace element are oysters, beef and mutton, pumpkin seeds and cocoa, dried herbs, most nuts and seeds. The daily requirement for zinc is 6-11 mg/day and depends on many physiological

factors, such as gender, physiological condition, physical activity, pregnancy [6-10].

Zinc oxide nanoparticles are broadly investigated, due to its properties. Previous researches on different cell lines showed that zinc oxide nanoparticles may possess pro-oxidative, cytotoxic activity or anti-oxidative activity [10]. Zinc oxide nanoparticles may show different activity in case of nanoparticles obtained by different synthesis method, different size, shape and electrochemical charge. Experiments were also conducted on rodents, where it was proved that zinc oxide nanoparticles showed anti-oxidative activity [6].

In the presented results zinc oxide nanoparticles in concentration 100 and 500 nmol did not show cytotoxic activity for the RAW 264.7. The obtained results are confirmed by the data obtained by Kim *et al* [11], where it was observed that zinc oxide nanoparticles in concentration range 1–5 μg/mL exceeded minimal influence on the cell viability. Presented results may suppose that zinc oxide nanoparticles acts as immunomodulating and anti-inflammatory agent in RAW 264.7 cells.

Conclusions

Our results suggested that ZnONP plays a role in enhancement of the anti-oxidant and anti-inflammatory factor and has a high therapeutic value. Supplementation of ZnONP has been beneficial for macrophage viability under LPS activation, and it remains the subject of future studies.

Resumo

Zinko estas spurelemento esenca por la korpo. Ĝi havas grandan efikon al la konvena evoluo kaj renovigo de histoj, reproduktadsistemo, haŭta funkciado aŭ imunaj procezoj. Zinko praktike influas preskaŭ ĉiujn aspektojn de la imuna sistemo kaj la produktadon kaj aktivigon de la blankaj sangĉeloj. Ĉi tiu laboro celis determini la efikon de zinko-oksidaj nanopartikloj (ZnONP) al la evoluado de pro-inflamaj proteinoj en makrofagoj de musoj RAW 264.7, aktivigita per lipopolisakarido (LPS). Uzante la teknikon de imuna malkaŝo estis difinita la evoluo de ciklooksigenazo 2 (COX-2), sintezo de E2 prostaglandino (cPGES), prostaglandina receptoro F2α (FP-receptoro) kaj nuklea faktoro Nrf2.

Statistike la plej alta evoluado de COX-2, cPGES, kaj FP-receptoro estis observita ĉe LPS-aktivigitaj makrofagoj. RAW 264.7 helpe de ZnONP 100 nmol kaj 500 nmol kaj LPS-aktivigo, kio rezultigis redukton de evoluado de COX-2 kaj cPGES, kaj pliigo de evoluado de Nrf2-proteino kompare kun kontrolaj bestoj.

La rezultoj sugestas kontraŭinflaman efikon kaj aktivigon de la antioksidanta sistemo de ZnONP en makrofagoj de RAW 264.7. Ŝajnas konvena fari pliajn esplorojn pri la molekula mekanismo de efiko de ZnONP en eŭkariotaj ĉeloj.

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