FP-FST-010-ID024

Anti-Inflammatory Effect of Lysozyme

Ayuka Tagashira [1], Kosuke Nishi [2,3], Shinya Matsumoto [2] and Takuya Sugahara [2,3,*]

[1] The United Graduate School of Agricultural Sciences, Ehime University, Matsuyama, Ehime 790-8566, Japan
[2] Graduate School of Agriculture, Ehime University, Matsuyama, Ehime 790-8566, Japan
[3] Food and Health Sciences Research Center, Ehime University, Matsuyama, Ehime 790-8566, Japan

* Correspondence: mars95@agr.ehime-u.ac.jp; Tel.: +81-89-946-9863

Received: 9 July 2018; Accepted: 4 December 2018; Published: 6 January 2020

Abstract: Lysozyme is an antibacterial protein that is widely distributed in nature including egg white, fish, and insects. Lysozyme from hen egg white has been reported to possess an anti-inflammatory effect; however, little is known about its detailed mechanism. We examined the effect of lysozyme on macrophages involved in inflammatory responses and on lipopolysaccharide (LPS)-induced inflammation model mice. Lysozyme significantly suppressed the production of interleukin (IL)-6 and tumor necrosis factor (TNF)-α by mouse peritoneal macrophages (P-mac) in a dose-dependent manner by suppressing their gene expression levels. Zymosan A-mediated phagocytosis activity of P-mac was not affected by lysozyme, suggesting that lysozyme shows the anti-inflammatory effect without affecting the phagocytotic response against microbes. In addition, lysozyme inhibited phosphorylation of c-jun N-terminal kinase (JNK) that is a member of mitogen-activated protein kinase (MAPK) involved in the production of inflammatory cytokines by macrophages. Oral administration of lysozyme at 2,250 mg/kg body weight/day significantly decreased the serum IL-6 and TNF-α levels in LPS-induced inflammation model mice. These findings suggest that lysozyme suppresses the gene expression of inflammatory cytokines by inhibiting MAPK signaling pathway in macrophages, and mitigates the hyperinflammatory condition in vivo.

Keywords: Anti-inflammatory effect; Lysozyme; Peritoneal macrophages; Tumor necrosis factor-α; Interleukin-6; Lipopolysaccharide-induced inflammation model mice

1. Introduction

Chronic inflammation closely related to the pathological base of chronic diseases such as cancer and lifestyle-related diseases has garnered attention [1,2]. Inflammation is a defensive reaction, which occurs when individuals are infected with pathogens such as viruses and bacteria. Macrophages involved in inflammation are multifunctional leucocytes related to innate immunity and remove foreign substances such as bacteria and dead cells. In addition, lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is recognized by macrophages and promotes the release of various mediators such as cytokines, chemokines, and prostaglandins. The recognition of LPS by macrophages is caused by binding to Toll-like receptors (TLR) 4 on the cell surface, which activates the cells and promotes the production of inflammatory mediators, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. On the other hand, the overexpression of these inflammatory cytokine genes causes rheumatoid arthritis, insulin resistance, and arteriosclerosis.

Lysozyme is an antibacterial protein that breaks down bacterial cell walls, and widely distributed in nature including egg white, fish, and insects. Hen egg white lysozyme is a basic protein (pI = 11) composed of a single polypeptide chain with 129 amino acid residues. Previous studies have been reported that hen egg white lysozyme promotes antibody production by lymphocytes [3,4]. In addition, heat-treated lysozyme enhances the anti-bacterial and immunostimulatory activities [5,6]. Heat treatment in the food processing step of hen egg white is considered to enhance the immunostimulatory activity. Thus, we examined the heath function of lysozyme derived from hen egg white in the immune system for application to functional foods. Lysozyme is well known to exhibit the anti-inflammatory

effect in addition to anti-bacterial and immunostimulatory activities [7-9]. Lysozyme has also been reported to attenuate inflammation in a porcine model of dextran sodium sulfate-induced colitis [10] and to suppress polyphosphate-mediated vascular inflammatory responses [11]. However, the detailed mechanism of the anti-inflammatory effect of lysozyme is still unknown. Hence, we examined the effect of lysozyme on macrophages involved in inflammatory responses and on LPS-induced inflammation model mice.

2. Materials and Methods

2.1. Reagents

Lysozyme from chicken egg white (≥ 90%), RPMI 1640 medium, penicillin, streptomycin, fetal bovine serum (FBS), and LPS from *Escherichia coli* 026/B6 were products from Sigma-Aldrich (St. Louis, Mo, USA). Goat anti-actin antibody and anti-goat IgG antibody labeled with horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-labeled anti-rabbit IgG antibody and rabbit antibodies against extracellular signal-regulated protein kinases (ERK) 1/2, phosphorylated ERK1/2, c-Jun N-terminal kinase (JNK), phosphorylated JNK, p38 mitogen-activated protein kinase (MAPK), and phosphorylated p38 MAPK, histone H3, and nuclear factor-κB (NF-κB) p65 were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise noted.

2.2. Animals

BALB/c mice were obtained from Japan SLC (Shizuoka, Japan) and kept in a temperature-controlled facility. All animals were maintained and examined according to the protocol approved by the Animal care and Use Committee of Ehime University.

2.3. Peritoneal macrophages

Peritoneal macrophages (P-Mac) were prepared as previously described [12] with some modifications. In brief, 8-week-old female BALB/c mice were injected with 3.0% thioglycolate medium (2 mL/body) into the peritoneum. Four days after injection, mice were sacrificed and injected with 3 mL of RPMI 1640 medium into the peritoneum to harvest thioglycollate-elicited P-Mac. Collected cells were centrifuged at 160 × *g* for 5 min at 4°C, and the cell pellet was washed with RPMI 1640 medium and centrifuged again. The cell pellet was then resuspended in RPMI 1640 medium supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% FBS and cultured in a culture dish (Corning, Corning, NY, USA). After incubation at 37°C for 1 h, the cells were washed with phosphate-buffered saline (PBS; pH 7.4) three times to remove unattached cells such as neutrophils. In the subsequent experiments, P-Mac were detached by pipetting in cold PBS.

2.4. Cytokine production assay

Lysozyme was dissolved in 10 mM sodium phosphate buffer (NaPB; pH 7.4) and sterilized by filtration. P-Mac suspended in 10% FBS-RPMI 1640 medium were seeded into a 96-well culture plate (Corning) at 6.0×10^4 cells/well and cultured at 37°C overnight under humidified 5% CO_2. After washing with PBS, P-Mac were pretreated with 100 ng/mL of LPS in 200 μL of 10% FBS-RPMI 1640 medium at 37°C. After incubation for 1 h, the cells were washed with PBS to remove LPS. P-Mac were then treated with various concentrations of lysozyme in 200 μL of 10% FBS-RPMI 1640 medium at 37°C. After incubation for 11 h, the concentrations of IL-6 and TNF-α in culture media were measured by enzyme-linked immunosorbent assay (ELISA) using mouse IL-6 ELISA MAX Standard (BioLegend, San Diego, CA, USA) and mouse TNF alpha ELISA Ready-SET-Go! (eBioscience, San Diego, CA, USA), respectively, according to the manufacturer's instructions.

2.5. Cell viability

Cytotoxicity of lysozyme to P-Mac was examined using a WST-8 assay kit (Nacalai Tesque) according to the manufacturer's instructions. P-Mac suspended in 10% FBS-PRMI 1640 medium were seeded into a 96-well culture plate at 6.0×10^4 cells/well and cultured at 37°C overnight under humidified 5% CO_2. After washing with PBS, P-Mac were pretreated with 100 ng/mL of LPS in 200 μL of 10% FBS-RPMI 1640 medium at 37°C. After incubation for 1 h, P-Mac were washed with PBS and treated with various concentrations of lysozyme in 200 μL of 10% FBS-RPMI 1640 medium at 37°C. After incubation for 11 h, the culture media were removed, and P-Mac were cultured in 100 μL of 10% FBS-RPMI 1640 medium containing 10% WST-8 solution for 40 min at 37°C under dark condition. The absorbance was then measured at 450 nm using a microplate reader.

2.6. Real-time RT-PCR

P-Mac suspended in 10% FBS-RPMI 1640 medium were seeded into a 24-well culture plate at 5.0×10^5 cells/well and cultured at 37°C overnight under humidified 5% CO_2. After washing with PBS, the cells were pretreated with 100 ng/mL of LPS in 1.0 mL of 10% FBS-RPMI 1640 medium at 37°C. After incubation for 1 h, the cells were washed with PBS and treated with 500 μg/mL of lysozyme in 1.0 mL of 10% FBS-RPMI 1640 medium at 37°C. After incubation for 5 h or 11 h, total RNA was isolated from the cells using Sepasol-RNA I Super G (Nacalai Tesque) according to the manufacturer's instructions and used as a template for cDNA synthesis with MML V-reverse transcriptase (Promega, Madison, WI, USA) and an oligo-$(dT)_{20}$ primer (Toyobo, Osaka, Japan). Real-time PCR was performed using Thunderbird SYBR qPCR Mix (Toyobo), 10 pmol of a forward primer, 10 pmol of a reverse primer, and 0.1 μg of a cDNA sample as previously described [13]. Thermal cycling conditions were 20 s at 95°C, and 40 cycles of 3 s at 95°C and 30 s at 60°C. PCR products were measured on a StepOnePlus Real-time PCR System (Applied Biosystems, Foster City, CA, USA), and relative gene expression was calculated based on the comparative CT method using StepOne Software v2.1 (Applied Biosystems). Expression of the β-actin gene was used as an endogenous control. Specific oligonucleotide sequences for each gene are as follows. Mouse β-actin: sense, 5'-CATCCGTAAAGACCTCTATGCCAAC-3' and antisense, 5'-ATGGAGCCACCGATCCACA-3'; mouse IL-6: sense, 5'-AAGCCAGATCCTTCAGAGAGAT-3' and antisense, 5'-TTGGATGGTCTTGGTCCTTAGC-3'; mouse TNF-α: sense, 5'-CTACTCCCAGGTTCTCTTCAA-3' and antisense, 5'-GCAGAGAGGAGGTTGACTTTC-3'.

2.7. Phagocytosis activity

Phagocytosis activity was measured as previously described [12] with some modifications. P-Mac suspended in 10% FBS-RPMI 1640 medium were seeded into a 48-well culture plate at 2.5×10^5 cells/well and cultured at 37°C under humidified 5% CO_2. After incubation for 3 h, the cells were pretreated with 100 ng/mL of LPS in 0.5 mL of 10% FBS-RPMI 1640 medium. After incubation at 37°C for 1 h, the cells were washed with PBS and treated with 500 μg/mL of lysozyme in 0.5 mL of 10% FBS-RPMI 1640 medium for 5 h or 11 h. After washing with PBS, 0.5 mL of 10% FBS-RPMI 1640 medium containing 500 μg of Texas Red-conjugated zymosan A (*Saccharomyces cerevisiae*) BioParticles (Molecular Probes, Eugene, OR, USA) were added to each well and incubated for 1 h under dark condition. After removing the culture medium, the cells were suspended in PBS and centrifuged at 160 × *g* for 5 min at 4°C. The cell pellet was suspended in 1 mL of 2% FBS-PBS, and phagocytosis activity was measured on a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA).

2.8. Immunoblot analysis

P-Mac suspended in 10% FBS-RPMI 1640 medium were seeded into a 35 mm culture dish (Corning) at 5.0×10^5 cells/dish and cultured at 37°C overnight under humidified 5% CO_2. After washing with PBS, the cells were pretreated with 100 ng/mL of LPS in 2 mL of 10% FBS-RPMI 1640 medium at 37°C. After incubation for 15 min, the cells were washed with PBS and treated with 500 μg/mL of lysozyme in 2 mL of 10% FBS-RPMI 1640 medium at 37°C. After incubation for 30 min,

cytosolic proteins were prepared using a CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich) according to the manufacturer's instructions. Denatured proteins were then separated using SDS-PAGE and transferred onto a PVDF membrane (Hybond-P; GE Healthcare, Buckinghamshire, UK). Immunoblotting with various antibodies was performed as previously described [14].

2.9. Lipopolysaccharide-induced inflammation model mice

The assay was performed by the method of [15] with some modifications. After acclimatization to their housing environment for 1 week, 8-week-old female BALB/c mice were placed into 5 groups (7 mice per group). On day 0, mice were orally administered using feeding needles (Natsume Seisakusho, Tokyo, Japan) with 200 μL of 10 mM NaPB for intact and control groups, 200 μL of lysozyme at 4.5 mg/kg body weight/day for the low-dose group, 200 μL of lysozyme at 450 mg/kg body weight/day for the middle-dose group, or 200 μL of lysozyme at 2,250 mg/kg body weight/day for the high-dose group for seven consecutive days from day 0 to day 6. Two hours after oral administration on day 6, the control, low-dose, middle-dose, and high-dose groups were injected with 20 μL of PBS containing LPS (5 mg/kg body weight) into the peritoneum to induce systemic inflammation. In contrast, the intact group was injected with 20 μL of PBS alone. Blood was taken by cardiac puncture 2 h after intraperitoneal administration, and sera were collected. The concentrations of IL-6 and TNF-α in the serum were measured by ELISA.

2.10. Statistical analysis

Data obtained were expressed as mean ± standard deviation. One-way ANOVA followed by Dunnett's test or Tukey-Kramer test was used to assess the statistical significance of the difference. Values with $^{*}p < 0.05$ or $^{**}p < 0.01$ were considered statistically significant.

3. Results

3.1. Effect of lysozyme on inflammatory cytokine production

Firstly, the effect of lysozyme on inflammatory cytokine production by P-Mac was examined. After pretreatment with 100 ng/mL of LPS for 1 h, P-Mac were treated with lysozyme at various concentrations for 11 h, and the cytokine concentration in the medium was measured by ELISA. As shown in Fig. 1A and 1B, lysozyme significantly inhibited the production of IL-6 and TNF-α in dose-dependent manners. When P-Mac were treated with 500 μg/mL of lysozyme, the production of IL-6 and TNF-α was suppressed by around 50% compared with control. The cytotoxicity of lysozyme was evaluated using the WST-8 assay. The result showed that lysozyme has no cytotoxicity to P-Mac at 1,000 μg/mL (Fig. 1C). From these results, further experiments were performed at 500 μg/mL of lysozyme.

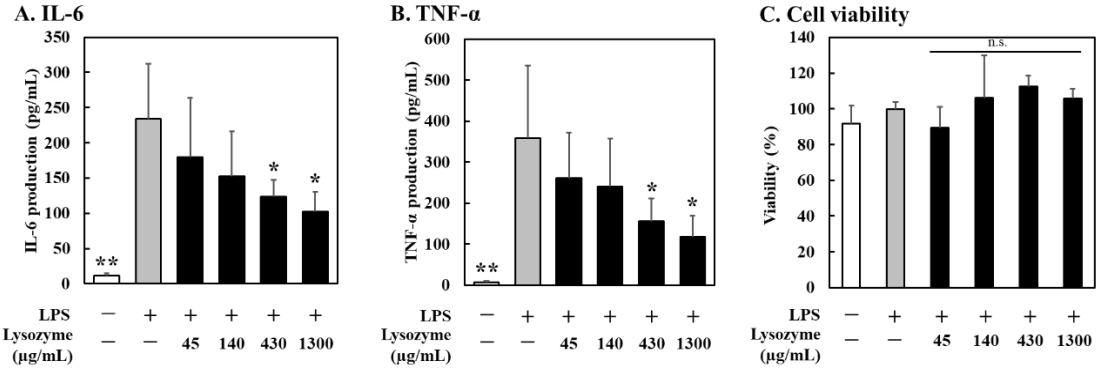

Figure 1. The effect of lysozyme on inflammatory cytokine production and viability of P-Mac. (A) and (B), for cytokine production assay, P-Mac were pretreated with 100 ng/mL of LPS or 10 mM NaPB. After washing, the cells were treated with various concentrations of lysozyme or with 10 mM NaPB. After incubation for 11 h, the concentrations of IL-6 and TNF-α in the culture medium were measured by ELISA. Data were represented as mean ± standard deviations ($n = 6$). *$p < 0.05$ or **$p < 0.01$ against LPS (+) / Lysozyme (−) by Dunnett's test. (C), for cell viability assay, P-Mac were pretreated with 100 ng/mL of LPS or 10 mM NaPB. After washing, the cells were treated with various concentrations of lysozyme or with 10 mM NaPB. After incubation for 11 h, cell viability was measured using a WST-8 assay kit. Data were represented as mean ± standard deviations (n = 9). n.s. indicates no statistical significance against LPS (+) / Lysozyme (−) by Dunnett's test.

3.2. Effect of lysozyme on cytokine gene expression

As described above, lysozyme inhibited cytokine production by P-Mac without cytotoxicity. Hence, the effect of lysozyme on cytokine gene expression was examined. After pretreatment with 100 ng/mL of LPS for 1 h, P-Mac were treated with lysozyme at 500 μg/mL for 5 h or 11 h. After that, the transcription level of the cytokine genes was evaluated by real-time RT-PCR. As a result, the expression of IL-6 and TNF-α genes was significantly inhibited by lysozyme in both treatment time (Data not shown). These results indicated that lysozyme downregulates the expression of IL-6 and TNF-α genes within 5 h after treatment, resulting in suppressed production of IL-6 and TNF-α. Furthermore, it was revealed that the effect of suppressed IL-6 and TNF-α gene expression by lysozyme continues at least for 11 h after treatment.

3.3. Effect of lysozyme on phagocytotic activity

Macrophages have an important role in innate immunity such as ingesting foreign materials and enhancing immune responses. We thus examined the effect of lysozyme on the phagocytosis activity of P-Mac using Texas Red-labeled zymosan A. P-Mac were treated with 500 μg/mL of lysozyme for 5 h or 11 h after pretreatment with 100 ng/mL of LPS for 1 h. The cells were then treated with Texas Red-labeled zymosan A for 1 h. The results showed that the zymosan A-mediated phagocytosis activity of the P-Mac is not affected by lysozyme compared with control. Thus, lysozyme inhibits LPS-induced cytokine production by P-Mac, but does not modulate phagocytotic activity. These results suggested that lysozyme has an anti-inflammatory effect without inhibiting the innate immune response by macrophages.

3.4. Effect of lysozyme on the signaling pathways in macrophages

Macrophages are induced the expression of inflammatory cytokine genes by activating the MAPK and the NF-κB signaling pathways. The effect of lysozyme on MAPK and NF-κB signaling was then examined. P-Mac were treated with 500 μg/mL of lysozyme for 30 min after pretreatment with 100 ng/mL of LPS for 15 min, and the cytosolic protein levels of the signal molecules were evaluated by immunoblot analysis. As summarized in Fig. 2A, the phosphorylation level of JNK was inhibited by lysozyme whereas those of ERK and p38 were not affected. In addition, lysozyme did not affect the

translocation of NF-κB from cytosol to the nucleus (Fig. 2B). These results suggest that lysozyme inhibits inflammatory cytokine production through inhibiting the phosphorylation of JNK.

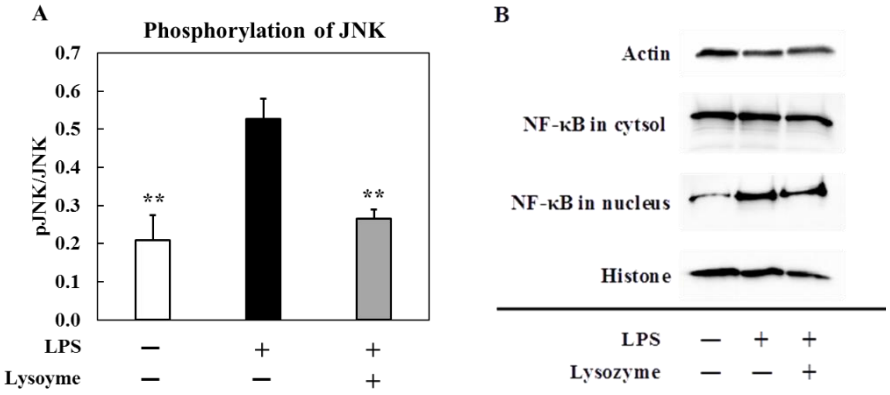

Figure 2. The effect of lysozyme on the signaling pathways of macrophage activation. P-Mac were pretreated with 100 ng/mL of LPS or 10 mM NaPB for 15 min. After washing, the cells were treated with 500 μg/mL of lysozyme or 10 mM NaPB for 30 min. (A), The result of densitometric analysis was expressed as the ratio of the amount of phosphorylated protein to that of whole protein. Data were represented as mean ± standard deviations of three independent experiments. $^{**}p < 0.01$ against LPS (+) / Lysozyme (−) by Tukey-Kramer test. (B), The protein level of NF-κB was evaluated by immunoblot analysis.

3.5. Effect of lysozyme in LPS-induced inflammation model mice

The effect of lysozyme *in vivo* was evaluated by LPS-induced inflammation model mice. Oral administration of lysozyme to mice for 7 consecutive days did not change body weight in all lysozyme-treated groups. In this experiment, systemic inflammation in mice was induced by LPS at 5 mg/kg body weight via intraperitoneal administration. As indicated in Fig. 3, the amounts of IL-6 and TNF-α in sera were significantly decreased in the high-dose group (2,250 mg/kg body weight/day) compared to the control group. Furthermore, the amounts of IL-6 and TNF-α tended to be suppressed in the middle-dose group (450 mg/kg body weight/day). These results suggest that lysozyme alleviates systemic inflammation *in vivo*.

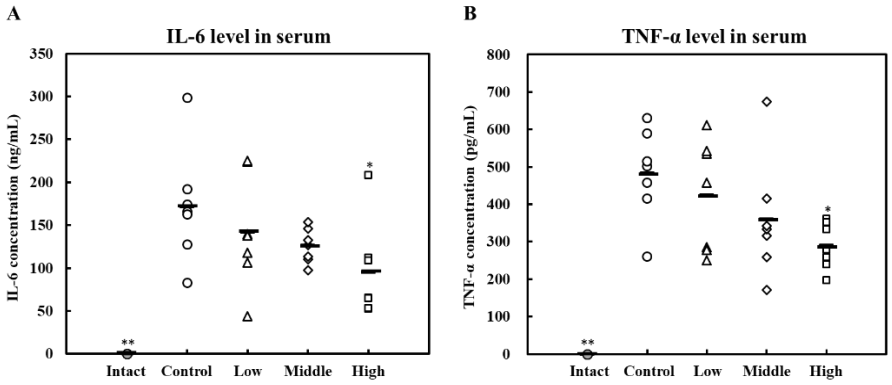

Figure 3. The effect of lysozyme on the amounts of inflammatory cytokines in serum. (A), (B), Seven-week-old female BALB/c mice were orally administered with 10 mM NaPB or lysozyme at various concentrations for seven consecutive days from day 0 to day 6. Two hours after oral administration on day 6, LPS (5 mg/kg body weight) in PBS was injected via peritoneum to induce systemic inflammation. In contrast, PBS was injected to intact group. Two hours after intraperitoneal administration, serum was collected. Then, the concentrations of IL-6 and TNF-α in serum were measured by ELISA. Data were represented as mean ± standard deviations of three independent experiments. $^{*}p < 0.05$ or $^{*}p < 0.01$ against control by Tukey-Kramer test.

4. Discussion

In this study, it was revealed that lysozyme suppresses production of IL-6 and TNF-α by suppressing their gene expression levels. It has been reported that lysozyme binds to LPS to form a complex and inhibits inflammatory reaction [16]. Moreover, several peptides derived from human lysozyme bind to TLR4 to show an antagonistic anti-inflammatory effect [17]. On the other hands, in this study, LPS was washed away with PBS before lysozyme treatment against P-Mac. Then, it is revealed that the suppressive effect of lysozyme on LPS-induced inflammation is not due to its antagonism activity against TLR4. Macrophages have an important role in innate immunity such as destroying microorganisms, ingesting foreign materials, removing dead cells, and enhancing acquired immune responses. As a result of examining the effect of lysozyme on the phagocytic activity of P-Mac, phagocytosis activity of P-Mac was not affected by lysozyme. These results suggested that lysozyme shows an anti-inflammatory effect without inhibiting the innate immune responses by macrophages. Next, the mechanism underlying the inhibition of inflammatory cytokine production by lysozyme was investigated. Macrophages are induced the expression of inflammatory cytokine genes by activating the MAPK and NF-κB cascades. LPS activates MAPK and NF-κB signaling to facilitate inflammatory cytokine production. To reveal how lysozyme affects these cascades, we examined the effect on the signal molecules in MAPK and NF-κB cascade. As summarized in Fig. 2, the phosphorylation level of JNK was significantly inhibited by lysozyme, whereas that of ERK or p38 was not affected, and lysozyme showed no effect on the translocation of NF-κB into the nucleus. These results suggest that lysozyme inhibits inflammatory cytokine production through inhibiting the phosphorylation of JNK. Finally, we investigated the effect of oral administration of lysozyme on LPS-induced inflammation model mice. As a result, oral administration of lysozyme significantly decreased the amounts of IL-6 and TNF-α in sera compared to the control group, suggesting that lysozyme mitigates the hyperinflammatory condition *in vivo*. These data indicate that lysozyme exhibits anti-inflammatory effect *in vitro* and *in vivo* and is expected to be effective as a functional food material.

5. Conclusions

Although lysozyme is well known to exhibit the anti-inflammatory effect, the detailed mechanism of its effect is still unknown. In this study, we found that lysozyme inhibits phosphorylation of JNK to suppress pro-inflammatory cytokines. Moreover, lysozyme suppressed inflammatory cytokine levels in sera from LPS-induced inflammation model mice. Taken together, our data indicate that lysozyme exerts anti-inflammatory effect *in vitro* and *in vivo*.

Acknowledgments: This work was supported by a JSPS KAKENHI Grant-in-Aid for Scientific Research C (15K07432). Animal experiments were accomplished at the Division of Genetic Research of the Advanced Research Support Center (ADRES), Ehime University.

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