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Anti-Inflammatory Effect of *o*-Vanillic Acid on Lipopolysaccharide-Stimulated Macrophages and Inflammation Models

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Abstract Inflammation is an important biological reaction in the body in response to external stimuli. Excessive inflammation causes various inflammatory disorders such as allergic hypersensitivity, autoimmune disease, rheumatoid arthritis, and cancer. Macrophages play a major role in the inflammatory response by producing inflammatory mediators such as nitric oxide, prostaglandin E_2, and pro-inflammatory cytokines. Natural products are often a source of bioactive compounds, which have great potential as novel therapeutic agents. *Amomum xanthoides* extract has been shown to possess various pharmacological activities including anti-inflammatory activity. This study evaluated the anti-inflammatory potential of *o*-vanillic acid (*o*-VA), a major compound in *A. xanthoides*, using lipopolysaccharide (LPS)-induced macrophages and *in vivo* animal models. *o*-VA decreased, in a concentration-dependent manner, the LPS-induced gene expression and production of inflammatory mediators, such as inducible nitric oxidase/cyclooxygenase-2 and pro-inflammatory cytokines, by reducing the nuclear factor-κB activation. In addition, *o*-VA dose-dependently ameliorated acetic acid-induced vascular permeability and zymosan-induced leukocyte migration. Thus, we suggest that *o*-VA can be used as a pharmacological agent or food supplement in the treatment of inflammatory conditions.

Keywords: *anti-inflammation, o-vanillic acid, macrophages, pro-inflammatory cytokines*

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1. Introduction

Inflammation is an essential biological reaction and the result of the normal defense mechanism of the immune system in response to external stimuli [1]. However, uncontrolled inflammation induces various diseases in humans, including asthma, rheumatoid arthritis, and cancer [2]. The hallmark of initial inflammation is increased vascular permeability, blood flow, and leukocytosis at the injured tissue site [3]. During an inflammatory response, intracellular signal events, driven by pro-inflammatory mediators and cytokines, are activated [4]. Among immune cells, macrophages play a major role in the inflammatory responses through the production of various mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), and pro-inflammatory cytokines [5]. NO produced by inducible nitric oxide synthase (iNOS) is indispensable to macrophages involved in the immunological function. However, excessive production of NO is involved in many physiological processes including the regulation of blood pressure, immune response, and neural communication [6]. Activated macrophages secrete pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 [7]. The nuclear factor (NF)-κB signaling pathway is involved in the regulation of pro-inflammatory cytokine expression during inflammatory responses [8].

Natural products derived from plants, animals, and microorganisms have been used as medicines in treatment of human diseases for a long time [9]. Natural products have pharmacological or biological activity that can be of therapeutic benefit. As such, natural products are the active components in most traditional medicines and modern medicines [10]. *Amomum xanthoides* is a traditional herbal medicine used across Asia for treatment of digestive system disorders [11]. Many studies have reported its pharmacological effects such as anti-allergy and anti-inflammatory activities [12,13]. Furthermore, several studies have indicated the anti-inflammatory effect of the active components in *Amomum xanthoides*, such as vanillic acid, tyrosol, and 1,2,4,5-tetramethoxybenxene [13,14]. *o*-Vanillic acid (2-hydroxy-3-methoxybenzoic acid, *o*-VA), also found in *Amomum xanthoides*, is a

derivate of vanillic acid [13]. The aim of this study was to evaluate the anti-inflammatory effect of *o*-VA and determine the underlying mechanisms.

2. Materials and Methods

2.1. Reagents and Cell Culture

LPS (from *E. coli* 026:B6), zymosan, dimethyl sulfoxide (DMSO), Evans blue, and aminopyrine were purchased from Sigma (St. Louis, MO). *o*-VA was procured from Santa Cruz Biotech (Santa Cruz, CA). For *in vitro* and *in vivo* experiments, *o*-VA was dissolved in dimethyl sulfoxide (DMSO) and diluted with Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Grand Island, NY) or phosphate-buffered saline (PBS, pH 7.4). Murine macrophage cell line J774A.1 (TIB-67, ATCC) was grown in DMEM supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and non-heat-inactivated 10% fetal bovine serum (GIBCO) in 5 % CO_2 at 37°C. Thioglycollate (TG)-elicited peritoneal macrophages were harvested 3 days after intraperitoneal injection of 3 ml of TG in mice and isolated, as previously reported [15]. Peritoneal lavage was performed using 8 ml of PBS. Then, the cells were distributed in the culture media.

2.2. Animals

Six-week-old male ICR mice were purchased from Dae-Han Biolink (Daejeon, Korea). The animals were housed in a laminar air flow room maintained at a temperature of 22 ± 2 °C and relative humidity of 55 ± 5 % throughout the study. All care and treatment of animals were performed in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and the study protocol was approved by the Institutional Animal Care and Use Committee of Kyungpook National University.

2.3. Cell Viability

Cells were seeded in 96-well plates at a density of 2 × 10^4 cells/well and allowed to attach overnight before the experiments were performed. Cells were then treated with various concentrations of *o*-VA for 24 hr. Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After incubation with *o*-VA, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well, and the plates were incubated at 37 °C for 2 hr. After the supernatant was removed from the plate, formazan byproduct in the cells was dissolved by the addition of 100 μl of DMSO to each well, and the absorbance was measured at 570 nm using a Versa Max ELISA Microplate Reader (Molecular Devices, Sunnyvale, CA). The absorbance of the untreated control wells was set to 100 % viability for all comparisons thereafter.

2.4. NO Assay

Cells were seeded in 96-well plates at a density of 2 × 10^4 cells/well and allowed to attach overnight before the experiments were performed. Then, the cells were pretreated with *o*-VA for 1 hr and activated with 100 ng/ml LPS for 24 hr. The amount of stable nitrite, the end product of NO generation by activated macrophages, was determined by a colorimetric assay as previously described [16]. In brief, 50 μl of culture supernatant was mixed with an equal volume of Griess reagent (1 % sulfanilamide, 0.1 % naphthylethylene diamine dihydrochloride). The absorbance was measured at 540 nm using a microplate reader. Nitrite concentration was determined by extrapolation from a sodium nitrite standard curve.

2.5. qPCR

Before isolation of the total cellular RNA, J774A.1 cells were seeded in 24-well plates at a density of 3 × 10^5 cells/well. After incubating overnight, the cells were pretreated with *o*-VA for 1 hr and activated with 100 ng/ml LPS for 6 hr. RNAiso Plus reagent (Takara Bio, Shiga, Japan) was used to extract total RNA according to the manufacturer's protocol. After confirming purity/concentration of the isolated total RNA at 260/280 nm using UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA), complementary DNA (cDNA) was synthesized from 2 μg total RNA using a Maxime RT-Pre-Mix Kit (iNtRON Biotechnology, Daejeon, Korea). qPCR was performed using the Thermal Cycler Dice TP850 (Takara) according to the manufacturer's protocol. The total reaction mixture (25 μl) was composed of the following: 1.5 μl of cDNA (150 ng), 1 μl of each forward and reverse primer (0.4 μM), 12.5 μl of SYBR Premix Ex Taq (Takara Bio), and 10 μl of dH_2O. The conditions for the qPCR steps were similar to those described in a previous study [15].

2.6. Enzyme-Linked Immunosorbent Assay

The secretion of TNF-α was measured by enzyme-linked immunosorbent assay (ELISA). Cells were seeded in 12-well plates at a density of 5 × 10^5 cells/well. After incubating overnight, the cells were pretreated with *o*-VA for 1 hr and activated with LPS (100 ng/ml) for 24 hr. ELISA was performed on a 96-well Nunc immuno plate using an ELISA kit (BD Biosciences, San Diego, CA) according to the manufacturer's protocol.

2.7. Western Blot

Nuclear and cytosolic proteins were extracted as described previously [17]. Before protein extraction, cells were seeded in 6-well plates at a density of 2 × 10^6 cells/well. After incubating overnight, the cells were pretreated with *o*-VA for 1 hr and activated with 100 ng/ml LPS. After suspension in 100 μl of cell lysis buffer A (0.5% Triton X-100, 150 mM NaCl, 10 mM HEPES, 1 mM EDTA, 1 mM Na_3VO_4, 0.5 mM phenylmethanesulfonylfluoride [PMSF], 0.5 mM dithiothreitol [DTT], 5 μg/ml leupeptin, and 5 μg/ml aprotinin), the cells were vortexed, incubated for 15 min on ice, and centrifuged at 400 *g* for 5 min at 4 °C. The supernatant was collected and used as the cytosolic protein extract. The remaining pellets were washed thrice with 1 ml of PBS and suspended in 25 μl of cell lysis buffer B

(25 % glycerol, 420 mM NaCl, 20 mM HEPES, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM Na_3VO_4, 0.5 mM PMSF, 0.5 mM DTT, 5 μg/ml leupeptin, and 5 μg/ml aprotinin), vortexed, sonicated for 30 sec, incubated for 30 min on ice, and centrifuged at 15,000 *g* for 15 min at 4°C. The resulting supernatant from this material was collected and used as the nuclear protein extract.

Proteins were separated on 8-12 % sodium dodecyl sulfate polyacrylamide gels by electrophoresis and transferred to nitrocellulose membrane. Immunodetection was performed using a chemiluminescent substrate (Thermo Scientific). The following antibodies were purchased from Santa Cruz Biotech; NF-κB (sc-109, rabbit polyclonal, 1:1,000), IκBα (sc-371, rabbit polyclonal, 1:1,000), β-actin (sc-8432, mouse monoclonal, 1:1,000), and Cell Signaling (Danvers, MA); COX-2 (#4842, rabbit polyclonal, 1:1,000), and iNOS (#2977, rabbit polyclonal, 1:1,000).

2.8. Acetic Acid-Induced Vascular Permeability

The effect of *o*-VA on acetic acid-induced vascular permeability was assessed by a modified method described by Whittles [18]. ICR mice (n = 35) were divided into seven groups of five mice each. Initially, the mice were orally administered with 100 μl of *o*-VA (2, 10, and 50 mg/kg), gallic acid (50 mg/kg), and aminopyrine (100 mg/kg). After 30 min, mice were intravenously injected with 1% Evans blue solution (at volume of 0.1 ml per 10 g). Vascular permeability was induced after 30 min by intraperitoneal (i.p.) injection of 0.6 % acetic acid (0.1 ml per 10 g). Mice were euthanized with carbon dioxide 20 min later, and their peritoneum was washed with 10 ml of PBS. The peritoneal fluid was centrifuged and the absorbance of the supernatant was measured at 630 nm using a spectrophotometer.

2.9. Zymosan-Induced Peritonitis

The effect of *o*-VA on zymosan-induced peritoneal inflammation was assessed by a modified method of Doherty [19]. ICR mice (n = 35) divided into seven groups of five mice each were used. Before treatment with zymosan, mice were orally administered with 100 μl of *o*-VA (2, 10, and 50 mg/kg), gallic acid (50 mg/kg), and aminopyrine (100 mg/kg). After 30 min, zymosan was prepared and was i.p. injected (1 mg/ml/mouse). After 6 hr, the peritoneal cavity was lavaged with 2 ml of PBS. After gentle manual massage, the peritoneal cavity was carefully opened and 1 ml of the exudate was retrieved. The number of leukocytes was measured using a scil Vet ABC hematology analyzer (Berlin, Germany)

2.10. Statistical Analysis

All data presented in the graphical format are mean ± SD of independent experiments. Statistical analyses were performed using Prism5 (GraphPad Software, San Diego, CA), and the treatment effects were analyzed using a one-way analysis of variance (ANOVA) followed by a Dunnett's test. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. o-VA Reduces Expression of Inflammatory Mediators in LPS-Induced Macrophages

To investigate the anti-inflammatory activity of *o*-VA *in vitro*, we measured the production of NO, TNF-α, IL-1β, iNOS, and COX-2 in LPS-stimulated J774A.1 cells after treatment with *o*-VA. To rule out the cytotoxic effect of *o*-VA, cell viability was analyzed using the MTT assay following exposure to *o*-VA. J774A.1 cells were exposed to *o*-VA at 0.1–1,000 μM for 24 h. There was no significant reduction in cell viability up to 10 μM *o*-VA (Figure 1A).

NO is an important inflammatory mediator produced by macrophages [20]. We investigated the effect of *o*-VA (0.001-10 μM) on the LPS-induced NO production. The known anti-inflammatory agent, gallic acid (GA), was used as the positive control [21]. In LPS-activated J774A.1 cells, *o*-VA concentration-dependently decreased NO production (Figure 1B). *o*-VA and GA have similar suppressive effects. Pro-inflammatory cytokines mediate inflammation by enhancing the recruitment and activation of immune cells [7]. To study the effect of *o*-VA on pro-inflammatory cytokines, we performed qPCR. This study demonstrated that *o*-VA significantly reduced the gene expression of TNF-α and IL-1β in LPS-activated J774A.1 cells. The expression of iNOS and COX-2 was also reduced by *o*-VA (Figure 1C). The secretion of LPS-induced TNF-α (Figure 1D) and protein production of iNOS/COX-2 (Figure 1E) were also reduced by *o*-VA. NF-κB is a major transcription factor regulating the expression of inflammatory cytokines and mediators [22]. To determine the mechanisms responsible for the reduction of inflammatory mediators by *o*-VA, the effect of *o*-VA on the activation of NF-κB was tested. *o*-VA inhibited LPS-induced degradation of IκBα and nuclear translocation of NF-κB in J774A.1 cells (Figure 1E).

The *in vitro* results were confirmed in TG-elicited peritoneal macrophages (TG-macrophages). Initially, peritoneal macrophages were exposed to *o*-VA at 0.1–100 μM during 24 h and no significant reduction in cell viability was observed up to 100 μM (Figure 2A). *o*-VA (0.001-10 μM) concentration-dependently inhibited NO production (Figure 2B) and TNF-α secretion (Figure 2C) in LPS-stimulated TG-elicited peritoneal macrophages.

3.2. o-VA Inhibits Capillary Permeability and Leukocyte Migration in Inflammation Models

The inflammatory response is characterized by the release of various mediators, such as histamine, serotonin, bradykinin, and prostaglandin, which results in capillary permeability and leukocyte migration at the inflammation site [23,24]. To study the effect of *o*-VA *in vivo*, acetic acid-induced capillary permeability and zymosan-induced leukocyte migration models were used. *o*-VA significantly reduced acetic acid-induced capillary permeability at 10

and 50 mg/kg (Figure 3A). Aminopyrine and GA were used as positive controls. The inhibitory effect of *o*-VA was similar to that of GA and greater than that of aminopyrine. Increased capillary permeability induces leukocyte migration, a central component of the inflammatory process, to the injured tissues [25]. *o*-VA significantly inhibited zymosan-induced leukocyte migration at 10 and 50 mg/kg (Figure 3B).

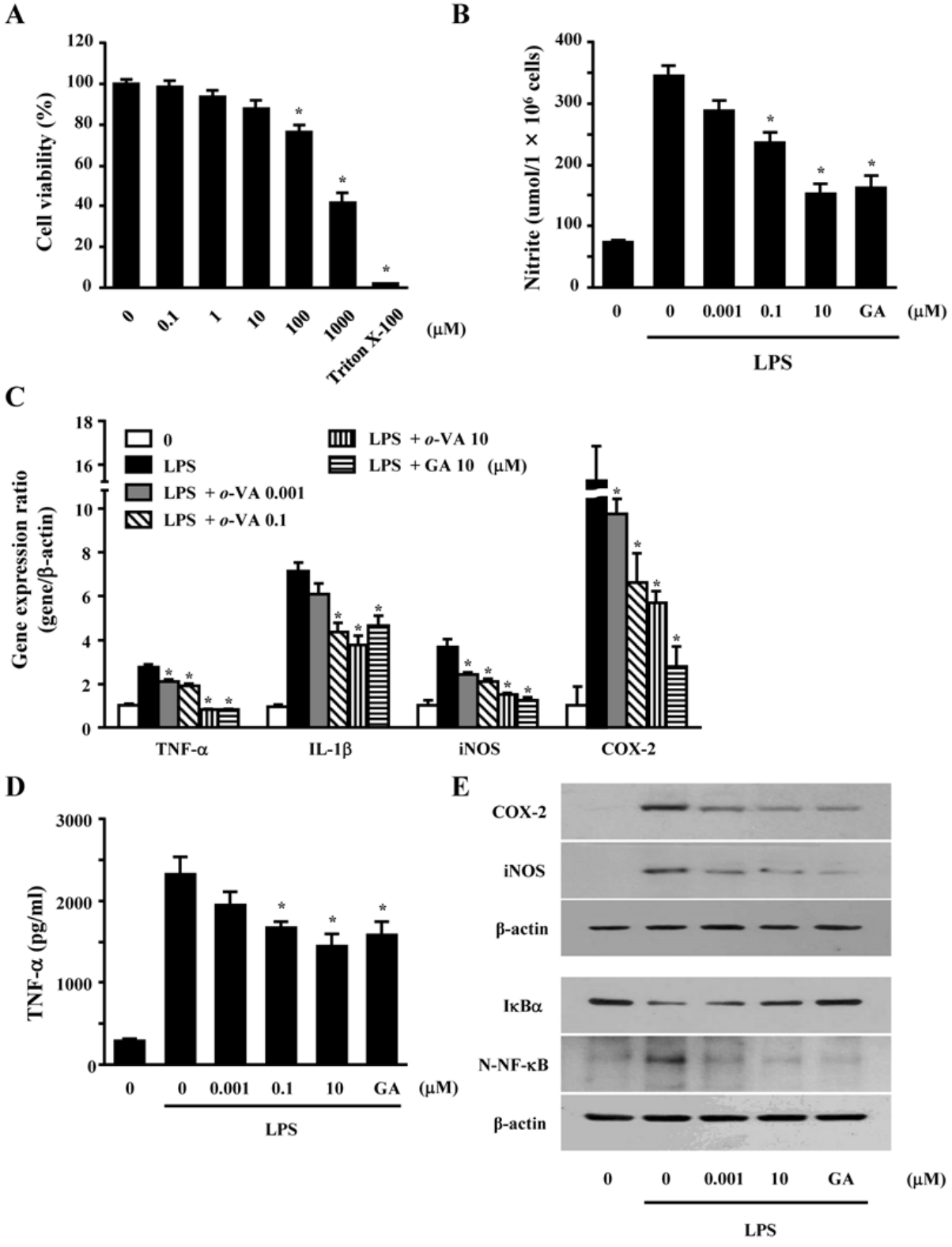

Figure 1. Effect of *o*-VA on cell viability, NO production, pro-inflammatory cytokine expression, and TNF-α secretion in J774A.1 cells**.** (A) Viability of J774A.1 cells exposed to various concentrations of *o*-VA (0.1–1,000 μM) for 24 hr. Cell viability was measured by MTT assay. Triton X-100 was used as a positive control. (B-C) Cells were incubated in the presence of LPS (100 ng/ml) and *o*-VA for 6 hr (qPCR) and 24 hr (NO assay and ELISA). The culture supernatant was harvested for measure of NO production (B), pro-inflammatory cytokines expression (C), and TNF-a secretion (D). (E) The activation of iNOS, COX-2, and NF-κB was assayed by Western blotting (1 hr). The bands of β-actin were used as the loading control. Data was presented as the mean ± SD of three independent experiments. *Significantly different from the LPS-stimulated and *o*-VA-treated group at $p < 0.05$. GA: gallic acid

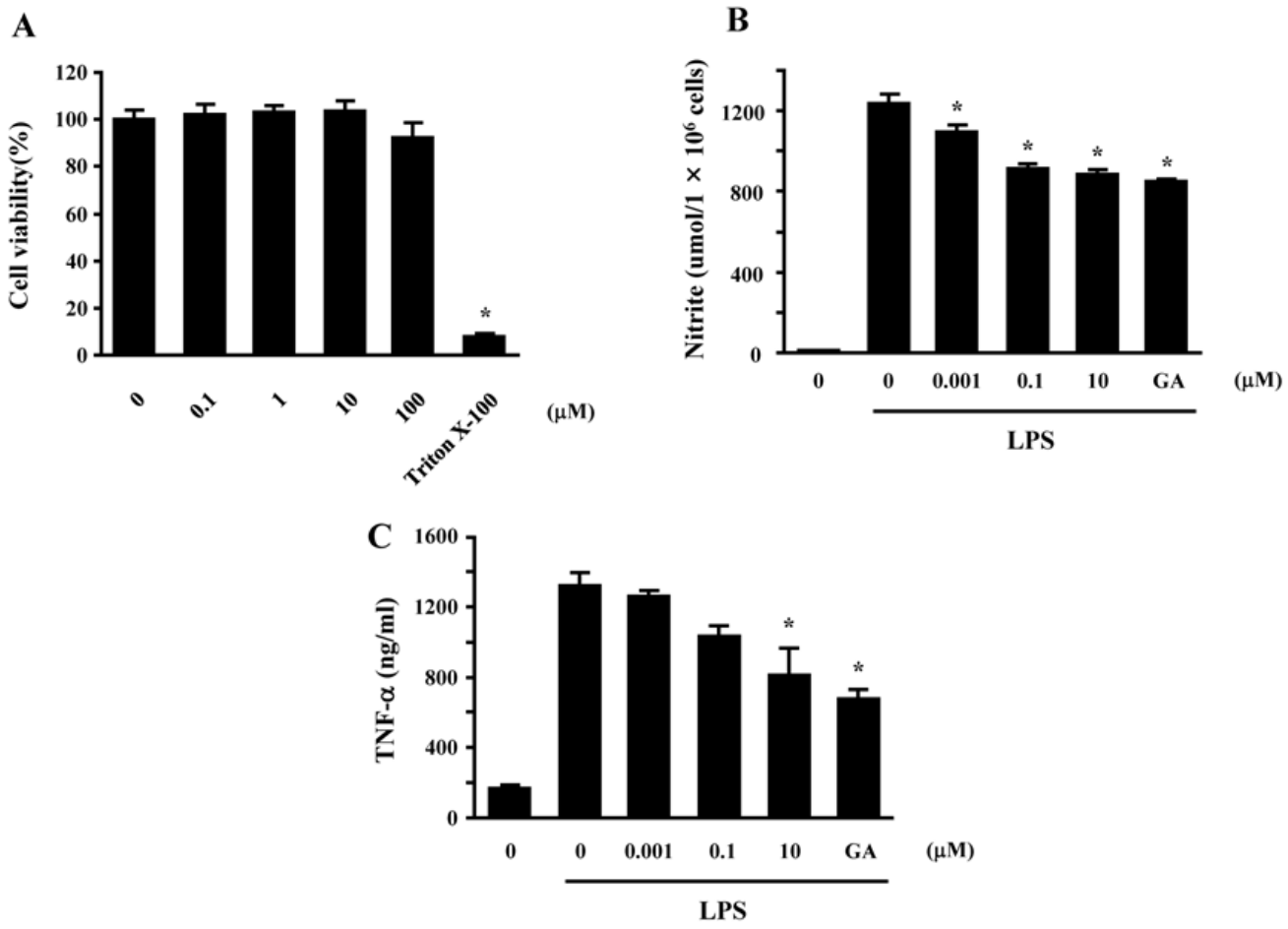

Figure 2. Effect of *o*-VA on cell viability, NO production, and TNF-α secretion in peritoneal macrophages**.** (A) Cell viability of peritoneal macrophages exposed to various concentrations of *o*-VA (0.1–1,000 μM) for 24 hr. Cell viability was measured by MTT assay. (B) The NO production was measured to identify *o*-VA effect in LPS-stimulated macrophages for 24 hr. (C) The secretion of TNF-α was measured by ELISA in LPS-stimulated macrophages for 24 hr. Data was presented as the mean ± SD of three independent experiments. *Significantly different from the LPS-stimulated and *o*-VA-treated group at $p < 0.05$. GA: gallic acid

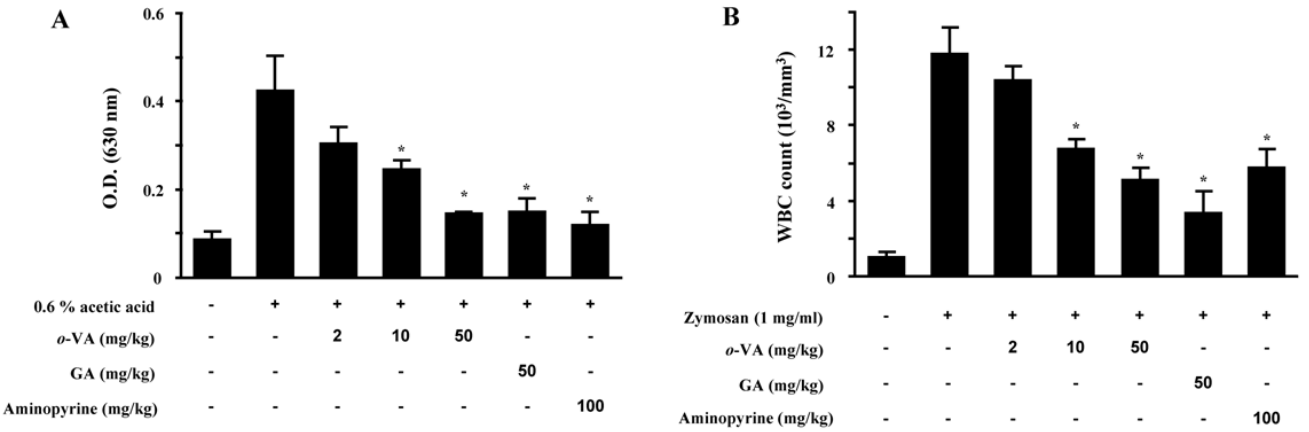

Figure 3. Effect of *o*-VA on capillary permeability and leukocyte migration in acetic acid-induced and zymosan-induced inflammatory models. (A) Anti-inflammatory effect of *o*-VA was measured by Whittles method. Capillary permeability induced injection of 0.6% acetic acid. Then, the peritoneal fluid was harvested for measure of absorbance at 630 nm using a spectrophotometer. (B) Anti-inflammatory effect of *o*-VA was measured by Doherty method. Leukocytes migration induced injection of zymosan. Then, the peritoneal fluid was harvested for measure of leukocyte number using a scil Vet ABC hematology analyzer. Data was presented as the mean ± SD of three independent experiments. *Significantly different from the LPS-stimulated and *o*-VA-treated group at $p < 0.05$. GA: gallic acid.

4. Discussion

Inflammatory responses to living tissue injuries involve enzyme activation, inflammatory mediator release, cell migration, fluid extravasation, tissue breakdown, and repair [26]. Inflammatory stimuli, such as LPS and pro-inflammatory cytokines, induce the activation of immune cells that release inflammatory mediators and free radicals [27]. The generated free radicals can enhance the production of inflammatory cytokines and cause extensive

damage to tissues and biomolecules leading to various diseases [28]. Many anti-inflammatory drugs, particularly nonsteroidal anti-inflammatory drugs (NSAIDs) are broadly used for controlling inflammation. However, the widespread use of NSAIDs increases the risk of adverse effects, such as gastrointestinal and kidney problems [29]. Therefore, study of natural products with anti-inflammatory activity has increased in the recent years.

The pro-inflammatory enzymes, such as iNOS and COX-2, play key roles in inflammatory processes [30]. NO produced by macrophages with iNOS is a crucial factor for inflammation and protects against bacteria, viruses, fungi, and tumor cells [5]. A previous study demonstrated that NO plays an important role in controlling vascular and vasodilation mediated by NO and may promote edema formation through neural stimulation [31]. Excessive NO production plays the main role in inflammation and arthritis. NO is an unstable molecule that triggers the formation of oxidative free radicals such as peroxynitrite ($ONOO^-$) [32]. Therefore, the reduction in production of NO by transcriptional downregulation of iNOS gene has appreciable therapeutic effect in the treatment of inflammation. COX-2 converts PGE2 from arachidonic acid. PGE2 plays a key role in the generation of an inflammatory response similar to that generated by NO [33]. Production of PGE2 is significantly increased in inflamed tissue and they contribute to the development of inflammation [34]. Thus, iNOS/COX-2 expression and NO levels are important indicators of inflammatory response. In the present study, we demonstrated that *o*-VA downregulated iNOS/COX-2 expression and NO production in LPS-stimulated macrophages. Consequently, these results suggest that *o*-VA controlled inflammatory response by inhibiting iNOS/COX-2.

Macrophages play a key role in driving the immune response by protective functions such as innate immunity and inflammatory reaction, foreign antigen presentation, and scavenging dead cells [35]. The mouse macrophage J774A.1 cell line consists of adherent slow-migrating monocyte-macrophages with the ability to phagocyte or kills foreign cells. Therefore, it has been widely used as an effective model in inflammation studies [36]. The production of pro-inflammatory cytokines from macrophages, such as TNF-α, IL-1β, and IL-6, take part in the process of inflammatory response [7]. Pro-inflammatory cytokines could act as a principal endogenous mediator in inflammatory response [37]. Therefore, the inhibition of pro-inflammatory cytokines synthesis or release could emerge as a potential therapeutic approach for inflammatory diseases [27]. NF-κB modulates the secretion of inflammatory cytokines and exerts a critical effect of regulating inflammatory responses to extracellular stimuli [38]. In this study, we used two types of macrophages (mouse macrophage J774A. l cell line and TG-macrophages) to assess the anti-inflammatory effect of *o*-VA. Our result demonstrated that *o*-VA inhibited LPS-induced degradation of IκBα and nuclear translocation of NF-κB. Thus, *o*-VA reduced pro-inflammatory cytokine levels by inhibiting NF-κB in LPS-stimulated macrophages.

Several diseases, including diabetes, hypertension, and autoimmune diseases, have shown an increase in vascular permeability [39]. Vascular change is induced by inflammatory mediators, such as serotonin, histamine, and bradykinin, to increase capillary permeability, leading to hyperemia and edema [40]. Permeability characterizes the capacity of a blood vessel wall to allow the flow of small molecules or lymphocytes to the site of inflammation [41]. Evans blue dye is widely used for measuring the increase in capillary permeability. By intravenously injecting Evans blue, we could monitor the vascular permeability to the site of inflammation [42]. Acetic acid causes peritoneal inflammation and induces an increase in PGE_2 and PGF_{2a} in the peritoneal fluid [43]. This capillary permeability assay is a typical model for acute phase of inflammation, where mediators of inflammation released following stimulation lead to the dilation of both arterioles and venules and increase capillary permeability [24]. The inflammatory response also induces the recruitment of leukocytes in response to inflammatory mediators including cytokines in tissue injury. Increased capillary permeability induces leukocyte migration to the injured tissues [25]. Zymosan, a polysaccharide cell wall component derived from *Saccharomyces cerevisiae*, is commonly used for the induction of peritonitis and can lead to inflammation [44]. Therefore, it has been widely used as a self-resolving model of inflammation within 30 min of the inflammatory stimulus due to the activation of macrophages and mast cells that release prostaglandins, particularly histamine and leukotrienes [45]. In this study, we used the acetic acid-induced vascular permeability model and zymosan-induced peritonitis model extensively to study the effects of *o*-VA. Our data demonstrated that *o*-VA could suppress the migration of leukocyte by inhibiting capillary permeability.

5. Conclusion

We demonstrated that *o*-VA inhibited LPS-induced inflammatory mediators by blocking NF-κB in macrophages. *o*-VA repressed vascular permeability and leukocyte migration, which are the initial inflammatory responses. Thus, *o*-VA could be effective in the treatment of inflammatory disorders, at least in the acute phase of inflammation, and could be used as a pharmacological agent or food supplement.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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