Thesis for the Degree of Master of Science

Anti-inflammatory effect of veratric acid through regulating of PI3K/Akt pathway and histone acetylation

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August 22, 2014

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(PI3K/Akt 경로와 histone의 아세틸화 조절을 통한 베라트릭 산의 염증 억제 효과)

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by

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CONTENTS

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ABSTARCT

Veratric acid, a simple benzoic acid, is known to exert several pharmacological effects including anti-oxidation, anti-inflammation and anti-hyperlipidemia. This study was focused on inhibitory effects of veratric acid on nitric oxide (NO) production in LPS-induced RAW264.7 cells. Veratric acid significantly suppressed mRNA and protein levels of iNOS resulting in the decrease of NO production in a dose-dependent manner. LPS-induced effects on PI3K subunits, including p85, p110α and p110β, and Akt were reduced by veratric acid. Also, veratric acid effectively decreased p300 acetylation and ATF-2 phosphorylation of what were consistently regulated by LY294002, a specific PI3K inhibitor. Moreover, LPS-induced HDAC3 expression and histone H4 acetylation were suppressed by veratric acid and LY294002. Our finding indicate

that inhibitory effects of veratric acid on iNOS expression require regulation of PI3K/Akt signaling inducing recovery of the basal level of histone H4 acetylation through suppression LPS-induced HAT activity of p300 and ATF-2, and HDAC3 expression.

INTRODUCTION

Macrophages are important for numerous inflammatory responses, including tissue repair and removal of pathogens, and host defense [9]. Their are cellular responses to occur, various molecular events, such as interactions between pathogen recognition receptors, including Toll-like receptor, and ligand [37]. The responses are released inflammatory substances, including cytokines, chemokines and nitric oxide (NO) in macrophages [12]. Above all, synthesis of NO is catalyzed from L-arginine by nitric oxide synthases (NOS), has at least three isoform: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) [10, 25, 30]. NO synthesized by iNOS is play the critical roles in the pathogenesis of inflammation [35]. Especially, synthesized NO shown regulation of the immune defense against pathogens during the early phase of inflammatory response [2]. During inflammatory responses, NO is related as an inflammatory regulator, inducing vasodilation, non-specific immune response, the resolution of the inflammation. The regulatory role of NO in gene expression and enzyme activity is attained through both autocrine and paracrine routes [5]. In the host defense mechanism, inflammatory agents, microbial invasion, and injury activate such pro-inflammatory cytokines as TNF-α, IL-1β and IL-6. Although the pro-inflammatory cytokines are necessary for inducing defense

mechanisms, the overexpression and abnormal regulation of pro-inflammatory cytokines damage the host tissues and perturb the immune system [21]. NO is related to the reduction of pro-inflammatory proteins and mRNAs by down-regulating NF-κB binding to the promoter region of pro-inflammatory cytokine genes [42]. Thus, the regulation of NO production can be a treatment for neutralizing excessive inflammatory responses.

Several studies have presented that inflammatory response also activates the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway in monocytes and macrophages [8, 13, 22]. PI3K are divided into three groups according to their substrate and structure property. Among them groups, class I_A PI3Ks are involved in receptor-mediated signaling in the immune system, composed of a heterodimer between a p110 catalytic subunit and a p85 regulatory subunit [39]. This kinase catalize the phosphorylation of $PI(4,5)P_2$ to the lipid second messenger $PI(3,4,5)P_3$ (PIP_3). Sequently, interaction of phosphoinositide-dependent protein kinase 1 (PDK-1) and Akt to PIP_3 occurs to the phosphorylation of Akt. The phosphorylation of Akt is down-regulated by the phosphatase and tensin homologue (PTEN), which changes PIP_3 to $PI(4,5)P_2$ [4]. Several studies reported that activation of PI3K by expression of the catalytically active p110 subunit inhibits the production of NO and expression of iNOS and thus important to the production of NO and induction of iNOS in the PI3K/Akt pathway [29].

Core histone modifications are reported to play an important role in regulation of transcription and chromatin structure [20]. The acetylation and methylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), two conserved histone enzymes, associate the addition and removal, respectively, of acetyl groups on histone lysine residues [31, 36]. HATs is composed into three families: the GCN5-related *N*-acetyltransferase (GNAT) family, represented by general control nonderepressible 5 (GCN5) and p300/CBP associated factor (PCAF); the p300/CBP family, including p300 and CREB-binding protein (CBP); and the MYST family, which includes TAT-interacting protein 60 (Tip60) [11]. In humans, HDACs are classified into three categories: the class I RPD3-like proteins (HDAC1, HDAC2, HDAC3, and HDAC8); the class II HDA1-like proteins (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10); and the class III SIR2-like proteins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7) [43]. HATs and HDACs are involved in the genome modification of acetyl groups on histones and, in addition, some also modify other factors. Through their interaction with sequence-specific transcription factors, they are also targeted to specific promoters, where they locally modify histones or transcription factors and thus regulate gene transcription. Because of the importance of histone modification in chromatin function, HATs and HDACs are closely linked to the induction of pro-inflammatory gene expression, and thus are supposed as a target for treatment of

inflammatory disoders [11, 23, 38].

The simple phenolic veratric acid is one of the major benzoic acid derivatives from *Sparassis crispa* and it also have been reported to anti-hyperlipidemia and anti-oxidation [14, 33]. Futhermore, veratric acid is known to have anti-inflammatory effect by inhibition of iNOS expression through inactivation of MAPKs and NF-κB in LPS-stimulated RAW264.7 cells [7]. In the present study, we investigated the down-regulating effects of veratric acid on PI3K/Akt pathway and histone modification, resulting in inhibitory effect of iNOS expression in LPS-stimulated RAW264.7 cells.

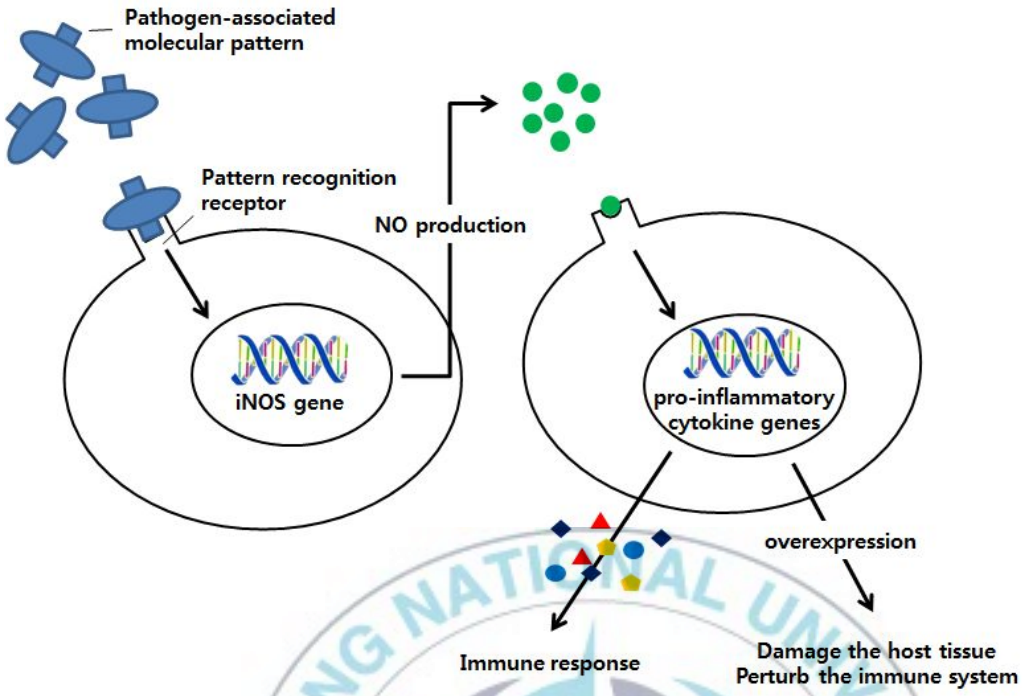

Fig. 1 Simplified conceptual model of the main steps in NO synthesis and thereby expressed the normal and abnomal regulatory effects of pro-inflammatory cytokines.

MATERIALS AND METHODS

Chemicals and Reagents

Veratric acid, LPS (Escherichia coli 0111:B4), Griess reagent and Tween-20 were purchased from Sigma Aldrich (St.Louis, MO, USA). Skim milk powder was purchased from Bioshop (Burlinton, ON, Canada). PI3K inhibitor LY294002 were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Cell culture

RAW264.7 mouse macrophage cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, U.S.A). The cell were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose with L-glutamine (Cellgro, Mediatech, Inc., Manassas, VA, USA) supplemented with 10% FBS (Cellgro) and 1% (v/v) penicillin (100 U/ml)/streptomycin (100 μg/ml) (PPA Laboratories GmbH, Pasching, Austria). The cells were incubated in humidified condition of 5% CO_2 at 37℃.

Cell viability assay

Cell viability was measured using the MTT assay. RAW264.7 cells were seeded in 96-well plate at 1 x 10^5 cells/well. After 24 h incubation,

cells were treated with veratric acid (100, 200 μM) and cultured for 24 h. Afterwards, 10 μl of WST-1® (Daeil Lab service, Seoul, Korea) was added to each well and after 3 h incubation at 37℃ for MTT-formazan formation. Absorbance at 460 nm was measured with ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Nitric Oxide (NO) assay

The RAW264.7 cells (5 x 10^5 cells/well) were cultured in 24-well plates for 24 h. The cells were pretreated with veratric acid (100, 200 μM) for 1 h, and were then induced with LPS (100 ng/ml) in the absence or presence of veratric acid for 24 h. NO in the culture medium was measured as an indicator of NO production, using Griess reagent. Briefly, 100 μl of culture supernatant was mixed with same volume of the Griess reagent, and the absorbance was determined at 540 nm by using a ELISA reader.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using a RNeasy kit (QIAGEN) from cells. The RNA was reverse transcribed using a AccuPower® RT premix (Bioneer, Daejeon, Korea) containing M-MLV reverse transcriptase using 1 μg of total RNA. The iNOS, GAPDH and HDACs genes were amplified from the cDNA by PCR. The primers (sense/antisense) were

iNOS, 5'-ATGTCCGAAGCAAACATCAC-3' and 5'-TAATGTCCAGGAAGTAGGTG-3';

GAPDH, 5'-AGGCCGGTGCTGAGTATGTC-3' and 5'-TGCCTGCTTCACCACCTTCT-3'; HDAC1, 5'-CGCATGACTCACAATTTGCT-3' and 5'-AAACACCGGACAGTCCTCAC-3'; HDAC2, 5'-AGACTGCAGTTGCCCTTGAT-3' and 5'-TTTGAACACCAGGTGCATGT-3'; HDAC3, 5'-CACCTTTTCCAGCCAGTCAT-3' and 5'-GTAGCCACCACCTCCCAGTA-3'. PCR products were electrophoresed in an agarose gel.

Isolation of cytosolic and nuclear protein extracts

Nuclear extraction of RAW264.7 cells was conducted by NE-PER Nuclear and cytoplasmic extraction reagents according to the manufacturer's instruction (Pierce, Rockford, IL, USA). Briefly, RAW264.7 cells were plated in 100 mm dishes, treated with veratric acid, stimulated with LPS for 1 h, scraped into 3 ml of cold PBS, and pelleted by centrifugation. The cell pellets were resuspended in ice-cold CER I buffer, and incubated on ice for 10 min. The cells were then mixed with ice-cold CER II buffer, and centrifuged at 16,000g for 5 min. The supernatant was transferred to a pre-chilled tube (cytosolic fraction). The insoluble fraction was resuspended in ice-cold NER buffer, incubated on ice for 40 min, and centrifuged at 16,000g for 10 min. The supernatant (nuclear fraction) was used for analyzing the nuclear protein expression.

Western blot analysis

For isolation of cell lysates, RAW264.7 cells were treated with veratric acid for 1 h, and induced with LPS for indicated time. After stimulation,

cells were lysed using lysis buffer (50 mM Tris-Cl [pH7.5], 150 mM NaCl, 1mM DTT, 0.5% NP-40, 1% Triton X-100, 1% Deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM EDTA, 1 μM Aprotinin, 1 μM Leupeptin, and 1 μM Pepstatin A) (Intron biotechnology, Seongnam, Korea). The protein concentration was determined using aProtein Quantification Kit (CBB solution®) (Dojindo Molecular Technologies, Rockvile, MD, USA) with bovine serum albumin (BSA) as standard. Equal volume of isolated proteins were separated on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane (PALL Life Sciences, Pensacola, MI, USA). the membrane was blocked with 5% skim milk in phosphate buffered saline Tween (PBST, 135 μM NaCl, 2.7 mM KCl, 4.3 mM NAPO4, 1.4 mM KH2PO4, and 0.5% Tween-20) for 1 h. After blocking, the membrane was incubated with the primary antibodies and washed three times with PBST. The membrane were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG as secondary antibodies (1:10000, Cell Signaling Technology, Beverly, MA, USA) and were washed three time with PBST. The immunoreactive proteins were visualized by an enhanced chemiluminescent (ECL) detection solution (AbFrontier, Gyeonggi, Korea)

Immunofluorescent staining

Cells grown on coverglass-bottomdishes (SPL Lifesciences, Gyeonggi, Korea) were incubated and treated with 200 μM of the veratric acid for

1 h and stimulated LPS for 3 h. After stimulated, the cells were pretreated with DAPI (4, 6-diamidino-2-phenylindole, Roche, Indianapolis, IN, USA) for 15 min at 37℃ and then fixed with 4% formaldehyde (Sigma, St. Louis, MO USA) and blocked for 1 h in 5% mouse and rabbit normal serum (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h. The blocked cells were incubated with 0.1 μg/ml of primary antibody (p-Akt) for 1 h and then incubated with anti-rabbit IgG tagged with Alexa Fluor 488 (Cell Signaling Technology) for 1 h and were washed with PBS. Stained cells were mounted on the slides with Prolong Gold Antifade Reagent (Invitrogen, Eugene, OR, USA) and obseeved in a fluorescent Nikon ECLIPS 50i microscope equipped with charged-couple device (CCD) camera (Nikon, Tokyo, Japan). Images were captured and processed with a High-Content Analysis Software (Cambeidge Healthtech Indtitute, Needham, MA, USA).

Statistical analysis

The data are expressed as the mean values ± standard errors of the mean (SEM) of triplicate experiments. Each untreated control group and LPS-stimulated group was measured for statistically significant values by t-tests. ANOVA post hoc tests and Dunnett's multiple comparison tests were used for evaluating the differences between the LPS-stimulated group and veratric acid and LPS-treated group. p-value less than 0.05 were considered statistically significant.

RESULTS

Inhibitory effects of veratric acid on NO production and iNOS expression in LPS-stimulated RAW264.7 cells

WST-1 assay was used to analyze the viability of RAW264.7 cells treated with veratric acid at 50, 100 and 200 μM. The cell viability was measured by WST-1® solution. The effects of veratric acid on the decreasing NO, the production of NO was determined by Griess solution and the expression of iNOS was evaluated by RT-PCR and western blot analysis. According to this results, veratric acid shown no cytotoxity effects and attenuation the NO production and iNOS expression in dose-dependent manner (Fig. 2).

Fig. 1

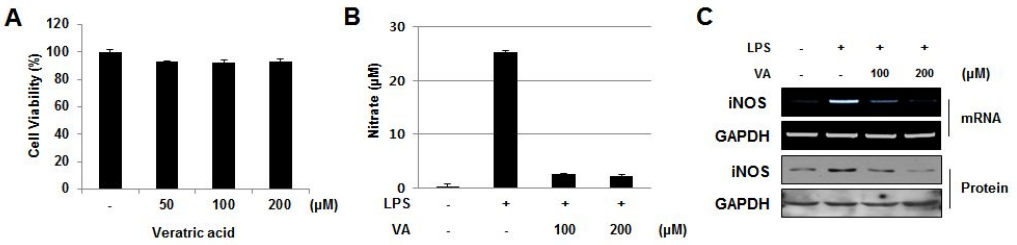

Fig. 2 Effects of veratric acid on cell viability and NO production in RAW264.7 cells. (A) The viability of RAW264.7 cells induced by veratric acid (50, 100 and 200 μM) was detected by MTT reagent. (B) NO production were pretreated (100 and 200 μM) for 1 h before the stimulation with LPS (100 ng). (C) The expression of iNOS was determined at the level of protein and mRNA using western blot analysis and RT-PCR, respectively. The data are representative of 3 independent experiments and expressed as mean ± SD.

Regulatory effect of veratric acid on PI3K subunits expression and PDK1 and Akt phosphorylation in LPS-stimulated RAW264.7 cells

Up-regulation of NO production activated to the PI3K and Akt in macrophages [32]. Therefore, we investigated effects of veratric acid on the regulation of activation of PI3K/Akt. First, we confirmed that phosphorylation of p85 and regulatory subunit of PI3K at protein level. These results shown, veratric acid regulated the inhibition of LPS-induced p85 phosphorylation and the increase p110α expression and reduction p100β expression in dose-dependent manner. (Fig. 3A, 3B, 3C). We examined regulatory effects of veratric acid in LPS-stimulated PI3K activation by PDK1 and Akt and it shows reduction of PDK1 and Akt phosphorylation (Fig. 3D). Veratric acid prevented Akt translocation whether or not which is investigated by immunofluorescent staining (Fig. 3E, 3F). These results indicated that veratric acid down-regulation on LPS-stimulated RAW264.7 cells.

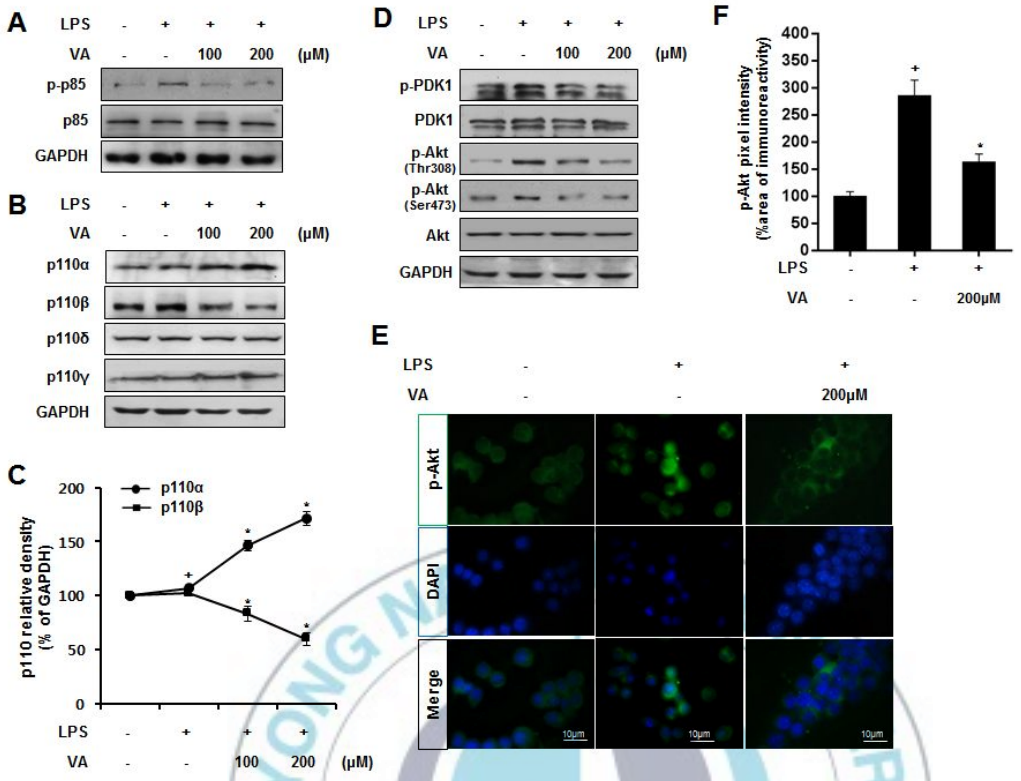

Fig. 3 Effects of veratric acid on LPS-induced PI3K/Akt pathway in RAW264.7 cells. The cells pretreated with veratric acid (100 and 200 μM) for 1h before the induction of LPS (100 ng/ml) for 30 min. (A) The expression of p85 in RAW264.7 cells was determined at the level of protein using western blot analysis. (B) RAW264.7 cells were treated with veratric acid (100 and 200 μM) for 1 h, and then were stimulated with LPS (100 ng/ml) for 8 h. The protein expression of p110 isoforms was detected by western blot analysis. (C) The intensity of the detected p110 isoforms was quantified using ImageJ. (D) Cells were treated with

veratric acid (100 and 200 μM) for 1h, after which LPS (100 ng/ml) for 30 min. The phosphorylation and dephosphorylation of PDK1 and Akt were detected by western blot analysis. (E) Immunofluorescent staining of p-Akt with a p-Akt antibody and an anti-rabbit IgG tagged with Alexa Flour 488 after fixation (*green*). The nuclei were counterstained by DAPI (*blue*). (F) The pixel intensity of immunostained p-Akt in the nucleus area was quantified using ImageJ. The values were normalized to 100% for the expression of the untreated group. Each value represents the mean ± SD for three separate experiments. $^{+}P < 0.01$ versus LPS-treated group, $^{*}P < 0.01$ versus veratric acid and LPS-treated group.

HATs is reduced by veratric acid in LPS-stimulated RAW264.7 cells

Akt phosphorylation enhanced the HAT activity of p300, its increased acetylation of histone H3 or H4 [34, 41]. Also, activation of HAT represented phosphorylation-induced conformational change that exposes the catalytic domain of ATF-2 [19]. We determined whether veratric acid controls the activation and expression of HAT, such as p300 and ATF-2 in LPS-stimulated RAW264.7 cells. Expression of p300 and ATF-2 was increased by LPS, but decreased by veratric acid in dose-dependent manner (Fig. 4A). Thus, we examined regulatory effects of veratric acid on acetylation of p300 and phosphorylation of ATF-2. Similarly, p300 acetylation and ATF-2 phosphorylation was inhibited by veratric acid in dose-dependent manner. We suspected whether PI3K/Akt pathway is associated to the p300 and ATF-2, because we treated specific PI3K inhibitor, LY29004, in LPS-stimulated RAW264.7 cells. As expected, alike reduced activation of p300 and ATF-2 in veratric acid-treated cells (Fig. 4C). These data suggest that veratric acid was down-regulated LPS-induced HAT activity of p300 and ATF-2.

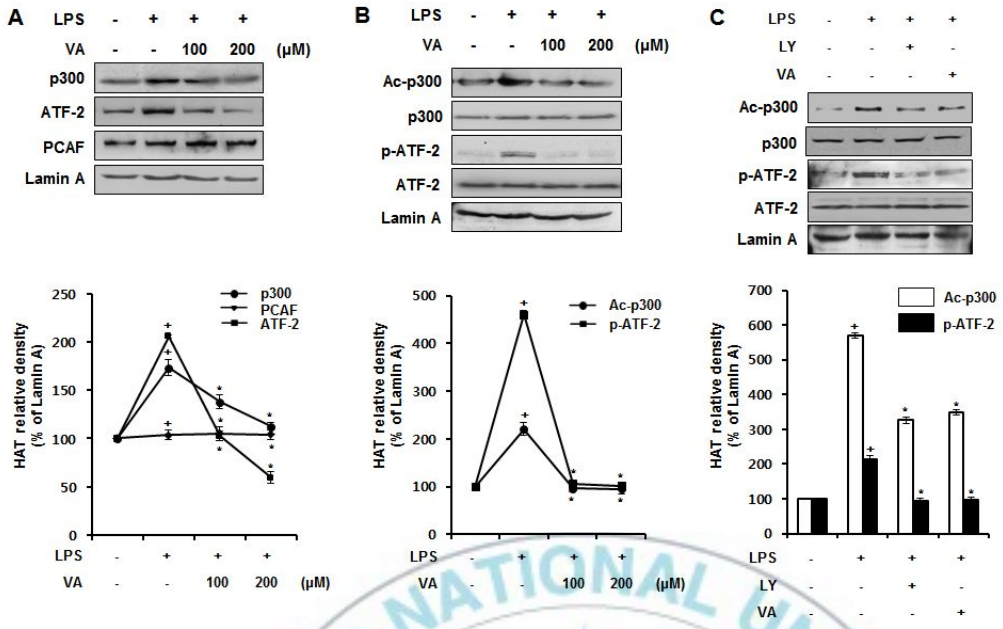

Fig. 4 Effecs of veratric acid on the expression and activation of HATs in LPS-stimulated RAW264.7 cells. The cells were pretreated with veratric acid (100 and 200 μM) for 1 h and then LPS (100 ng/ml) for 1 h. Effects of veratric acid on (A) expression of p300 and ATF-2, (B) acetylation of p300 and phosphorylation of ATF-2 and (C) inhibitory effects of LY294002 in HATs activation were detected western blot analysis. The pixel intensity was quantified using ImageJ. Each value represents the mean ± SD for three separate experiments. $^{+}P < 0.01$ versus LPS-treated group, $^{*}P < 0.01$ versus veratric acid and LPS-treated group.

HDAC expression is down-regulated by veratric acid in LPS-stimulated RAW264.7 cells

The regulation of HATs for HDACs can mediated gene expression including inflammatory genes [27]. We objected to determine whether veratric acid can regulate HDACs expression at mRNA and protein level detected by RT-PCR and western blot analysis. Fig. 5A is shown that veratric acid only reduced HDAC3 expression in dose-dependent manner. The Fig. 5B is show that veratric acid inhibits the HDAC3 expression in LPS-stimulated RAW264.7 cells as the LY294002. These results imply that HDAC3 expression inhibitory effects of veratric acid can regulate the PI3K/Akt pathway.

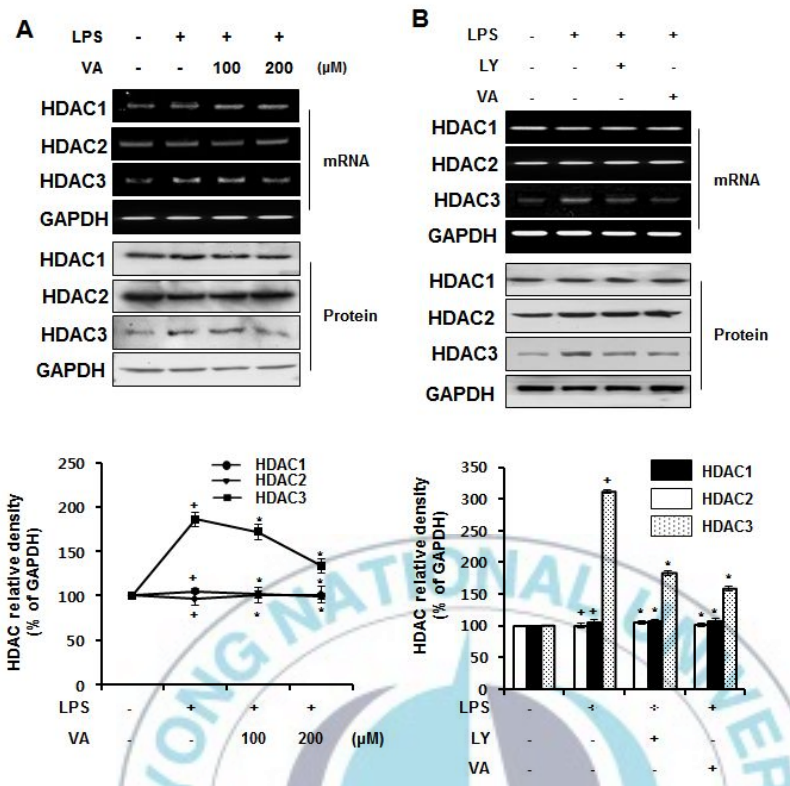

Fig. 5 Effects of veratric acid on the HDACs expression in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated veratric acid (100 and 200 μM) or LY294002 (20 μM) for 1 h, and then were stimulated with LPS (100 ng/ml) for 8 h. The expression of HDACs was detected at the level of mRNA and protein using RT-PCR and western blot analysis, respectively. The intensity were detected by ImageJ. Each value represents the mean ± SD for three separate experiments. $^{+}P < 0.01$ versus LPS-treated group, $^{*}P < 0.01$ versus LY294002, veratric acid and LPS-treated group.

Histone acetylation is inhibited by veratric acid in LPS-stimulated RAW264.7 cells

Inhibition of PI3K leading to attenuate in HAT activity, p300 and ATF-2 [16]. We examined the acetylation levels of histone for identify wether the control of HATs and HDACs by veratric acid. The veratric acid reduced the LPS-stimulated acetylation of histone H4 in dose-dependent manner, but histone H3 is not changed (Fig. 6A). Thus, we compared with the acetylation of histone H3 and H4 in the LY294002-treated or veratric acid-treated cells using western blot analysis. Expectably, veratric acid is down-regulated the acetylation of histone H4 as the LY294002 (Fig. 6B). The our results suggested that veratric acid is regulation of PI3K/Akt pathway via attenuation of LPS-stimulated histone H4 acetylation.

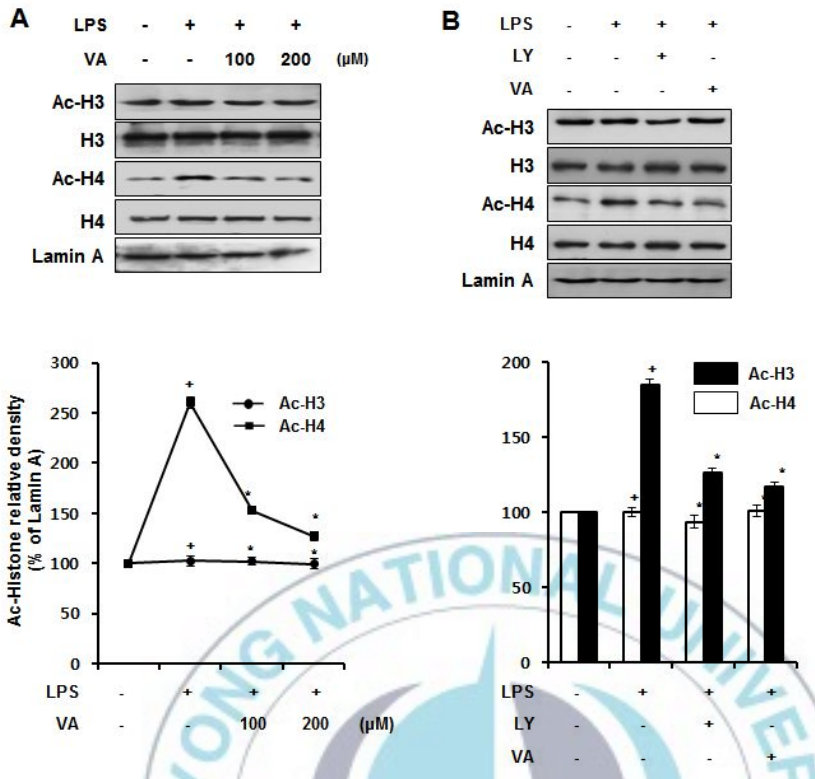

Fig. 6 Effects of veratric acid on histone acetylation in LPS-stimulated RAW264.7 cells. The nuclear protein were isolated from the cells treated with veratric acid (100 and 200 μM) or LY294002 (20 μM) for 1 h, and then were stimulated with LPS (100 ng/ml) for 1 h. Histone acetylation was detected at the protein level using western blot analysis. Pixel intensity were detected by ImageJ. Each value represents the mean ± SD for three separate experiments. $^{+}P < 0.01$ versus LPS-treated group, $^{*}P < 0.01$ versus LY294002, veratric acid and LPS-treated group.

DISCUSSION

Veratric acid was reported anti-oxidant, anti-inflammation [7] and anti-hyperlipidemia effects [33]. Also, veratric acid has inhibitory effects MAP kinases and transcription factors such as STAT1, STAT3 and NF-κB, thus down-regulated iNOS expression in LPS-stimulated RAW264.7 cells [7]. We study the effects of veratric acid on Akt, PI3K subunit including p85 and p110, and histone acetylation in LPS-stimulated RAW264.7 cells.

In previous studies demonstrated that NO plays a critical role in immune response and host defense and is produced by iNOS through the recognition of microbial molecular pattern [2]. Therefore, these results means that veratric acid has a anti-inflammatory effect by negative regulation of iNOS expression. Our results showed that veratric acid down-regulrated NO production via the inhibition of iNOS expression (Fig. 2). Moreover, NO production is interrelate PI3K. Macrophages deficient for class I_A PI3K are decreased in NO production [32]. The PI3K catalytic subunit p110β-deficient RAW264.7 cells was unable to activate Akt [40]. Our studied demonstrated that veratric acid reduced the phosphorylation of LPS-induced p85, PDK-1, Akt and p110β and whereas up-regulated the p110α (Fig. 3A, 3B, 3D). Taken together, veratric acid has inhibitory effect of iNOS expression that decreases the phosphorylation of Akt by p85 and regulation of p110 subunit in

LPS-stimulated RAW264.7 cells.

Furthermore, Akt phosphorylation was regulated of HAT activity of p300. HDAC inhibitors stimulate Akt phosphorylation of p300, recruitment of p300 to the chromatin, and modulation of its HAT activity in non-small-cell lung cancer cell lines [26]. Similarly, in the present studies shown that significantly reduction of p300 acetylation in veratric acid-treated cells, thus we suggest that veratric acid may impair the p300 acetylation through down-regulation of PI3K/Akt pathway (Fig. 4). ATF-2 was shown to interact and cooperate with p300 [18]. Moreover, ATF-2 phosphorylation at Thr 71 is associated iNOS expression via its HAT function [3]. Fig. 4 demonstrated that treatment of veratric acid was weakened LPS-induced ATF-2 phosphorylation. These results indicated, veratric acid is critical roles the PI3K/Akt-mediated iNOS expression by p300 acetylation and ATF-2 phosphorylation in LPS-stimulated RAW264.7 cells.

Class I HDACs, including HDAC1, -2 and -3, is interrelate expression of pro-inflammatory products, such as iNOS, IL-1β and TNF-α [24]. Above all, HDAC3 is closely related to regulation of PI3K/Akt pathway. Depletion of HDAC3 by RNA interference significantly down-regulated TGF-β-induced Akt phosphorylation [1]. Also, deacetylation of TNF-α -induced RelA, NF-κB p65 subunit, by HDAC3 controls the translocation between nucleus and cytoplasm [6]. Our results detected that inhibitory effect of veratric acid on the HDAC3 expression (Fig. 4). Thus,

inhibition of HDAC3 expression by veratic acid may negative regulator of iNOS related to impair of Akt phosphorylation.

Phosphorylation of p300 by Akt mediates two effects. Its increased the recruitment of p300 to the *ICAM-1* promoter and the acetylation of histone H3 or H4 at *ICAM-1* promoter. The acetylation process mediated by the HAT activity of p300 [16]. The HAT activity of ATF-2 have key roles that lysine acetylate at H2B, and H4. Furthermore, histone H4K8 and H4K12 acetylation is related to regulation of inflammatory genes in A549 cells [17]. The histone H3K14 and H4K8 hyperacetylation is increses the *IL12a*, *IL13* and *TLR13* and up-regulates the *IL12a* and *TLR13* in *E. coli* infected mice mammary tissue, respectively [28]. In addition, acetylated histone H4 to influence the activity of iNOS gene through PI3K/p70s6 pathway in mesanginal cells. Moreover, LY294002 blocked iNOS expression in murine macrophages in response to LPS or cytokines [44]. A similar effect of LY294002 on iNOS expression was detected in vescular smooth muscle cells [15]. Our present study demonstrated that acetylation of histone H4 was reduced by veratric acid, the similar to LY294002 (Fig. 6). Therefore, these results suggest that veratric acid regulates the iNOS expression via modulation of histone H4 acetylation.

Our results shown that decreses Akt phosphorylation by p85 and p110β in veratric acid-treated cells. Veratric acid treatment was down-regulated the HAT activity through reduction of p300 acetylation and ATF-2

phosphorylation, and suppressed HDAC3 expression, thereby inhibited the actylation of histone H4. These results was also presented that regulation of protein expression and activation by veratric acid in LPS-stimulated RAW264.7 cells is similar effects by LY294002. In conclusion, our study demonstrated that the inhibitory effects of veratric acid on PI3K/Akt pathway expected down-regulation of iNOS expression by inhibition of histone H4 acetylation through suppression of HAT and HDAC activity. Thus, these results suggest that veratric acid may be a potential anti-inflammatory reagent via regulation of iNOS expression.

국문초록

베라트릭 산은 간단한 구조의 benzoic acid 중 하나로서, 항산화, 항염증, 항고지혈증 등을 포함하는 약리학적 효과를 가지는 것으로 알려져 있다. 본 연구는 LPS에 의해 자극된 RAW264.7 세포주에서 베라트릭 산이 nitric oxide (NO) 생성을 억제하는지에 확인하기 위하여 진행하였다. 베라트릭 산은 농도 의존적으로 iNOS의 mRNA와 protein level을 억제하였고, 이로 인해 NO의 생성이 감소하는 것을 확인할 수 있었다. 또한 베라트릭 산을 처리한 경우, LPS에 의해 유도된 효과가 PI3K subunit들과 Akt에 미치는 영향을 감소시켰다. 그리고 베라트릭 산은 PI3K inhibitor인 LY294002와 유사하게 p300의 아세틸화와 ATF-2의 인산화를 효과적으로 억제하였다. 마찬가지로 LPS에 의해 유도된 HDAC3의 발현과 histone H4의 아세틸화 역시 억제하였다. 이러한 결과는 베라트릭 산이 LPS에 의해 유도된 p300과 ATF-2의 HAT activity와 HDAC3의 발현을 억제하여 histone H4 아세틸화를 기본적인 수준으로 회복시키고, 이를 통해 iNOS 발현에 필요한 PI3K/Akt signaling을 조절을 억제하는 효과를 가진다는 것을 나타낸다.

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