Phosphatase inhibition by sodium orthovanadate displays anti-inflammatory action by suppressing AKT-IKKβ signaling in RAW264.7 cells

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1. Introduction

The immune system protects the body from harmful substances such as pathogens, toxins, or allergens. Innate immunity, the initial line of defense, is not specific but initiates quickly upon recognition of pathogen-associated molecular patterns (PAMPs) [1]. Host organisms express receptors that specifically recognize PAMPs called pattern recognition receptors (PRRs) [2]. Toll-like receptors (TLRs), one of the PRRs activated at the onset of infection, have been studied extensively in humans and rats. When ligands bind to TLRs (e.g., lipopolysaccharide (LPS) binding to TLR4), TLR dimerization recruits the adaptor molecule myeloid differentiation factor 88 (MyD88) to activate downstream signal cascades, such as NF-κB signaling [3]. In the absence of stimulation, NF-κB is bound to its inhibitor κB (IκB) and remains in the cytoplasm in an inactive state [4]. IκB kinase (IKK), which is activated in response to external stimuli such as pathogens and cytokines, phosphorylates IκB, which targets proteolysis of IκB in a ubiquitin-dependent manner. The free NF-κB subunits (p65 and p50) then migrate into the nucleus and act as transcription factors [5]. TLRs activated through NF-κB signaling ultimately stimulate the production of immune substances such as chemokines, cytokines, and inflammatory mediators and thereby maintain immunological defense [6]. IKK contains three subunits (IKKα, IKKβ, and IKKγ) that are tightly associated with each other. IKKγ functions as a catalytic subunit, whereas IKKα and IKKβ function as a regulatory subunit [7]. The kinase activity of the catalytic subunits can be positively or negatively regulated by phosphorylation at serine or threonine residues. Phosphorylation of IKKβ in the activation loop (S177 and S181 in human IKKβ) allows entry

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ABSTRACT

Sodium orthovanadate (Na₃VO₄) is an inhibitor of phosphatases that acts as a phosphate analog and is being developed as an anti-diabetes drug. Phosphatases play important roles in inflammatory signal pathways by modulating the removal of phosphate moieties of key signaling proteins. However, the role of protein phosphatases on the inflammatory response has not been fully established. In this study, we investigated how phosphatases can control the inflammatory response using Na₃VO₄ in LPS-stimulated RAW264.7 cells and explored the molecular mechanisms by NO assay, mRNA analysis, immunoblotting analysis, kinase assay, luciferase reporter gene assay, and mutation strategy. Na₃VO₄ decreased the release of nitric oxide (NO) and suppressed the expression of pro-inflammatory genes at the transcriptional level, without cytotoxicity. The translocation of nuclear factor (NF)-κB subunits into the nucleus and the level of p-IκBα were reduced by Na₃VO₄, as was IKKβ activity. Na₃VO₄ inhibited NF-κB-Luc activity under AKT1/2 and IKKβ overexpression. However, the inhibitory effect of Na₃VO₄ against NF-κB-Luc was not observed in the group overexpressing both AKT2 and IKKβ-M10, a mutant in which the 10 serine residues in the autophosphorylated region of the C-terminal were replaced with alanine. Na₃VO₄ directly decreased the activity of protein phosphatase 1a (PP1α) and protein phosphatase 2 A (PP2A) by 95%. Phosphatase inhibition by Na₃VO₄ also selectively suppressed AKT-IKKβ signaling by directly blocking the phosphatase activity of PP1 and PP2A, consequently down-regulating NF-κB and inflammatory gene expression. Therefore, these results suggest that vanadium compounds including Na₃VO₄ can be developed as anti-inflammatory drugs.

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and binding of ATP and substrates by repelling the activation loop from the catalyst pocket, activating IKK [8,9]. The kinases that regulate IKK include PDK1 and AKT. PDK1 directly phosphorylates IKKβ at serine 181, but it does not phosphorylate IKKα [10]. AKT induces phosphorylation of IKKα at threonine 23 residue to activate NF-κB in tumor necrosis factor (TNF)-α-stimulated conditions [11,12]. Autophosphorylation in the helix-loop-helix (HLH) motif and C-terminal down-regulates IKK kinase activity [13]. Previous studies reported that IKK subunits are autophosphorylated at the C-terminus when IκB is quantitatively reduced by IKK activation. This negative feedback plays a vital role in timely activation and deactivation of the inflammatory reaction.

Sodium orthovanadate (Na₃VO₄), a complex of vanadate with hydrogen peroxide, is an inorganic salt of vanadium (Fig. 1A) and broadly used as an inhibitor of protein phosphatases (protein serine/threonine phosphatases (PS/TPs) and protein tyrosine phosphatases (PTPs)) with structural properties similar to phosphate [14,15]. Vanadium-based compounds, including Na₃VO₄, are attracting attention for their insulin-mimetic effect. Na₃VO₄ inhibits the activity of PTP1B and moves the glucose transporter type 4 (GLUT4) channels to the cell membrane, ultimately increasing sugar uptake [16,17]. In addition, administration of Na₃VO₄ lowers elevated blood sugar level in

![Fig. 1. Effect of Na₃VO₄ on LPS-triggered inflammatory reactions in RAW264.7 cells. (A) The skeletal structural formula of Na₃VO₄. (B) RAW264.7 cells were pre-treated with Na₃VO₄ (0–400 μM) for 30 min and then stimulated with LPS (1 μg/ml) (left panel), Poly(I:C) (right panel), or Pam3CSK4 (right panel) for 24 h. NO release from RAW264.7 cells was examined by Griess assay. (C) RAW264.7 cells and HEK293 cells were treated with Na₃VO₄ (0–400 μM) for 24 h, and cytotoxicity was determined by MTT assay. (D) RAW264.7 cells pre-treated with Na₃VO₄ (0–400 μM) were incubated with LPS (1 μg/ml) for 6 h. The gene expression levels of iNOS and TNF-α were examined by real-time PCR (left panel) and semi-quantitative PCR (right panel). The data are representative of three independent experiments. (E) Inhibitory activity of prednisolone on NO production was examined with pretreatment of prednisolone (0–200 μM) for 30 min and then stimulation with LPS (1 μg/ml). The data presented in (B), (C), (D), and (E) are shown as the mean ± standard deviation (SD) (n = 3 independent experiments). # and ## indicate p values less than 0.05 and 0.01, respectively, compared with the normal group. * and ** indicate that p values are less than 0.05 and 0.01, respectively, compared with the LPS, poly(I:C), or Pam3CSK4 group alone.

various diabetic rat models [18]. Bis(ethylmalolato)oxovanadium (IV) (BEOV), an organic vanadium compound synthesized to improve bioavailability of inorganic vanadium, was explored in phase I clinical trials and toxicity studies. BEOV was administered orally in doses ranging from 25 mg to 90 mg, and adverse effects were not reported in healthy volunteers [19]. In a phase IIa trial, BEOV (20 mg for 28 days) reduced blood glucose level in type 2 diabetic patients [20]. These promising clinical data suggest the potential of vanadate in therapeutic applications. In addition to its potential antidiabetic effects, Na$_2$VO$_4$ has demonstrated effects in diverse physiological and pathological processes including fertilization, apoptosis, cell cycle, and neuronal death [21–23]. Despite the important role of phosphatases in regulating the immune response [24], the immunosuppressive effect of Na$_2$VO$_4$ has not been clearly elucidated.

Therefore, in this study, we explored how protein phosphatases control inflammatory responses using Na$_2$VO$_4$ in LPS-treated macrophages, RAW264.7 cells. We identified the direct target of Na$_2$VO$_4$ by examining its regulatory mechanism at the molecular level in HEK293 cells.

2. Materials and methods

2.1. Materials

Na$_2$VO$_4$ prednisolone (Pred), LPS, poly(I:C), pam3CSK$_4$, poly-ethylenimine (PEI), tetrazol 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyloxazol-3-yl (MTT), phorbol 12-myristate 13-acetate (PMA), sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), pam3CSK$_4$, and LPS (E. coli 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin, streptomycin, TRIZol reagent, and RPMI 1640 and DMEM/F12 media were obtained from Gibco; Thermo Fisher Scientific, Inc. (Grand Island, NY, USA). RAW264.7 and HEK293 cells were purchased from ATCC (Rockville, MD, USA). All other chemicals used in this study were of analytical grade and obtained from Sigma Chemical Co. Primary antibodies against p-p65, total p65, p50, p-IκB, total IκB, p-IKKα/β, IKKα/β, IKKα, p-AKT, AKT, p-AKT1, AKT1, p-AKT2, AKT2, protein phosphatase 1 (PP1), Flag, β-actin, HA, and Lamin A/C were purchased from Cell Signaling (Beverly, MA, USA) or Santa Cruz Biotechnology (Santa Cruz, CA, USA). Constructs of MyD88-Flag, CFP-TRIF, Flag-IKKα, Myc-PDK1, Flag-IKKα AKT1-HA, AKT2-HA, Flag-IKKβ-M10, and NF-κB-Luc were used as reported previously (REVs). Polyvinylidene difluoride (PVDF) membranes and an enhanced chemiluminescent (ECL) kit were purchased from Thermo Scientific (Waltham, MA, USA). Premixed reagents for real-time PCR (SYBR Premix Ex Taq) and RT-PCR (Bio-D) were obtained from Takara (Tokyo, Japan) and Bioneer (Daejeon, Korea), respectively.

2.2. Cell culture

RAW264.7 cells were cultured and maintained in RPMI 1640 media supplemented with 10% FBS and 1% penicillin/streptomycin as described previously [25]. Gene overexpression and luciferase assays were performed using HEK293 cells cultured and maintained in DMEM/F12 media supplemented with 10% FBS and 1% penicillin/streptomycin as described previously [25].

2.3. Nitric oxide (NO) assay

RAW264.7 cells were plated in a 96-well cell culture plate at a density of 1 × 10$^5$ cells/well. For reliability of the results, each experimental group consisted of 10 wells (n = 10). After 18 h of incubation, the culture media was replaced with 100 μl of RPMI 1640 medium. RAW264.7 cells were treated for 30 min with Na$_2$VO$_4$ (0–400 μM) dissolved in 50 μl of RPMI 1640 medium and then incubated for 24 h after adding LPS (1 μg/ml) dissolved in 50 μl RPMI 1640 without medium exchange. For the control group, 50 μl of medium was added instead of LPS. NO concentration in the supernatant (100 μl) was measured and calculated using Griess reactions as previously reported [26].

2.4. Real-time PCR and semi-quantitative PCR

RAW264.7 cells pre-treated with Na$_2$VO$_4$ for 30 min were stimulated with LPS for 6 h. Total RNA was prepared using TRIzol, according to the manufacturer’s instructions. Real-time PCR was conducted using premix reagent (SYBR Premix Ex Taq) as described previously [27]. RT-PCR was performed using premix (Bio-D) as described previously [28]. The ImageJ program was used to measure and quantify band intensity. The sequences of primers used for PCR analysis are listed in Table 1.

2.5. Tetrazole 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyloxazol (MTT) assay

Cells were plated in 96-well plates and then incubated with various doses of Na$_2$VO$_4$ (0–400 μM). For reliability of the results, each experimental group consisted of 10 parallel wells. After 24 h, MTT reagent (10 μl) was added to each well. After 3 h, the reaction was stopped using stop solution, and the absorbance of formazan crystal was measured as previously described [29].

2.6. Extraction of whole lysates

RAW264.7 cells and HEK 293 cells were treated with Na$_2$VO$_4$ and then incubated with LPS or transfected with plasmids as indicated in figures. The detailed protocol for extracting whole lysates is described in our previous report [30]. Briefly, cells were collected and washed once with PBS. Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4; 1% Triton X-100; 10% glycerol; 10 μg/ml aprotinin; 10 μg/ml pepstatin; 1 μM benzamide; and 2 μM PMSF) using a cell sonicator (Thermo Fisher Scientific, Waltham, MA, USA) for 15 s, 3 times, on ice. The lysates were centrifuged at 12,000 rpm for 5 min at 4 °C, and the supernatant was transferred to fresh tubes.

2.7. Preparing nuclear lysates

RAW264.7 macrophages were cultured with LPS for 24 h in the presence or absence of Na$_2$VO$_4$ (400 μM). The protocol for extracting nuclear fractions was described in a previous report [26]. Briefly, the nuclear fraction was prepared using a three-step method. First, the cells were collected and washed with cold PBS. The cells were lysed using 500 μl of nuclear lysis buffer (50 mM KCl, 0.5% Nonidet P (NP) – 40, 25 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 100 μM 1,4-dithiothreitol) and then incubated on ice for 4 min. Next, the lysates were centrifuged at 16,000g for 1 min, Table 1

| Name  | Sequence (5’–3’) |
|-------|----------------|
| Semi-quantitative PCR | |
| GADPH | F CAAAGCAGGGGCTACAGGCAAC |
| R AGGAGATCCTCAGTGT GG |
| PP1CA | F AAGCAGACCTGCTGGTGGGG |
| R GTCTGGCAGATAGGGTCAAA |
| PP2CA | F GATCAAGGGGCTGCGGAGAA |
| R TGCTGGCAGATAGGGTCAAG |
| Real-time PCR | |
| iNOS | F CTCCTCGGAGTTCTCAGGACGAC |
| R GGCTGTCAGGCTCTGGTGGT |
| TNF-α | F AGTCTGACGTCGTCATG |
| R CCTCGTACCAGACCTGAG |
| GAPDH | F CACTCAGCGGCAAATTCAGGGCAC |
| R GACCTCAGACATATCTCAAGGCA |
and the pellet was washed with nuclear lysis buffer without NP-40. Finally, the washed pellet was lysed using extraction buffer (nuclear lysis buffer with 500 mM KCl and 10% glycerol) at −80 °C until frozen and then centrifuged at 16,000g for 5 min. The supernatant was used as the nuclear extract.

2.8. Western blotting

Western blotting analysis was performed as described previously [30]. Briefly, lysates were separated by 7–15% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 3% BSA and then incubated with primary antibody overnight at 4 °C. After washing three times with Tris-buffered saline with Tween 20, the membrane was incubated with secondary antibody conjugated with horseradish peroxidase in 3% BSA for 2 h at room temperature. The immunoreactive bands were detected by an ECL system. The ImageJ program was used to measure and quantify band intensity.

2.9. Immunoprecipitation assay

Whole cell lysates were immunoprecipitated according to the protocol reported in our previous publication [30]. Briefly, cell lysates were precleared with 10 μl of protein A–coupled Sepharose beads (50% v/v) for 3 h at 4 °C and then incubated with antibodies for 24 h at 4 °C. The immunocomplex was incubated with 10 μl of protein A–coupled Sepharose beads (50% v/v) for 4 h at 4 °C. The beads were washed with PBS 5 times, and the samples were boiled in 2 × Laemmli buffer from BioRad (Hercules, CA, USA) for 2 min.

2.10. Luciferase assay

HEK293 cells were plated in 6-well cell culture plates and transfected with MyD88-Flag, CFP-TRIF, Flag-IKKβ, Myc-PDK1, Flag-IRKα, AKT1-HA, AKT2-HA, Flag-IRKβ-M10, and NF-κB luciferase reporter plasmids for 24 h as indicated in the figures. Cells were then treated with Na3VO4 (400 μM) for an additional 24 h. For reliability of the results, each experimental group consisted of 6 parallel wells. The assay was performed using a luciferase assay kit (Promega, Madison, WI, USA) as described in the manufacturer’s instructions and a previous publication [31].

2.11. Kinase assays

For kinase assays, we used a commercial service platform measuring activity profiles of kinases or phosphatases (Millipore, Burlington, MA, USA). This platform uses recombinant enzymes IKKα, IKKβ, PDK1, AKT1, AKT2, PP1α, and protein phosphatase 2 A (PP2A), and Na3VO4 was prepared as a 50 × stock solution in DMSO. The reaction procedure for the in vitro enzyme assay has been detailed in our previous publication [30].

For in vitro kinase assays, endogenous IKKβ or exogenous Flag-IKKβ was immunoprecipitated from LPS-stimulated RAW264.7 cells and AKT2 overexpressing HEK293 cells, respectively, and used as enzyme sources. Immunoprecipitated IkBα protein was used as a substrate. The kinase reaction was performed with enzyme sources and substrates for 30 min at 30 °C in 50 μl of kinase reaction buffer (Upstate Biotechnology, Lake Placid, NY, USA). The incubated mixture was analyzed by western blotting to determine the phosphorylated substrates, as described previously [30].

2.12. Cellular thermal shift assay (CETSA)

HEK293 cells treated with DMSO or Na3VO4 (400 μM) for 24 h were collected in PBS. The resuspended cells were divided equally into 7 tubes and heated for 3 min at 57–72 °C. After cooling for 3 min at 25 °C, the cells were lysed using liquid nitrogen in three freeze-thaw cycles. The lysates were mixed with 2 × Laemmli buffer and analyzed by western blotting. Methods for CETSA have been detailed in our previous report [32]. Protein phosphatase 1 (PP1) antibody used for CETSA was obtained from Cell Signaling (Beverly, MA, USA).

2.13. RNA interference

For small interfering RNA (siRNA)-mediated knockdown of PP1α or PP2A, cells were transfected with 20 nM of either targeting or control siRNA (GenoLife, Seoul, Korea) using Lipofectamine RNAiMax (Invitrogen) for 72 h. The sequence of siRNA targeting mouse PP1α (NM_031868) was 5′-CCGGAGAATTTCTTTCTACTT-3′, and the sequence of siRNA targeting mouse PP2A (NM_019411) was 5′-CCAGATACAAATTACCTGT-3′.

2.14. Statistical analysis

All data in this study are shown as mean ± standard deviation (SD) of three independent experiments. To assess the significant differences between the data, the results were analyzed by analysis of variance (ANOVA), Scheffe’s post hoc test, and Kruskal–Wallis/Mann–Whitney tests. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Na3VO4 suppresses the inflammatory response in RAW264.7 cells

To assess the anti-inflammatory efficacy of Na3VO4, the release of inflammatory mediator including NO was evaluated in RAW264.7 cells. Na3VO4 (0–400 μM) dose-dependently suppressed the secretion of NO in LPS-, Poly(I:C)-, and Pam3CSK4-stimulated RAW264.7 cells (Fig. 1B, left and right panels). LPS, Poly(I:C), and Pam3CSK4 are ligands for TLR4, TLR3, and TLR2, respectively. The IC50 values for NO production were 148.7 μM, 122.5 μM, and 98.2 μM in LPS, Poly(I:C), and Pam3CSK4 responses, respectively. However, cytotoxicity of Na3VO4 was rarely observed at any treated concentration (up to 400 μM) in RAW264.7 cells and HEK293 cells (Fig. 1C), suggesting that the inhibitory effect against NO is not due to cytotoxicity.

The production of NO is mainly regulated by inducible nitric oxide synthase (iNOS), and the expression of the iNOS gene was evaluated using real-time PCR analysis and semi-quantitative PCR. Gene expression of TNF-α, a well-known pro-inflammatory cytokine, was also tested to confirm the anti-inflammatory property of Na3VO4. Both iNOS and TNF-α mRNA levels were remarkably up-regulated by LPS and were down-regulated by Na3VO4 (50, 100, 200, and 400 μM) (Fig. 1D). The standard drug, prednisolone, exhibited inhibitory activity on NO production in a dose-dependent manner (Fig. 1E) as reported previously [33].

3.2. Na3VO4 inhibits NF-κB signaling by targeting IKKα/β

Next, we examined the molecular mechanism by which Na3VO4 suppresses inflammatory responses. Because Na3VO4 suppressed the expression of inflammatory genes at the transcriptional level, the effect of Na3VO4 on the NF-κB transcriptional factor was assessed by measuring the levels of nuclear translocated p65 and p50 (subunits of NF-κB). The translocation of p65 and p50 into the nucleus was inhibited by treatment with 400 μM of Na3VO4 after 60 min and 90 min of LPS stimulation (Fig. 2A). Na3VO4 also inhibited the phosphorylation of IkBα, an upstream regulator of NF-κB, from 30 min after LPS treatment (Fig. 2B). However, the phosphorylation of p65(IKKβ) was clearly increased at 15, 60, 90, and 120 min after LPS stimulation in Na3VO4-treated RAW264.7 cells (Fig. 2C, left and middle panels). Na3VO4 also elevated the phosphorylation level of IkBα at 15 min and 60 min after LPS treatment (Fig. 2C, left and middle panels). Moreover, Na3VO4 enhanced
the autophosphorylation level of IKKβ under conditions where IKKβ is overexpressed (Fig. 2C, right panel). IKKα was not autophosphorylated (Fig. 2C, right panel). Interestingly, kinase assay showed that Na$_3$VO$_4$ inhibited IKKβ kinase activity despite increasing phosphorylation of IKKβ (Fig. 2D). The inhibitory efficacy of Na$_3$VO$_4$ against IKKβ activity was further confirmed by a luciferase assay (Fig. 2E). HEK293 cells were used for the luciferase assay because of the low DNA transfection efficacy of RAW264.7 cells [34]. In addition, due to the absence of TLR4, either TRIF or MyD88, which are adaptor molecules of TLR4, was overexpressed in HEK293 cells. Consistent with the results of the kinase assay, Na$_3$VO$_4$ decreased the transcriptional activity of NF-κB stimulated by IKKβ (Fig. 2E, left and right panels), indicating that Na$_3$VO$_4$ inhibits NF-κB signaling by targeting IKKβ. However, Na$_3$VO$_4$ did not block the kinase activity of IKKα/β in an in vitro kinase assay, indicating that

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**Fig. 2.** Effect of Na$_3$VO$_4$ on LPS-induced IKKα/β activation. (A) RAW264.7 cells treated with Na$_3$VO$_4$ (400 μM) for 30 min were cultured with LPS (1 μg/ml) for 30, 60, and 90 min. To measure the nuclear translocation levels of NF-κB subunits (p65 and p50), western blot analysis was performed with nuclear fractions. Lamin A/C was used as a loading control. (B, C, left panel and C, middle panel) RAW264.7 cells were pre-treated with 400 μM of Na$_3$VO$_4$ and stimulated with LPS (1 μg/ml) for the time indicated. The phosphorylated and total levels of IκBα (B) and IKKα/β (C, left and middle panels) were measured by western blot analysis with whole lysates. β-Actin was used as a loading control. (C, right panel) HEK293 cells were transfected with Flag-IKKα and/or Flag-IKKβ as indicated and then incubated with Na$_3$VO$_4$ (400 μM) for 24 h. The phosphorylation level of IKKα/β and total protein levels of Flag and β-actin were detected in whole lysates by western blotting. (D) A kinase assay was performed using immunoprecipitated IKKα and IκBα proteins prepared from LPS-stimulated RAW264.7 cells as enzyme and substrate sources, respectively. The phosphorylation level of IκBα was analyzed by western blot analysis. (E) HEK293 cells were transfected with NF-κB-Luc constructs and activated with the overexpression of MyD88-Flag (left panel), CFP-TRIF (right panel), and Flag-IKKβ as indicated. β-Gal plasmid was used for normalization. HEK293 cells were incubated with Na$_3$VO$_4$ (400 μM) for 24 h. Luciferase activity was measured by a luminometer and normalized using β-gal values. (F) An in vitro kinase assay was carried out with purified IKKα and IKKβ. The data presented in (A), (B), (C), and (D) are representative of three independent experiments. The graphs presented in (A), (B), (C), (D), (E) and (F) show the mean ± SD of three independent experiments. # and ## indicate p values less than 0.05 and 0.01, respectively, compared with the normal group. * and ** indicate p values less than 0.05 and 0.01, respectively, compared with the control group.
IKKα/β are not direct targets of Na₃VO₄ (Fig. 2F).

3.3. Na₃VO₄ suppresses IKKβ in an AKT1/2-dependent manner

We then investigated the regulatory mechanism of Na₃VO₄ against IKKα/β. To identify the target of Na₃VO₄ in IKKα/β regulation, the effect of Na₃VO₄ was examined under conditions in which IKKα/β were activated with various upstream regulatory enzymes, such as PDK1, AKT1, and AKT2 [10,35]. NF-κB-Luc activity, which was elevated by co-transfection of PDK1 and IKKα/β, was not decreased by Na₃VO₄ (Fig. 3A, left panel). In contrast, Na₃VO₄ significantly (p < 0.01) down-regulated NF-κB-Luc activity, which was induced by the co-transfection of IKKβ and AKT1/2 (Fig. 3A, middle and right panels). These results indicate that Na₃VO₄ may inhibit IKKβ activity by targeting AKT signaling. To explore this hypothesis, we examined whether Na₃VO₄ regulates AKT1/2. We found that phosphorylated levels of AKT1/2 at Ser 473 and Thr 308 were significantly (both p < 0.05 and p < 0.01) up-regulated by Na₃VO₄ treatment in LPS-treated RAW264.7 cells (Fig. 3B). However, phosphorylation level of mTOR, an enzyme to phosphorylate Thr 308 residue of AKTs, was not regulated by Na₃VO₄ (Fig. 3B). Na₃VO₄ suppressed the phosphorylation level of IκBα, which was induced by co-transfection of AKT2 and IKKβ (Fig. 3C). Furthermore, Na₃VO₄ inhibited IKKβ activity, which was induced by AKT2 overexpression, according to a kinase assay (Fig. 3D). These results imply that Na₃VO₄ suppresses IKKβ activation through AKT dependent regulation. However, in the in vitro kinase assay, PDK1 and AKT activities were not affected by Na₃VO₄ (Fig. 3E), suggesting that Na₃VO₄ does not directly modulate AKT1/2. These results support that the phosphorylation of AKT induced by Na₃VO₄ was not regulated by PDK1 and mTOR.

3.4. Na₃VO₄ suppresses AKT-induced IKKβ activation via modulation of autophosphorylation

IKKβ is autophosphorylated at a number of serine residues between the HLH motif and the C-terminus, and autophosphorylation at these sites has been reported to negatively modulate the kinase activity of IKKβ [36]. Indeed, we confirmed that IKKβ, not IKKα, is autophosphorylated by overexpression of IKKβ, and the autophosphorylated level was increased by Na₃VO₄ treatment (Fig. 2C, right panel). Thus, we hypothesized that autophosphorylation may be involved in the mechanism by which Na₃VO₄ regulates IKKβ activity. To prove this hypothesis, we constructed a substitution mutant in which the 10 serine residues in the HLH motif and the C-terminus, and autophosphorylation at these sites has been reported to negatively modulate the kinase activity of IKKβ [36]. Indeed, we confirmed that IKKβ, not IKKα, is autophosphorylated by overexpression of IKKβ, and the autophosphorylated level was increased by Na₃VO₄ treatment (Fig. 2C, right panel). These results suggest that Na₃VO₄ targets autophosphorylation in the AKT-IKKβ signaling regulatory mechanism.

3.5. Na₃VO₄ inhibits the activity of PP2A and PP1

PP1α and PP2A serine/threonine phosphatases dephosphorylate AKT [37-39]. LPS promoted the transcription of PP2A and PP1α, the catalytic subunits of PP2A and PP1α, after 30 min (Fig. 5A), suggesting that these phosphatases are involved in the inflammatory reaction induced by LPS. Therefore, we evaluated the effect of Na₃VO₄ on these enzymes. In CETSA, Na₃VO₄ significantly (p < 0.01) increased the stability of PP1α at high temperatures (59–65 °C) compared with the vehicle treatment, indicating an interaction between Na₃VO₄ and PP1α.
Fig. 3. Effect of Na<sub>3</sub>VO<sub>4</sub> on AKT-induced IKKβ activation. (A) HEK293 cells were transfected with NF-κB-Luc plasmid and activated with overexpression of PDK1 (A, left panel), AKT1-HA (A, middle panel), and AKT2-HA (A, right panel), Flag-IKKα, or Flag-IKKβ. β-Gal plasmid was used for normalization. HEK293 cells were incubated with Na<sub>3</sub>VO<sub>4</sub> (400 μM) for 24 h. Luciferase activity was quantified using a luminometer and normalized using β-gal values. The data are shown as the mean ± SD, n = 6. (B) RAW264.7 cells were pre-treated with 400 μM of Na<sub>3</sub>VO<sub>4</sub> and stimulated with LPS (1 μg/ml) for the time indicated. The phosphorylated and total levels of AKT1 and AKT2 were measured in whole lysates by western blot analysis. β-Actin was used as a loading control. (C) HEK293 cells overexpressing AKT2-HA, Flag-IKKα, and Flag-IKKβ were incubated with Na<sub>3</sub>VO<sub>4</sub> (400 μM) for 24 h. The phosphorylation level of IκBα and the expression of HA, total IκBα, and β-actin were detected in whole lysates by western blotting. (D) Kinase assay was performed using immunoprecipitated Flag-IKKβ and IκBα proteins from AKT2-transfected HEK293 cells as enzyme and substrate sources, respectively. (E) In vitro kinase assay was performed with purified PDK1 and AKT in the absence or presence of Na<sub>3</sub>VO<sub>4</sub> (400 μM). The data presented in (B), (C), and (D) are representative of three independent experiments. The graphs presented in (A), (B), (C), (D), and (E) show the mean ± SD of three independent experiments. * and ** indicate p values less than 0.05 and 0.01, respectively, compared with the normal group. # and ## indicate p values less than 0.05 and 0.01, respectively, compared with the control group.
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4. Discussion

The inflammatory response is activated by intracellular signaling that is induced by external stimuli. The cellular signaling pathway is generally regulated through phosphorylation and dephosphorylation of key factors [40]. Since kinases and phosphatases are responsible for these phosphorylation processes, kinases and kinase inhibitors have been the focus of multiple studies. In contrast, there has been a lack of interest in phosphatases because they are considered constitutively active enzymes with little regulation [41]. However, several studies have refuted this concept by showing that phosphatases are involved in regulatory mechanisms [42,43]. Indeed, in our study, the transcription of PP2A and PP1 increased time-dependently in response to LPS (Fig. 5A). We also found that PP2A was up-regulated by LPS (data not shown). These results imply that activation of phosphatase fluctuates under LPS stimulation and eventually can induce an inflammatory response through control of the signaling pathway. In this study, the anti-inflammatory action of Na3VO4, a phosphatase inhibitor, was examined using LPS-treated RAW264.7 cells, and its mechanism was investigated.

In Na3VO4-treated RAW264.7 cells, the activity of IKKα/β was decreased but the phosphorylated level was elevated (Fig. 2C–E). In general, phosphorylation positively regulates IKKα/β activity, but autophosphorylation of IKKβ at a serine-rich region located in carboxy-terminal can negatively regulate IKKβ activity [36]. In this study, we confirmed by an IKKβ-M10 construct that autophosphorylation of IKKβ is required for Na3VO4-derived anti-inflammatory activity. In some kinases, autophosphorylation is involved in negative feedback mechanisms. For example, c-Src is inactivated by autophosphorylation at tyrosine 527 [44]. In addition, autophosphorylation of calmodulin-dependent protein kinase IV (CaMKIV) at serine 332 is associated with negative feedback [45]. We speculate that AKT, which is up-regulated by Na3VO4, induces autophosphorylation of IKKβ, ultimately reducing the activity of IKKβ through a negative feedback loop.

The activities of AKT, IKKα, and IKKβ were not influenced by Na3VO4 in the in vitro kinase assay, suggesting that Na3VO4 does not directly target these kinases (Fig. 2F and 3E). Instead, Na3VO4 directly blocked the phosphatase activity of PP1α and PP2A by 95% in an in vitro phosphatase assay, suggesting that these phosphatases are targets of Na3VO4 (Fig. 5C). Finally, phosphatase inhibition by knockdown using PP1α- or PP2A-specific siRNA (siPP1α and siPP2A) also abolished the effect of Na3VO4 on the phosphorylation of IkBα in LPS-stimulated RAW264.7 cells (Fig. 5D). These results suggest that PP1α and PP2A are the specific targets of Na3VO4 in macrophages during inflammatory responses.

5. Conclusion

In this study, we showed that Na3VO4 selectively inhibits AKT1/2-activated IKKβ by directly blocking the phosphatase activity of PP1 and PP2A. Na3VO4 downregulated NF-κB-mediated signaling and expression levels of pro-inflammatory genes (Fig. 6). Our results suggest that blocking phosphatases such as PP1 and PP2A using vanadium compounds such as Na3VO4 may be an effective therapeutic approach for infectious and inflammatory diseases.
CRediT authorship contribution statement

Hang Gyung Kim, Seong-Gu Jeong, Ji Hye Kim, and Jae Youl Cho planned the experiments. Han Gyung Kim and Seong-Gu Jeong carried out the laboratory assays. Ji Hye Kim and Jae Youl Cho analyzed the data and wrote the manuscript. Jae Youl Cho reviewed and revised the manuscript. The manuscript has been read and approved by all authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial
interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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