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Abstract: Diospyrin, plant-derived bisnaphthoquinonoid, is known to have anticancer activity. However, pharmacological activity of diospyrin on viral infection is not well known. We investigated effects of diospyrin on macrophages induced by polyinosinic-polycytidylic acid (poly I:C), a mimic of double-stranded viral RNA. Various cytokines, intracellular calcium, nitric oxide (NO), phosphorylated p38 MAPK, and phosphorylated ERK1/2 as well as mRNA expressions of transcription factors were evaluated. Diospyrin significantly reduced NO production, granulocyte-macrophage colony-stimulating factor production, and intracellular calcium release in poly I:C-induced RAW 264.7. The phosphorylation of p38 MAPK and ERK1/2 was also significantly suppressed. Additionally, diospyrin inhibited mRNA levels of nitric oxide synthase 2, C/EBP homologous protein (CHOP), calcium/calmodulin dependent protein kinase II alpha, signal transducers and activators of transcription 1 (STAT1), STAT3, STAT4, Janus kinase 2, first apoptosis signal receptor, c-Jun, and c-Fos in poly I:C-induced RAW 264.7. Taken together, this study represents that diospyrin might have the inhibitory activity against viral inflammation such as excessive production of inflammatory mediators in poly I:C-induced RAW 264.7 via ER stress-induced calcium-CHOP pathway.

**Keywords:** diospyrin; poly I:C; viral infection; p38 MAPK; ERK1/2; CHOP; STAT; ER stress; calcium; cytokine

1. Introduction

Innate immune activity is thought to be essential for overcoming infectious diseases [1]. Gilroy reported that immune reaction and inflammatory activity are important against infection [2]. Recently, Nahrendorf and Swirski reported that when stimuli persist and inflammation is not resolved, monocytes might cause a chronic inflammation [3]. Then, it is reasonable that the regulation of inflammatory responses is becoming more meaningful [4].

Macrophages are important in immunity. Zong et al. reported that various inflammatory diseases are related with a complex reaction generated by macrophages [5]. In inflammatory processes, macrophages and monocytes not only produce cytokines and nitric oxide (NO) but also release intracellular calcium.

Cho et al. reported that viral infection can activate immune responses and trigger inflammatory diseases [6]. Alexopoulou et al. reported that double-stranded RNA (dsRNA) induces macrophages [7].

Diospyrin (Figure 1), the plant-derived bisnaphthoquinonoid, is known to have anti-leishmanial effect [8]. In the previous report, we already reported the anti-inflammatory activity of diospyrin on RAW 264.7 stimulated by lipopolysaccharide (LPS) [9]. However, the activity of diospyrin on viral inflammation is not yet reported.
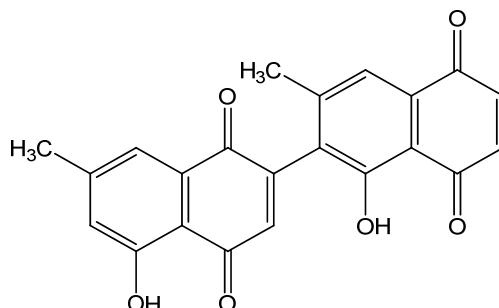
Figure 1. Chemical structure of diospyrin.

In this experiment, we investigated inhibitory effects of diospyrin on RAW 264.7 induced by polyinosinic-polycytidylic acid (poly I:C), a mimic of double-stranded viral RNA. Diospyrin significantly reduced productions of GM-CSF and NO as well as calcium release in RAW 264.7 induced with poly I:C. The phosphorylation of p38 MAPK and ERK1/2 was also significantly suppressed. The mRNA expressions of nitric oxide synthase 2 (NOS2), C/EBP homologous protein (CHOP), calcium/calmodulin dependent protein kinase II alpha (Camk2a), signal transducers and activators of transcription 1 (STAT1), STAT3, STAT4, Janus kinase 2 (Jak2), first apoptosis signal receptor (Fas), c-Jun, and c-Fos were reduced. Data means diospyrin might have the inhibitory activity against viral inflammation such as excessive production of inflammatory mediators in dsRNA-stimulated macrophages via ER stress-induced calcium-CHOP pathway.

2. Materials and Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), other cell culture reagents, and MILLIPLEX MAP Mouse Cytokine/Chemokine assay kits were obtained from Millipore (Billerica, MA, USA). Indomethacin, baicalein, chrysin, and poly I:C were obtained from Sigma-Aldrich (St. Louis, MO, USA). Diospyrin was isolated from *Diospyros lotus* by Dr. Inamullah Khan. Phospho-ERK1/2 Antibody (Thr202, Tyr204) (eBioscience 12-9109-42), Phospho-p38 MAPK Antibody (T180/Y182) (eBioscience 17-9078-42), Mouse IgG1 kappa Isotype Control (eBioscience 12-4714-81), and Mouse IgG2b kappa Isotype Control (eBioscience 12-4732-81) were from Life Technologies Corporation (Carlsbad, CA, USA).

2.2. Cell Culture

RAW 264.7 was purchased from Korea Cell Line Bank (Seoul, Korea). In the previous study [9], we reported that diospyrin did not show any cytotoxicity up to a concentration of 10 μM, which was chosen for subsequent experiments.

2.3. NO Production

RAW 264.7 was seeded in a 96-well plate (1×10^4 cells/well) and treated with diospyrin and poly I:C for 24 h. After treatment, supernatants were collected, and NO content was measured using the modified Griess reagent assay kit (Millipore) [9,10].

2.4. Calcium Release

RAW 264.7 was seeded in a 96-well plate (1×10^5 cells/well) and treated with diospyrin and poly I:C for 18 h. Then, calcium release was measured using Fluo-4 NW Calcium Assay Kits (Thermo Fisher Scientific, Waltham, MA, USA) by a spectrofluorometer (Dynex, West Sussex, UK) at 485 nm (excitation) and 535 nm (emission) [11–13]

2.5. Cytokines Production

RAW 264.7 was seeded in a 96-well plate (1×10^4 cells/well) and treated with diospyrin and poly I:C. After 24 h treatment, the production of cytokines was measured using MILLIPLEX MAP Mouse Cytokine/Chemokine assay kits and Bio-Plex 200 suspension array system (Bio-Rad) [9–13].

2.6. Quantitative Polymerase Chain Reaction

RAW 264.7 was treated with diospyrin and poly I:C for 18 h in a six-well plate (1×10^6 cells/well). After 18 h treatment, RNA quantity of each well was measured using NucleoSpin RNA kit (Macherey-Nagel, Duren, Germany) and Experion RNA StdSens Analysis kit (Bio-Rad) with Experion Automatic Electrophoresis System (Bio-Rad). Each cDNA was produced using iScript cDNA Synthesis kit (Bio-Rad). Then, gene expressions were measured using quantitative polymerase chain reaction with iQ SYBR Green Supermix (Bio-Rad) [14,15]. The target genes are listed in Table 1. The *β-actin* was used as a housekeeping gene.

Table 1. Primers used for quantitative PCR.

Name [1]	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
NOS2	TGGAGGTTCTGGATGAGAGC	AATGTCCAGGAAGTAGGTGAGG
CHOP	CCACCACACCTGAAAGCAG	TCCTCATACCAGGCTTCCA
Camk2a	AGCCATCCTCACCACTAT	ATTCCTTCACGCCATCATT
STAT1	TGAGATGTCCCGGATAGTGG	CGCCAGAGAGAAATTCGTGT
STAT3	GTCTGCAGAGT TCAAGCACCT	TCCTCAGTCACGATCAAGGAG
STAT4	TTCAGAGCAGCTCAACATGC	GGTGAGGTGACCATCATTGTAG-3
Jak2	TTGGTTTTGAATTATGGTGTCTGT	TCCAAATTTTACAAATTCTTGAACC
Fas	CGCTGTTTTCCCTTGCTG	CCTTGAGTATGAACTCTTAACTGTGAG
c-Jun	ACTGGGTTGCGACCTGAC	CAATAGGCCGCTGCTCTC
c-Fos	AGAGCGGGAATGGTGAAGA	TCTTCCTCTTCAGGAGATAGCTG
β-Actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA

[1.] Primer names; nitric oxide synthase 2 (NOS2), C/EBP homologous protein (CHOP), calcium/calmodulin dependent protein kinase II alpha (Camk2a), signal transducers and activators of transcription 1 (STAT1), STAT3, STAT4, Janus kinase 2 (Jak2), first apoptosis signal receptor (Fas), c-Jun, c-Fos, and β-Actin.

2.7. Flow Cytometry Assay

RAW 264.7 was seeded in a six-well plate (1×10^6 cells/well) and treated with diospyrin and poly I:C for 15 min. After incubation, cells were harvested and washed with Flow Cytometry Staining Buffer. Prior to antibody staining, cells were fixed and permeabilized using Fix Buffer I and Perm Buffer III, respectively. RAW 264.7 stained with Phospho-ERK1/2 (Thr202, Tyr204) Antibody (eBioscience 12-9109-42) and phospho-p38 MAPK (T180/Y182) Antibody (eBioscience 17-9078-42) were analyzed on the Attune NxT flow cytometer (Thermo Fisher Scientific) using Attune NxT software.

2.8. Statistics

Data are presented as mean ± SD. All data were analyzed by one-way analysis of variance test followed by Tukey's multiple comparison test using GraphPad Prism (ver. 4; GraphPad Software, San Diego, CA, USA).

3. Results

3.1. NO Production from RAW 264.7

NO production from RAW 264.7 after 24 h treatment of diospyrin at concentrations of 1, 5, and 10 μM were 92.02 ± 6.15%, 93.52 ± 4.44%, and 93.27 ± 4.99% of media group (Figure 2A). In the case of poly I:C stimulation, diospyrin at concentrations of 1, 5, and 10 μM showed 88.58 ± 3.24%, 48.4 ± 8.08%, and 45.48 ± 3.95% of the poly I:C alone ($p < 0.001$) (Figure 2B).
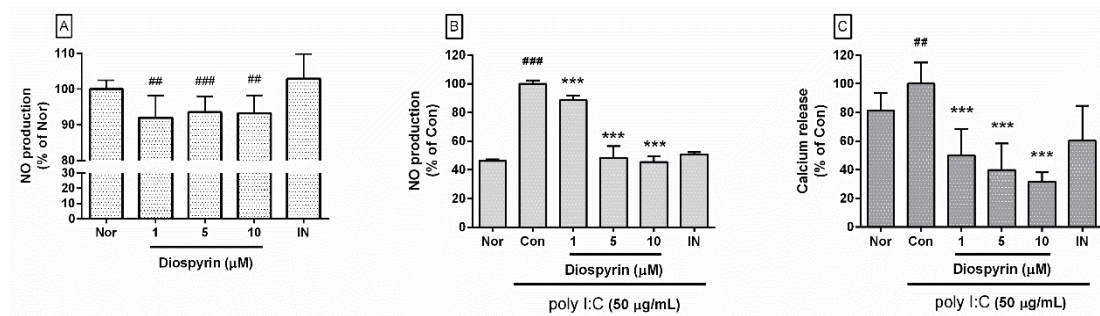
Figure 2. Effect of diospyrin on production of nitric oxide (**A**,**B**) and calcium release (**C**). Values are mean ± SD of the three independent experiments. Nor, normal group (media only); Con, control group (poly I:C alone); IN, indomethacin (0.5 μM). [##] $p < 0.01$ vs. Nor; [###] $p < 0.001$ vs. Nor; [***] $p < 0.001$ vs. Con.

3.2. Calcium Release in RAW 264.7

Calcium release with diospyrin at concentrations of 1, 5, and 10 μM were 50.07 ± 18.52%, 39.82 ± 18.39%, and 31.61 ± 6.69% of the poly I:C alone ($p < 0.001$) (Figure 2C).

### 3.3. Cytokine Production from RAW 264.7

Diospyrin significantly reduced GM-CSF production in RAW 264.7 stimulated by poly I:C. Concretely, GM-CSF production from RAW 264.7 with diospyrin at concentrations of 0.1, 1, and 5 μM were 49.46 ± 6.26%, 38.45 ± 6.45%, and 5.37 ± 0.58% of the poly I:C alone; MCP-1 were 70.66 ± 4.79%, 55.11 ± 9.17%, and 89.39 ± 8.72%; IL-6 were 99.15 ± 1.98%, 96.72 ± 1.98%, and 68.5 ± 5.13%; LIF were 86.75 ± 6.77%, 85.79 ± 6.03%, and 73.29 ± 2.74%; VEGF were 97.94 ± 11.18%, 96.66 ± 7.7%, and 58.73 ± 0.48%; IL-15 were 80 ± 6.44%, 74.09 ± 5.51%, and 60.91 ± 1.56%; Eotaxin were 84.62 ± 9.24%, 84.16 ± 11.11%, and 85.97 ± 7.84%; IP-10 were 84.62 ± 9.24%, 84.16 ± 11.11%, and 85.97 ± 7.84% (Figure 3).

3.4. mRNA Expression of NOS2, CHOP, Camk2a, STAT1, STAT3, STAT4, Jak2, Fas, c-Jun, and c-Fos

Diospyrin inhibited expressions of *NOS2, CHOP, Camk2a, STAT1, STAT3, STAT4, Jak2, Fas, c-Jun*, and *c-Fos* in RAW 264.7 stimulated by poly I:C ($p < 0.05$) (Figure 4). Concretely, *NOS2* expression in RAW 264.7 treated with diospyrin at concentrations of 1, 5, and 10 μM were 18.9 ± 1.33%, 0.05 ± 0.01%, and 38.72 ± 4.72% of the poly I:C alone; *CHOP* were 31.05 ± 2.64%, 17.47 ± 1.87%, and 21.43 ± 1.69%; *Camk2a* were 7.64 ± 2.28%, 8.93 ± 0.43%, and 9.44 ± 0.69%; *STAT1* were 13.58 ± 4.69%, 24.54 ± 8.59%, and 21.68 ± 2.62%; *STAT3* were 12.01 ± 1.36%, 22.9 ± 7.41%, and 16.37 ± 6.56%; *STAT4* were 8.89 ± 1.35%, 2.31 ± 0.82%, and 37.79 ± 11.83%; *Jak2* were 11.97 ± 3.03%, 15.41 ± 3.99%, and 27.1 ± 7.84%; *Fas* were 14.39 ± 5.29%, 10.98 ± 1.26%, and 21.98 ± 4.75%; *c-Jun* were 13.99 ± 4.17%, 16.82 ± 11.55%, and 17.71 ± 5.93%; *c-Fos* were 5.04 ± 1.94%, 14.15 ± 2%, and 26.54 ± 2.44%.
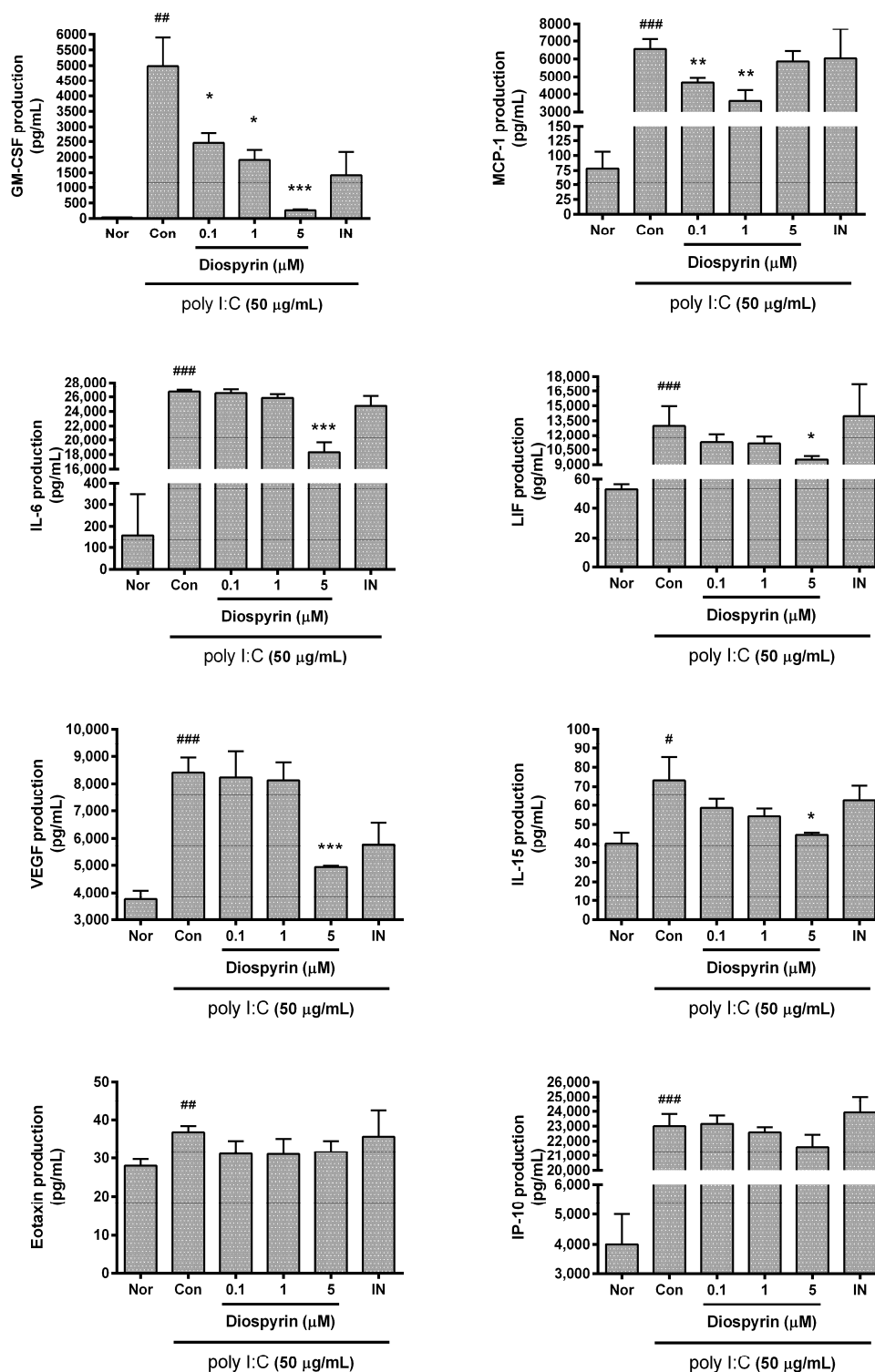
Figure 3. Effect of diospyrin on cytokine production from RAW 264.7. Values are the mean ± SD of the three independent experiments. Nor, media alone; IN, indomethacin (0.5 μM). [#] $p < 0.05$ vs. Nor; [##] $p < 0.01$ vs. Nor; [###] $p < 0.001$ vs. Nor; * $p < 0.05$ vs. Con; ** $p < 0.01$ vs. Con; *** $p < 0.001$ vs. Con.
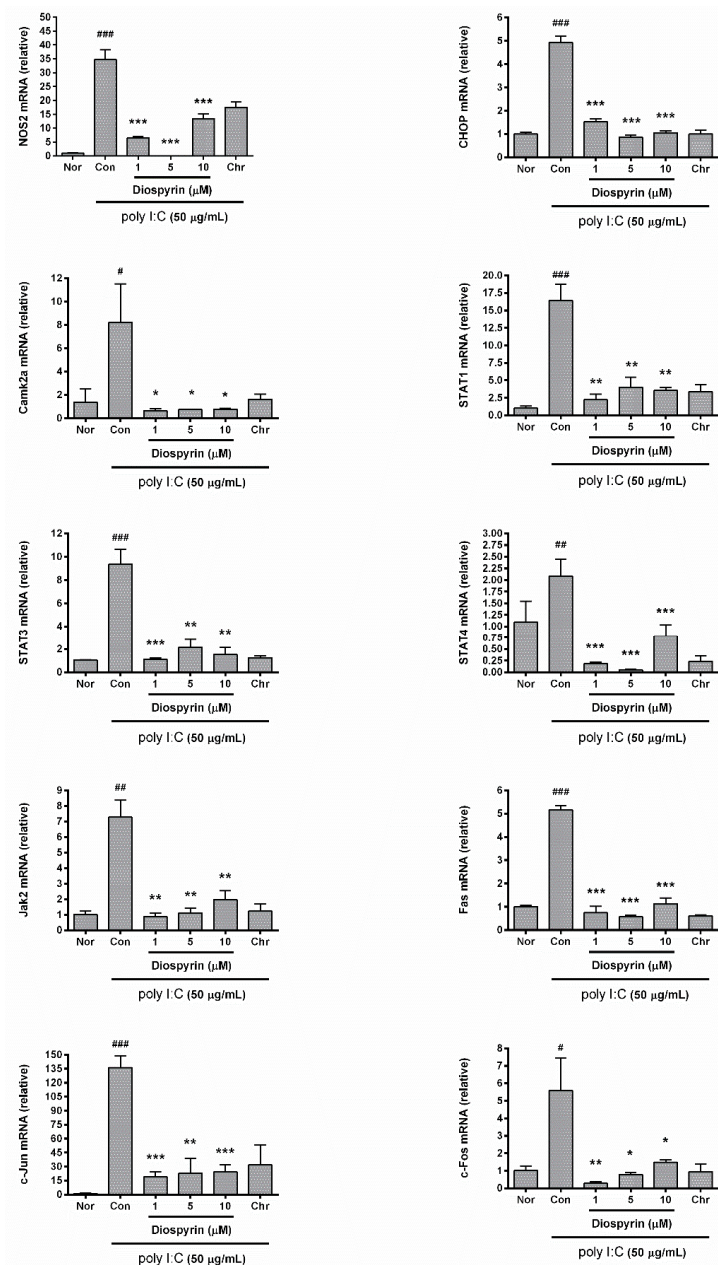
Figure 4. Expressions of *NOS2*, *CHOP*, *Camk2a*, *STAT1*, *STAT3*, *STAT4*, *Jak2*, *Fas*, *c-Jun,* and *c-Fos* mRNA in RAW 264.7. mRNA expressions were normalized to *β-actin*. Values are the mean ± SD of the three independent experiments. Nor, media alone; Chr, Chrysin (10 μM). [#] $p < 0.05$ vs. Nor; [##] $p < 0.01$ vs. Nor; [###] $p < 0.001$ vs. Nor; * $p < 0.05$ vs. Con; ** $p < 0.01$ vs. Con; *** $p < 0.001$ vs. Con.

3.5. Phosphorylation of p38 MAPK and ERK1/2 in RAW 264.7

The phosphorylation of p38 MAPK and ERK 1/2 was inhibited by diospyrin significantly (Figure 5). To be concrete, p38 MAPK phosphorylation in RAW 264.7 with diospyrin at concentrations of 1, 5, and 10 μM were 91.38 ± 1.64%, 48.69 ± 1.58%, and 41.73 ± 0.46% of the poly I:C alone; ERK 1/2 were 91.23 ± 3.59%, 53.79 ± 1.74%, and 31.64 ± 0.34%.
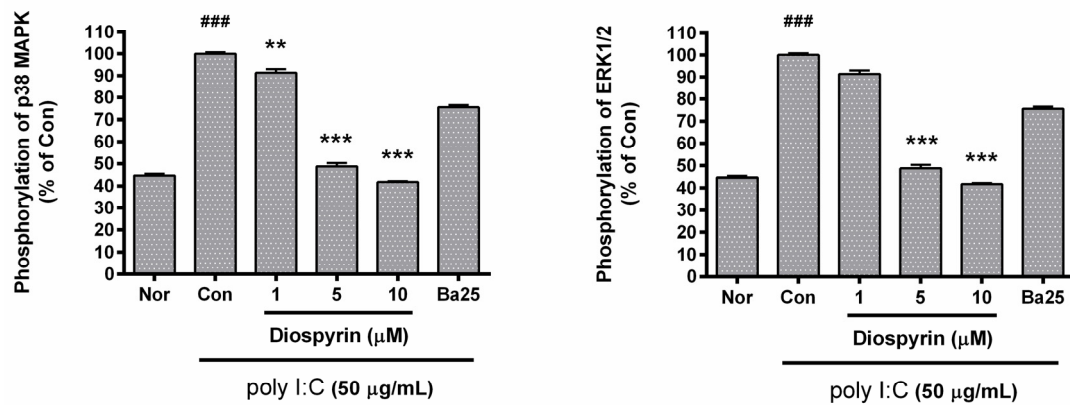
Figure 5. The phosphorylation of p38 MAPK and ERK 1/2 in RAW 264.7. Values are the mean ± SD of the three independent experiments. Nor, media alone; Ba25, Baicalein (25 μM). [###] $p < 0.001$ vs. Nor. [**] $p < 0.01$ vs. Con; [***] $p < 0.001$ vs. Con.

4. Discussion

Diospyrin, the medicinal plant (i.e., species of Diospyros such as *Diospyros lotus* and *Diospyros montana*)-derived bisnaphthoquinonoid, is known to suppress cancer [16], tuberculosis [17], and leishmaniasis [18,19]. In detail, Bailly reported in 2000 that diospyrin exerts the activity of a topoisomerase I suppressor, resulting in anti-tumor effect [16]. Lall et al. reported in 2005 that diospyrin exerted the inhibitory activity against *Mycobacterium tuberculosis* [20]. In 2013, Hazra et al. reported that diospyrin represented the anti-leishmanial activity [19]. But effects of diospyrin on viral inflammation have been rarely reported so far.

Although the innate immunity is essential for overcoming hazardous infections, the uncontrolled immuno-inflammatory reaction such as cytokine storm also might be fatal for human life. Thus, the resolution of excessive inflammation is being required continuously because there is no pertinent therapeutics against the newly emerged pandemic viral infection (such as Coronavirus Disease-19), which sometimes provokes cytokine storm.

Karpuzoglu and Ahmed reported that excessive NO production might be concerned with inflammatory diseases such as inflammatory autoimmune diseases [21]. Moreover, it is well reported that excessive production of NO contributes to septic shock [22]. Cooper et al. reported that viral infections might cause airway inflammation and increase the production of various cytokines excessively [23]. Interestingly, respiratory tract viral infections such as Influenza A and Parainfluenza virus were reported to show higher levels of GM-CSF, IL-17A, and IL-22 [24]. In this year, Crisci et al. reported that cytokines such as GM-CSF, VEGF, MCP-1, IL-6, and LIF were increased in severe patients with COVID-19 [25]. It is reasonable that excessive production of cytokines becomes a major target for relieving inflammatory illnesses [26]. In this study, we tried to find a material able to inhibit hyper-production of cytokines in macrophages induced with virus-like particles. During viral infection, dsRNA is produced in host cells and brings about viral inflammatory reaction in macrophages producing NO, ILs, TNF-α, and other cytokines [27]. Then, multiplex cytokine assay is regarded to be effective for checking anti-inflammatory activity of drug candidates [28–32]. For example, Kim et al. reported that baicalein showed inhibitory effects on productions of various cytokines in poly I:C–induced RAW 264.7 [14]. Meanwhile, Alexopoulou et al. reported in 2001 that dsRNA recognized by mammalian Toll-like receptor 3 might provoke inflammatory response via activation of p38 MAPK and ERK1/2 [7]. However, it is not yet fully reported for effects of diospyrin on viral inflammation.

In this experiment, bio-activity of diospyrin in poly I:C–induced RAW 264.7 was evaluated using multiplex cytokine assay, flow cytometry assay, etc. Experimental data means that diospyrin reduces the production of NO and GM-CSF as well as phosphorylation of p38 MAPK and ERK1/2 in poly I:C–induced RAW 264.7. As NO is related with septic shock, diospyrin could be tested for relieving cytokine storm with viral sepsis.

Recently, pandemic viral infection COVID-19 has produced a global threat. Interestingly, TNF-α-converting enzyme (TACE) was reported to be involved in the cell entry of SARS-CoV [33,34]. Xiao et al. reported that SARS-CoV-2 binds angiotensin-converting enzyme 2 (ACE2) for cell entry [35]. ACE2 is known to be shed by TACE and Palau et al. suggested that TACE inhibition may be important for protecting COVID-19 [36]. Additionally, Scott et al. reported in 2011 that TACE activity is upregulated in LPS-induced human monocytes through p38 activation [37]. In this study, the authors presented TACE inhibitory potential of diospyrin predicted by structure-based virtual screening in Figure 6. However, more studies are needed to ascertain whether diospyrin exerts a meaningful effect on TACE activity.
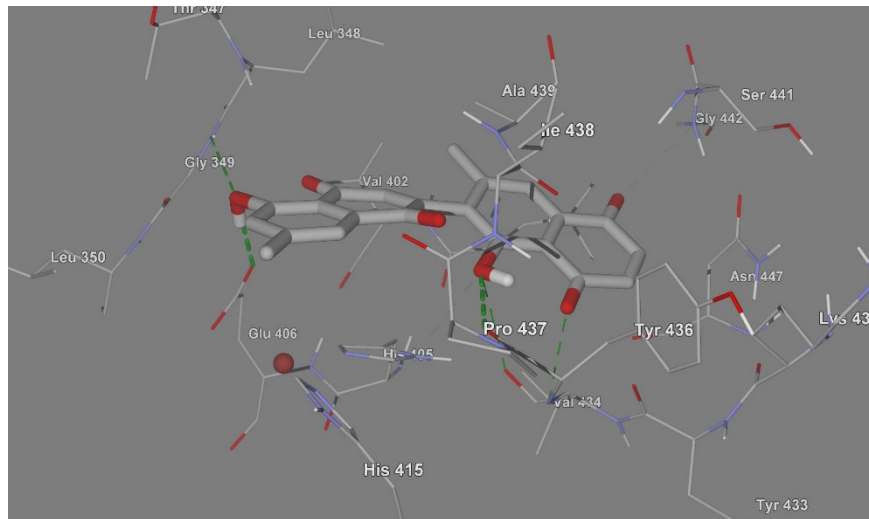
Figure 6. Predicted binding mode of lowest energy conformation of diospyrin inside the active site of TNF-α-converting enzyme (TACE).

With an infection of microorganisms, macrophages show a distinct reaction, called pyroptosis, which is different from both apoptosis and necrosis. In pyroptosis, various inflammatory mediators are produced from infected macrophages [38]. In 2016, Broz and Dixit also reported about pyroptotic cells to produce many kinds of cytokines [39]. In 2019, Goddard et al. reported that microbial infections can stimulate macrophages [40]. However, it is not yet fully reported for virus-induced pyroptosis accompanying massive production of inflammatory mediators with the calcium signaling pathway.

With ER stress, NO is well known to increase CHOP expression with releasing calcium from ER calcium store and leads to macrophage apoptosis [41–44]. In 2009, Timmins et al. suggested in their impressive report that ER stress-induced Camk2a activation might enable macrophage apoptosis through Fas induction and/or activation of STAT1 in macrophages [43]. In 2006, Endo et al. reported that LPS causes the overexpression of CHOP, which mediates apoptosis in macrophages and ER stress [45]. Interestingly, Stout et al. reported in 2007 that a brief activation of STAT1 induces ER stress and calcium release from ER calcium store in IFN-gamma-induced airway epithelial cell death with increase of STAT1 protein level [46]. In 2008, Lim et al. reported atheromata-related macrophage apoptosis might be provoked by ER stress through a rise in cytosolic calcium [47].

In the current study, poly I:C-induced RAW 264.7 represents propyrototic ER stress with enhancing mRNA expression of *NOS2, CHOP, Camk2a, STAT1, STAT3, STAT4, Jak2, Fas, c-Jun*, and *c-Fos*. Additionally, diospyrin modulates calcium release and mRNA expression of *NOS2, CHOP, Camk2a, STAT1, STAT3, STAT4, Jak2, Fas, c-Jun*, and *c-Fos* in poly I:C-induced RAW 264.7. This data means diospyrin could regulate macrophage activation in viral infection via ER stress-induced calcium-CHOP pathway.

However, this study could not elucidate whether intracellular calcium level is increased through influx of extracellular calcium or ER calcium store depletion in poly I:C-induced RAW 264.7. Further

study is needed for the exact mechanism for anti-viral activity of diospyrin and medicinal usefulness of diospyrin on viral inflammatory diseases.

5. Conclusions

Diospyrin inhibits excessive production of pro-inflammatory mediators such as GM-CSF and NO via ER stress-induced calcium-CHOP pathway in poly I:C-induced RAW 264.7. Further study is required to clarify pharmacological usefulness for virus-triggered hyper-inflammation such as cytokine storm syndromes from SARS-CoV-2 infection.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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