Full Paper

 α -Viniferin Suppresses the Signal Transducer and Activation of Transcription-1 (STAT-1)–Inducible Inflammatory Genes in Interferon- γ –Stimulated MacrophagesEun Yong Chung¹, Eunmiri Roh¹, Jeong-Ah Kwak¹, Heun-Sik Lee¹, Seung Ho Lee², Chong-Kil Lee¹, Sang-Bae Han¹, and Youngsoo Kim^{1,*}¹College of Pharmacy, Chungbuk National University, Cheongju 361-763, Korea²College of Pharmacy, Yeungnam University, Kyeongsan 712-749, Korea

Received September 4, 2009; Accepted February 8, 2010

Abstract. α -Viniferin, an oligostilbene of trimeric resveratrol, has been reported to have anti-inflammatory potential in carrageenin-induced paw edema or adjuvant-induced arthritis in animal models. However, little is known about the molecular basis. In this study, α -viniferin at 3 – 10 μ M dose-dependently inhibited interferon (IFN)- γ –induced Ser⁷²⁷ phosphorylation of the signal transducer and activation of transcription-1 (STAT-1), a pivotal transcription factor controlling IFN- γ –targeted genes, in RAW 264.7 macrophages, and also IFN- γ –induced activation of the extracellular signal-regulated kinase (ERK)-1, a protein kinase upstream of the Ser⁷²⁷ phosphorylation of STAT-1. However, α -viniferin, only at a higher concentration of 10 μ M, inhibited Janus kinase 2–mediated Tyr⁷⁰¹ phosphorylation of STAT-1 in the cells. To understand STAT-1–dependent inflammatory responses, we quantified nitric oxide (NO) or chemokines. α -Viniferin at 3 – 10 μ M dose-dependently inhibited IFN- γ –induced production of NO, IFN- γ –inducible protein-10 (IP-10), or the monokine induced by IFN- γ (MIG) in RAW 264.7 cells and also that of NO in primary macrophages-derived from C57BL/6 mice. Furthermore, α -viniferin diminished IFN- γ –induced protein levels of inducible NO synthase (iNOS), attenuated mRNA levels of iNOS, IP-10, or MIG as well as inhibited promoter activity of the iNOS gene. In conclusion, this study proposes an anti-inflammatory mechanism of α -viniferin, down-regulating STAT-1–inducible inflammatory genes via inhibiting ERK-mediated STAT-1 activation in IFN- γ –stimulated macrophages.

Keywords: α -viniferin, anti-inflammation, signal transducer and activation of transcription-1 (STAT-1) activation, interferon (IFN)- γ , macrophage

Introduction

Interferon (IFN)- γ , produced mainly from Th1 and NK cells, is a pleiotropic cytokine associated with antiviral activity and immune surveillance (1). Biological responses to IFN- γ are predominantly mediated by the signal transducer and activator of transcription-1 (STAT-1)–dependent regulation of gene expression (2, 3). Physiological relevance of IFN- γ –induced STAT-1 signaling has been further established by targeted gene disruption,

in which STAT-1–null mice develop normally, but lack many biological responses to IFN- γ , causing extreme sensitiveness to microbial and viral infection (4, 5).

Upon binding to ligand, the IFN- γ receptors are oligomerized and the receptor-associated Janus kinase (JAK)-1 and -2 are activated via auto- or trans-phosphorylation (6, 7). These activated JAKs phosphorylate the intracellular domain of IFN- γ receptor, which serves as docking site of STAT-1 or other signal molecules, also leading to phosphorylation of STAT-1 at the Tyr-701 residue (7, 8). The Tyr⁷⁰¹–phosphorylated STAT-1 is released from the IFN- γ receptor, undergoing dimerization through Src-homology-2 (SH-2) domains and then translocates into the nucleus, where it specifically binds to the

*Corresponding author. youngsoo@chungbuk.ac.kr

Published online in J-STAGE

doi: 10.1254/jphs.09247FP

IFN- γ -activated sequence (GAS) motif on promoter regions (2, 9). At some early phase after IFN- γ stimulation, STAT-1 is also phosphorylated at the Ser-727 residue, important for its transcriptional activity and IFN- γ -mediated antiviral action without impact on the dimerization and nuclear import of STAT-1 (10 – 12). Nuclear STAT-1 collaborates with other transcription factors, such as the mini-chromosome maintenance protein 5 (MCM5) and the breast cancer susceptibility gene 1 (BRCA1) interacting with the Ser⁷²⁷-phosphorylated STAT-1, for optimization of its transcriptional activity (13, 14).

STAT-1-regulated gene transcription has been evidenced to mediate many, but not all, of the immune and inflammatory actions of IFN- γ (3, 15). IFN regulatory factor (IRF)-1 is another transcription factor, rapidly inducible upon IFN- γ stimulation, and its expression is dependent upon STAT-1 transcriptional activity (16). Nuclear IRF-1 binds to the IFN-stimulated regulatory element (ISRE), participating in the transcriptional control of IFN- γ -regulated secondary genes (17). Thereby, IFN- γ -regulated gene transcription occurs through the STAT-1-responsive GAS and/or IRF-1-responsive ISRE motifs on promoter regions of target genes, including inducible nitric oxide (NO) synthase (iNOS), IFN- γ -inducible protein-10 (IP-10), and the monokine induced by IFN- γ (MIG) (18 – 20).

α -Viniferin (Fig. 1A) is an oligostilbene of trimeric resveratrol and was firstly isolated as the anti-inflammatory constituent of *Caragana chamlagu* (21), an herbal medicine used for anti-arthritis purpose in Korea. We also isolated α -viniferin as an inhibitor of prostaglandin H₂ synthase from another plant, *Carex humilis* (22), and demonstrated its anti-inflammatory efficacy on carrageenin-induced paw edema in mice (23). Others have reported that α -viniferin decreased paw edema and bone damage in adjuvant-induced arthritis in rats (24) and also affected keratinocyte functions, being useful for ameliorating skin inflammation (25). However, little is known about the molecular basis for these pharmacological properties. In the present study, we demonstrated that α -viniferin inhibited IFN- γ -induced STAT-1 activation in macrophages, preferentially affecting Ser⁷²⁷ phosphorylation, which contributed to its down-regulatory mechanism on STAT-1-dependent expression of inflammatory genes.

Material and Methods

Materials

α -Viniferin (>97% purity) was isolated from *Carex humilis*, a plant of the Cyperaceae family, as described previously (22). IFN- γ and the mitogen-activated protein

kinase (MAPK) inhibitors of 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene (U0126), 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059), and 1,9-pyrazoloanthrone (SP600125), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against JAK-1, JAK-2, IRF-1, pY⁷⁰¹-STAT-1, and pS⁷²⁷-STAT-1 were purchased from Cell Signaling Tech (Danvers, MA, USA); those against iNOS, STAT-1, extracellular signal-regulated kinase (ERK)-1/2, and glyceraldehydes 3-phosphate dehydrogenase (GAPDH), from Santa Cruz Biotech (Santa Cruz, CA, USA); and those against p-ERK-1 or phosphotyrosine (4G10), from Upstate Tech (Charlottesville, VA, USA). STAT-1-dependent reporter luciferase (Luc) plasmid of pGAS-Luc was purchased from BD Biosciences Clontech (Palo Alto, CA, USA). Promoter-dependent reporter plasmid of piNOS-Luc has been previously described (18).

Macrophage culture

RAW 264.7 macrophages were purchased from American Type Culture Collection (Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's media supplemented with heat-inactivated 10% FBS, benzylpenicillin potassium (143 U/ml) and streptomycin sulfate (100 μ g/ml) at 37°C and under a 5% CO₂ atmosphere. C57BL/6 mice were obtained from Korea Research Institute of Bioscience Biotechnology (KRIBB) (Ochang, Korea). Macrophages were derived from bone marrows of the male mice (5 – 6 weeks of age). Briefly, bone marrow cells were flushed out from femurs and tibias. After removing red blood cells, whole bone marrow cells (2×10^6 cells/ml) were cultured in complete media containing macrophage-colony stimulating factor (M-CSF, 10 ng/ml). On culture day 3, the media were replaced with fresh complete media containing M-CSF (10 ng/ml), and on day 6, half of the media was refreshed in the presence of M-CSF (10 ng/ml). On day 8, adherent cells were harvested and then used as primary macrophages. This animal experiment protocol was approved by the Ethics Committee of our institute.

Western blot analysis

The cells were pretreated with α -viniferin for 2 h and stimulated with IFN- γ (3 ng/ml) for the indicated times, in the presence of α -viniferin. Cell extracts were resolved on SDS-acrylamide gels by electrophoresis and then transferred to a polyvinylidene difluoride membrane. Either 5% non-fat milk in phosphate-buffered saline containing Tween 20% or 5% bovine serum albumin in Tris-buffered saline containing Tween 20 was used as the blocking buffer. The blots were usually incubated at

4°C overnight with primary antibody and then further incubated at room temperature for 2–5 h with appropriate horseradish peroxidase-labeled secondary antibody. Immune complexes on the blots were finally visualized by exposure to X-ray film after reacting with an enhanced chemiluminescence reagent (GE Healthcare, Chalfont, UK). Positive signals were quantified by Kodak 1D 3.6 image analysis software (Eastman Kodak Co., New Haven, CT, USA).

Immunoprecipitation

The cells were pretreated with α -viniferin for 2 h and stimulated with IFN- γ (3 ng/ml) for 10 min. Cell extracts (500 μ g) were incubated with anti-JAK-1 or anti-JAK-2 antibody (1–2 μ g) at 4°C overnight and then precipitated with protein G-agarose beads (GE Healthcare).

Nitrite quantification

The cells were stimulated with IFN- γ (3 ng/ml) for 24 h, in the presence of α -viniferin, and then quantified for nitrite, a stable metabolite of NO, as described previously (26). Aliquots of the culture media were mixed with an equal volume of Griess reagent [0.1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in 5% phosphoric acid], and absorbance values were then measured at 540 nm with sodium nitrite as a standard.

Enzyme-linked immunosorbent assay (ELISA)

The cells were stimulated with IFN- γ (3 ng/ml) for 24 h in the presence of α -viniferin. Amounts of IP-10 or MIG in the culture media were determined using appropriate ELISA kits (R&D Systems, Minneapolis, MN, USA).

Cell viability assay

The cells were incubated with various concentrations of α -viniferin for 24 h in the presence of IFN- γ (3 ng/ml). They were exposed to a water-soluble WST-1 of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (Dojindo Lab., Kumamoto) for 3 h, and absorbance values were then measured at 450 nm.

Reverse transcription-polymerase chain reaction (RT-PCR)

The cells were pretreated with α -viniferin for 2 h and stimulated with IFN- γ (3 ng/ml) for 4–6 h in the presence of α -viniferin. Total RNA of the cells was subjected to semi-quantitative RT-PCR using an RNA PCR kit (Solgent Co., Daejeon, Korea). Briefly, total RNA (1 μ g) was reversely transcribed at 42°C for 1 h and then subjected to 25–30 cycles of PCR (GenePro model, Hang Bioer Technology Co., Hangzhou, China), consisting of

30-s denaturation at 94°C, 30-s annealing at 50°C–60°C, and 90-s extension at 72°C. The sequences of PCR primers and the sizes of PCR products are as follows: iNOS (457 bp): sense, 5'-GTCAACTGCAAGAGAACGGA GAAC-3' and antisense, 5'-GAGCTCCTCCAGAGGG TAGGCT-3'; IP-10 (430 bp): sense, 5'-ACTCACT CAGTTTGTGAGTCATTC-3' and antisense, 5'-TTT GATTAGTACTGTAGGGTTAATG-3'; MIG (421 bp): sense, 5'-CTTCAGCCCCAGCAGTGTATTCTTT-3' and antisense, 5'-AGAGAACCTGGGAGTAGACAA GGTA-3'; IRF-1 (470 bp): sense, 5'-ACATAACTC CAGCACTGTCACCGTG-3' and antisense, 5'-GGT CAGAGACCCAACTATGGTGCA-3'; β -actin (745 bp): sense, 5'-CACCACACCTTCTACAATGAGCTGC -3' and antisense, 5'-GCTCAGGAGGAGCAATGATCT TGAT-3'. RT-PCR products were finally resolved on agarose gels by electrophoresis and stained with ethidium bromide. Positive signals were quantified by the Kodak 1D 3.6 image analysis software (Eastman Kodak Co.).

DNA transfection and Luc reporter assay

The cells were transiently transfected with Luc reporter constructs of piNOS (–1592/+183)–Luc (18) or pGAS (3 copies)–Luc (BD Biosciences Clontech) using Lipofectamine, according to the manufacturer's recommendations. In brief, the cells (2×10^6) in 1.8 ml of media were incubated for 2 h and then gently mixed with the reporter construct (2 μ g) – Lipofectamine (6 μ l) complex in 0.2 ml of media. After incubation for 6 h, transfection reactions were pooled and then redistributed at 5×10^5 cells per well. The transfected cells were stimulated with IFN- γ (3 ng/ml) for 16 h in the presence of α -viniferin. Luc activity was measured with cell extracts using an assay kit (Promega, Madison, WI, USA). Total proteins were determined using a dye-based assay kit (Bio-Rad Lab., Hercules, CA, USA).

Statistical analyses

Data were subjected to the one-way analysis of variances followed by the Dunnett's test and are represented as the mean $\pm$ S.D. Values of $P < 0.05$ were considered as significantly different.

Results

In the present study, we investigated the effects of α -viniferin on IFN- γ receptor-mediated STAT-1 signaling and also on STAT-1-inducible inflammatory genes. α -Viniferin is a moderately water-insoluble oligostilbene compound (Fig. 1A). Stocks (100 mM each) of α -viniferin or MAPK inhibitors were prepared in 100% dimethylsulfoxide as a vehicle, and freshly diluted with media to be less than 0.03% vehicle concentrations, insignificantly

Fig. 1. IFN- γ -induced STAT-1 phosphorylation. A) Chemical structure of α -viniferin (AVF). RAW 264.7 cells were pretreated with AVF for 2 h and then stimulated with IFN- γ for 15 min, and cell extracts were then subjected to Western blot analysis (WB) with anti-pY⁷⁰¹-STAT-1 antibody (B) or anti-pS⁷²⁷-STAT-1 antibody (C). One of the similar blots is represented and its relative ratio is also indicated, normalizing to the total STAT-1. Each reported data value is the mean $\pm$ S.D. from three separate experiments. # P < 0.05 vs. media alone-added group. * P < 0.05 vs. IFN- γ alone-stimulated group.

affecting experimental parameters (Table 1). For most studies, RAW 264.7 macrophages were used because these cells express IFN- γ receptors at high levels, and IFN- γ -induced inflammatory gene expression in these cells is relatively well characterized.

Effect of α -viniferin on STAT-1 phosphorylation in IFN- γ -activated macrophages

We first examined whether α -viniferin could affect the Tyr⁷⁰¹ phosphorylation of STAT-1 in IFN- γ -activated RAW 264.7 cells, since this tyrosine phosphorylation is indispensable for homodimer formation of STAT-1 and

then nuclear translocation for transcriptional activity (2, 9). The cells were stimulated with IFN- γ in the presence of α -viniferin. Cell extracts were assessed by Western blot analysis with a specific antibody against Tyr⁷⁰¹-phosphorylated STAT-1. This tyrosine phosphorylation as an activation index of STAT-1 signaling was hardly detectable in the resting cells and markedly increased upon exposure to IFN- γ alone (Fig. 1B). Treatment of α -viniferin inhibited IFN- γ -induced levels of Tyr⁷⁰¹-phosphorylated STAT-1 only at a higher concentration of 10 μ M (Fig. 1B). As expected, cellular levels of total STAT-1 were not changed by treatment of IFN- γ and α -viniferin (Fig. 1: B and C). Another phosphorylation of STAT-1 at Ser-727 residue is also required for its full transcriptional activity and IFN- γ -mediated antiviral function (10, 27). In a parallel experiment with another antibody, α -viniferin at 1–10 μ M inhibited IFN- γ -induced levels of Ser⁷²⁷-phosphorylated STAT-1 in a dose-dependent manner (Fig. 1C). These results clearly indicate that α -viniferin was effective for inhibiting STAT-1 activation in response to IFN- γ , in which Ser⁷²⁷ phosphorylation of STAT-1 was much more sensitive than Tyr⁷⁰¹ phosphorylation.

Effect of α -viniferin on IFN- γ -induced activation of JAKs or ERK

We next focused on protein kinases upstream of STAT-1 phosphorylation at the Tyr-701 or Ser-727 residue. RAW 264.7 cells were stimulated with IFN- γ in the presence of α -viniferin. Cell extracts were immunoprecipitated with antisera against JAK-1 or -2, followed by Western blot analysis with anti-phosphotyrosine antibody

Table 1. IFN- γ -induced IP-10 or MIG production in RAW 264.7 cells

Sample	IP-10 (ng/ml)	MIG (pg/ml)
Media alone	0.1 $\pm$ 0.3	15 $\pm$ 8
AVF (10 μ M) alone	0.2 $\pm$ 0.4	n.d.
IFN- γ alone	18.6 $\pm$ 2.3 [#]	237 $\pm$ 24 [#]
IFN- γ + Vehicle	19.0 $\pm$ 1.7	n.d.
IFN- γ + AVF (1 μ M)	14.8 $\pm$ 1.8	198 $\pm$ 11
IFN- γ + AVF (3 μ M)	11.0 $\pm$ 0.7*	169 $\pm$ 15*
IFN- γ + AVF (5 μ M)	8.1 $\pm$ 1.5*	135 $\pm$ 26*
IFN- γ + AVF (10 μ M)	3.1 $\pm$ 2.1*	66 $\pm$ 9*

The cells were stimulated with IFN- γ (3 ng/ml) for 24 h, in the presence of vehicle (0.01% dimethylsulfoxide) only or with α -viniferin (AVF) in the vehicle. Amounts of IP-10 or MIG were determined by ELISA. Data are each the mean $\pm$ S.D. from three to five separate experiments. # P < 0.05 vs. media alone-added group. * P < 0.05 vs. IFN- γ alone-stimulated group. n.d. denotes "not determined".

Fig. 2. IFN- γ -induced activation of JAKs or ERK. RAW 264.7 cells were pretreated with α -viniferin (AVF) for 2 h and then stimulated with IFN- γ for 10 min. Cell extracts were subjected to the immunoprecipitation (IP) with anti-JAK-1 antibody (A) or anti-JAK-2 antibody (B), followed by Western blot analysis (WB) with anti-phosphotyrosine antibody 4G10. One of the similar blots is represented and relative ratio % of p-JAK-1 or p-JAK-2 is also indicated, normalizing to the total JAK-1 or JAK-2. C) Cell extracts were assessed by WB with anti-p-ERK antibody. One of the similar blots is represented and its relative ratio % is also indicated, normalizing to the total ERK-1/2. Each reported data value is the mean $\pm$ S.D. from three separate experiments. # P < 0.05 vs. media alone-added group. * P < 0.05 vs. IFN- γ alone-stimulated group.

4G10. JAK-1 or -2 was very weakly phosphorylated in the resting cells, and their phosphorylated levels were markedly increased upon exposure to IFN- γ alone (Fig. 2: A and B). Treatment of α -viniferin did not affect IFN- γ -induced JAK-1 activation (Fig. 2A), but significantly inhibited IFN- γ -induced JAK-2 activation, only at a higher concentration of 10 μ M (Fig. 2B).

The Ser-727 residue of STAT-1 lies within a potential phosphorylation consensus of MAPKs, and IFN- γ -induced ERK activation has been known to affect the Ser⁷²⁷ phosphorylation of STAT-1 (12, 28, 29). The cells were stimulated with IFN- γ in the presence of α -viniferin, and cellular extracts were then assessed by Western blot analysis with anti-p-ERK antibody. Upon exposure to IFN- γ alone, cellular levels of phosphorylated ERK-1 were markedly increased from the basal levels (Fig. 2C). Treatment of α -viniferin at 3 – 10 μ M significantly inhibited IFN- γ -induced ERK-1 activation in a dose-dependent manner, without affecting the cellular levels of total ERK-1/2 (Fig. 2C).

Effect of α -viniferin on IFN- γ -induced production of NO or chemokine in macrophages

To investigate STAT-1-dependent inflammatory responses, we first quantified NO in IFN- γ -activated RAW 264.7 cells or primary macrophages from bone marrow of C57BL/6 mice. Amounts of nitrite, a stable metabolite of NO, were quite low with 7.5 ± 3.2 μ M in the resting cells, but markedly increased up to 28.9 ± 2.1 μ M, upon

exposure to IFN- γ alone (Fig. 3A). Treatment of α -viniferin at 3 – 10 μ M significantly inhibited IFN- γ -induced nitrite production in RAW 264.7 cells, showing an IC₅₀ value of 2.6 μ M (Fig. 3A). Primary macrophages from C57BL/6 mice also increased nitrite production upon exposure to IFN- γ alone (Fig. 3B). This nitrite induction by IFN- γ was consistently inhibited in the presence of α -viniferin, showing an IC₅₀ value of 3.7 μ M (Fig. 3B).

We next examined whether α -viniferin could affect chemokine IP-10 or MIG production. Expression of these chemokines is also dependent upon STAT-1 transcriptional activity in IFN- γ -activated macrophages (19, 20). Upon exposure to IFN- γ alone, RAW 264.7 cells markedly increased IP-10 production over the basal levels (Table 1). Treatment of α -viniferin at 3 – 10 μ M dose-dependently inhibited IFN- γ -induced IP-10 production, showing an IC₅₀ value of 4.6 μ M, but did not affect basal levels of IP-10 (Table 1). In a parallel experiment, α -viniferin inhibited IFN- γ -induced MIG production with an IC₅₀ value of 5.7 μ M (Table 1). However, α -viniferin at the concentrations for inhibition of inflammatory mediator production did not affect the viability of RAW 264.7 cells (Fig. 3C), excluding its possible non-specific cytotoxicity.

Effect of α -viniferin on IFN- γ -induced iNOS expression

To understand whether the effect of α -viniferin on nitrite production was associated with its influence on iNOS synthesis, RAW 264.7 cells were stimulated with

Fig. 3. IFN- γ -induced NO production. RAW 264.7 cells (A) or primary macrophages derived from C57BL/6 mice (B) were stimulated with IFN- γ for 24 h, in the presence of α -viniferin (AVF). Amounts of nitrite, a stable metabolite of NO, were determined with sodium nitrite as a standard. C) RAW 264.7 cells were incubated with various concentrations of AVF for 24 h, in the presence of IFN- γ and then subjected to the WST-1 method, measuring optical density at 450 nm (OD₄₅₀) as a parameter of cell viability. Each reported data value is the mean $\pm$ S.D. from three to five separate experiments. [#] P < 0.05 vs. media alone-added group. ^{*} P < 0.05 vs. IFN- γ alone-stimulated group.

IFN- γ in the presence of α -viniferin, and cell extracts were then assessed by Western blot analysis. Protein levels of iNOS were quite low in the resting cells, but markedly increased upon exposure to IFN- γ alone (Fig. 4A). Treatment of α -viniferin at less than 10 μ M decreased IFN- γ -induced protein levels of iNOS in a dose-dependent manner (Fig. 4A). We next performed an RT-PCR analysis to investigate mRNA levels of iNOS. Consistent with iNOS protein data, treatment of α -viniferin dose-dependently attenuated cellular induction of iNOS mRNA in response to IFN- γ (Fig. 4B), and its semi-quantitative determination is also shown in Fig. 4C. In a parallel experiment, treatment of α -viniferin differentially attenuated IFN- γ -induced mRNA levels of chemokine IP-10 or MIG (Fig. 4: B and C). To delineate whether this suppressive action of α -viniferin takes place at the transcription level, we transfected RAW 264.7 cells with piNOS-Luc construct, encoding iNOS promoter (-1592/+183) fused to Luc gene as a reporter (18). Upon exposure to IFN- γ alone, the transfected cells increased Luc expression up to about 12-fold over the basal levels (Fig. 4D). This Luc induction by IFN- γ was also inhibited by treatment of α -viniferin, showing an IC₅₀ value of 4.2 μ M (Fig. 4D). These results indicate that α -viniferin could suppress IFN- γ -induced expression of iNOS or chemokine gene at the transcription level.

Effect of α -viniferin on IFN- γ -stimulated IRF-1 induction

IRF-1 is another transcription factor, rapidly inducible

in response to IFN- γ , in which STAT-1-responsive GAS motifs play a pivotal in the IRF-1 induction (16). IRF-1-responsive ISRE motifs, in collaboration with STAT-1-responsive GAS motifs, are essential for IFN- γ -induced maximal induction of iNOS, IP-10, or MIG gene (18–20). To further investigate the contribution of STAT-1-dependent action of α -viniferin, we examined IRF-1 induction in IFN- γ -activated RAW 264.7 cells. Upon exposure to IFN- γ alone, protein levels of IRF-1 were markedly increased, which was inhibited by treatment with α -viniferin at less than 10 μ M (Fig. 4A). Consistently, mRNA levels of IRF-1 induced by IFN- γ were also attenuated by treatment with α -viniferin (Fig. 4B), and its semi-quantitative determination is also shown in Fig. 4C.

Effects of MAPK inhibitors on IFN- γ -induced STAT-1 transcriptional activity and NO production

The results above indicate that α -viniferin inhibited IFN- γ -induced ERK-1 activation, leading to the Ser⁷²⁷ phosphorylation of STAT-1 for induction of inflammatory genes. To clarify which MAPKs are involved in the STAT-1 activation, we used the specific inhibitor U0126 or PD98059 affecting the ERK-1/2 pathway and the JNK inhibitor SP600125. RAW 264.7 cells were transfected with pGAS-Luc reporter construct, encoding 3 copies of STAT-1-responsive GAS motifs fused to Luc gene as a reporter. The transfected cells were stimulated with IFN- γ , in the presence of each MAPK inhibitor, and Luc expression was then evaluated. Consistently, the cells

Fig. 4. IFN- γ -induced expression of iNOS, IRF-1, or chemokine. A) RAW 264.7 cells were stimulated with IFN- γ for 24 h in the presence of α -viniferin (AVF). Cell extracts were subjected to Western blot analysis (WB) with anti-iNOS antibody or anti-IRF-1 antibody. One of the similar blots is represented and its relative ratio % is also indicated, normalizing to the GAPDH. B and C) RAW 264.7 cells were pretreated with AVF for 2 h and then stimulated with IFN- γ for 4–6 h, in the presence of AVF. Total RNA of the cells was subjected to the RT-PCR analysis to detect cellular levels of iNOS, IP-10, MIG, or IRF-1 transcript. One of similar results is represented (B) and its semi-quantitative determination is also shown as relative ratio %, normalizing to β -actin (C). D) RAW 264.7 cells were transfected with pNOS-Luc reporter construct and then stimulated with IFN- γ for 16 h in the presence of AVF. Cell extracts were subjected to dual-Luc assay. Luc expression is represented as a relative fold, in which firefly Luc activity was normalized to the *Renilla* activity. Each reported data value is the mean $\pm$ S.D. from three separate experiments. $^{\#}P < 0.05$ vs. media alone-added group. $^{*}P < 0.05$ vs. IFN- γ alone-stimulated group.

produced pronounced levels of Luc, upon exposure to IFN- γ alone, over the basal levels. Treatment of U0126 or PD98059 inhibited IFN- γ -induced Luc expression with IC₅₀ values of 3.7 or 10.6 μ M, respectively, but that of SP600125 at 5–30 μ M was not effective (Fig. 5A). To further understand the contribution of MAPKs on STAT-1-mediated induction of inflammatory mediators, RAW 264.7 cells were stimulated with IFN- γ in the presence of MAPK inhibitor (30 μ M each). Consistently, treatment of U0126 or PD98059 inhibited IFN- γ -induced NO production in the cells, but that of SP600125 could not significantly affect it (Fig. 5B).

Discussion

IFN- γ is a potent activator of macrophages, playing an important role in inflammatory responses, as well as the control of specific immune processes (1). However, dys-

regulated overexpression and hyper-responsiveness to IFN- γ are implicated in deleterious conditions, including autoimmune diseases or tissue damages secondary to excessive inflammatory responses (30–33). Much attention has been focused on the pharmacological bases that restrain IFN- γ action, thereby securing the host from excessive cell-activating properties. This study was aimed to investigate the effect of α -viniferin (Fig. 1A), an oligostilbene of trimeric resveratrol, on STAT-1-inducible inflammatory genes in IFN- γ -activated macrophages and also to propose a molecular basis underlying the anti-inflammatory action.

Numerous studies have established the physiological importance of STAT-1 in the inflammatory and immune responses of IFN- γ (2–5). We first examined STAT-1 activation in IFN- γ -activated macrophages. Treatment of α -viniferin inhibited IFN- γ -induced Tyr⁷⁰¹ phosphorylation and also Ser⁷²⁷ phosphorylation of STAT-1, in which

Fig. 5. Effects of MAPK inhibitors on IFN- γ -induced STAT-1 transcriptional activity and NO production. A) RAW 264.7 cells were transfected with pGAS-Luc reporter construct. The transfected cells were stimulated with IFN- γ for 16 h in the presence of MAPK inhibitor of U0126 (solid circle), PD98059 (open circle), or SP600125 (solid triangle). Luc expression, as a reporter of STAT-1 transcription, was determined and is represented as relative % compared with those of the transfected cells treated with IFN- γ plus vehicle alone. B) RAW 264.7 cells were stimulated with IFN- γ for 24 h in the presence of vehicle (0.03% dimethylsulfoxide) or MAPK inhibitor (30 μ M each) in the vehicle. Amounts of nitrite were determined with sodium nitrite as a standard. Each reported data value is the mean $\pm$ S.D. from three to five separate experiments. # P < 0.05 vs. media alone-added group. * P < 0.05 vs. IFN- γ alone-stimulated group (B).

the serine phosphorylation was much sensitive (Fig. 1: B and C). We then focused on protein kinases upstream of STAT-1 phosphorylation in the cells. Treatment with α -viniferin at only the higher concentration of 10 μ M inhibited IFN- γ -induced activation of JAK-2 but not JAK-1 (Fig. 2: A and B), correlating with its effect on the Tyr⁷⁰¹ phosphorylation of STAT-1. Moreover, treatment of α -viniferin at 3 – 10 μ M was very effective, producing dose-dependent inhibition of IFN- γ -induced ERK-1 activation (Fig. 2C), consistent with its effect on the Ser⁷²⁷

phosphorylation of STAT-1 (Fig. 1C). Similarly, vitisin A, an oligostilbene of tetrameric resveratrol, was reported to inhibit protein kinase B-mediated Ser⁷²⁷ phosphorylation of STAT-1, inducing chemokine RANTES expression in influenza A virus H1N1-infected alveolar epithelial A549 cells (34). Resveratrol with the monomeric moiety of α -viniferin was reported to inhibit JAK-mediated Tyr⁷⁰¹ phosphorylation of STAT-1 as a molecular basis of its anti-proliferation and apoptosis effects on human epidermoid carcinoma A431 cells (35, 36); and it was also reported to inhibit JAKs and Src kinase, causing sequentially loss of Tyr⁷⁰¹ phosphorylation of STAT-1, for apoptosis induction in docetaxel-resistant prostate tumor PC3-DR cells (37).

Since α -viniferin inhibited STAT-1 activation in which Ser⁷²⁷ phosphorylation was much more sensitive than Tyr⁷⁰¹ phosphorylation (Fig. 1: B and C), we tested whether the compound could affect STAT-1-inducible inflammatory mediators in response to IFN- γ . Treatment of α -viniferin at 3 – 10 μ M dose-dependently inhibited IFN- γ -induced production of NO, IP-10, or MIG in RAW 264.7 cells (Fig. 3A and Table 1) and also that of NO in primary macrophages derived from C57BL/6 mice (Fig. 3B). Moreover, treatment with α -viniferin at 3 – 10 μ M diminished IFN- γ -induced protein levels of iNOS in the cells (Fig. 4A), differentially attenuated IFN- γ -induced mRNA levels of iNOS, IP-10, or MIG (Fig. 4: B and C), as well as inhibited IFN- γ -induced promoter activity of iNOS gene (Fig. 4D). These results indicate that α -viniferin could restrain IFN- γ -induced expression of inflammatory genes at the transcription level. Furthermore, these down-regulatory actions of α -viniferin on inflammatory mediator production were consistently observable within effective concentrations inhibiting ERK-mediated Ser⁷²⁷ phosphorylation for STAT-1 activation (Figs. 1C and 2C), excluding its mode of the action from JAK-2-mediated Tyr⁷⁰¹ phosphorylation (Figs. 1B and 2B).

IRF-1, another transcription factor acting on ISRE motifs, is rapidly inducible upon exposure to IFN- γ , in which STAT-1-responsive GAS motifs are essential for maximal IRF-1 expression (16). Treatment of α -viniferin at 3 – 10 μ M dose-dependently suppressed IFN- γ -induced protein and mRNA levels of IRF-1 in RAW 264.7 cells (Fig. 4: A – C), further supporting that α -viniferin's action is dependent upon the Ser⁷²⁷ phosphorylation for STAT-1 activation. The STAT-1-responsive GAS or IRF-1-responsive ISRE motifs could be identified in the promoter regions of inflammatory genes: iNOS with three GAS sites and two ISRE sites upstream from -722 relative to the transcription start; IP-10 at -224/-212; MIG at -222/-198 and -99/-85 (18 – 20).

Inflammation involves a complex process of intracel-

lular and intercellular cytokine signals, as well as other components of signaling networks, including MAPK pathways. As described above, α -viniferin inhibited ERK-mediated Ser⁷²⁷ phosphorylation of STAT-1 in IFN- γ -activated macrophages, contributing to its suppression of STAT-1-inducible inflammatory genes. Treatment with U0126 or PD98059, specifically inhibiting the ERK pathway, diminished the IFN- γ -induced STAT-1 transcriptional activity in RAW 264.7 cells, but the JNK inhibitor SP600125 was not effective (Fig. 5A). Consistently, treatment with U0126 or PD98059 inhibited IFN- γ -induced NO production in the cells, but that of SP600125 could not significantly affect it (Fig. 5B). In an analogous manner, IFN- γ induced ERK activation, requiring serine- but not tyrosine-phosphorylated STAT-1, in which IFN- γ -stimulated gene expression was abrogated in the presence of dominant-negative ERK mutants but not p38 MAPK mutants (28).

Taken together, this study proposes an anti-inflammatory mechanism of α -viniferin, down-regulating STAT-1-inducible inflammatory genes via inhibiting ERK-mediated Ser⁷²⁷ phosphorylation of STAT-1, an index of STAT-1 activation, in IFN- γ -stimulated macrophages.

Acknowledgments

We appreciate Dr. C.J. Lowenstein (Johns Hopkins University School of Medicine, Baltimore, MD, USA) for kindly supplying us with piNOS-Luc reporter construct. This work supported by a research grant of Chungbuk National University in 2008.

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