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RESEARCH ARTICLES

Resveratrol down-regulates interferon-γ-inducible inflammatory genes in macrophages: molecular mechanism via decreased STAT-1 activation☆

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Abstract

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is one of nonflavonoid polyphenolic phytoalexins found in various plant species, a number of which are components of human diet including grapes and red wines. Resveratrol has exerted several beneficial effects with anti-inflammation, cardioprotection and cancer chemoprevention. However, its mechanisms of action are not completely understood. In this study, we investigated effects of resveratrol on inflammatory gene expression in interferon (IFN)-γ alone-stimulated macrophages and proposed a molecular basis underlying the action. Resveratrol inhibited IFN-γ-induced production of nitric oxide (NO), IFN-γ-inducible protein-10 (IP-10), or the monokine induced by IFN-γ (MIG) in RAW 264.7 macrophages and also that of NO in primary macrophages derived from bone marrows of C3H/HeJ (toll-like receptor-4$^{-/-}$) mice. Moreover, resveratrol diminished IFN-γ-induced protein levels of inducible NO synthase (iNOS), attenuated mRNA levels of iNOS, IP-10 or MIG as well as inhibited IFN-γ-induced promoter activity of iNOS gene, indicating that the phytoalexin could down-regulate inflammatory genes at the transcription level. To understand a mechanism of the action, we tested resveratrol could affect the signal transducers and activation of transcription-1 (STAT-1), a pivotal transcription factor in IFN-γ-induced expression of inflammatory genes. Resveratrol inhibited IFN-γ-induced transcriptional activity of STAT-1 in macrophages and also IFN-γ-induced Tyr^{701} or Ser^{727} phosphorylation of STAT-1. We then focused on protein kinases upstream STAT-1 phosphorylation. Resveratrol inhibited IFN-γ-induced activation of Janus kinase-2 (JAK-2) and also the extracellular signal-regulated kinase, in which JAK-2 was more sensitive. Taken together, this study proposes a new mechanism of resveratrol, blocking JAK/STAT-1 pathway that controls inflammatory responses in IFN-γ-activated macrophages.
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Keywords: Phytoalexin; Resveratrol; Inflammatory macrophages; Interferon-γ; STAT-1

1. Introduction

Resveratrol, two phenol rings linked by a styrene *trans*-double bond to be 3,4′,5-trihydroxystilbene (Fig. 1A), is a naturally occurring phytoalexin abundant in grapes and red wines. Even though considerably depending on the grape variety, environmental factors in the vineyard and wine processing techniques, resveratrol content reaches about 50–100 μg/g fresh weight in the grape skins [1] and is in the range of 1.5–3 mg/L of red wines [2]. A primary interest on resveratrol has come from epidemiological association of a low incidence of cardiovascular diseases with intake of red wines, so called “French paradox” [3]. Also, one of the striking beneficial activities of resveratrol has been its cancer-chemopreventive potential by blocking multiple process of carcinogenesis such as tumor initiation, promotion and progression [4]. One of the possible mechanisms for its nutraceutical activities has been proposed to restrain inflammatory responses [5]. Resveratrol has been evidenced to show anti-inflammatory activities through inhibition of synthesis and release of inflammatory mediators via transcription factors such as nuclear factor (NF)-κB and the activator protein (AP)-1 [6,7], modification of eicosanoid metabolism [8] or inhibiting the activated immune cells [9,10]. Despite theses important advances, molecular mechanisms by which resveratrol exerts its various biological effects are not yet clear.

The signal transducers and activation of transcription (STAT) proteins are major cytokine-activated transcription factors that play an important role in the biology of immune system [11]. Interferon (IFN)-γ stimulation causes the activation of receptor-associated Janus kinase (JAK)-1 and -2 [12,13]. Activated JAKs phosphorylate the intracellular domain of IFN-γ receptor, serving as a docking site of STAT-1 or other signal molecules, and lead to the phosphorylation of STAT-1 at Tyr-701 residue [12,14]. Upon tyrosine phosphorylation, STAT-1 is released from the receptor, forms a homodimer to function as a transcription factor [15,16] and enters into the nucleus [17,18], where it specifically binds to the IFN-γ-activated sequence (GAS)

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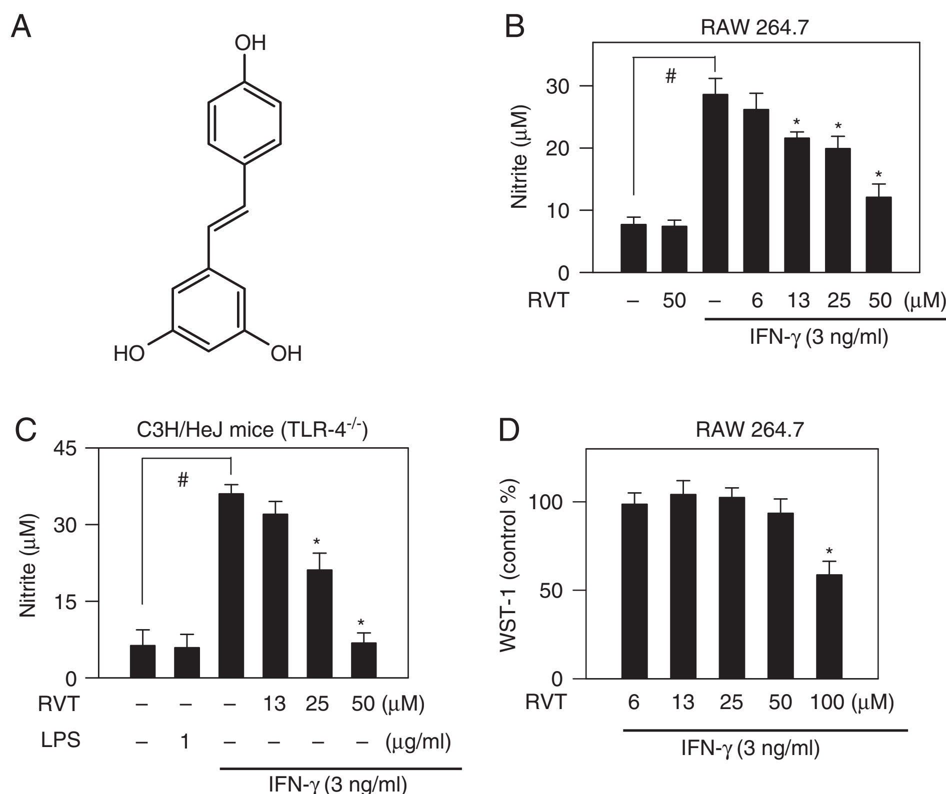

Fig. 1. Effect of resveratrol on IFN-γ-induced NO production. (A) Chemical structure of resveratrol (RVT). RAW 264.7 cells (B) or primary macrophages-derived from bone marrows of C3H/HeJ (TLR-$4^{-/-}$) mice (C) were stimulated with IFN-γ or LPS for 24 h, in the presence of RVT. Amounts of nitrite, a stable metabolite of NO, were determined with sodium nitrite as a standard. (D) RAW 264.7 cells were incubated with RVT plus IFN-γ for 24 h. After exposure to WST-1 solution for 3 h, absorbance values were measured at 450 nm. Effect of RVT on the cell proliferation is represented as control %, compared with IFN-γ alone-treated group. Data are means±SD from 3 to 5 separate experiments. #P<.05 vs. media alone-added group; *P<.05 vs. IFN-γ alone-stimulated group.

motif for transcriptional control 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) [19–21].

IFN-γ stimulation also generates Ser^{727}-phosphorylated STAT-1, important for enhancing transcriptional activity of STAT-1 and IFN-γ-mediated antiviral function [22,23]. However, Ser^{727} phosphorylation has no impact on homodimer formation of STAT-1, Tyr^{701} phosphorylation and nuclear translocation [24,25]. Thereby, STAT-1 requires JAK-mediated Tyr^{701} phosphorylation and another kinase-mediated Ser^{727}-phosphorylated status to achieve full transcriptional activity. The identity of kinase inducing Ser^{727} phosphorylation of STAT-1 is not fully resolved. In particular to mitogen-activated protein kinase (MAPK) pathway, IFN-γ triggers the activation of extracellular signal-regulated kinase (ERK)-1/2 via multiple signaling dependent or independent of ERK kinase (MEK)-1/2, leading to Ser^{727} phosphorylation of STAT-1 [26,27]. However, IFN-γ-induced activation of p38 MAPK or c-Jun N-terminal kinase (JNK) is somewhat controversial [28,29].

IFN-γ is a potent activator of macrophages for inflammatory response and cellular immunity [30]. However, overproduction and hyper-responsiveness to IFN-γ are implicated in the pathophysiologic conditions, including autoimmunity and tissue damage secondary to excessive inflammation [31–33]. Much attention has been focused on molecular mechanisms that restrain or attenuate IFN-γ action, thus protecting the host from inflammatory and immune disorders. Resveratrol was previously reported to inhibit IFN-γ production in phytohaemaglutinin-activated human peripheral blood mononuclear cells and also in concanavalin A-activated splenic lymphocytes through inhibition of NF-κB activation [34,35]. This study was aimed to investigate effects of resveratrol on IFN-γ receptor-mediated expression of inflammatory genes in macrophages and to elucidate a molecular basis underlying the effects.

2. Materials and methods

2.1. Materials

Antisera against iNOS, STAT-1, ERK-1/2 or glyceraldehydes 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA), those against p-ERK-1 or phosphotyrosine (4G10) from Upstate Tech (Charlottesville, VA, USA), and those against Janus kinase (JAK)-2, JAK-1, pY-STAT-1 or pS-STAT-1 from Cell Signaling Tech (Danvers, MA, USA). STAT-1-dependent luciferase reporter plasmid of pGAS-Luc and NF-kB-dependent secretory alkaline phosphatase (SEAP) reporter plasmid of pκB-SEAP were purchased from BD Biosciences Clontech (Palo Alto, CA, USA). Promoter-dependent luciferase reporter plasmid of piNOS(-1592/+183)-Luc has been previously described [19]. Fetal bovine serum (FBS) and other culture materials were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals, including resveratrol (>97% purity) and IFN-γ, were otherwise purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

RAW 264.7 macrophages were cultured in Dulbecco's modified Eagle's media supplemented with heat-inactivated 10% FBS, benzylpenicillin potassium (143 U/ml) and streptomycin sulfate (100 µg/ml) under 37°C and 5% CO_2 atmosphere. C3H/HeJ [toll-like receptor (TLR)-$4^{-/-}$] mice were obtained from KRIBB (Ochang, Korea). Macrophages were derived from bone marrows of the male mice (5-6 weeks of age), following protocols approved by the Animal Experimentation Ethics Committee in my institute. 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 changed in the presence of M-CSF (10 ng/ml). On Day 8, adherent cells were harvested and then used as primary macrophages.

2.3. Measurement of NO release

RAW 264.7 cells or primary macrophages from mice were stimulated with IFN-γ (3 ng/ml) or lipopolysaccharide (LPS, 1 μg/ml) for 24 h, in the presence of resveratrol. Aliquots of the culture media were mixed with the same volume of 0.05% sulfanilamide and 0.05% *N*-(1-naphthyl)ethylenediamine, and absorbance values were then measured at 540 nm with sodium nitrite as a standard.

2.4. Measurement of IP-10 or MIG release

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

2.5. Cell proliferation assay

The cells were incubated with various concentrations of resveratrol 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, Japan) for 3 h, and absorbance values were then measured at 450 nm.

2.6. Immunoblotting analysis

The cells were pretreated with sample for 2 h and stimulated with IFN-γ (3 ng/ml) for indicated times, in the presence of sample. The cells were disrupted in a lysis buffer (20 mM HEPES, pH 7.9, 1% Triton X-100, 20% glycerol, 1 mM EGTA, 20 mM NaF, 1 mM DTT, 1 mM Na_3VO_4, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Cell extracts were resolved on sodium dodecyl sulfate acrylamide gel by electrophoresis and then transferred to a polyvinylidene difluoride membrane. Either 5% nonfat 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 incubated at 4°C overnight with primary antisera and further reacted with appropriate horseradish peroxidase-labeled secondary antisera at room temperature for 2–5 h. 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).

2.7. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

The cells were pretreated with resveratrol for 2 h, and stimulated with IFN-γ (3 ng/ml) for 4–6 h, in the presence of resveratrol. Total RNA of the cells was subjected to semi-quantitative RT-PCR, using an RNA PCR kit (Solgent, Daejeon, Korea). Briefly, total RNA was reversely transcribed at 42°C for 1 h and then subjected to 25–30 cycles of PCR consisting of 30-s denaturation at 94°C, 30-s annealing at 50–60°C and 90-s extension at 72°C. The sequences of primers for RT-PCR and the sizes of PCR products are as follow: iNOS (457 bp), sense 5′-GTCAACTGCAAGAGAACGGAGAAC-3′ and antisense 5′-GAGCTCCTCCAGAGGGTAGGCT-3′; IP-10 (430 bp), sense 5′-ACTCACT-CAGTTTGTTGAGTCATTC-3′ and antisense 5′-TTTGATTAGTACTGTAGGGTTAATG-3′; MIG (421 bp), sense 5′-CTTCAGCCCCAGCAGTGTATTCTTT-3′ and antisense 5′-AGA-GAACCTGGGAGTAGACAAGGTA-3′; β-actin (745 bp), sense 5′-CACCACACCTTCTA-CAATGAGCTGC-3′ and antisense 5′-GCTCAGGAGGAGCAATGATCTTGAT-3′. RT-PCR products were finally resolved on agarose gels by electrophoresis and stained with ethidium bromide.

2.8. Luciferase reporter assay

The cells were transiently transfected with a *Renilla* control vector in combination with each luciferase (Luc) reporter construct of piNOS (−1592/+183)-Luc or pGAS (3 copies)-Luc, using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. In brief, the cells (2×10^6) in 1.8 ml of Opti-MEM were incubated for 2 h and then gently mixed with the reporter construct (2 μg)-Lipofectamine (6 μl) complex in 0.2 ml of Opti-MEM. 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 resveratrol. Luciferase activity was measured with cell extracts using a dual assay kit (Promega, Madison, WI, USA). Protein levels were measured using a dye-based assay kit (Bio-Rad Lab., Hercules, CA, USA).

2.9. Immunoprecipitation

The cells were pretreated with resveratrol for 2 h and stimulated with IFN-γ (3 ng/ml) for 10 min. Cell extracts (500 μg) were incubated with antisera (1–2 μg) against JAK-1 or JAK-2 at 4°C overnight and then precipitated with protein G-agarose beads (GE Healthcare, Chalfont, UK).

2.10. SEAP reporter assay

The cells were transiently transfected with a pSV-β-galactosidase control vector in combination with NF-κB-dependent reporter construct of pκB (3 copies)-SEAP, using Lipofectamine (Invitrogen) according to the manufacturer's recommendations as described above. The transfected cells were stimulated with LPS (1 μg/ml) for 18 h, in the presence of resveratrol. Aliquots of the culture media were incubated at 65°C for 5 min and reacted with 4-methylumbellifery phosphate (500 μM) in the dark for 1 h. SEAP activity was measured relative fluorescence units with emission 449 nm and excitation 360 nm. β-Galactosidase activity were measured with cell extracts using an assay kit (Promega, Madison, WI).

2.11. Statistical analysis

Data are expressed as means±S.D. and were subjected to the one-way analysis of variances followed by the Dunnet's test. Values of $P<.05$ were considered as significantly different.

3. Results

In this study, we examined effects of resveratrol on IFN-γ-inducible inflammatory gene and also on IFN-γ receptor-mediated STAT-1 signaling. Resveratrol is a moderately water-soluble hydroxystilbene compound (Fig. 1A). For most studies, RAW 264.7 macrophages were used because these cells express IFN-γ receptor-1 and receptor-2, and inflammatory responses in these cells are relatively well characterized.

3.1. Resveratrol inhibited IFN-γ-induced production of NO or chemokine in macrophages

We first demonstrated anti-inflammatory activity of resveratrol by NO quantification in IFN-γ-activated RAW 264.7 cells or primary macrophages from C3H/HeJ mice ($TLR\text{-}4^{-/-}$). Amounts of nitrite, a stable metabolite of NO, were quite low with 6-9 μM in the resting RAW 264.7 cells but markedly increased up to 26-31 μM upon exposure to IFN-γ alone (Fig. 1B). Treatment of resveratrol inhibited IFN-γ-induced nitrite production in a dose-dependent manner, showing an IC_{50} value of 31 μM but did not affect basal levels of NO in the cells (Fig. 1B). Previous studies have proven that resveratrol can inhibit NF-κB-activating pathway, resulting in the decrease of TLR-4-mediated NO production in LPS-activated macrophages [36–38]. To exclude TLR-4 signaling in response to LPS, primary macrophages-derived from bone marrows of C3H/HeJ ($TLR\text{-}4^{-/-}$) mice were then used. These primary cells increased nitrite production by stimulation with IFN-γ but not by LPS alone (Fig. 1C). The nitrite production in response to IFN-γ was also inhibited in the presence of resveratrol (Fig. 1C).

We next examined whether resveratrol could affect IFN-γ-induced production of chemokines. Upon exposure to IFN-γ alone, RAW 264.7 cells released pronounced amounts of IP-10 (16.5±1.3 ng/ml) from the basal levels of 1.1±0.8 ng/ml (Table 1). Treatment of resveratrol inhibited IFN-γ-induced IP-10 production in a dose-dependent manner, showing an IC_{50} value of 24 μM (Table 1). In a parallel experiment, resveratrol inhibited IFN-γ-induced MIG production with an IC_{50} value of 35 μM (Table 1). However, resveratrol at the effective concentrations for anti-inflammatory responses did not affect the proliferation of RAW 264.7 cells (Fig. 1D), excluding its

Table 1
Effect of resveratrol on IFN-γ-induced production of IP-10 or MIG

Treatment	IP-10 (ng/ml)	MIG (pg/ml)
Media alone	1.1±0.8	17±13
IFN-γ alone	16.5±1.3[a]	250±19[a]
IFN-γ+RVT (13 μM)	14.1±0.7	227±33
IFN-γ+RVT (25 μM)	8.5±0.9[b]	188±15[b]
IFN-γ+RVT (50 μM)	5.3±1.2[b]	51±16[b]

RAW 264.7 cells were stimulated with IFN-γ (3 ng/ml) for 24 h, in the presence of resveratrol. Amounts of IP-10 or MIG in the culture media were quantified by ELISA. Data are means±SD from 3 separate experiments.
RVT, resveratrol.
[a] $P<.05$ vs. media alone-added group.
[b] $P<.05$ vs. IFN-γ alone-treated group.

nonspecific cytotoxicity. These results clearly indicate that resveratrol was effective in the dose-dependent inhibition of inflammatory mediator production in response to IFN-γ.

3.2. Resveratrol down-regulated IFN-γ-induced iNOS expression at the transcription level

To investigate whether the inhibitory action of resveratrol on IFN-γ-induced nitrite production was attributable to its influence on iNOS synthesis, RAW 264.7 cells were stimulated with IFN-γ in the presence of resveratrol and then subjected to Western blot analysis. Protein levels of iNOS were hardly detectable in the resting cells but markedly increased upon exposure to IFN-γ alone (Fig. 2A). Treatment of resveratrol dose-dependently decreased IFN-γ-induced protein levels of iNOS (Fig. 2A). We next performed an RT-PCR analysis to investigate mRNA levels of iNOS. Consistent with iNOS protein data, treatment of resveratrol attenuated cellular induction of iNOS mRNA in response to IFN-γ (Fig. 2B). In a parallel experiment, treatment of resveratrol differentially decreased IFN-γ-induced mRNA levels of chemokine IP-10 or MIG (Fig. 2B).

To delineate whether this suppressive action of resveratrol could be taken place at the transcription level, we transfected RAW 264.7 cells with piNOS-Luc construct, encoding iNOS promoter (−1592/+183) that is fused to luciferase gene as a reporter [19]. Upon exposure to IFN-γ alone, the transfected cells increased luciferase expression up to about 13 fold over the basal levels (Fig. 2C). This luciferase induction in response to IFN-γ was consistently inhibited by treatment of resveratrol in a dose-dependent manner with an IC_{50} value of 27 μM (Fig. 2C).

3.3. Resveratrol inhibited IFN-γ-induced STAT-1 signaling

Even though the 5′-flanking region of murine iNOS gene contains at least 10 copies of IFN-γ response elements, three copies of the GAS, two copies of the IFN-stimulated response element (ISRE) and two copies of NF-κB [19,39], STAT-1-responsive GAS motifs appear to be essential for maximal iNOS induction in response to IFN-γ. To address a mechanism of resveratrol down-regulating IFN-γ-induced transcription of iNOS or chemokine genes, we first investigate whether resveratrol could affect STAT-1 transcriptional activity or not. RAW 264.7 cells were transfected with pGAS-Luc construct, containing three copies of STAT-1-responsive GAS motif fused to luciferase gene as a reporter. Upon exposure to IFN-γ alone, we observed profound increases of luciferase expression in the cells, harboring pGAS-Luc reporter construct, over the basal levels (Fig. 3A), indicating that cellular STAT-1 is functional. Treatment of resveratrol dose-dependently inhibited IFN-γ-induced transcriptional activity of STAT-1 with an IC_{50} value of 29 μM (Fig. 3A).

The transcriptional activity of STAT-1 in response to IFN-γ indispensably requires JAK-mediated phosphorylation event of STAT-1 at Tyr-701 residue, within the intracellular domain of IFN-γ receptors [12,13]. To investigate whether resveratrol could affect the tyrosine phosphorylation of STAT-1, the cells were stimulated with IFN-γ in the presence of resveratrol. Cell extracts were assessed by Western blot analysis using specific antibody against Tyr^{701}-phosphorylated STAT-1. The tyrosine phosphorylation of STAT-1 was quite low in the resting cells and markedly increased upon exposure to IFN-γ alone (Fig. 3B). Treatment of resveratrol dose-dependently inhibited IFN-γ-induced levels of Tyr^{701}-phosphorylated STAT-1 (Fig. 3B). Another phosphorylation at Ser-727 residue is also involved in the transcriptional activity of STAT-1 [22,23]. In a parallel experiment with another antibody, resveratrol at higher concentration of 50 μM inhibited IFN-γ-induced levels of Ser^{727}-phosphorylated STAT-1 significantly (Fig. 3C). As expected, cellular levels of total STAT-1 proteins were not changed by treatment of IFN-γ and resveratrol (Fig. 3B and C).

3.4. Resveratrol inhibited IFN-γ-induced JAK-2 or ERK activation

We next examined whether resveratrol's action on the Tyr^{701} phosphorylation of STAT-1 was due to its primary effect on IFN-γ-induced activation of JAKs. RAW 264.7 cells were stimulated with IFN-γ in the presence of resveratrol. Cell extracts were immunoprecipitated with anti-JAK-2 or anti-JAK-1 antibody, followed by Western blot analysis with an anti-phosphotyrosine antibody (4G10). JAK-2 and JAK-1 were weakly phosphorylated in the resting cells, and their phosphorylated levels were markedly increased upon exposure to IFN-γ alone (Fig. 3D and E). Treatment of resveratrol consistently reduced IFN-γ-induced tyrosine phosphorylation of JAK-2 (Fig. 3D) but could not affect that of JAK-1 (Fig. 3E).

The Ser-727 residue of STAT-1 lies within a potential MAPK-phosphorylation motif, and IFN-γ-stimulated ERK activation is known to affect the serine phosphorylation of STAT-1 [26,27]. To understand whether resveratrol could affect ERK activation, the cells were

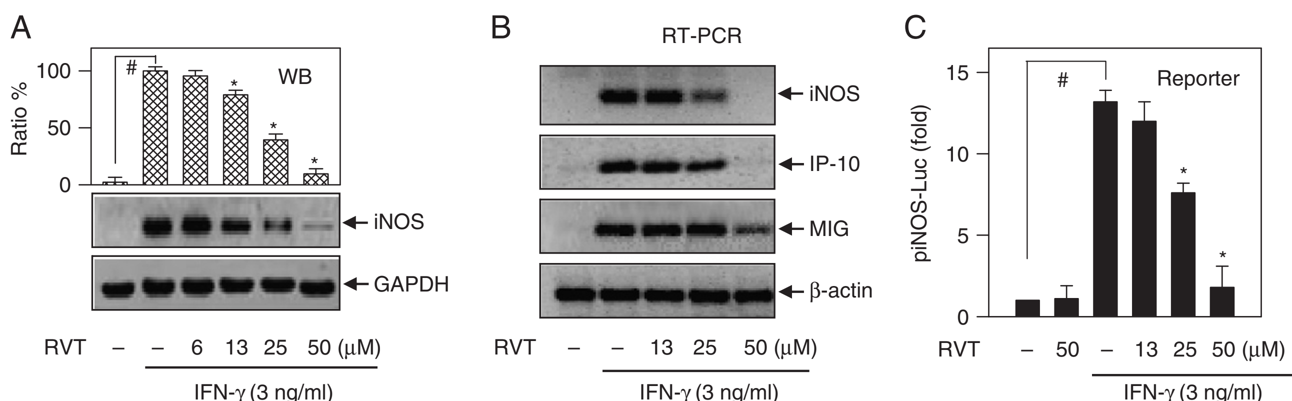

Fig. 2. Effect of resveratrol on IFN-γ-induced iNOS expression. (A) RAW 264.7 cells were stimulated with IFN-γ for 24 h, in the presence of RVT. Cell extracts were subjected to Western blot (WB) analysis with anti-iNOS or anti-GAPDH antibody. One of similar blots is represented, and relative ratio % is also indicated, in which iNOS signal was normalized to GAPDH signal. (B) The cells were pretreated with RVT for 2 h and then stimulated with IFN-γ for 4-6 h, in the presence of RVT. Total RNA of the cells was subjected to an RT-PCR analysis with β-actin gene as an internal control. One of similar results is represented. (C) The cells were transfected with *Renilla* control vector in combination with piNOS-Luc reporter construct and then simulated with IFN-γ for 16 h in the presence of RVT. Cell extracts were subjected to dual-luciferase assay. Luciferase expression is represented as a relative fold, in which firefly luciferase activity was normalized to *Renilla* activity as the transfection efficiency. Data are means±S.D. from three separate experiments. #*P*<.05 vs. media alone-added group. **P*<.05 vs. IFN-γ alone-stimulated group.

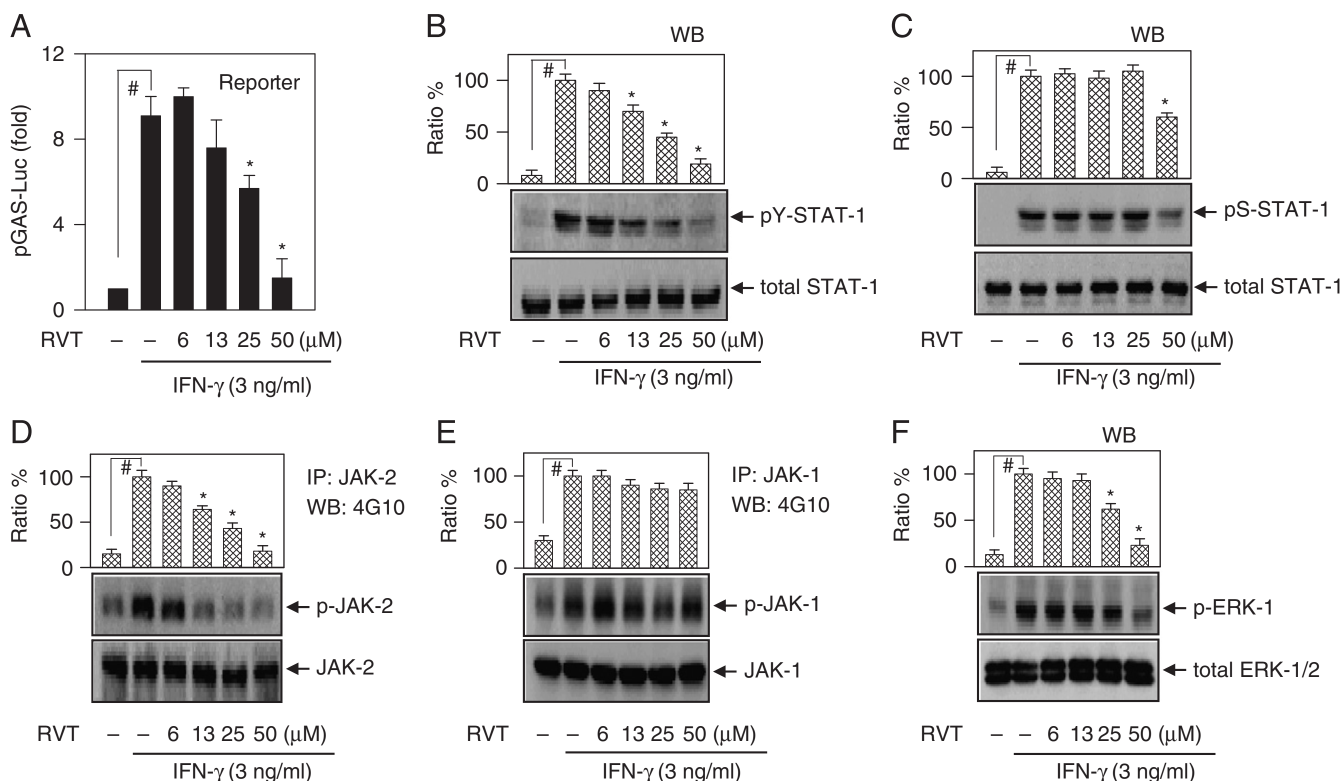

Fig. 3. Effect of resveratrol on IFN-γ-induced STAT-1 activating pathway. (A) RAW 264.7 cells were transfected with *Renilla* control vector in combination with pGAS-Luc reporter construct, and then stimulated with IFN-γ for 16 h in the presence of RVT. Cell extracts were subjected to dual-luciferase assay. Luciferase expression is represented as a relative fold, in which firefly luciferase activity was normalized to the *Renilla* activity. (B and C) The cells were pretreated with RVT for 2 h and stimulated with IFN-γ for 15 min. Cell extracts were subjected to WB analysis with antisera against pY-STAT-1 (Tyr-701) (B) or pS-STAT-1 (Ser-727) (C). One of similar blots is represented, and relative ratio % is also indicated, normalizing to total STAT-1 signal. (D-F) The cells were pretreated with RVT for 2 h and stimulated with IFN-γ for 10 min. Cell extracts were subjected to immunoprecipitation (IP) with anti-JAK-2 (D) or anti-JAK-1 antibody (E), followed by WB analysis with anti-phosphotyrosine antibody 4G10. Cell extracts were also subjected to WB analysis with anti-p-ERK-1 antibody (F). One of similar results is represented, and relative ratio % is also indicated, normalizing to total JAKs or total ERK-1/2 signal. Data are means±S.D. from three to five separate experiments. #P<.05 vs. media alone-added group. *P<.05 vs. IFN-γ alone-stimulated group.

stimulated with IFN-γ in the presence of resveratrol, and their cellular extracts were then subjected to Western blot analysis with anti-p-ERK antibody. Upon exposure to IFN-γ alone, cellular levels of phosphorylated ERK were markedly increased from the basal levels (Fig. 3F). Resveratrol at 25–50 μM significantly reduced IFN-γ-induced ERK activation without affecting the cellular levels of total ERKs (Fig. 3F).

3.5. IFN-γ-induced STAT-1 transcriptional activity is controlled by tyrosine kinase and ERK

The collected results indicate that resveratrol inhibited IFN-γ-induced activation of JAK-2 and also ERK, which could be associated with STAT-1-dependent induction of inflammatory genes. To identify which protein kinase is involved in IFN-γ-induced STAT-1

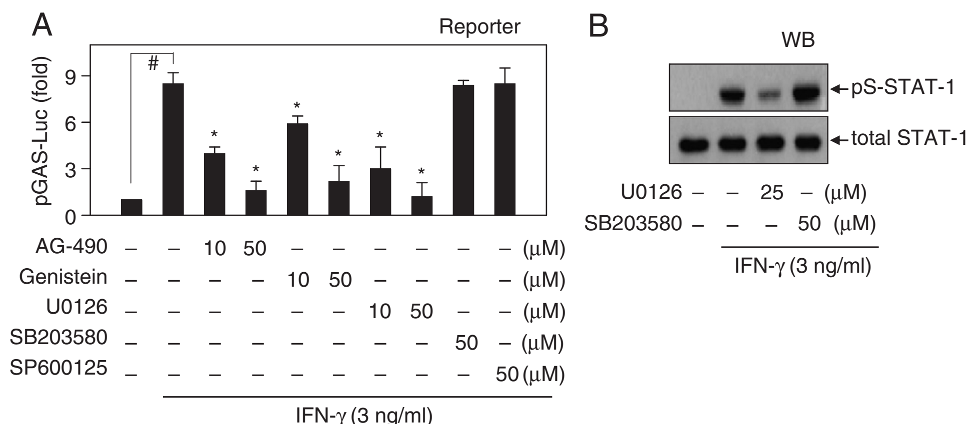

Fig. 4. Effects of pharmacological inhibitors of tyrosine kinase or MAPK on IFN-γ-induced transcriptional activity and serine phosphorylation of STAT-1. (A) RAW 264.7 cells were transfected with *Renilla* control vector in combination with pGAS-Luc reporter construct and then stimulated with IFN-γ for 16 h in the presence of each pharmacological inhibitor. Cell extracts were subjected to dual-luciferase assay. Luciferase expression is represented as a relative fold, in which firefly luciferase activity was normalized to the *Renilla* activity. Data are means±S.D. from three separate experiments. #P<.05 vs. media alone-added group; *P<.05 vs. IFN-γ alone-stimulated group. (B) The cells were pretreated with MAPK inhibitor U0126 or SB203580 for 2 h and stimulated with IFN-γ for 10 min. Cell extracts were subjected to WB analysis with anti-pS-STAT-1 (Ser-727) antibody.

transcriptional activity, we first used the specific JAK-2 inhibitor AG490 or tyrosine-specific protein kinase inhibitor genistein. RAW 264.7 cells were stimulated with IFN-γ in the presence of AG490 or genistein and then evaluated for luciferase expression in the cells harboring pGAS-Luc reporter construct. Treatment of either AG490 or genistein inhibited IFN-γ-induced STAT-1 transcriptional activity (Fig. 4A). We next investigated effects of MAPKs using specific MEK-1/2 inhibitor U0126, p38 MAPK inhibitor SB203580 or JNK inhibitor SP600125. The cells harboring pGAS-Luc reporter construct increased the luciferase expression upon exposure to IFN-γ alone, and this luciferase induction was inhibited by treatment of U0126 but not affected by SB203580 or SP600125 (Fig. 4A). The differential actions of MAPKs were further confirmed, due to the upstream target of serine phosphorylation of STAT-1. IFN-γ induced the Ser^{727} phosphorylation of STAT-1, which was consistently inhibited by treatment of U0126 but not by SB203580 (Fig. 4B).

4. Discussion

This study was designed to investigate effects of resveratrol (Fig. 1A) on IFN-γ receptor-mediated expression of inflammatory genes and to elucidate a molecular basis of the action. We first quantified inflammatory mediators in IFN-γ-activated macrophages. Treatment of resveratrol inhibited IFN-γ-induced production of NO, IP-10 or MIG in RAW 264.7 cells (Fig. 1B and Table 1) and also NO production in primary macrophages-derived from bone marrows of C3H/HeJ (TLR-$4^{-/-}$) mice (Fig. 1C), in which LPS-induced NF-κB activation is excluded. Moreover, treatment of resveratrol diminished protein levels of iNOS in IFN-γ-activated RAW 264.7 cells (Fig. 2A), differentially attenuated mRNA levels of iNOS, IP-10 or MIG (Fig. 2B) as well as inhibited promoter activity of iNOS gene (Fig. 2C). These results indicate that resveratrol could restrain inflammatory responses in IFN-γ-activated macrophages through down-regulating the expression of inflammatory genes at the transcription level. This mode of resveratrol's action is consistent with previous effects on iNOS expression reported in LPS-activated macrophages or microglia systems [36,37]. However, this contrasts with the effect reported in LPS-activated Kupper cells, in which iNOS transcription was not affected by treatment of resveratrol, but posttranscriptional process for enzyme activity was reduced [40].

Gene-knockout studies provide a physiologic importance of STAT-1 in the inflammatory and immune responses of IFN-γ [41]. Since resveratrol down-regulated IFN-γ-induced transcription of inflammatory genes, we next tested whether resveratrol could affect STAT-1 activation in macrophages. Supporting the hypothesis, treatment of resveratrol inhibited IFN-γ-induced transcriptional activity of STAT-1 (Fig. 3A), and also IFN-γ-induced Tyr^{701} or Ser^{727} phosphorylation, as the activation index of STAT-1 (Fig. 3B and C). We then focused on protein kinases upstream of STAT-1 phosphorylation. Treatment of resveratrol inhibited IFN-γ-induced activation of JAK-2 and also ERK, in which JAK-2 was more sensitive (Fig. 3D, F) but could not affect that of JAK-1 (Fig. 3E). Further supporting, IFN-γ-induced STAT-1 transcriptional activity was consistently inhibited by treatment of JAK-2 inhibitor or MEK-1/2-mediated ERK activation inhibitor (Fig. 4A). Similarly, resveratrol functions as an effective tyrosine kinase inhibitor affecting Src and JAKs, thus resulting in loss of STAT-1 activation for sensitizing docetaxel-resistant prostate tumor cells to the tumor necrosis factor (TNF)-related apoptosis inducing ligand [42].

Taken together, resveratrol inhibited STAT-1 phosphorylation in response to IFN-γ, which could contribute to its down-regulatory mechanism on iNOS or chemokine expression in macrophages. STAT-1-responsive GAS motifs have been identified in the promoter regions of inflammatory genes: iNOS with 3 GAS sites upstream from −722 relative to the transcription start; IP-10 with one site at −224/−212; MIG with two sites at −222/−198 and −99/−85 [19–21]. Treatment of resveratrol suppressed IFN-γ-induced mRNA levels of MIG to a considerably less extent than those of iNOS and IP-10 (Fig. 2B). This differential action could speculate the possibility that additional promoter elements may be required to recapitulate maximal MIG responsiveness in IFN-γ-activated macrophages, since the 5′-flanking region of MIG gene contains three copies of the ISRE, three copies of the κB and one copy of the AP-1 in addition to the GAS motifs [21]. Recently, resveratrol has been reported to inhibit JAK-mediated STAT-1 phosphorylation for apoptosis regulation in docetaxel-resistant PC3-DR and DU145-DR prostate tumor cells and A431 epidermoid carcinoma cells [42,43].

There has been a great interest in investigating the effects of resveratrol on transcription factors, particularly NF-κB and AP-1 that regulate the expression of inflammatory genes. Resveratrol was reported to affect NF-κB activation in concanavalin A-activated spleen cells, resulting in inhibition of IFN-γ production [35]. Resveratrol has evidenced to down-regulate iNOS expression via NF-κB and/or AP-1 in cell-based models of LPS-activated macrophages (RAW 264.7, J774, mouse peritoneal cells), LPS-activated N9 microglial cells [36–38] and β-amyloid-activated C6 glioma cells and also in animal models of 12-*O*-tetradecanoylphorbol-13-acetate-applied mouse epidermis and sodium taurocholate-induced acute pancreatitis in rats [44,45]. In this study, resveratrol also inhibited NF-κB activation in LPS-activated RAW 264.7 cells with an IC_{50} value of 40 μM (Fig. 5). However, STAT-1 activation in the same cells was more sensitively affected by resveratrol, showing an IC_{50} value of 29 μM in IFN-γ-induced STAT-1 transcriptional activity (Fig. 3A).

IFN-γ activates some transcription factors, equivalently in wild-type and STAT-$1^{-/-}$ cells [46]. These transcription factors appear to function in parallel with STAT-1 to mediate inflammatory responses and cellular immunity in response to IFN-γ. IFN-γ induces NF-κB activation in 2fTGH fibrosarcoma cells lacking STAT-1 [47]. IFN-γ-dependent gene expression in mouse embryo fibroblasts requires inhibitory IκB kinase activity, in addition to STAT-1 activation, but bypasses the conventional NF-κB-mediated pathway [48]. IFN-γ rapidly and transiently induces AP-1 activation in mouse embryo fibroblasts, HeLa epithelial carcinoma cells, and HL-60 promyelomonocytic cells [28,49]. IFN-γ-mediated AP-1 activation is independent of JAK or STAT-1, because it can still be observable in JAK-$1^{-/-}$ or STAT-$1^{-/-}$ fibroblasts [28]. Interestingly, Vila-del Sol et al. delineated that IFN-γ generated an early response depending upon STAT-1 activation, using a model of iNOS induction in RAW 264.7 cells and

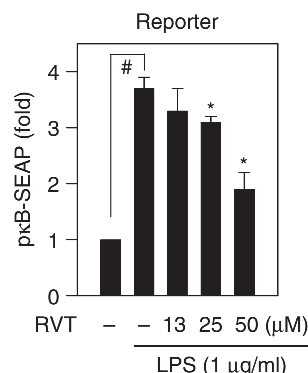

Fig. 5. Effect of resveratrol on NF-κB activation. RAW 264.7 cells were transfected with pSV-β-galactosidase control vector in combination with pκB-SEAP reporter construct, and then stimulated with LPS for 18 h in the presence of RVT. SEAP activity was measured with the culture media and β-galactosidase activity with cell extracts. SEAP expression is represented as a relative fold, in which SEAP activity was normalized to β-galactosidase activity as the transfection efficiency. Data are means±S.D. from three separate experiments. #P<.05 vs. media alone-added group; *P<.05 vs. LPS alone-stimulated group.

peritoneal macrophages from TNF-α-deficient (TNF-$\alpha^{-/-}$) mice, and TNF-α secretion in response to IFN-γ activated NF-κB, in an autocrine manner, for additional responses [50]. In general, NF-κB or AP-1 activation in response to IFN-γ could be uncoupled with conventional STAT-1 activation.

Resveratrol has been known to inhibit NF-κB or AP-1 activation, demonstrating its effects in TNF or LPS-stimulated macrophages, lymphoid and epithelial cells [7,42]. This study firstly proposes another mechanism of resveratrol to inhibit IFN-γ-induced STAT-1 activation, which contributes to its down-regulatory effects on STAT-1-regulated expression of iNOS, IP-10 and MIG, restraining inflammatory responses in IFN-γ alone-stimulated macrophages. Taken together, this study may explain, at least in part, the resveratrol's effects reducing cardiovascular diseases and preventing oncogenesis by attenuation of the early stage of inflammation.

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