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The TRIF/TBK1/IRF-3 activation pathway is the primary inhibitory target of resveratrol, contributing to its broad-spectrum anti-inflammatory effects

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Resveratrol, a stilbene type compound identified in wine and fruit juice, has been found to exhibit various pharmacological activities such as anti-oxidative, anti-cancerous, anti-inflammatory and anti-aging effects. Although numerous papers have explored the pharmacology of resveratrol in one particular cellular action, how this compound can have multiple effects simultaneously has not been fully addressed. In this study, therefore, we explored its broad-spectrum inhibitory mechanism using lipopolysaccharide (LPS)-mediated inflammatory responses and reporter gene assays involving overexpression of toll like receptor (TLR) adaptor molecules. Co-transfection of adaptor molecules such as (1) myeloid differentiation primary response gene 88 (MyD88), (2) Toll/4Il-1 Receptor-domain-containing adapter-inducing interferon-β (TRIF), (3) TRIF-related adaptor molecule (TRAM), or (4) TANK-binding kinase (TBK) 1 strongly enhanced luciferase activity mediated by transcription factors including nuclear factor (NF)-κB, activator protein (AP)-1, and interferon regulatory factor (IRF)-3. Of the adaptor proteins, TRIF and TBK1 but not MyD88 and IKK enhanced luciferase activity mediated by these transcription factors. Resveratrol dose-dependently suppressed LPS-induced NO production in macrophages. It also blocked the increases in levels of mRNA for IFN-β, tumor necrosis factor (TNF)-α, and inducible nitric oxide synthase (iNOS) that were induced by LPS. Resveratrol diminished the translocation or activation of IRF-3 at 90 min, c-Jun, a subunit of AP-1, and STAT-1 at 120 min, and p50, a subunit of NF-κB, at 60 and 90 min. Resveratrol strongly suppressed the up-regulation of luciferase activity induced by these adaptor molecules with IC_{50} values of 5 to 65 μM. In particular, higher inhibitory effects of resveratrol were when TRIF or TBK1 were overexpressed following cotransfection of luciferase constructs with IRF-3 binding sequences. Taken together, our data suggest that the suppression of TRIF and TBK1, which mediates transcriptional activation of NF-κB, AP-1, and IRF-3, contributes to resveratrol's broad-spectrum inhibitory activity, and that this compound can be further developed as a lead anti-inflammatory compound.

1. Introduction

Inflammation is a natural defensive response mediated by various immune cells such as macrophages, neutrophils and eosinophils (Nagata 2005). Through experiments with macrophages, a variety of molecular and cellular events have been identified. For example, macrophages produce large amounts of various inflammatory mediators [nitric oxide (NO), prostaglandin E_2 (PGE_2) and some cytokines such as TNF-α] (Lee et al. 2008). Interactions between surface receptors [pattern recognition receptor (PPR)] such as toll-like receptor (TLR)-4 or TLR-2 and their ligands derived from bacterial products such as lipopolysaccharide (LPS) are very critical initiation points for the activation of macrophages (Takeuchi and Akira 2001). Then, various adaptor molecules such as myeloid differentiation primary response gene 88 (MyD88), Toll/Il-1 Receptor-domain-containing adapter-inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM), and their associated enzymes such as TANK-binding kinase (TBK) 1 are continuously activated so that they eventually induce translocation and activation of transcription factors such as nuclear factor (NF)-κB, activator protein (AP)-1, and interferon regulatory factor (IRF)-3 (Takeuchi and Akira 2001; Yamamoto and Akira 2009). To date, it has been reported that MyD88 is an important adaptor molecule activating NF-κB- and AP-1-mediated transcriptional events, while the TRIF/TBK1 pathway is critical for IRF-3-mediated transcription (Kawai and Akira 2005). After all, these factors contribute to the production of vastly different inflammatory cytokines, mediators, and chemokines which mediate the total inflammatory response. Surprisingly, accumulated studies strongly suggest that pro-

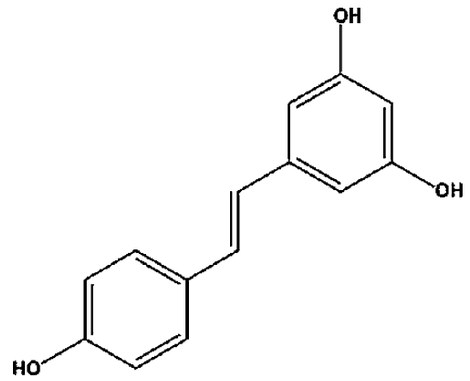


Fig. 1: Chemical structure of resveratrol

longed inflammatory states in the body can cause or contribute to various chronic and acute diseases such as cancer, diabetes, atherosclerosis, and osteoporosis (Ferencik et al. 2007; Hogg 1998). This notion encouraged us to try to develop drugs with anti-inflammatory properties for the treatment or prevention of such serious diseases.

Resveratrol (Fig. 1) is a polyphenol compound isolated from grapes and other plants (Bavaresco et al. 1999). Like other polyphenol compounds, resveratrol displays beneficial, e.g., anti-oxidative properties (Guerrero et al. 2009). Furthermore, resveratrol has been reported to modulate various defensive cellular responses such as inhibition of cancer cell proliferation, induction of apoptosis, and suppression of inflammatory responses (Dulak 2005; Meeran and Katiyar 2008; Surh et al. 2001; Yadav et al. 2009). At the molecular level, this compound appears to block signal transduction processes required for new protein synthesis by suppressing transcriptional processes essential for proliferation, survival, apoptosis, and inflammation. For example, most previous papers reported that resveratrol inhibits the activation of NF-κB and AP-1, critical transcription factors required for the above-noted cellular events (Kundu and Surh 2004, 2008). As a result, resveratrol can suppress the expression of various cytokines, inflammatory mediators, and anti-apoptotic proteins in macrophages and cancer cell lines (Manson 2005; Surh et al. 2001). Nonetheless, which inflammatory pathway is the most sensitive target in resveratrol pharmacology, and how this compound displays multiple actions at the same time (explaining its broad-spectrum pharmacology) has not yet been elucidated. In this study, therefore, we addressed these issues using lipopolysaccharide (LPS)-mediated inflammatory responses and a reporter gene assay established using overexpression of TLR adaptor molecules.

2. Investigations, results and discussion

Overexpression of signaling molecules linked to TLR activation upon cotransfection with reporter gene (luciferase) constructs with inflammatory transcription factor binding sites strongly enhanced NF-κB-, AP-1-, and IRF-3-mediated luciferase activities (Fig. 2) as reported previously (Ahn et al. 2009; Youn et al. 2009). Thus, the overexpression of MyD88 and IKKβ up-regulated luciferase activity mediated by NF-κB activation up to 100 fold (Figs. 2 and 4). TRIF and TBK1 cotransfection enhanced NF-κB-mediated luciferase activity 2,500-fold (Figs. 2 and 4). The overexpression of MyD88, TRIF, and TBK1 also increased AP-1-mediated luciferase activity 5 to 16 fold (Figs. 2 and 5), although the levels induced were not striking compared to the NF-κB-mediated response. As reported previously (Youn et al. 2009, 2006), co-transfection of TRIF or TBK1 with an IFN-β-promoter containing luciferase constructs powerfully enhanced IRF-3-mediated luciferase activity by 1,000-fold (Figs. 2 and 6). These results indicate that TLR adaptor molecules share the machinery for activation of transcription factors and that a TRIF/TBK1 pathway seems to play a critical role in multiple transcriptional activations that contribute to inflammatory responses.

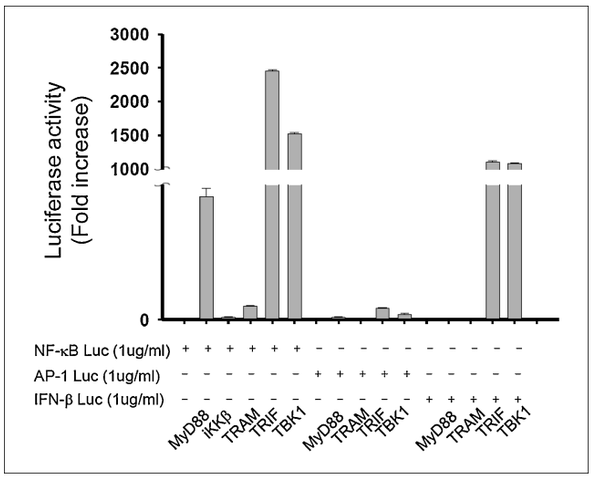


Fig. 2: The effect of adaptor proteins on the up-regulation of luciferase activity mediated by NF-κB, AP-1, and IRF-3. HEK293 cells (1×10^6 cells/ml) were co-transfected with constructs expressing adaptor molecules (MyD88, TRAM, TRIF, IKK or TBK1) and promoter binding site-containing reporter genes [NF-κB-Luc, AP-1-Luc, CREB-Luc (each 1 μg/ml)] and β-gal (0.1 μg/ml, as a transfection control). After 24 h incubation, luciferase activity from lysed samples was measured by a luminometer

Even though a variety of transfection reagents with less cytotoxicity and higher efficiency have been developed, not many papers have published data obtained from transfection of DNA constructs into RAW264.7 cells, due to low efficiency. In contrast, HEK293 cells are the most widely used cell line for transfection experiments (Lee et al. 2004), although this cell line is quite different from macrophages. Nonetheless, cotransfection of such adaptor molecules with promoter constructs with DNA binding sites for NF-κB, AP-1, and IRF-3 significantly and dramatically enhanced reporter gene (luciferase)-derived enzyme activity. The fact that IKKβ never up-regulated AP-1- or IRF-3-induced luciferase activity, and that MyD88 cotransfection with an IFN-γ-promoter construct failed to enhance IRF-induced luciferase activity (Fig. 2), indicate that the results from our cell system are not nonspecifically generated merely by overexpression conditions. Indeed, because of their specificity, these conditions have been widely used for anti-inflammatory drug screening experiments (Kim et al. 2006; Lee et al. 2004; Youn et al. 2006). Unexpectedly, we also found that overexpression of TRIF and TBK1 enhanced AP-1 mediated luciferase activity. However, it is not clear how these proteins can activate the translocation and activation of AP-1. To further probe the molecular mechanism underlying this finding, we analyzed the involvement of amino acid residues of these proteins and other molecules that act as a bridge between TRIF/TBK1 and mitogen-activated protein kinases (MAPKs). These other molecules include extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase JNK and p38, which are required for the phosphorylation and translocation of AP-1 (c-Jun/c-Fos), as suggested by the analysis of structural determinants of TRIF essential for the activation of IRF-3 (Tatematsu et al. 2010).

It was reported that resveratrol blocks inflammatory signaling events (Lu et al. 2010; Oh et al. 2009). This compound was then shown to suppress the expression of inflammatory genes such as IFN-β, TNF-α, and iNOS under our conditions (Fig. 3A). This compound also significantly blocked the

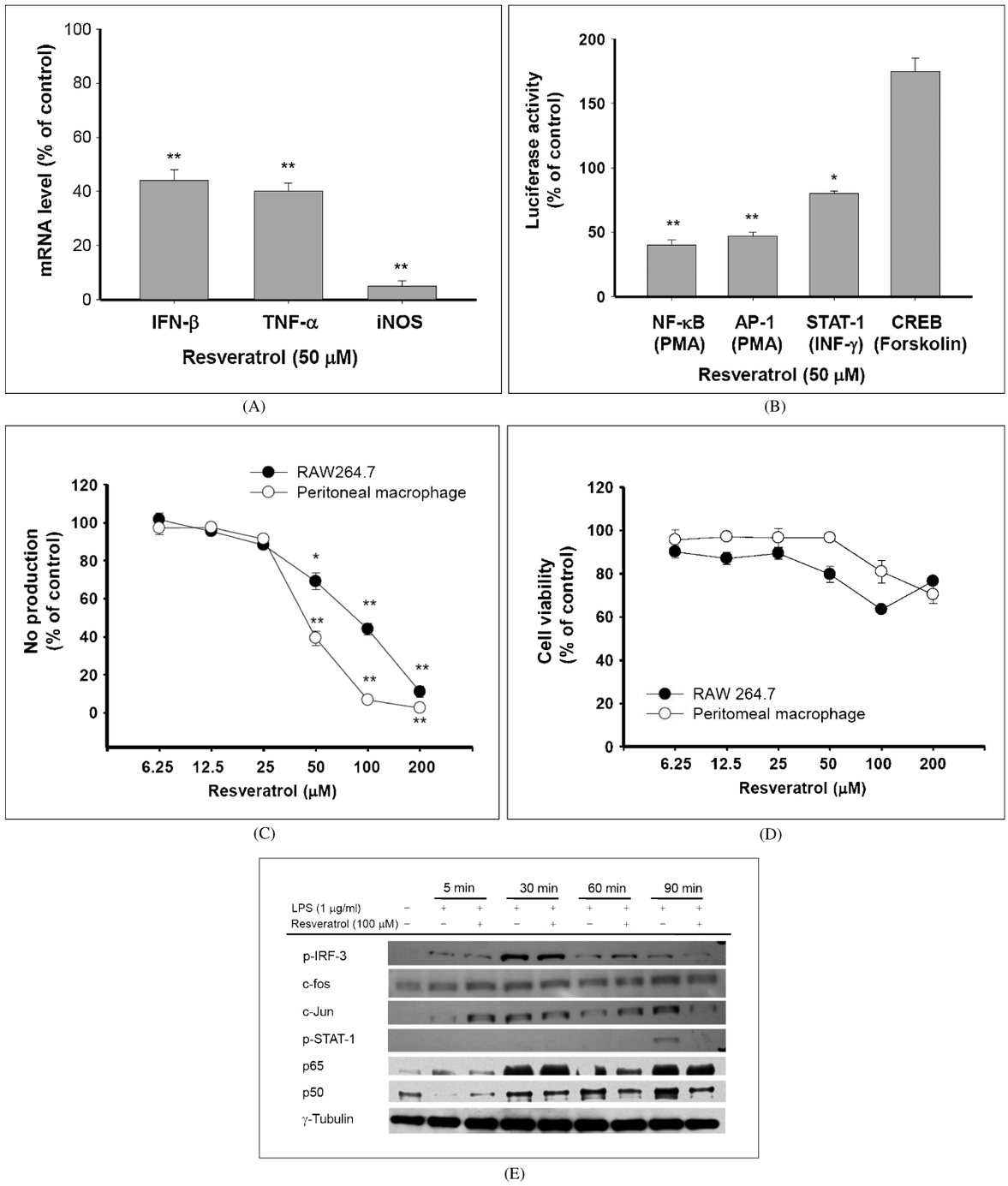


Fig. 3: The inhibitory effect of resveratrol on LPS-mediated inflammatory responses in RAW264.7 cells and transcriptional activation in HEK293 cells. (A) RAW264.7 cells (1×10^6 cells/ml) were treated with resveratrol in the presence or absence of LPS (1 μg/ml) for 6 h. mRNA levels of IFN-β, TNF-α, and iNOS were analyzed by real-time PCR. (B) HEK293 cells (1×10^6 cells/ml) co-transfected with plasmid constructs NF-κB-Luc, STAT-1-Luc, AP-1-Luc, CREB-Luc (each 1 μg/ml) and β-gal (0.1 g/ml, as a transfection control) were treated with resveratrol in the presence or absence of PMA (100 nM, 8 h), IFN-γ (250 U/ml,24 h), and forskolin (2 μM, 8 h). Luciferase activity was measured by a luminometer. (C) RAW264.7 cells (1×10^6 cells/ml) were treated with resveratrol in the presence or absence of LPS (1 μg/ml) for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined by Griess reagent. (D) Cell viability was determined by MTT assay, as described in Materials and Methods. (E) Levels of transcription factors translocated into nucleus was analyzed by immunoblotting *: $P < 0.05$ and **: $P < 0.01$ compared to the control group

activation of redox-sensitive transcription factors such as NF-κB, AP-1, and STAT-1, but not CREB (Fig. 3B). Indeed, resveratrol dose-dependently (0 to 200 μM) inhibited NO production (Fig. 3C) without altering cell viability (Fig. 3D). In agreement with luciferase assays, this compound diminished the activation and translocation of transcription factors including phospho-IRF-3, c-Jun/AP-1, and phospho-STAT-1 at 90 min, and p50, a component of NF-κB, at 60 and 90 min (Fig. 3E). Taken together with previous reports that this compound inhibits MAPK, and NF-κB and AP-1 activation induced by LPS from *E. coli* and *Porphyromonas gingivalis* in microglia and peritoneal macrophages (Huang et al. 2008; Kang et al.

Table 1: Inhibitory potency (IC_{50} value) of resveratrol in the upregulation of luciferase activity induced by various adaptor molecules and their downstream kinases

Molecule	IC_{50} value (μM)		
	NF-κB	AP-1	IRF-3
MyD88	20.9 ± 3.7	67.5 ± 2.7	NA
IKK	30.6 ± 0.7	NA	NA
TRAM	17.5 ± 1.1	NA	NA
TRIF	14.9 ± 1.2	NA	6.2 ± 3.1
TBK1	11.2 ± 0.2	17.1 ± 0.1	8.9 ± 2.4

NA: Not available

2009; Kundu and Surh 2004), our results strongly suggest that resveratrol can suppress several inflammatory responses by blocking the activation of multiple inflammatory transcription factors.

Recently, a growing number of reports have suggested the functional importance of adaptor molecules associated with TLRs. Indeed, it has been suggested that TLRs can serve as an anti-inflammatory drug target using adaptor molecules (Akira 2000; Takeda and Akira 2005). Examples of these molecules are MyD88 and TRIF. Knockout of these molecules leads to a defect in bacterial and viral immunity and to inflammatory responses such as cytokine production, lethality, and inflammatory mediator production mediated by NF-κB, AP-1, and IRF-3 (Akira 2000; Takeuchi and Akira 2002). Therefore, we next examined whether functional activation of these mediators can be suppressed by resveratrol treatment using conditions in which adaptor molecules such as TRAM, IRIF, and MyD88 are transfected and overexpressed, and in which there is co-transfection of reporter gene (luciferase) constructs with NF-κB, AP-1, and IRF-3 binding sites.

Our data strongly suggested that inflammatory responses induced by MyD88, IKKβ, TRAM, TRIF, and TBK1 can be targeted by resveratrol. Thus, NF-κB activation generated by these molecules was dose-dependently suppressed by resveratrol with IC_{50} values ranging from 11 to 30 μM (Fig. 4). AP-1 activation induced by overexpression of MyD88, TRIF, and TBK1 was diminished by resveratrol, being 2 to 3 fold lower than under NF-κB activation conditions (Fig. 5). Interestingly, the strongest inhibitory potency of resveratrol was seen under IRF-3 activation conditions in which IC_{50} values of 6 to 9 μM were observed (Fig. 6). Given these inhibitory potencies, these data implied that IRF-3 activation conditions could be the primary target of resveratrol, and that a series of signaling cascades containing TRIF and TBK1 could be a more sensitive and stronger inhibitory target in resveratrol pharmacology.

How resveratrol simultaneously suppresses the activation of NF-κB, AP-1, and IRF-3 (Table 1) is not clear yet. Considering that overexpression of TRIF and TBK1 is able to enhance the transcriptional activation of transcription factors (Fig. 2), TRIF and TBK1 and their downstream proteins could be important components in the general expression of inflammatory genes mediated by NF-κB, AP-1, and IRF-3. Indeed, several TLR ligands such as poly(I:C), CpG, LPS, pam3CSK, and some live or dead bacteria enhanced the functional activation of the TRIF/TBK1 pathway (Aksoy et al. 2005; De Trez et al. 2005). Furthermore, a report that resveratrol strongly suppressed kinase activity of recombinant TBK1 also support this possibility (Youn et al. 2005). Such findings seem to explain why resveratrol can have a broad-spectrum anti-inflammatory response. Nonetheless, the inhibitory effect of resveratrol on IKK-induced NF-κB luciferase activity suggests that it can act as a direct inhibitor of IKK, although the activity under IKK overexpression conditions was 3 to 4 fold weaker than when TRIF- or TBK1-mediated luciferase activity. These broad-spectrum inhibitory properties of resveratrol seem to be due to its simple chemical structure, since compounds with a stilbene type structure such as piceatannol have broad inhibitory activity against various enzymes (Billack et al. 2008; Kim et al. 2008; Lee et al. 2009). Resveratrol has been taken in the form of wine for a long time (Wallerath et al. 2002), and it can be considered as a good leading compound for the development of anti-inflammatory drugs (Chen et al. 2005; Cho et al. 2002). So far, we have synthesized 150 different derivatives of resveratrol and have found few compounds with 10 to 100 fold upregulated activity (data not shown). Therefore, we are currently testing the possibility that these novel compounds can be developed as therapeutic drugs against several inflammatory diseases.

In conclusion, we found that resveratrol can act as a broad-spectrum anti-inflammatory drug. It blocked the up-regulation of luciferase-activity induced by NF-κB, AP-1, and STAT-1 and nuclear translocation or activation of these transcription factors. In particular, in a reporter gene luciferase assay cotransfected with various TLR adaptor proteins such as TRIF, MyD88, and TRAM, we found that TRIF/TBK1-mediated transcriptional up-regulation is blocked by resveratrol and that the IRF-3-mediated activation pathway can be a predominant inhibitory target of resveratrol. Therefore, our data suggest that the broad inhibitory activity of resveratrol is due to suppression of the TRIF/TBK1 pathway and that novel, multi-targeted anti-inflammatory drugs can be further developed by chemical optimization of resveratrol.

3. Experimental

3.1. Materials

Resveratrol, interferon (IFN)-γ, forskolin, and lipopolysaccharide (LPS, *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO). LY294002 (LY), wortmannin (Wort), parthenolide (Parth), and BAY11-7082 (BAY) were obtained from Calbiochem (La Jolla, CA). Fetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY). RAW264.7 and HEK293 cells were purchased from the American Tissue Culture Center (Rockville, MD). All other chemicals were of Sigma grade. Phospho- or total antibodies to IRF-3, c-Fos, c-Jun, STAT-1, p65, p50, and γ-tublin were purchased from Cell Signaling (Beverly, MA) or Santa Cruz (Santa Cruz, CA).

3.2. Plasmids

Luciferase constructs containing NF-κB, CREB, STAT-1, IRF-3, and AP-1 binding promoters were used as previously reported (Kim et al. 2010; Lee et al. 2008). Wild types of MyD88, IKKβ, TRIF, TBK1, and IRF-3 were obtained from Addgene (Cambridge, MA). The wild-type IRF3 was obtained from G. Cheng (University of California, Los Angeles, CA). All DNA constructs were prepared in large scale using EndoFree Plasmid Maxi kit (Qiagen) for transfection.

3.3. Mice

Six-week old, C57BL/6 male mice (6 weeks old, 17–21 g) were obtained from DAEHAN BIOLINK (Chungbuk, Korea) and maintained in plastic cages under conventional conditions. Water and pelleted diets (Samyang, Daejeon, Korea) were supplied *ad libitum*.

3.4. Cell culture

RAW264.7 and HEK293 cells were maintained in RPMI1640 or DMEM supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum. Cells were grown at 37 °C with 5% CO_2.

3.5. Preparation of peritoneal macrophages

Preparation of peritoneal macrophages was performed in accordance with guidelines established by the Kangwon University Institutional Animal Care

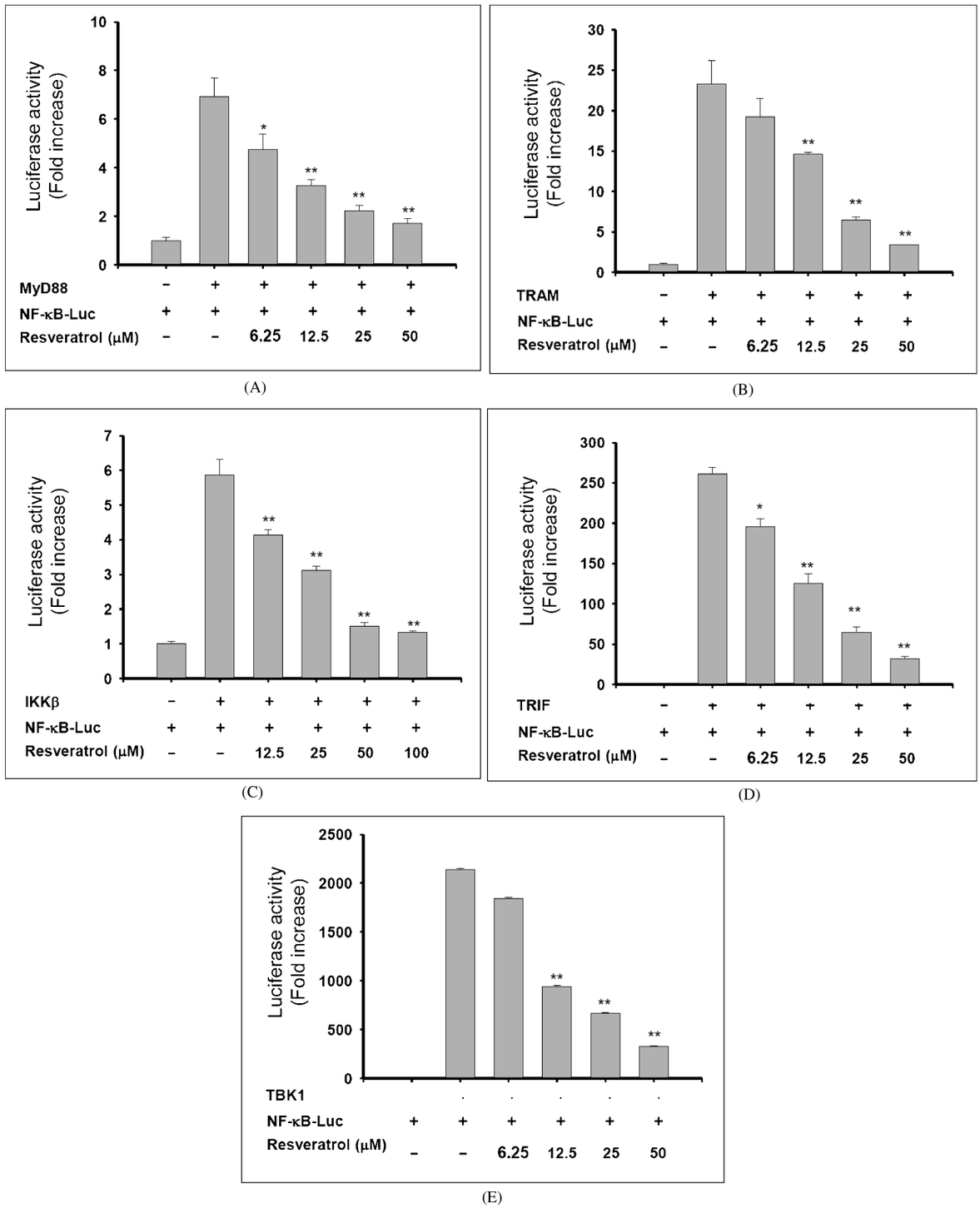
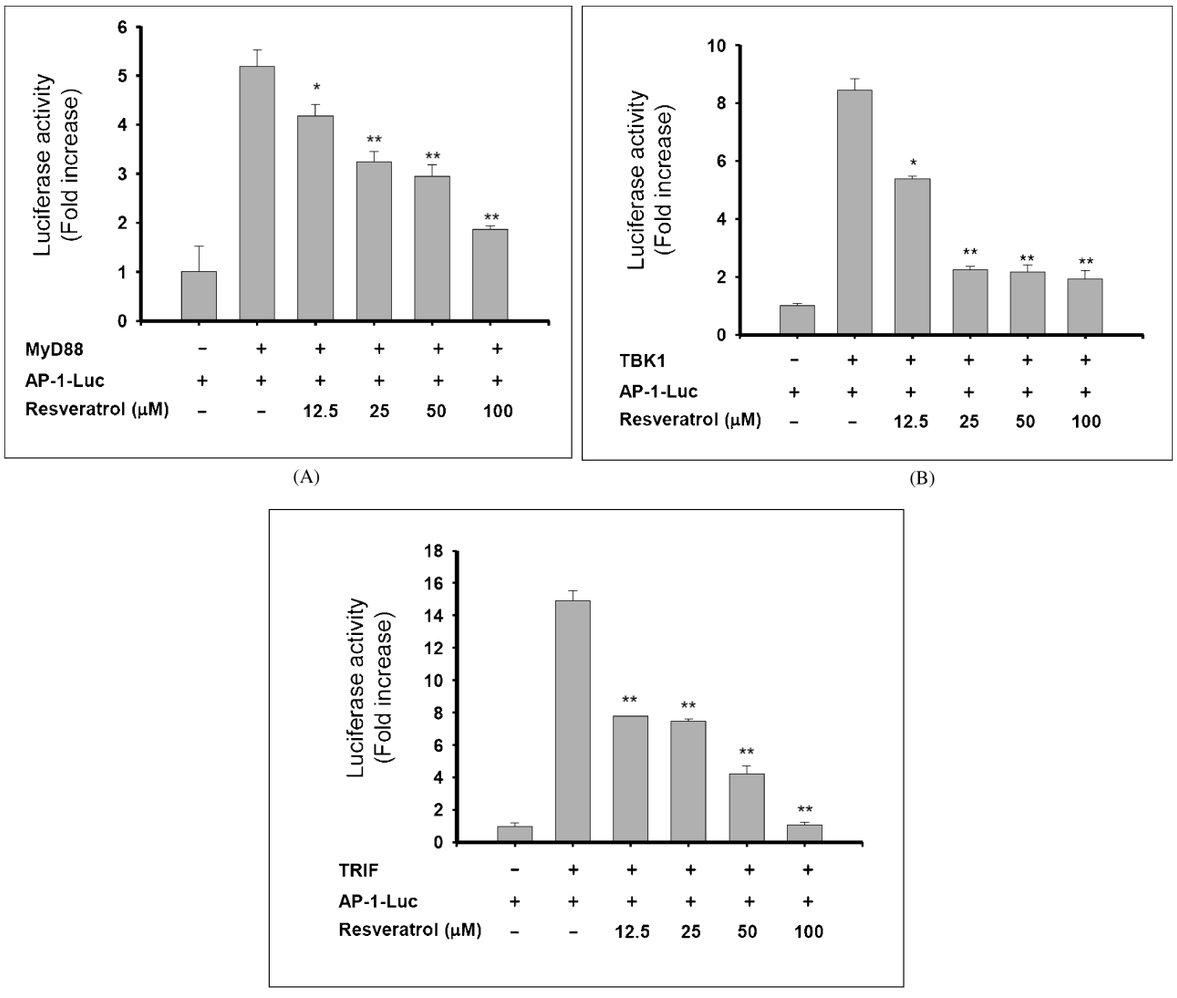


Fig. 4: The effect of resveratrol on the up-regulation of NF-κB-mediated luciferase activity induced by adaptor molecules. (A, B, C, D, and E) HEK293 cells (1×10^6 cells/ml) were co-transfected with constructs expressing adaptor molecules (MyD88, TRAM, TRIF, IKK or TBK1) and NF-κB-Luc (1 μg/ml)] and β-gal (0.1 μg/ml, as a transfection control). After 24 h incubation, luciferase activity from lysed samples was measured by a luminometer. *: $P < 0.05$ and **: $P < 0.01$ compared to control

and Use Committee. Peritoneal exudates were obtained from C57BL/6 male mice (7–8 weeks old, 17–21 g) by lavaging 4 days after intraperitoneal injection of 1 ml of sterile 4% thioglycollate broth (Difco Laboratories, Detroit, MI). After washing with RPMI 1640 medium containing 2% FBS, peritoneal macrophages (1×10^6 cells/ml) were plated in 100 mm tissue culture dishes for 4 h at 37 °C in a 5% CO_2 humidified atmosphere (Kim et al. 2009).

3.6. mRNA detection by real-time reverse transcription-PCR

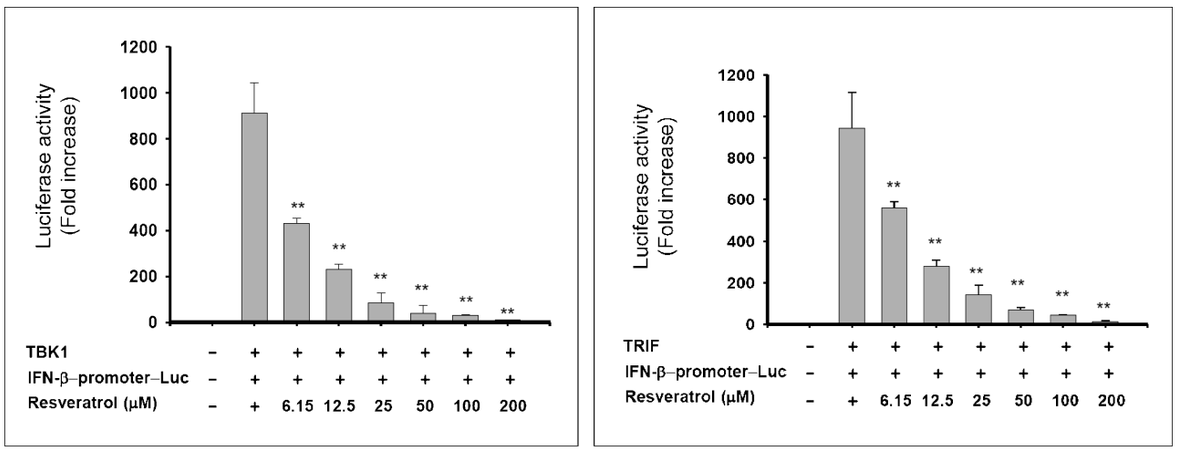
Total RNA from LPS-treated RAW264.7 cells (5×10^6 cells/ml) was prepared by adding TRIzol Reagent (Gibco BRL) according to the manufacturer's protocol. Total RNA (1 μg) was incubated with oligo-dT15 for 5 min at 70 °C and mixed with a 5 × first-strand buffer, 10 mM dNTPs, and 0.1 M DTT. The reaction mixture was further incubated for 5 min at 37 °C



(A) (B)

(C)

Fig. 5: The effect of resveratrol on the up-regulation of AP-1-mediated luciferase activity induced by adaptor molecules. (A, B, and C) HEK293 cells (1×10^6 cells/ml) were co-transfected with constructs expressing adaptor molecules (MyD88, TRIF, or TBK1) and AP-1-Luc (1 μg/ml)] and β-gal (0.1 μg/ml, as a transfection control). After 24 h incubation, luciferase activity from lysed samples was measured by a luminometer. *: $P < 0.05$ and **: $P < 0.01$ compared to control



(A) (B)

Fig. 6: The effect of resveratrol on the up-regulation of IRF-3-mediated luciferase activity induced by adaptor molecules. (A and B) HEK293 cells (1×10^6 cells/ml) were co-transfected with constructs expressing adaptor molecules (TRIF, or TBK1) and IRF-3-containing IFN-β-promoter-Luc (1 μg/ml)] and β-gal (0.1 μg/ml, as a transfection control). After 24 h incubation, luciferase activity from lysed samples was measured by a luminometer. *: $P < 0.05$ and **: $P < 0.01$ compared to control

Table 2: PCR primers used in this experiment

Name		Sequence (5' to 3')
iNOS	F	GGA GCC TTT AGA CCT CAA CAG A
	R	TGA ACG AGG AGG GTG GTG
TNF-α	F	TGC CTA TGT CTC AGC CTC TTC
	R	GAG GCC ATT TGG GAA CTT CT
IFN-β	F	TCC AAG AAA GGA CGA ACA TTC G
	R	TGA GGA CAT CTC CCA CGT CAA
GAPDH	F	CAA TGA ATA CGG CTA CAG CAA C
	R	AGG GAG ATG CTC AGT GTT GG

and for 60 min after the addition of MuLV reverse transcriptase (2 U). Reactions were terminated after 10 min at 70 °C, and total RNA was depleted by adding RNase H. For real-time PCR analysis, one microgram of RNA was submitted to reverse transcription with the Molony murine leukemia virus reverse transcriptase (Invitrogen). Two microliters of cDNA obtained for each sample were submitted to a qPCR protocol using the SYBR green Master mix method (Applied Biosystems, Foster City, CA) in the ABO sequence detection system, The results were normalized with the 18S transcript. The primers (Bioneer, Daejeon, Korea) used in this experiment are indicated in Table 2.

3.7. Transfection and luciferase reporter gene activity assay

HEK293 cells (1×10^6 cells/ml) were transfected with 1 μg of plasmids with NF-κB-Luc or AP-1-Luc as well as β-galactosidase using a calcium phosphate method in a 12-well plate according to the manufacturer's protocol. The cells were used for experiments 48 h after transfection. Luciferase assays were performed using the Luciferase Assay System (Promega) (Jeon et al. 2009; Kim et al. 2010). Briefly, the transfected cells treated with testing compounds in the presence of PMA (20 ng/ml) or TNF-α (20 ng/ml) were lysed in the culture dishes with reporter lysis buffer. Lysates were centrifuged at maximum speed for 10 min in an Eppendorf microcentrifuge. Ten μl of the supernatant fraction were incubated with 50 μl of luciferase substrate, and the relative luciferase activity was determined with a Luminoskan Ascent (Thermo Labsystems Oy, Helsinki, Finland). Luciferase activity was normalized to β-galactosidase activity.

3.8. Determination of NO production

RAW 264.7 cells (1×10^6 cells/ml) were preincubated with each compound for 30 min and continuously activated with LPS (1 μg/ml) for 24 h (Lee et al. 2008). Nitrite in culture supernatants was also measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μl samples of medium for 10 min at room temperature. The OD at 570 nm (OD570) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA). A standard curve of NO was made with sodium nitrite. The detection limit of the assay was 0.5 μM.

3.9. MTT assay (colorimetric assay) for measurement of cell viability

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (Lee et al. 2008; Kong et al. 2009). RAW 264.7 cells (1×10^6 cells/ml) were cultured in flat bottom 96 well microtiter plates with testing compounds for 24 h. At 4 h prior to culture termination, 10 μl MTT solution (10 mg/ml in phosphate buffered-saline, pH 7.4) was added to culture in each well and cells were continuously cultured until termination of the experiment. The culture was stopped by addition of 15% sodium dodecyl sulfate (SDS) dissolved in 1.5N HCl into each well for solubilization of formazan. The OD570 as in 3.7.

3.10. Preparation of cell lysates and nuclear fraction, and immunoblotting

RAW264.7 cells (5 × 106 cells/ml) were washed 3 times in cold PBS with 1 mM sodium orthovanadate and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglycotetraacetic acid, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzimide, and 2 mM PMSF) for 30 min with rotation at 4 °C. The lysates were clarified by centrifugation at 16,000 g for 10 min at 4 °C and stored at –20 °C until needed (Yuan et al. 2010).

Nuclear lysates were prepared with a three-step procedure. After treatment, cells were collected with a rubber policeman, washed with 1 × PBS, and lysed in 500 μl of lysis buffer containing 50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES (pH 7.8), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 100 μM 1,4-dithiothreitol (DTT) on ice for4 min. Cell lysates were then centrifuged at 14,000 rpm for 1 min in a microcentrifuge. In the second step, the pellet (the nuclei fraction) was washed once in washing buffer, which was the same as the lysis buffer without Nonidet P-40. In the final step, nuclei were treated with an extraction buffer containing 500 mM KCl, 10% glycerol, and several other reagents as in the lysis buffer. The nuclei/extraction buffer mixture was frozen at-80 °C, and then thawed on ice and centrifuged at 14,000 rpm for 5 min. Supernatant was collected as nuclear extract.

Whole cell or nuclear lysates were then analyzed by immunoblotting. Proteins were separated on 10% SDS-polyacrylamide gels and transferred by electroblotting to polyvinylidenedifluoride (PVDF) membrane. Membranes were blocked for 60 min in Tris-buffered saline containing 3% bovine serum albumin, 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20 at room temperature. The membrane was incubated for 60 min with specific primary antibody at 4 °C, washed 3 times with the same buffer, and incubated for an additional 60 min with HRP-conjugated secondary antibody. The total and phosphorylated levels of ERK, p38, JNK, IκBα, IKKα/β, p85, Akt, Src, γ-tublin, cytoplasmic actin (actin) and β-actin were visualized using the ECL system (Amersham, Little Chalfont, Buckinghamshire, UK).

3.11. mRNA detection by quantitative real-time reverse transcription-PCR

Total RNA from LPS-treated-RAW264.7 cells (5×10^6 cells/ml) was prepared by adding TRIzol Reagent (Gibco BRL) according to the manufacturer's protocol. The total RNA solution was stored at –70 °C until used. Semi-quantitative RT reactions were conducted using MuLV reverse transcriptase. Total RNA (1 μg) was incubated with oligo-dT15 for 5 min at 70 °C and mixed with a 5 × first-strand buffer, 10 mM dNTPs, and 0.1 M DTT. The reaction mixture was further incubated for 5 min at 37 °C and for 60 min after the addition of MuLV reverse transcriptase (2 U). Reactions were terminated after 10 min at 70 °C, and total RNA was depleted by adding RNase H. The PCR reaction was conducted with the incubation mixture (2 μl cDNA, 4 μM 5' and 3' primers, a 10 × buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100], 250 μM of dNTP, 25 mM of $MgCl_2$, and 1 unit of Taq polymerase [Promega, USA]). The following incubation conditions were used: a 30 s denaturation time at 94 °C, an annealing time of 30 s between 55 and 60 °C, an extension time of 45 s at 72 °C, and a final extension of 5 min at 72 °C. For real-time PCR analysis (ln et al. 2010; Sun et al. 2010), one microgram of RNA was submitted to reverse transcription with the Molony murine leukemia virus reverse transcriptase (Invitrogen). Two microliters of cDNA obtained for each sample were submitted to a qPCR using the SYBR green Master mix method (Applied Biosystems, Foster City, CA) in the ABO sequence detection system, The results were normalized with the 18S transcript. The primers (Bioneer, Daejeon, Korea) used in this experiment are indicated in Table 2.

3.12. Statistic analysis

The Student's *t*-test and a one–way ANOVA were used to determine the statistical significance between mean ? values of the various experimental and control groups. P values of 0.05 or less were considered to be statistically significant.

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