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Resveratrol inhibits NF-κB signaling through suppression of p65 and IκB kinase activities

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Resveratrol has been shown to possess multiple pharmacological activities including anti-tumor, anti-inflammation and immunomodulation, and participates in the regulation of the NF-κB signaling pathway. However, the mechanism of the NF-κB signaling pathway inhibited by resveratrol remains obscure. In this study, we first examined the effect of resveratrol on endogenous and TNF-α-induced NF-κB activation, and found that resveratrol suppressed NF-κB activation in a dose dependent manner. Resveratrol reduced the transcriptional activity of p65, but neither affected the DNA-binding activity of NF-κB nor blocked the nuclear translocation of p65. Moreover, resveratrol had no effect on the expression level of IκBα protein and inhibited IκBα degradation. Further investigation revealed that resveratrol blocked the ubiquitination of NEMO and inhibited IκB kinaseβ-mediated NF-κB activation. These results demonstrated that resveratrol effectively suppressed NF-κB signaling through inhibiting the activities of NF-κB and IκB kinase. Therefore, resveratrol may provide a novel approach to treating inflammation-associated diseases and cancer.

1. Introduction

Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a natural polyphenolic compound derived from grapes, berries, peanuts, and other plant sources (Csaki et al. 2008). It possesses multiple pharmacological activities, such as anti-tumor (Aziz et al. 2005; Jang et al. 1997; Shukla and Singh 2011; Singh et al. 2011), anti-oxidant (Miller and Rice-Evans 1995), and cardioprotective (Gusman et al. 2001; Li et al. 2012) capabilities. Recently, resveratrol has also been found to exhibit anti-inflammatory and immunomodulatory effects through regulating the NF-κB signaling pathway. By inhibiting the NF-κB cascade, resveratrol mitigates LPS-mediated microglial inflammation (Capiralla et al. 2012). Through regulating the NF-κB activation, resveratrol reduces TNF-α-induced U373MG human glioma cell invasion (Ryu et al. 2011), and inhibits tumor TNF-α-mediated matrix metalloproteinase-9 expression in human hepatocellular carcinoma cells (Yu et al. 2008). Resveratrol can suppress the UVB induced NF-κB activation by blocking the activation of IKKα (Adhami et al. 2003). Resveratrol not only inhibits NF-κB -mediated cytokine expression in adipocytes (Gonzales and Orlando 2008) but also suppresses iNOS expression and nitric oxide generation (Cao et al. 2011; Lei et al. 2012; Qureshi et al. 2012).

The NF-κB family is a group of closely related transcription factors consisting of five members, including p65, Rel B, c-Rel, p52 and p50 (Hayden and Ghosh 2012). The precursor proteins p100 (NF-kB2) and p105 (NF-kB1) gives rise to p52 and p50, respectively (Hayden and Ghosh 2011, 2012; Sears et al. 1998). All members contain an N-terminal Rel homology domain (RHD) which serves as their dimerization and makes contact with DNA, and the most common dimer is formed by p65 and p50 (Hayden and Ghosh 2012). NF-kB is in complex with IκBα which is a member of IκB proteins, and predominantly resides in the cytoplasm in an inactive state (Hayden and Ghosh 2011, 2012). Treatment of cells with pro-inflammatory cytokines, such as TNF-α, or with microbial danger signals, such as LPS, leads to the activation of IκB kinase (IKK) complex which phosphorylates IκB and tags it for proteasomal degradation (Ghosh and Hayden 2008; Hayden and Ghosh 2011, 2012). After the degradation of IκB, the released NF-kB proteins translocate to the nucleus and bind to the κB site of DNA so as to modulate target gene expression (Hayden and Ghosh 2011, 2012). The IKK complex is made of two kinase subunits (IKKβ and IKKα) and a regulatory subunit (NEMO). NF-κB, IκB and IKK are the basic components in the NF-κB signaling pathway (Hayden and Ghosh 2011, 2012).

Despite the tremendous progress that has been made in understanding the anti-inflammatory and immunomodulatory capabilities of resveratrol in regulating the NF-κB signaling pathway, there is much that remains to be understood. The detailed molecular mechanism for the effect of resveratrol on

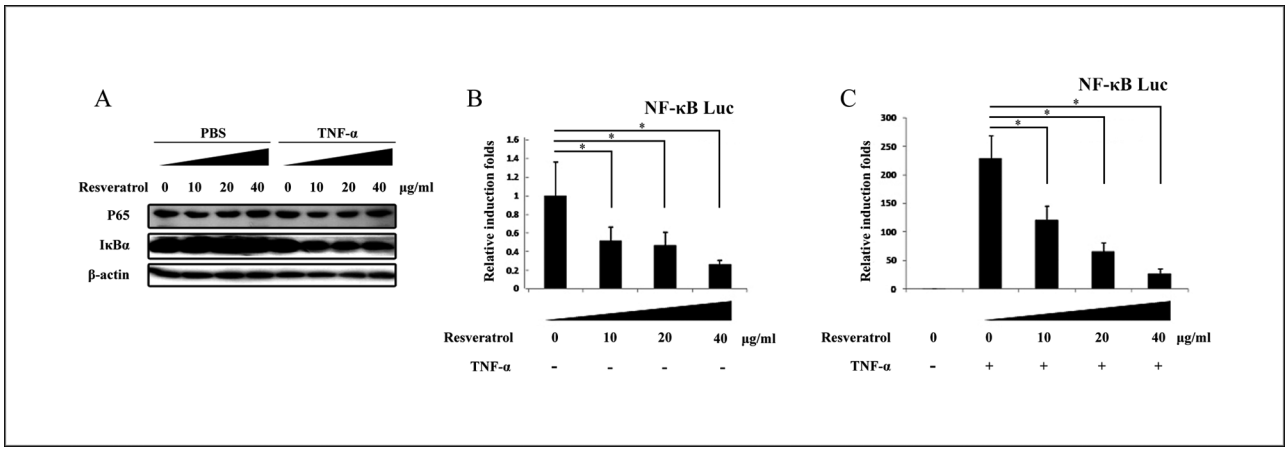

Fig. 1: Resveratrol inhibited endogenous and TNF-α-induced NF-κB activation. (A) Under normal conditions, the protein levels of p65 and IκBα were not changed significantly. With TNF-α over stimulation, the expression of p65 decreased at low concentration of resveratrol (less than 20 μg/ml resveratrol), and the decline in IκBα protein was not very significant. (B) Resveratrol suppressed endogenous NF-κB activation. (C) Resveratrol blocked TNF-α-induced NF-κB activation in a dose-dependent manner. Data were showed by mean ± S.D. *: $p < 0.05$

the function of the basic components in the NF-κB signaling pathway remains to be explored. In the present study, we investigated how resveratrol suppresses the NF-κB signaling pathway via inhibiting the function of NF-κB, IκB and IKK. Our findings provide further support for the beneficial effects of resveratrol on treating inflammatory diseases and cancer.

2. Investigations and results

2.1. Resveratrol inhibits endogenous NF-κB activity

To investigate the effect of resveratrol on endogenous NF-κB activity, the expression of both p65 and IκBα was evaluated by Western Blot. Compared with mock control, the protein levels of both p65 and IκBα were not significantly changed in the presence of resveratrol, implying that resveratrol could not induce NF-κB activation (Fig. 1A, indicated as PBS). To assess whether resveratrol inhibits endogenous NF-κB activity, HEK-293T cells transfected with NF-κB Luc vector were treated with different amounts of resveratrol. Compared with corresponding mock control, the activity of NF-κB Luc was decreased significantly in the presence of resveratrol (Fig. 1B), indicating that resveratrol suppressed endogenous activity of NF-κB.

2.2. Resveratrol suppressed TNF-α-induced NF-κB activation

TNF-α has been shown as an inductor of NF-κB activation. To distinguish whether resveratrol was involved in the regulation of NF-κB signaling pathway, HEK-293T cells, which were transfected with NF-κB Luc vector, were stimulated by TNF-α and treated by resveratrol at the same time. Compared with to the mock control, the NF-κB Luc activity with TNF-α stimulus was increased more than 200 times in the absence of resveratrol (presented as negative control) (Fig. 1C). After TNF-α stimulation, resveratrol treatment resulted in a substantial reduction of NF-κB Luc activity compared with negative control and presented in a dose-dependent manner (Fig. 1C). Interestingly, the expression of p65 protein was decreased at low concentrations of resveratrol (10 μg/ml resveratrol) (Fig. 1A, indicated as TNF-α). Moreover, the DNA binding activity of NF-κB was not affected by resveratrol with TNF-α induction (Fig. 2B, indicated as TNF-α). These results promoted us to investigate the effect of resveratrol on p65 in NF-κB signaling pathway.

2.3. Resveratrol reduces p65 transcriptional activity without blocking TNF-α-induced nuclear translocation of p65

When NF-κB is activated, NF-κB is translocated from the cytoplasm to the nucleus. P65 was an important member of the Rel family in NF-κB signaling pathway and mainly resided in the cytoplasm in an inactive state (Fig. 2A, Control). After TNF-α stimulation, nuclear accumulation of p65 was strongly induced (Fig. 2A, TNF-α). However, nuclear translocation of p65 was not observed in the presence of resveratrol (Fig. 2A, resveratrol), implying resveratrol could not induce the activation of NF-κB alone. Importantly, the shift of p65 to the nucleus was not blocked by resveratrol upon TNF-α stimulation (Fig. 2A, TNF-α and resveratrol), suggesting that the suppression of TNF-α-induced NF-κB activation by resveratrol did not result from blockade of p65 nuclear translocation.

When NF-κB is translocated to the nucleus, it is required for NF-κB to bind to a κB DNA site before fulfilling its transcriptional function. To determine the effect of resveratrol on the DNA binding activity of NF-κB, p65 was over expressed and EMSA was conducted. Results showed that resveratrol did not affect the DNA binding activity of p65 (Fig. 2B, indicated as Flag-P65). P105 is a member of the Rel family, and resveratrol did not decrease p105 DNA binding activity (Fig. 2B, indicated as Flag-P105).

To confirm the transcriptional activity of NF-κB suppressed by resveratrol, p65 was over-expressed in HEK-293T cells, and then the cells were treated with resveratrol. Compared with control, the NF-κB Luc activity was increased more than 250 times when exogenous p65 was expressed without resveratrol treatment (Fig. 2C). However, increasing amounts of resveratrol resulted in a dose-dependent reduction in NF-κB Luc activity (Fig. 2C), indicating that resveratrol inhibited p65 transcriptional activity in a dose-dependent manner. A similar experiment was performed in which p105 took the place of p65. When p105 was over-expressed, the NF-κB Luc activity was increased, and resveratrol decreased the NF-κB Luc activity in a dose-dependent manner (Fig. 2D).

2.4. Resveratrol does not affect IκBα expression

IκB inhibits NF-kB activation. We evaluated the effect of resveratrol on IκBα expression. Compared with untreated cells, resveratrol did not change the expression of IκBα protein

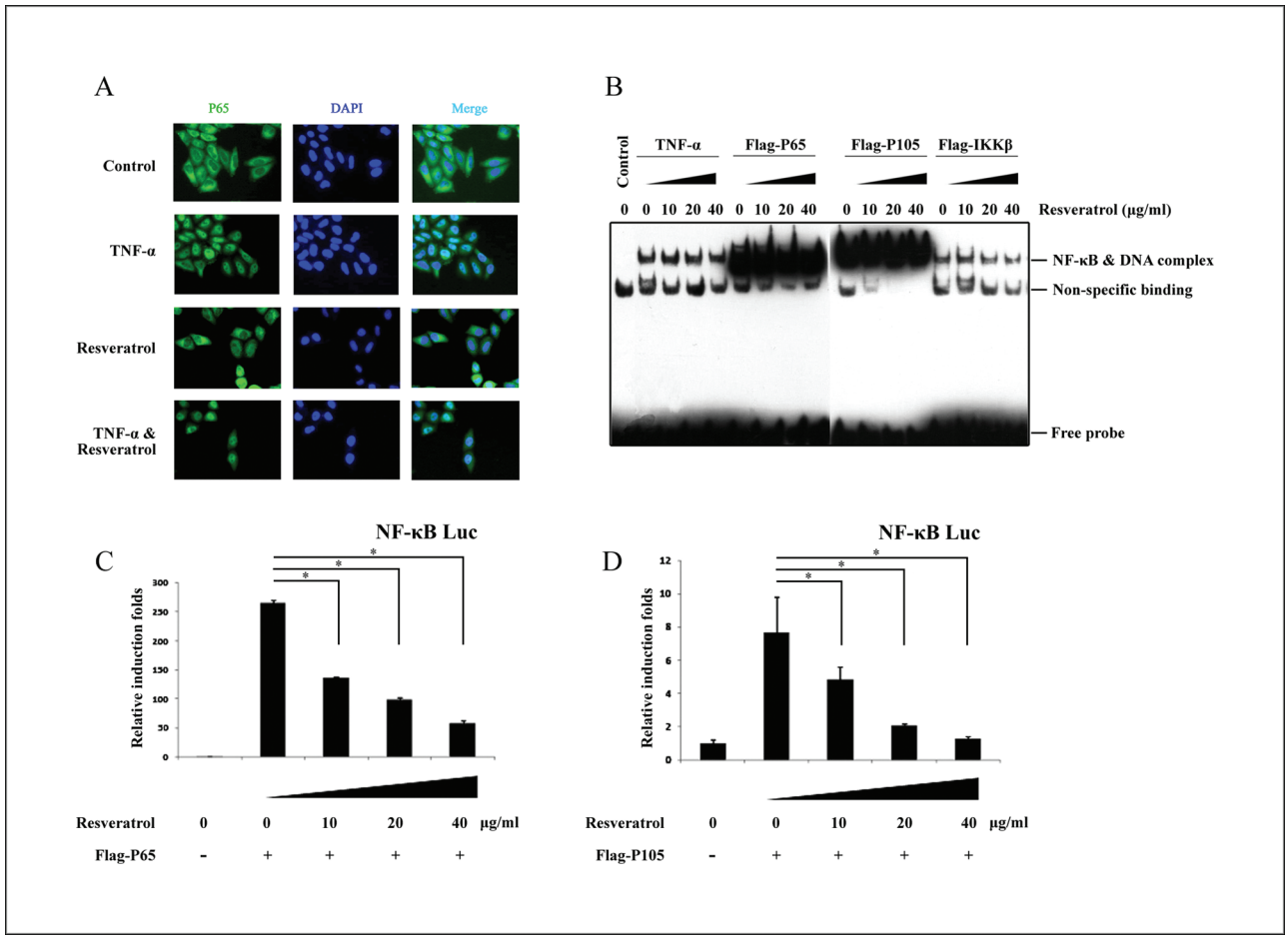

Fig. 2: Resveratrol suppressed NF-κB transcriptional activity without blocking the shift of p65 to the nucleus by TNF-α stimulus. (A) Resveratrol did not block the TNF-α-induced nuclear translocation of p65. (B) Resveratrol did not affect the DNA binding activity of NF-κB but decrease the amount of NF-κB which translocated into nucleus caused by IKKβ over expression. (C) Resveratrol reduced exogenous p65 induced NF-κB activation. (D) Resveratrol inhibited NF-κB activation with p105 over expression. Data were showed by mean ± S.D. *: $p < 0.05$

(Fig. 1A, indicated as PBS). With TNF-α over stimulation, the modest reduction of IκBα was not significant after the treatment with different amounts of resveratrol (Fig. 1A, indicated as TNF-α). When p65 or p105 was over expressed, the protein level of IκBα with the treatment of resveratrol was the same as usual (Fig. 3A) . In general, the activated IKK induces the degradation of IκBα. However, the expression of IκBα protein was not changed by resveratrol treatment with IKKβ or IKKα over-expression (Fig. 3A), implying resveratrol blocked the IKK mediated IκBα degradation.

2.5. Resveratrol suppresses IKK-mediated NF-κB activation

The IKK complex is the core element of the NF-kB cascade, and it consists of IKKβ, IKKα, and NEMO. Exogenous IKKβ effectively induced NF-kB activation (Fig. 4A). Results of NF-κB luciferase assays revealed that resveratrol exerted a dose-dependent inhibition on NF-κB Luc activity by suppressing IKKβ (Fig. 4A). Furthermore, resveratrol decreased the DNA binding activity of NF-κB by inhibiting IKKβ at high concentration (40 μg/ml) (Fig. 2D, indicated as Flag- IKKβ), implying

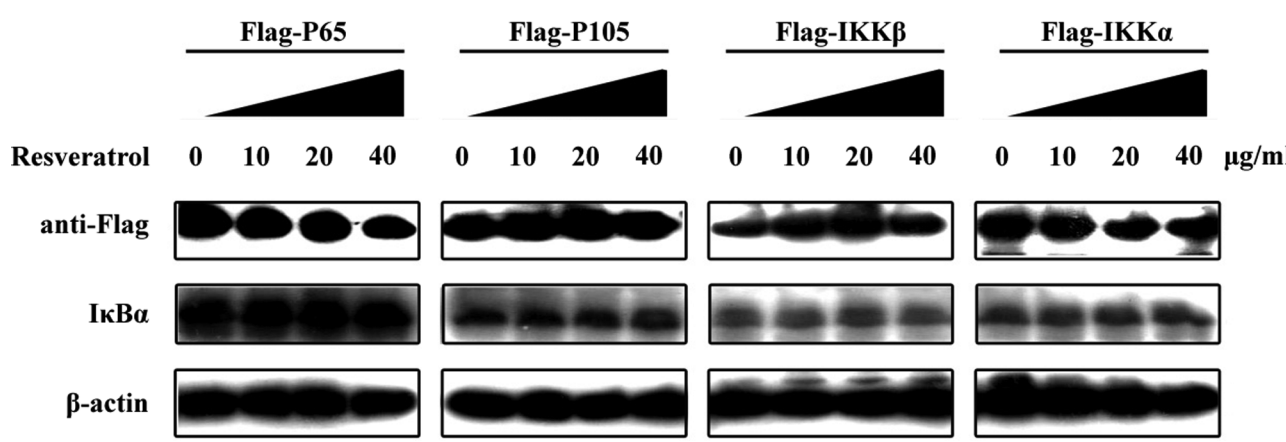

Fig. 3: The effect of resveratrol on the expression of IκBα. The expression of IκBα protein was not changed with resveratrol treatment when p65, p105, IKKβ, or IKKα was over expression

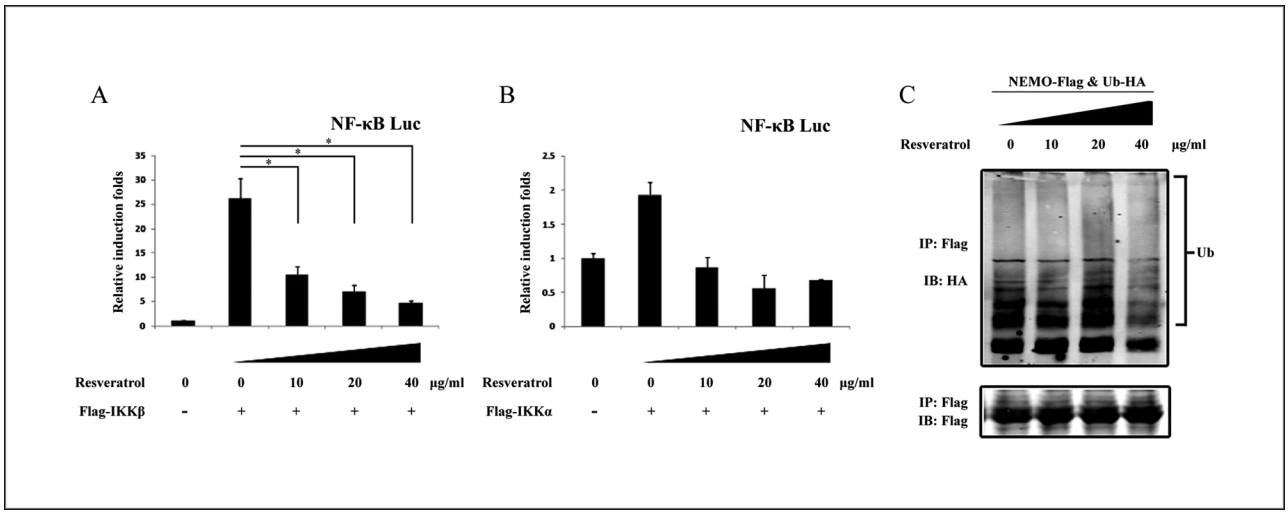

Fig. 4: Resveratrol inhibited IKK-mediated NF-κB activation. (A) Resveratrol resulted in a dose-dependent reduction in NF-κB Luc activity when IKKβ was over-expressed. (B) The NF-κB Luc activity was decreased by resveratrol treatment with IKKα over expression. (C) Resveratrol inhibited the ubiquitination of NEMO. Data were showed by mean ± S.D. *: $p < 0.05$

that resveratrol suppressed IKKβ activity so as to decrease the amount of NF-κB shifted from cytoplasm into nucleus. The similar NF-κB luciferase assays were performed in which IKKα replaced IKKβ. To a certain extent, resveratrol decreased the NF-κB Luc activity, too (Fig. 4B).

To further confirm whether resveratrol inhibited the ubiquitination of NEMO to modulate NF-κB activation, ubiquitination assay was carried out. When NEMO-Flag and Ub-HA were co-expressed in HEK-293T cells, the ubiquitination of NEMO was observed, and resveratrol inhibited the ubiquitination of NEMO at high concentration (40 μg/ml) (Fig. 4C). This result indicated that resveratrol blocked NEMO ubiquitination so as to regulate the NF-κB signaling pathway.

3. Discussion

Accumulating evidence suggests that resveratrol is a potential therapeutic agent for inflammatory disease and cancer. For example, resveratrol suppresses NF-κB-dependent COX-2 activation (Yi et al. 2011), NO generation (Cho et al. 2002; Zhong et al. 2012), iNOS expression (Lei et al. 2012; Tsai et al. 1999; Zhong et al. 2012), and TNF-α–induced matrix metalloproteinase expression (Lee and Moon 2005). These data show that resveratrol is involved in the regulation of the NF-κB signaling pathway. However, the effect of resveratrol on the key components (NF-κB, IκB, and IKK) in NF-κB signaling pathway seems ambiguous in previous reports, and further studies are needed to understand the molecular mechanistic details.

Using a NF-κB luciferase reporter assay, we demonstrated that resveratrol inhibits both endogenous and TNF-α–induced NF-κB activation in a dose-dependent manner in 293T cells, and these results are consistent with previous studies which show that resveratrol blocks NF-κB activation induced by TNF-α in U373MG cells (Ryu et al. 2011) and human $CD34^+$ cells (Jeong et al. 2011), by anoxia/reoxygenation injury in cardiomyocytes (Zhang et al. 2012), and by receptor activator of NF-κB ligand (RANKL) in bone-derived cells (Shakibaei et al. 2011). These data suggest that resveratrol exerts inhibitory function on NF-κB dependent gene transcription. To transcribe NF-κB target genes, NF-κB must be translocated from cytoplasm to the nucleus. Using immunofluorescence, our data show that resveratrol does not induce the nuclear translocation of p65 in Hela cells, and this is correspondent with the fact that is reported in U-937 cells (Manna et al. 2000). However, Shu Wen et al. reported that p65 nuclear translocation was observed in UW228-2 and UW228-3 medulloblastoma cells treated with resveratrol alone (Wen et al. 2011). Furthermore, in agreement with previous findings in human umbilical vein endothelial cells (Pellegatta et al. 2003), our immunofluorescence result indicated that the suppression of NF-κB activity is not the result of the blockage of NF-κB nuclear translocation. This seems inconsistent with several reports. For instance, resveratrol treatment blocks NF-κB translocation in TNF-α induced PC-3 cells (Benitez et al. 2009), human $CD34^+$ cells (Jeong et al. 2011), and U-937 cells (Manna et al. 2000). One reason why resveratrol has a different effect on NF-κB translocation may be attributed to the different cell lines. From another perspective, our immunofluorescence result implies that resveratrol does not affect the transportation of NF-κB from cytoplasm to nucleus and NF-κB cytoplasmic retention may be due to the suppression of signaling molecules upstream of NF-κB such as IKK. When NF-κB is translocated to the nucleus, NF-κB binds to the κB site of DNA to transcribe target genes. As demonstrated by EMSA, our result reveals that resveratrol has no effect on the DNA binding activity of NF-κB with TNF-α stimulation in 293T cells, and this result is in agreement with the previous investigation in TNF-α treated U-937 cells (Manna et al. 2000). Moreover, resveratrol does not inhibit the DNA binding activity of NF-κB when p65 or p105 is over-expressed. Based on the similarity in structure between p65 and p105, this result implies that RHD is not the target domain with which resveratrol interacts. Interestingly, the reduction of NF-κB luciferase activity is observed when p65 or p105 is over-expressed. Combined with the results of EMSA, immunofluorescence and luciferase reporter assay, we speculate that resveratrol reduces NF-κB activation is mainly by suppression of NF-κB transcriptional activity.

IκB is in complex with NF-κB dimer in cytoplasm to act as an inhibitor of NF-κB activation. In this study, our result shows that resveratrol does not suppress IκBα expression. This observation is in agreement with the common belief that the degradation of IκBα results in NF-κB activation (Hayden and Ghosh 2012). The inhibitory effect of resveratrol on IκBα degradation was observed by different external stimuli in several recent reports (Capiralla et al. 2012; Roy et al. 2009; Shakibaei et al. 2011; Zhong et al. 2012), and our result confirms these observations. However, other reports show that resveratrol suppresses TNF-α induced NF-κB activation without blockage of the degradation of IκBα (Manna et al. 2000; Pellegatta et al. 2003). On the other hand, it is described that oxidative stress induces NF-

κB nuclear translocation without degradation of IκBα (Canty et al. 1999), and resveratrol even enhances NF-κB activity in cytokine-exposed mesangial cells (Uchida et al. 2005). These contradictory results may be attributed to the signaling crosstalk which is triggered by different external stimuli.

It is well known that the activated IKK complex catalyzes the phosphorylation of IκBα to activate NF-κB (Hayden and Ghosh 2011, 2012). Until now, there is little conclusive evidence to elucidate such a basic problem whether resveratrol suppresses NF-κB activation through regulating IKK activity. In the present study, our result reveals that resveratrol blocks NF-κB activation via suppressing the activities of IKKβ and IKKα. The effective suppression of IKKβ-mediated NF-κB activation by resveratrol indicates that resveratrol exhibits a remarkable inhibitory effect on the canonical NF-κB signaling pathway. It has already been shown that the activation of IKK is required for the ubiquitination of NEMO (Hayden and Ghosh 2012; Rothwarf et al. 1998), and we provide the first evidence that resveratrol inhibits the ubiquitination of NEMO. In addition to the suppression of the NF-κB signaling pathway, IKKα and IKKβ mediate cross-talk with additional signaling pathways such as the p53 and MAP kinase (MAPK) pathways (Oeckinghaus et al. 2011), implying that resveratrol regulates aspects of transcription and possesses a broad spectrum of anti-inflammation and anti-cancer activities. This study has provided important insights into the molecular mechanism by which resveratrol suppresses the NF-κB signaling pathway. We have further demonstrated that resveratrol can exert inhibitory effects on NF-κB and IKK activities. Resveratrol suppresses the ubiquitination of NEMO to inhibit the catalytic activities of IKKβ and IKKα. Reduced activities of IKKβ and IKKα block the degradation of IκBα protein, leading to the inactivation of NF-κB. Meanwhile, resveratrol directly inhibits the transcriptional activity of p65. These results provide the theoretical basis for a novel approach to treating inflammatory disorders using resveratrol, and chemical optimization of resveratrol can be further developed for the prevention and treatment of cancer.

4. Experimental

4.1. Plasmid construction

The coding region of genes including human p65 (GenBank ID: NP_068810.3), p105 (GenBank ID: NP_003989.2), IKKα (GenBank ID: NP_001269.3), IKKβ (GenBank ID: NP_001547.1), and NEMO (GenBank ID: NP_001093326.2) were inserted into the multiple cloned site of pcDNA3.1 plasmid (Invitrogen). These genes were fused with Flag tag and named as Flag-P65, Flag-P105, Flag- IKKα, Flag- IKKβ, and NEMO-Flag, respectively. The recombinant pcDNA3.1 vector coding human ubiquitin fused with HA tag was constructed, too.

4.2. Cell culture and luciferase reporter assay

Human embryonic kidney (HEK) 293T cells and Hela cells were grown in DMEM supplemented with 10% fetal calf serum at 37 °C in 5% CO_2. HEK-293T cells were transfected with DNA mixture using lipofectamine 2000 (Invitrogen) in 48-well plates. The DNA mixture contained the indicated amount of plasmids: 5 ng of the *Renilla* luciferase reporter plasmid (Promega), 100 ng of the NF-κB response promoter luciferase reporter (NF-κB Luc), and 400 ng of the recombined gene expression vector (or empty pcDNA3.1 vector used as negative control). At 24 h after transfection, resveratrol was added to culture medium. Luciferase assays were performed 12 h later using dual-luciferase reporter assay system (Promega). In another assay, cells were transfected with both *Renilla* and NF-κB Luc, and treated by resveratrol with or without TNF-α stimulation 24 h later. After 12 h treatment, the luciferase assays were conduced. NF-κB Luc activity was normalized to *Renilla* luciferase activity and indicated as relative induction folds (mean ± S.D.). Data are means of triplicate determinations from a representative experiment repeated at least three times with similar results. Results were analyzed by Student's t-test, and significance is indicated when P values were less than 0.05.

4.3. Western blotting

HEK-293T cells were treated as described above and harvested. The proteins of cell extracts were separated by SDS-PAGE and then transferred to a nitrocellulose membrane (Millipore). Following a blocking step in 10% skimmed milk solution for 2 h, membranes were incubated with primary antibody at 4 °C overnight and a secondary antibody for 1 h in the dark. Membranes were then visualized by the Odyssey IR imaging system (LI-COR).

4.4. Immunofluorescence microscopy

Hela cells were seeded in a 12-well plate for 12 h, then treated with water, TNF-α, resveratrol, or both TNF-α and resveratrol. Twenty four hours later, cells were fixed in a 4% formaldehyde/1 × PBS solution, washed with 0.02% TritonX-100/1 × PBS, and blocked with 1% BSA/1 × PBS. Then the primary antibody (anti-p65) was added (1:200) into blocking buffer and incubated at 4 °C overnight. The samples were subsequently incubated with secondary antibody (anti-mouse immunoglobulin G; Sigma) (1:500) in the dark for 1 h, and stained with 0.2 μg/mL DAPI in PBS. After the usual washings, the samples were examined under a fluorescence microscope (Zeiss AxioVision 4 microscope, Germany).

4.5. Nuclear extraction and electrophoretic mobility shift assay (EMSA)

A double-stranded oligonucleotide containing the NF-κB binding element (AGT TGA GGG GAC TTT CC CAG GC) was labeled with biotin. HEK-293T cells were treated as described above. Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Scientific), and protein concentration was measured according to manufacturer's instruction of the Pierce BCA Protein Assay Kit (Thermo Scientific). Binding reactions that probe was incubated with nuclear extracts were performed for 20 min at room temperature. The DNA and protein complexes were resolved on 10% non-denaturing polyacrylamide gel, and transferred to a Biodyne B Nylon membrane (Thermo Scientific). Signals were detected on X-ray film with streptavidin-horseradish peroxidase conjugates that bound to biotin and chemi-luminescent substrate.

4.6. Co-immunoprecipitation and ubiquitination assay

The NEMO-Flag plasmid was transfected into HEK-293T cells with HA-tagged ubiquitin (Ub) vector. After 24 h, the indicated amount of resveratrol was added into the culture medium. Twelve hours later, whole cell extracts were incubated with anti-Flag antibody in immunoprecipitation lysis buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid sodium salt, and cocktail protease inhibitor) at 4 °C for 1 h. Protein G Sepharose (Roche) was added and incubated at 4 °C overnight. After centrifugation, NEMO-Flag was immunoprecipitated (IP) and immunoblotted (IB) with anti-HA antibody to examine the ubiquitination of NEMO.

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