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Inhibitory effects of flavonoids on TNF-α-induced IL-8 gene expression in HEK 293 cells

Soohyoung Lee[1], Young-Jin Kim[1], Sanghoon Kwon[1], Younghee Lee[2], Soo Young Choi[3], Jinseu Park[3] & Hyung-Joo Kwon[1,4,]*

[1]Department of Microbiology, College of Medicine, Hallym University, Chuncheon 200-702, [2]Department of Biochemistry, College of Natural Sciences, Chungbuk National University, Cheongju 361-763, [3]Department of Biomedical Science, [4]Center for Medical Science Research, College of Medicine, Hallym University, Chuncheon 200-702, Korea

Due to their multiple biological activities, flavonoids have gained attention as potentially useful therapeutics for a variety of diseases including cancer, cardiovascular diseases, and autoimmune diseases. In this study, we demonstrated that several flavonoids, including kaempferol, quercetin, fisetin, and chrysin block TNF-α induced IL-8 promoter activation and gene expression in HEK 293 cells. In addition, phosphorylation and degradation of IκBα and translocation of NF-κB p65 were inhibited by these flavonoids in TNF-α-stimulated HEK 293 cells. Furthermore, generation of reactive oxygen species (ROS) in response to TNF-α was reduced by the flavonoids. Moreover, although pretreatment with fisetin, quercetin, or chrysin decreased cell viability, kaempferol did not. Taken together, these findings suggest that kaempferol would be useful for the treatment of TNF-α-induced inflammatory diseases. [BMB reports 2009; 42(5): 265-270]

INTRODUCTION

Flavonoids are a group of natural polyphenolic compounds that are found in a wide variety of crop plants as well as in tea and wine (1, 2). The fundamental structure of polyphenolic flavonoids contains a diphenylpropane skeleton that includes monomeric flavonols, flavonols, flavonones and flavones (3, 4). Many flavonoids have diverse biological activities that include anti-oxidant function, anti-inflammatory effects, and inhibition of cell-signaling proteins (3, 5, 6). These multiple biological activities have resulted in flavonoids gaining attention as potentially useful therapeutics for a variety of diseases such as cancer, cardiovascular diseases, autoimmune diseases and infectious diseases (7). However, despite the diverse beneficial biological and pharmacological effects of flavonoids, their use for the treatment of human diseases is not popular due to their high effective concentrations and poor absorptive effects and because their detailed molecular mechanisms have not yet been elucidated (7, 8). Therefore, better understanding of their activities, action mechanisms and interaction with other compounds is necessary (7, 9).

TNF-α is a pleiotropic pro-inflammatory cytokine that is primarily produced by activated macrophages and many other cell types including lymphocytes, endothelial cells, and mast cells (10, 11). The upregulated production of TNF-α has been found to be associated with a variety of inflammatory diseases including rheumatoid arthritis, psoriasis, Crohn's disease, and refractory asthma (10, 12-14). TNF-α induces the activation of several transcriptional factors, including NF-κB, through TNF-α receptor (TNFR)-dependent signaling pathways (9-11, 15, 16). Flavonoids are widely used to inhibit the activation of NF-κB (17, 18), and several studies have shown that flavonoids inhibit the expression of proinflammatory genes in response to inflammatory mediators such as TNF-α (6, 19). However, the potential involvement of flavonoids in the regulation of TNF-α-induced IL-8 gene expression in HEK 293 cells has not yet been fully explored.

Therefore, to elucidate the effect of flavonoids in TNF-α-induced IL-8 gene expression, we analyzed NF-κB-responsive IL-8 gene expression and NF-κB activation in the presence of several flavonoids. We found that IL-8 promoter activation and gene expression was inhibited by flavonoids in TNF-α-stimulated HEK 293 cells.

RESULTS AND DISCUSSION

Effects of flavonoids on TNF-α-induced IL-8 promoter activity and IL-8 expression

To identify flavonoids that specifically inhibit TNF-α-inducible gene expression, the IL-8 promoter-reporter construct was transiently transfected into HEK 293 cells that had been pretreated with flavonoids at 37°C for 1 h. Next, the cells were cultured with TNF-α (5 ng/ml) for 8 h, after which the luciferase activity was monitored. Of the 20 flavonoids we evaluated, kaempferol, quercetin, fisetin, and chrysin were found to exert a strong inhibitory effect and were selected for subsequent analysis. As shown in Fig. 1A, pretreatment with each of the four flavonoids resulted in a dose-dependent decrease in TNF-α-in-

*Corresponding author. Tel: 82-33-248-2635; Fax: 82-33-241-3640; E-mail: hjookwon@hallym.ac.kr

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duced IL-8 promoter activation. Interestingly, fisetin and chrysin exerted a dramatic inhibitory effect on promoter activity at concentrations of over 20 μM. However, pretreatment with kaempferol only partially inhibited the TNF-α-induced IL-8 promoter activation (approximately 60% at 80 μM). These results indicate that the various flavonoids evaluated here exert different inhibitory effects on the TNF-α-induced IL-8 promoter activation.

To further determine if each flavonoid could inhibit IL-8 gene expression in TNF-α-treated HEK 293 cells, we pre-incubated the cells with flavonoids (40 μM) for 1 h prior to TNF-α treatment and then evaluated the IL-8 mRNA expression by RT-PCR. Treatment with TNF-α greatly enhanced the IL-8 mRNA expression in HEK 293 cells in a time-dependent manner (Fig. 1B). Specifically, the IL-8 expression in TNF-α-treated cells was dramatically reduced in the presence of fisetin, whereas the inhibitory effects of kaempferol, quercetin and chrysin were not as strong (Fig. 1C). Taken together, these results indicate that IL-8 promoter activation and gene expression is differentially regulated by each flavonoid in TNF-α-stimulated HEK 293 cells.

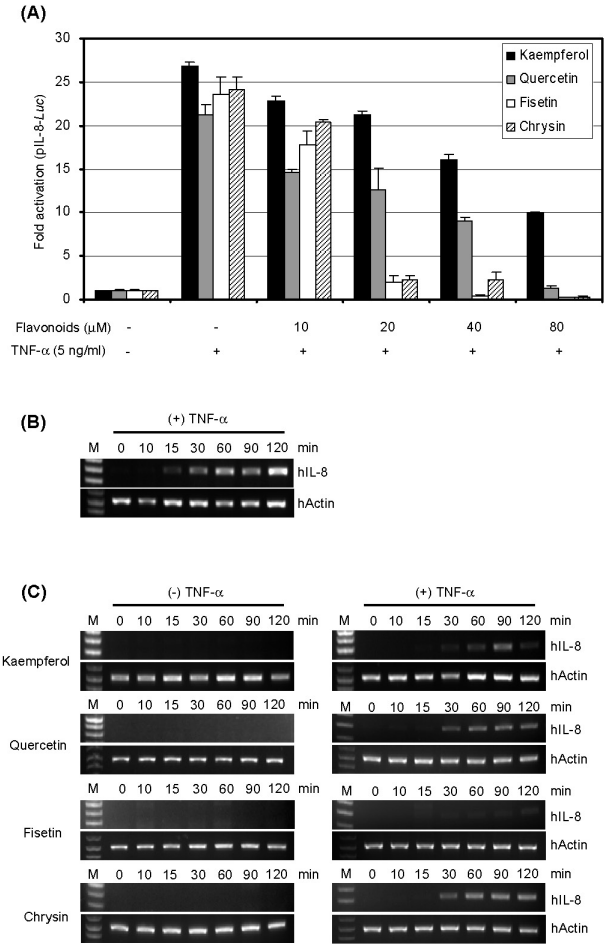

Fig. 1. Effects of flavonoids on TNF-α-induced IL-8 promoter activation and gene expression in HEK 293 cells. (A) HEK 293 cells were transiently transfected with an IL-8 promoter-reporter construct for 24 h. The cells were then stimulated with TNF-α for 8 h in the presence of increasing amounts of flavonoids. Next, a luciferase assay were performed as described in the Materials and Methods. (B) HEK 293 cells were incubated in the presence of TNF-α (5 ng/ml) for the indicated time periods. (C) HEK 293 cells were treated with TNF-α in the presence of the described flavonoids (40 μM) for the indicated time periods. After extraction of the total RNA, the expression of the IL-8 gene was analyzed by RT-PCR analysis. M denotes DNA standard marker.

Inhibition of TNF-α-induced NF-κB activation by flavonoids in HEK293 cells

Exposure to TNF-α activates IKKs, which subsequently phosphorylate IκB. Phosphorylation of IκB results in its degradation and the subsequent release and translocation of NF-κB to the nucleus, where it activates various genes (11, 20). Therefore, we evaluated IκBα degradation and NF-κB activation to determine if they were modulated by flavonoids in TNF-α-stimulated HEK 293 cells. To evaluate the effects of flavonoids on TNF-α-mediated IκBα degradation, we incubated the cells in the presence of flavonoids (40 μM) for 1 h prior to TNF-α treatment and then monitored the phosphorylation and degradation of IκBα by Western blotting analysis. The exposure of the HEK 293 cells to 5 ng/ml TNF-α resulted in the phosphorylation and degradation of IκBα within 5 min (Fig. 2A, control). However, as shown in Fig. 2A (lower panel), the TNF-α-induced phosphorylation and degradation of IκBα was strongly attenuated by pretreatment of the cells with fisetin. In addition, when the cells were treated with TNF-α in the presence of kaempferol, quercetin, or chrysin, the phosphorylation and degradation of IκBα was reduced.

To further explore the effects of flavonoids on TNF-α-induced NF-κB activation, the signal-induced subcellular localization of NF-κB was evaluated by indirect immunofluorescence analysis. Confocal images revealed that NF-κB p65 was normally sequestered in the cytoplasm and that nuclear accumulation of NF-κB p65 was strongly induced by the stimulation of HEK 293 cells with TNF-α (Fig. 2B). However, the TNF-α-induced nuclear accumulation of NF-κB p65 was completely abolished by pretreatment of the cells with fisetin, whereas pretreatment with kaempferol, quercetin and chrysin were less effective at suppressing the TNF-α-induced nuclear accumulation of NF-κB p65. Furthermore, the degradation of IκBα and the translocation of NF-κB p65 was not induced in cells that were treated with flavonoids but not subjected to TNF-α stimulation. Taken together, these results indicate that each flavonoid inhibited the phosphorylation and degradation of IκBα and the translocation of NF-κB p65 in TNF-α-stimulated HEK 293 cells, but that the efficacy of the individual flavonoids varied.

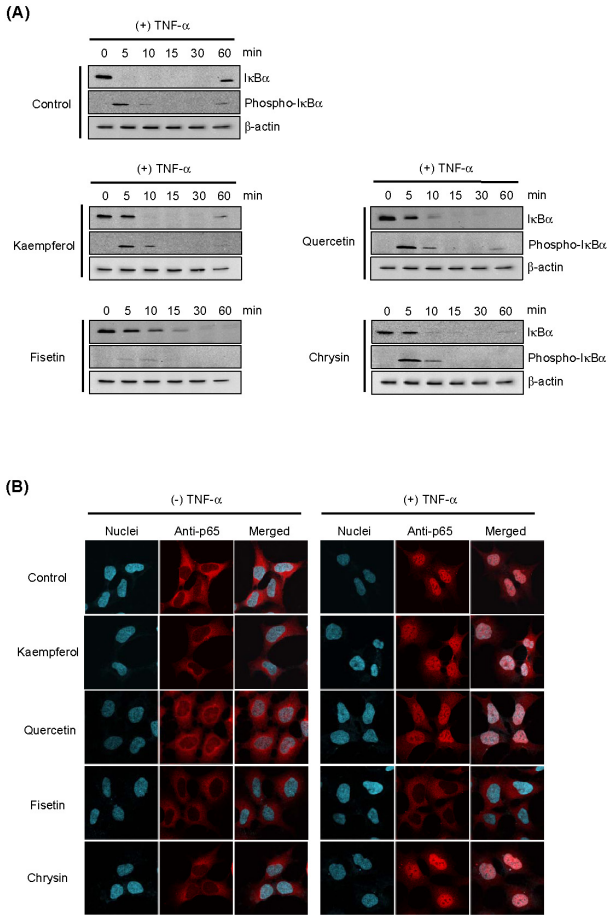

Fig. 2. Effects of flavonoids on TNF-α-induced IκBα degradation and NF-κB p65 nuclear localization. HEK 293 cells were pretreated with the indicated flavonoids (40 μM) for 1 h, after which they were stimulated with TNF-α for the indicated time periods. (A) The phosphorylation and degradation of IκBα were then analyzed by Western blotting with IκBα and phosphor-IκBα antibody. (B) Localization of NF-κB p65 was visualized by confocal microscopy after immunofluorescence staining with NF-κB p65 antibody. The cells were then stained with Hoechst No. 33258 to visualize the nuclei.

Effect of flavonoids on TNF-α-induced ROS production

It is known that the production of ROS occurs in TNF-α-stimulated cells (6, 21). Therefore, we evaluated TNF-α-induced ROS generation to determine if it was modulated by pretreatment with flavonoids. To accomplish this, confocal microscopy was used to evaluate HEK 293 cells that had been pretreated with flavonoids at a concentration of 40 μM for 1 h prior to treatment with TNF-α for 30 min As shown in Fig. 3, ROS production was detected in TNF-α-treated HEK 293 cells, but pretreatment with flavonoids resulted in a decrease in dihydrorhodamine 123 fluorescence. Furthermore, the results revealed that kaempferol more effectively suppressed the ROS

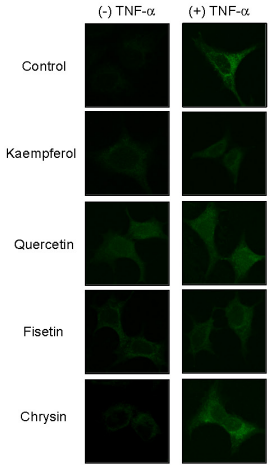

Fig. 3. Effects of the flavonoids on TNF-α-induced ROS production in HEK 293 cells. HEK 293 cells were treated with TNF-α (5 ng/ml) for 30 min in the presence of the indicated flavonoids (40 μM). The intracellular ROS levels were then measured after staining with dihydrorhodamine 123.

generation than the other flavonoids.

Effect of flavonoids on HEK293 cells viability

To determine if the treatment of the cells with flavonoids induced cell death and influenced TNF-α-induced IL-8 expression, we investigated the effect of flavonoids on the viability of HEK 293 cells using an MTT assay. As shown in Fig. 4, the viability of the cells decreased dramatically in response to treatment with greater than 40 μM fisetin (Fig. 4C). In addition, treatment of the cells with quercetin or chrysin resulted in decreased viability in a dose-dependent manner at 48 h (Fig. 4B and D). However, the viability of the cells was not affected by treatment with kaempferol (Fig. 4A).

TNF-α is pleiotropic pro-inflammatory cytokine that is produced by a wide range of activated cells (1, 8). TNF-α stimulation results in the activation of IKK, which then phosphorylates IκBα at serine 32 and 36. The phosphorylation of IκBα results in its degradation and the subsequent release and translocation of NF-κB to the nucleus. In the nucleus, NF-κB facilitates transcriptional regulation of the gene downstream of the κB motif. Here, we demonstrated that several flavonoids block TNF-α-induced NF-κB activation and IL-8 gene expression in HEK 293 cells. In addition, the results of this study revealed that the phosphorylation and degradation of IκBα and the translocation of NF-κB p65 was differentially regulated by each flavonoid in TNF-α-stimulated HEK 293 cells. Furthermore, IL-8 promoter activation and gene expression was also differentially regulated by each flavonoid in TNF-α-stimulated HEK 293 cells. Although fisetin showed the strongest inhibitory activity, the viability of the cells decreased dramatically in the presence of fisetin. Additionally, treatment with

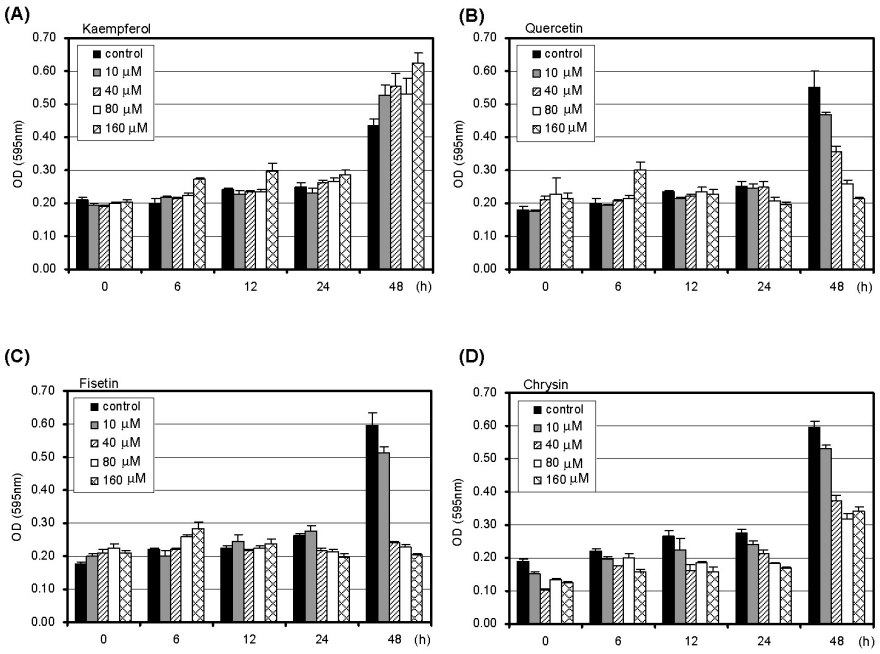

Fig. 4. Effect of flavonoids on the cell viability. HEK 293 cells were incubated in the presence of increasing amounts of kaempferol (A), quercetin (B), fisetin (C), or chrysin (D). Cell viability was measured by MTT after the indicated time periods.

quercetin and chrysin decreased the viability of the HEK 293 cells in a dose-dependent manner. However, kaempferol did not affect the viability of HEK 293 cells. Additionally, kaempferol most effectively suppressed the production of ROS that was induced by TNF-α. Taken together, these results indicate that kaempferol shows the highest potential of the flavonoids evaluated here for use in the treatment of TNF-α-induced inflammatory diseases. In conclusion, this study elucidated a mechanism by which flavonoids involved in the inhibition of IL-8 gene expression function in TNF-α-treated HEK 293 cells.

MATERIALS AND METHODS

Reagents

Kaempferol and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA), while fisetin and chrysin were purchased from Fluka (St. Louis, MO, USA) and then dissolved in dimethyl sulfoxide (DMSO). Recombinant human TNF-α was derived from *E. coli* obtained from R&D system (Minneapolis, MN, USA). Antibodies against IκBα and phospho-IκBα were obtained from Cell Signaling Technology, Inc (Beverly, MA, USA) and monoclonal anti-β actin was purchased from Sigma- Aldrich.

Cell culture

HEK 293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbelcco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under 95% air and 5% CO_2. The viability of the untreated cells, which was assayed using trypan blue dye exclusion, was typically greater than 95%.

Cell viability as determined by MTT assay

The cell growth and viability of HEK 293 cells that were treated with flavonoids was determined by an MTT assay as described previously (22). Briefly, HEK 293 cells were seeded in 96-well plates and then treated with each of the flavonoids at the indicated concentrations. After the cells were treated with the flavonoids for the indicated times, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) solution was added to each well and the plates were incubated for an additional 4 h at 37°C. Next, the medium was removed, after which the formazan crystals were solubilized in DMSO. The quantity of formazan was then determined based on the color development, which was measured using a spectrophotometer (Spectra Max250, Molecular Devices, Downingtown, PA, USA) at 570 nm with a reference wavelength of 650 nm.

Construction of the luciferase reporter plasmid and luciferase assay

The IL-8 promoter fragment −135 to +46 was amplified from human genomic DNA (Clonetech, Palo Alto, CA, USA) using the following primers as described previously (23): 5'primer, 5'-GTGAGATCTGAAGTGTGATGACTCAGG-3'; 3' primer, 5'-GTGAAGCTTGAAGCTTGTGTGTGCTCTGC-3'. The promoter fragment was then ligated into the BglII and HindIII restriction sites of the luciferase reporter plasmid pGL-3-Basic vector (Promega, Madison, WI, USA), which yielded the reporter construct pIL-8-Luc. The transfection assay and the luciferase assay

were then performed as described elsewhere (24). One day prior to the transfection, HEK 293 cells were seeded at a density of 5×10^4 cells/well in 12-well plates. After transfection, the cells were placed in complete medium for 24 h before being treated with TNF-α (5 ng/ml) for 8 h. To detect the effects of flavonoids on TNF-α-induced IL-8 promoter activation, we preincubated the cells with flavonoids at the indicated concentrations for 1 h prior to treatment with TNF-α. The cells were then harvested, washed, and lysed by freeze-thawing three times, after which the luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) in conjunction with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) in accordance with the manufacturer's specifications.

Total RNA isolation and Reverse-transcription PCR analysis

The HEK 293 cells were pretreated with flavonoids (kaempferol, quercetin, fisetin, chrysin) at a concentration of 40 μM for 1 h, after which the cells were treated with TNF-α (5 ng/ml) for the indicated time periods. Next, the total RNA was extracted using an RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA). Five micrograms of total RNA were then reverse-transcribed in first-strand synthesis buffer that contained 6 μg/ml of oligo (dT) primer, 50 U of reverse transcriptase, 4 mM of dNTP, and 40 U of RNase inhibitor. One microliter of the cDNA mixture was then subjected to a standard PCR reaction for 25 cycles with the following primer sets: human β-actin, 5'-GGGTCAGAAGG ATTCCTATG-3' (sense), 5'-CCTTAATGTCACGCACGATTT-3' (anti-sense) (500 bp); human IL-8, 5'-ATGACTTCCAAGCTGG CCGTGGCT-3' (sense), 5'-TCTCAGCCCTCTTCAAAAACTTCT-3' (anti-sense) (292 bp). The PCR products were then visualized with UV light after being resolved on a 1.2% agarose gel.

Western blotting

We performed SDS-PAGE and Western blot analysis as described elsewhere (25). Briefly, HEK 293 cells were preincubated with flavonoids (kaempferol, quercetin, fisetin, or chrysin) at a concentration of 40 μM for 1 h prior to TNF-α treatment for the indicated time periods. After treatment, the cells were harvested and lysed in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 1% NP-40, and complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Equal amounts of protein were then resolved by 10% SDS-PAGE, after which they were electrotransferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The immunoreactive proteins were then detected by horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence reagent (Animal Genetics Inc., Korea).

Indirect immunofluorescence assay and confocal microscopy

The effects of flavonoids on TNF-α-induced NF-κB p65 nuclear localization were detected by an indirect immunofluorescence assay using confocal microscopy as described previously (25). Briefly, HEK 293 cells (1×10^5 cells/well) were cultured in 24-well plates and then preincubated with flavonoids (kaempferol, quercetin, fisetin, or chrysin) at a concentration of 40 μM for 1 h prior to TNF-α treatment for 30 min. After stimulation, the cells were washed with PBS and then fixed and permeabilized with 0.2% triton X-100 in PBS for 20 min at 4°C. Next, the cells were blocked with 1.5% normal donkey serum (Sigma), after which they were incubated with polyclonal antibody to NF-κB p65 for 1 h followed by incubation with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) for 1 h. DNA staining (0.5 μg/ml Hoechst No. 33258; Sigma) was then used to identify cell nuclei. Briefly, the cells were mounted on coverslips using Fluoromount-G (Southern Biotechnology Associates Inc, Birmingham, AL) and then scanned with a Zeiss LSM 510 laser scanning confocal device attached to an Axiovert 100 microscope using a Plan-Apochromat 100X/Oil DIC objective (Carl Zeiss, Germany).

Measurement of intracellular ROS levels

The oxidation-sensitive fluorescent probe, dihydrorhodamine 123 (Molecular Probes, Eugene, OR, USA), was used to evaluate the intracellular ROS levels in accordance with the manufacturer's specifications (26). Briefly, HEK 293 cells (1×10^5 cells/well) were cultured in 24-well plates and then preincubated with flavonoids (kaempferol, quercetin, fisetin, or chrysin) at a concentration of 40 μM for 1 h prior to TNF-α treatment for 30 min. Next, the cells were incubated with dihydrorhodamine 123, after which they were fixed with 4% paraformaldehyde (USB Corp., Cleveland, OH, USA) for 10 min. The cells were subsequently transferred to coverslips, mounted in Fluoromount- G, and then scanned with a confocal device (Zeiss LSM 510).

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REFERENCES

1. Chen, C. C., Chow, M. P., Huang, W. C., Lin, Y. C. and Chang, Y. J. (2004) Flavonoids inhibit tumor necrosis factor-alpha-induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells through activator protein-1 and nuclear factor-kappaB: structure-activity relationships. *Mol. Pharmacol.* **66**, 683-693.
2. Lotito, S. B. and Frei, B. (2006) Dietary flavonoids attenuate tumor necrosis factor alpha-induced adhesion molecule expression in human aortic endothelial cells. *J. Biol. Chem.* **281**, 37102-37110.
3. Comalada, M., Ballester, I., Bailón, E., Sierra, S., Xaus, J.,

Gálvez, J., de Medina, F. S. and Zarzuelo, A. (2006) Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure-activity relationship. *Biochem. Pharmacol.* **72**, 1010-1021.
4. Fang, S. H., Rao, Y. K. and Tzeng, Y. M. (2005) Inhibitory effects of flavonol glycosides from Cinnamomum osmophloeum on inflammatory mediators in LPS/IFN-gamma-activated murine macrophages. *Bioorg. Med. Chem.* **13**, 2381-2388.
5. Kempuraj, D., Madhappan, B., Christodoulou, S., Boucher, W., Cao, J., Papadopoulou, N., Cetrulo, CL. and Theoharides, T. C. (2005) Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. *Br. J. Pharmacol.* **145**, 934-944.
6. Pang, J. L., Ricupero, D. A., Huang, S., Fatma, N., Singh, D. P., Romero, J. R. and Chattopadhyay, N. (2006) Differential activity of kaempferol and quercetin in attenuating tumor necrosis factor receptor family signaling in bone cells. *Biochem. Pharmacol.* **71**, 818-826.
7. Hu, M. (2007) Commentary: bioavailability of flavonoids and polyphenols: call to arms. *Mol. Pharm.* **4**, 803-806.
8. Lee, E. R., Kang, Y. J., Kim, J. H., Lee, H. T. and Cho, S. G. (2005) Modulation of apoptosis in HaCaT keratinocytes via differential regulation of ERK signaling pathway by flavonoids. *J. Biol. Chem.* **280**, 31498-31507.
9. Lin, H. Y., Juan, S. H., Shen, S. C., Hsu, F. L. and Chen, Y. C. (2003) Inhibition of lipopolysaccharide-induced nitric oxide production by flavonoids in RAW264.7 macrophages involves heme oxygenase-1. *Biochem. Pharmacol.* **66**, 1821-1832.
10. Bradley, J. R. (2008) TNF-mediated inflammatory disease. *J. Pathol.* **214**, 149-160.
11. Rahman, M. M. and McFadden, G. (2006) Modulation of Tumor Necrosis Factor by Microbial Pathogens. *PLoS Pathog.* **2**, 66-77.
12. Kim, H. J., Hwang, J. S., Woo, C. H., Kim, E. Y., Kim, T. H., Cho, K. J., Seo, J. M., Lee, S. S. and Kim, J.H. (2008) TNF-α-induced up-regulation of intercellular adhesion molecule-1 is regulated by a Rac-ROS-dependent cascade in human airway epithelial cells. *Exp. Mol. Med.* **40**, 167-175.
13. Muppidi, J. R., Tschopp, J. and Siegel, R. M. (2004) Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. *Immunity* **4**, 461-465.
14. Palladino, M. A., Bahjat, F. R., Theodorakis, E. A. and Moldawer, L. L. (2003) Anti-TNF-α therapies: the next generation. *Nat. Rev. Drug Discov.* **9**, 736-746.
15. Zhang, G. (2004) Tumor necrosis factor family ligand-receptor binding. *Curr. Opin. Struct. Biol.* **2**, 154-160.
16. Karin, M. and Greten, F. R. (2005) NF-κB: linking inflammation and immunity to cancer development and progression. *Nat. Rev. Immunol.* **10**, 749-759.
17. Park, J. S., Rho, H. S., Kim, D. H. and Chang, I. S. (2006) Enzymatic preparation of kaempferol from green tea seed and its antioxidant activity. *J. Agric. Food Chem.* **54**, 2951-2956.
18. Samhan-Arias, A. K., Martín-Romero, F. J. and Gutiérrez-Merino, C. (2004) Kaempferol blocks oxidative stress in cerebellar granule cells and reveals a key role for reactive oxygen species production at the plasma membrane in the commitment to apoptosis. *Free Radic. Biol. Med.* **37**, 48-61.
19. Park, H. H., Lee, S., Son, H. Y , Park, S. B., Kim, M. S., Choi, E. J., Singh, T. S., Ha, J. H., Lee, M. G., Kim, J. E., Hyun, M. C., Kwon, T. K., Kim, Y. H. and Kim, S. H. (2008) Flavonoids inhibit histamine release and expression of proinflammatory cytokines in mast cells. *Arch. Pharm. Res.* **31**, 1303-1311.
20. Chen, Z. J. (2005) Ubiquitin signalling in the NF-κB pathway. *Nat. Cell Biol.* **7**, 758-765.
21. Hoek, J. B. and Pastorino, J. G. (2004) Cellular signaling mechanisms in alcohol-induced liver damage. *Semin. Liver Dis.* **24**, 257-272.
22. Alley, M. C., Scudiero, D. A., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., Abbott, B. J., Mayo, J. G., Shoemaker, R. H. and Boyd, M. R. (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* **48**, 589-601.
23. Kim, D. S., Han, J. H. and Kwon, H. J. (2003) NF-κB and c-Jun-dependent regulation of macrophage inflammatory protein-2 gene expression in response to lipopolysaccharide in RAW 264.7 cells. *Mol. Immunol.* **40**, 633-643.
24. Choi, Y. J., Lee, K. W., Kwon, H. J. and Kim, D. S. (2006) Identification of immunostimulatory oligodeoxynucleotide from Escherichia coli genomic DNA. *J. Biochem. Mol. Biol.* **39**, 788-793.
25. Lee, K. W., Lee, Y., Kwon, H. J. and Kim, D. S. (2005) Sp1-associated activation of macrophage inflammatory protein-2 promoter by CpG-oligodeoxynucleotide and lipopolysaccharide. *Cell Mol. Life Sci.* **62**, 188-198.
26. Park, L. J., Ju, S. M., Song, H. Y., Lee, J. A., Yang, M. Y., Kang, Y. H., Kwon, H. J., Kim, T. Y., Choi, S. Y. and Park, J. (2006) The enhanced monocyte adhesiveness after UVB exposure requires ROS and NF-kB signaling in human keratinocytes. *J. Biochem. Mol. Biol.* **39**, 618-625.