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Suppression of TNFα-Mediated NFκB Activity by Myricetin and Other Flavonoids Through Downregulating the Activity of IKK in ECV304 Cells

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Abstract Flavonoids are a group of naturally-occurring phenolic compounds in the plant kingdom, and many flavonoids are found with vascular protective properties. Nevertheless how the protective response is exerted by flavonoids is not well characterized. In view of the nuclear factor-κB (NFκB) may play a central role in the initiation of atherosclerosis, prevention of the activation of NFκB represents an important role in protecting vascular injury. In this study, the effects of flavonoids on NFκB/inhibitor-κB (IκB) system in ECV304 cells activated with tumor necrosis factor-α (TNFα) were examined. We investigated the inhibitory action of six flavonoids on IκB kinase (IKK) activity, an enzyme recently found to phosphorylate critical serine residues of IκB for degradation. Of six flavonoids tested, myricetin was found to strongly inhibit IKK kinase activity, and prevent the degradation of IκBα and IκBβ in activated endothelial cells. Furthermore, myricetin was also found to inhibit NFκB activity correlated with suppression of monocyte adhesion to ECV304 cells. Therefore we conclude that flavonoids may be of therapeutic value for vascular disease through down regulation of NFκB/IκB system. J. Cell. Biochem. 74:606–615, 1999. © 1999 Wiley-Liss, Inc.

Key words: myricetin; flavonoids; IκB kinase; NFκB; ECV304 cell

The transcription factor nuclear factor-κB (NFκB) is a pleiotropic regulator of many genes included in immune and inflammatory response, including the leukocyte adhesion molecules [Grilli et al., 1993]. NFκB exits in a latent form in the cytoplasm of unstimulated cells comprising a transcriptionally active dimer bound to an inhibitor, inhibitor-κB (IκB) [Liou et al., 1993]. These IκB proteins contain ankyrin repeat motifs that mask the nuclear localization sequence of NFκB subunits [Beg et al., 1992; Hatada et al., 1993]. Following cytokine stimulation such as tumor necrosis factor-α (TNF-α), the NFκB is activated which involves the phosphorylation and degradation of IκB proteins [Baeuerle et al., 1996; Zabel et al., 1990]. Recently, a high molecular mass (500–900 kDa) multisubunit IκB kinase (IKK) has been found to phosphate at Ser-32 and Ser-36 of IκBα and Ser-19 and Ser-23 in the N terminus of IκBβ. Two catalytic subunits (termed IKK1 and IKK2) of IKK have been identified, cloned and shown to be widely expressed in human tissues. IKK1 and IKK2 form homo- and heterodimers with each other, but the active complex appears to be the heterodimer. It has been demonstrated that IKK is the kinase involved in the signal-inducible degradation of IκB [Didonato et al., 1997; Maniatis et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Zandi et al., 1997].

Previous reports have been shown that NFκB/IκB may play a key role in regulating vascular pathophysiology [Lindner et al., 1996]. Functional NFκB elements can be found in many genes whose expression is increased in vascular cells at sites of inflammatory responses [Collins, 1993; Collins et al., 1993a]. NFκB can be activated by a variety of signals relevant to induce leukocyte adhesion and transmigration in the vessel wall, thus results in endothelial

Abbreviations used: IκB, inhibitor κB; IKK, IκB kinase; NFκB, nuclear factor-κB; TNF-α, tumor necrosis factor-α.

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pathology [Collins et al., 1995]. A wide variety of activities have been attributed to flavonoids such as anti-inflammatory, antimicrobial, hepato-protective, and vascular protective activities [Cody et al., 1988; Middleton et al., 1992]. In this study, we examined the possible effects of flavonoids on IKK activity and NFκB/IκB system in endothelial ECV304 cells. We investigated the inhibitory action of six flavonoids on IKK activity, myricetin was the most potent one.

MATERIALS AND METHODS

Reagents

Kaempferol, quercetin, myricetin, resveratrol, apigenin, and genistein were purchased from Sigma Chemical (St. Louis, MO). Human recombinant TNFα were purchased from R&D Systems (Minneapolis, MN). Isotopes were obtained from Amersham (Arlington Heights, IL).

Cell Culture

The immortalized human endothelial cell line ECV304 cells was obtained from the Culture Collection and Research Center of Food Industry Research and Development Institute. ECV304 cells were cultured in medium 199 (HEPES modification) containing 10% endotoxin-free heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, and cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. When the cells reached a confluent monolayer, they were activated by incubation in medium containing TNFα(25 ng/ml). Various concentrations of the tested compounds dissolved in DMSO were added together with TNFα. The human promyelomonocytic HL-60 cells was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 supplemented with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin.

IκB Kinase Assay

The IκB kinase assay was performed as described by Spiecker et al. [1998] with some modifications. Whole cell extracts were lysed with Gold lysis buffer (10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris-HCl pH7.9, 100 μM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for 30 min at 4°C. The cell lysate was clarified by centrifugation at 12,000*g* for 10 min at 4°C. The IKK1 and IKK2 were immunocipitated with IKK-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 18 h at 4°C. The IKK-antibody complex was then precipitated with protein-A agarose and washed three times with PD-buffer (40 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.1% Nonidet P40, 6 mM EDTA, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM sodium orthovanadate, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM DTT) and once with kinase buffer (without ATP). The purified enzyme was incubated with an IκBα-GST fusion protein (Santa Cruz Biotechnology) as a substrate in kinase buffer containing HEPES (20 mM pH 7.7), 10 mM $MgCl_2$, 10 μM ATP, [γ-^{32}P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM sodium orthovanadate, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM DTT. The reaction was terminated by the addition of 5 × SDS-polyacrylamide gel electrophoresis (PAGE) sample-buffer and boiling for 10 min. Proteins were separated on 12% SDS-PAGE and autoradiography of the dried gel was performed at –70°C.

Western Blots

Total cellular extract was prepared using Gold lysis buffer (10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris-HCl pH 7.9, 100 μM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Total proteins (for IKK1, IKK2, IκBα, IκBβ and α-tubulin) containing 30–50 μg of protein were separated on sodium dodecyl sulfate-polyacrylamide minigels (8% for IKK1, IKK2, and 10% for IκBα, IκBβ) and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membrane was incubated overnight at 37°C with 10% bovine serum albumin in phosphate-buffered saline to block nonspecific immunoglobulins and then incubated with anti-IκBα, IκBβ, IKK1, and IKK2 polyclonal antibodies (Santa Cruz Biochemicals), or anti-α- tubulin monoclonal antibody (Oncogene Science, Cambridge, UK). IKK1, IKK2, IκBα, IκBβ, and α-tubulin protein were detected by chemiluminescence

(ECL, Amersham), or by incubation with coloregenic substrates nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as suggested by the manufacture (Sigma Chemical Co.).

Electrophoretic Mobility Shift Assays for NFκB

Nuclear and cytoplasmic extracts were prepared according to a modified method of Lin et al. [1997]. At the end of culture, the cells were suspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice and vortexed for 10 sec. Nuclei were pelleted by centrifugation at 12,000*g* for 20 sec. The supernatants containing cytosolic proteins were collected. A pellet containing nuclei was suspended in buffer C (20 mM HEPES, pH 7.6, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 12,000*g* for 20 min and stored at –20°C. For electrophoretic mobility shift assay, each 2 μg of nuclear extract was mixed with the labeled double-stranded NFκB oligonucleotide, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, and incubated at room temperature for 20 min. The incubation mixture included 1 μg of poly (dI-dC) in a binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, 50 mM NaCl). The DNA/protein complex was electrophoresed on 6% nondenaturing polyacrylamide gels in 0.5 × Tris/borate/EDTA buffer (0.0445 M Tris, 0.0445 M borate, 0.001 M EDTA). The specificity of binding was also examined by competition with the unlabeled oligonucleotide. Radioactive bands were detected by autoradiography.

Transient Transfection and Luciferase Assay

The luciferase assay was performed as described by George et al. [1997] with some modification. ECV304 cells were seeded in a 24-well tissue culture plate. When the cells are confluent, medium was replaced the serum-free medium. Cells were transfected with the pNFκB-Luc plasmid (Stratagene, La Jolla, CA) reporter gene using LipofectAMINE™ reagent (Gibco, NRL, Life Techhnologies, Bethesda, MD). As an internal control for transfection efficiency, pGF-Pemd-cmv control plasmid (Packard, Meriden, CT) was co-transfected in the experiment. After 24 h, replace the medium fresh, complete medium for 24 h. Cells were then incubated with the TNFαand myricetin for 4 h before the adding of 200 μl of lysis buffer (0.5 M HEPES pH 7.8, 5% Triton N-101, 1 mM $CaCl_2$, 1 mM $MgCl_2$). Cells were shaken for 5 min to ensure complete lysis before transfer 100μl to a white (opaque) 96-well plate, then the Packard Luclite reagent were added to 96-well plate. Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence Counter (Packard 9912V) in singlephoton counting mode for 0.1 min/well, following a 10 min adaptation in the dark.

Monocyte Adherence to ECV304 Cells

Monocyte adherence to ECV304 cells was assayed according to a protocol described earlier [Lafrenie et al., 1996]. Briefly, promyelomonocytic HL-60 cells were suspended at 0.75 × 10^6 cells/ml in RPMI 1640 medium containing 10% FBS and incubated with 1 μCi of [^{3}H]thymidine overnight at 37°C. Labeled monocytes were washed and resuspended to approximately 10^6 cells/ml in basal medium199. ECV304 cell monolayers were incubated without or with indicated concentrations of myricetin and TNFα (25 ng/ml) for 12 h at 37°C. The medium was aspirated, and 500 μl of monocyte suspension was added to each ECV304 cell monolayer-containing well and incubated for 60 min at 37°C. Nonadherent cells were aspirated and the monolayers were washed three times with HBSS, pH 7.4. The adherent cells were solubilized and counted in a beta scintillation counter (Packard Co.). The percent binding was calculated and the data were normalized (TNF = 100%). Results are the means of three independent experiments and the data are expressed as means ± SD.

RESULTS

Inhibition of IKK1 Activity by Selected Flavonoids on ECV304 Cells

Flavonoids comprise a large group of naturally occurring low molecular weight substances that are present in a wide variety of fruits and vegetables. The selected flavonoids were apigenin, genistein, kaempferol, myricetin, quercetin, and resveratrol. Structure of the selected flavonoids is shown in Figure 1.

ECV304 cells were exposed to each of the selected flavonoids and TNFα (25 ng/ml) for 15

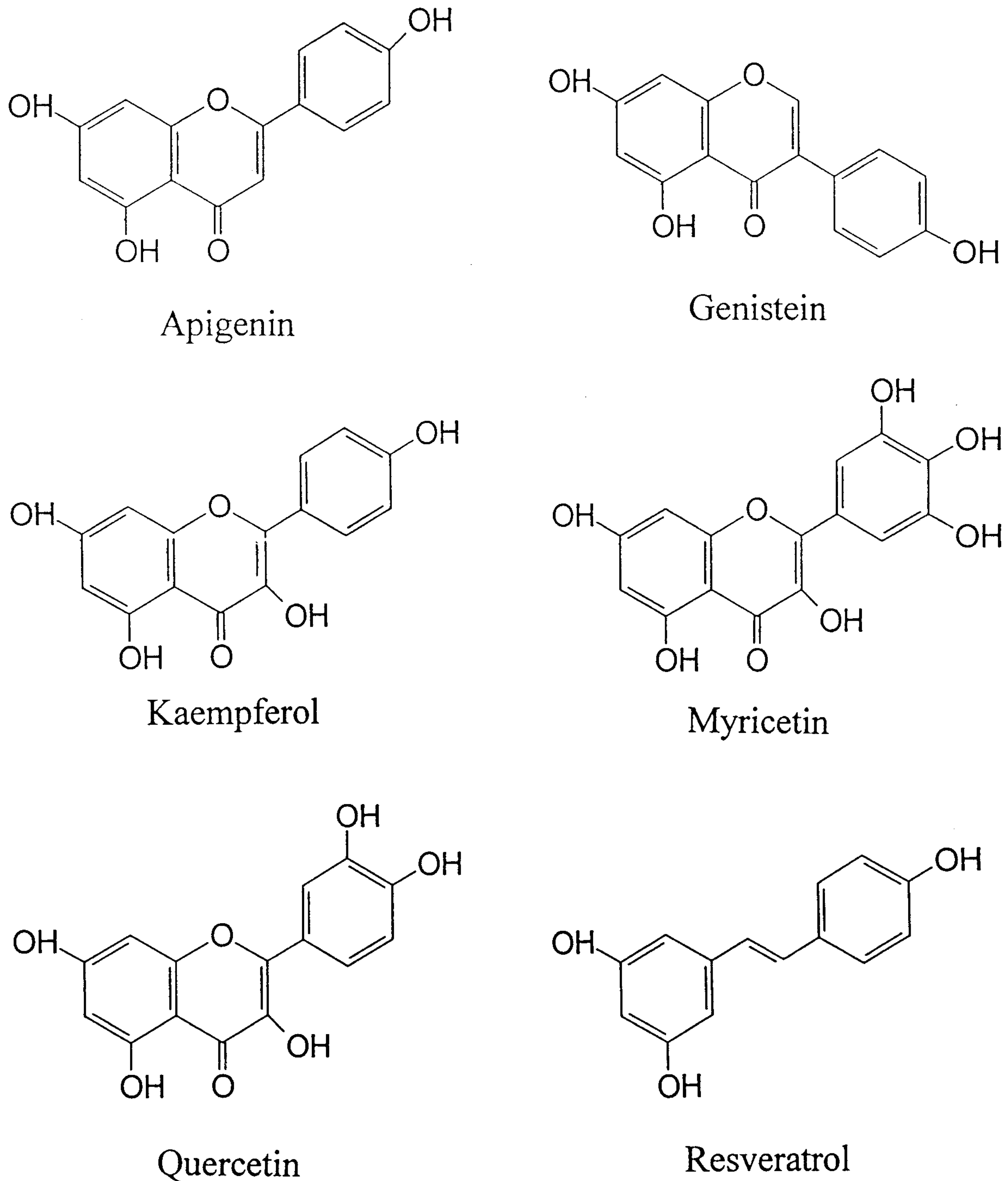

Fig. 1. Structures of selected flavonoids.

min. In order to directly measure IKK1 activity in ECV304 cells, IKK1 protein was immunoprecipitated from cell extracts and the kinase activity in the immunocomplex assayed using recombinant GST-IκBα (1–317) as a substrate. Figure 2 illustrates the relative effect on IKK1 activity by selected flavonoids at a concentration of 30 μM. After stimulation with TNFα (25 ng/ml), GST-IκBα fusion protein was strongly phosphorylated, indicating stimulation of IKK1 activity, and the minimal basal IKK1 activity in unstimulated ECV304 cells was also found. Some of the selected flavonoids were shown to inhibit the activity of IKK1 induced by TNFα at

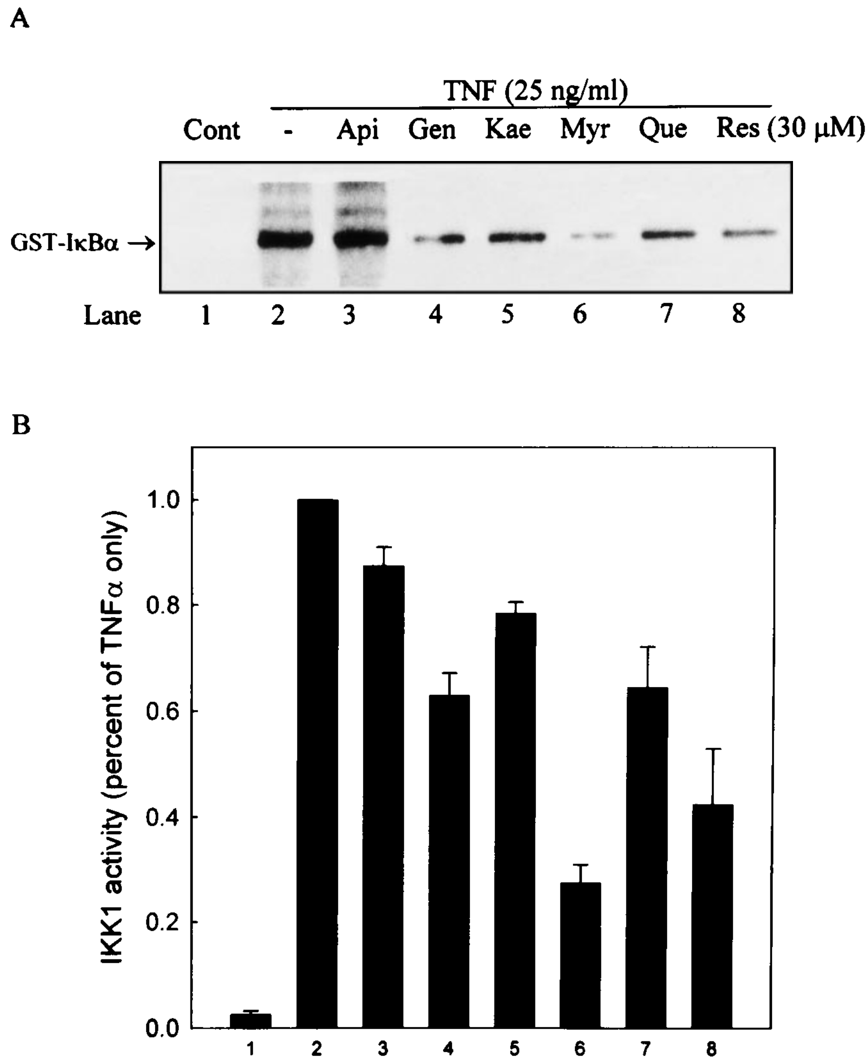

Fig. 2. Analysis of IKK1 expression and its activation in ECV304 cells treated with TNFα and various flavonoids. **A**: Immunoprecipitation, kinase assays on whole cell extracts. ECV304 cells were treated with various flavonoids for 15 min. Cells were harvested and IKK1 activity in the soluble fractions were analyzed using immune complex kinase assays as described in Materials and Methods. IKK1 was immunoprecipitated using the anti-IKK1 antibody, and the activity in the immune complexes was assayed using GST-IκBα(1–317) as a substrate. Api, apigenin; Gen, genistein; Kae, kaempferol; Myr, myricetin; Que, quercetin; Res, resveratrol. **B**: Quantification of the phosphorylated-GST-IκBα was performed by densitometetric analysis (IS-1000 Digital System) of the kinase assay. Data are expressed as the mean ± SE of the percentage of maximal phosphorylated-GST-IκBα observed with TNFα as determined by three independent experiments.

30 μM, the most effective compound was myricetin, which inhibited IKK1 activity by 70%. The inhibitory activities of resveratrol, genistein, and quercetin, however, were lower, and apigenin, kaempferol had little effect on TNFα-induced IKK1 activity. Because the result showed that some flavonoids can inhibit the IKK1 activity in ECV304 cells, we sought to determine whether this effect is mediated by changes in the level of IKK1 complex. Western blot analysis for IKK1 indicated that the level of IKK1 protein was not changed (data not shown). This result suggested that the inhibition of flavonoids on TNFα-induced IKK1 activity was not due to decrease expression of IKK1.

Inhibition of Kinase Activity of IKK1 and IKK2 by Myricetin on ECV304 Cells

Of the flavonoids tested, myricetin was the most potent inhibitor of IKK1 in ECV304 cells. Therefore, we studied the effect of different concentration of myricetin on IKK1 activity. Since it has been reported that IKK1 and IKK2 need to form a heterodimer for maximal en-

zyme activity [Moureo et al., 1997], we also tested the effect of myricetin on the IKK2 activity of ECV304 cells using an immunocomplex kinase assay. Stimulation of ECV304 cells with TNFα caused a marked increase in IKK1 and IKK2 activity, as measured after 10 min. Myricetin, at 10–50 μM, dose dependently inhibited IKK1 and IKK2 activity, and the kinase activity of IKK2 was less affected compared to that of IKK1 (Fig. 3). Western blot analysis for IKK1 and IKK2 showed that the protein level of IKK1 and IKK2 were not changed (data not shown). These results further to confirm that myricetin could inhibit the activity of IKK complex (IKK1 and IKK2) in TNFα-stimulated ECV304 cells, and these effects were not due to decrease the protein level of IKK.

Inhibition of TNFα-Induced Degradation of IκBs and NFκB Activity in ECV304 Cells

It has been reported that IKK could phosphorylate IκBα and IκBβ then trigger the degradation of IκBs. We then studied whether myricetin affects degradation of IκBs. After 15 min activation of ECV304 cells by TNFα, IκBα and IκBβ were detected by specific IκBα and IκBβ antibodies. The results are illustrated in Figure 4. Western blotting of cell extracts showed that stimulation with TNFα alone caused a reduction in the levels of both IκBα and IκBβ. Myricetin blocked the TNF-α-mediated degradation of IκBα and IκBβ proteins with a dose-dependent manner.

The degradation of IκBs results in the NFκB activation, we then investigated the effect of myricetin on NFκB activation by electrophoretic mobility shift assay and transient transfection. As shown in Figure 5A, gel mobility shift assay revealed that myricetin inhibited TNFα-stimulated NFκB binding activity, and the specific of this protein-DNA complex for the NFκB sequence was determined by competition binding experiments (data not shown). Moreover, similar results were obtained with transient transfection. A pNFκB-Luc reporter was cotransfected with pGFPemd-cmv control plasmids into ECV304 cells. Figure 5B showed that myricetin blocked induction of NFκB reporter activity with a dose-dependent manner. Together with the results, myricetin inhibited TNFα-stimulated NFκB activation, consistent with their effect on TNFα-mediated IκBs degradation and IKK activity. These results suggested that myricetin could inhibit IKK activity

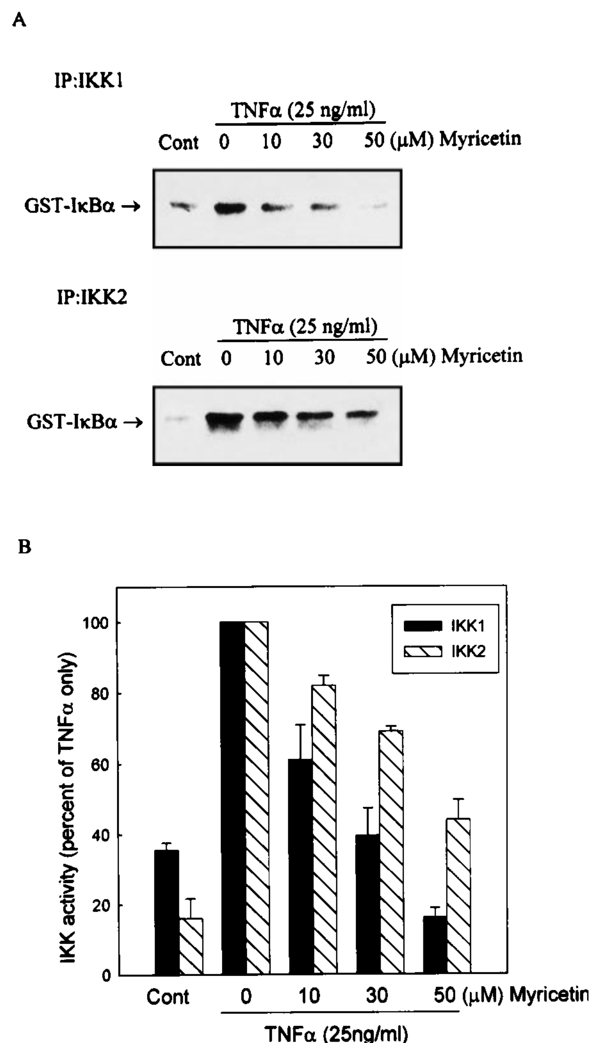

Fig. 3. Inhibition of TNFα- induced dose-dependent IKK kinase activity in ECV304 cells. Whole cell extracts were prepared from ECV304 cells treated with the indicated concentration of myricetin and 25 ng/ml TNFα for 10 min. IKK1 and IKK2 kinase activity were carried out as described in Materials and Methods. **A**: Effects of myricetin concentration on IKK1 kinase activity. A part of each extract was used to measure IKK1 activity (upper panel), and another part was used to measure IKK2 activity by immune complex kinase assay (bottom panel). **B**: Quantification of the phosphorylated-GST-IκBα was performed by densitometetric analysis (IS-1000 Digital System) of the kinase assay. Data are expressed as the mean ± SE of the percentage of maximal phosphorylated-GST-IκBα observed with TNFα as determined by three independent experiments.

stimulated with TNFα in ECV304 cells, then perturbed the degradation of IκBα and IκBβ. Finally, the NFκB activation was blocked.

Inhibition of TNFα-Induced Monocyte Adhesion to Endothelial ECV304 Cells

The results described here identify myricetin as an inhibitor of IKK, then results in perturb-

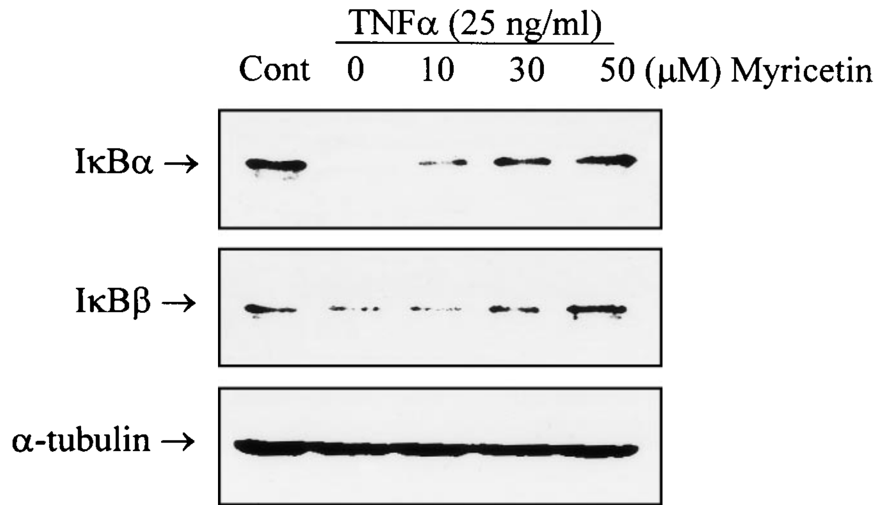

Fig. 4. Inhibition of TNFα-induced dose-dependent stabilization of IκBα and IκBβ in ECV304 cells. Western blot analysis of total protein extracts from ECV304 cells treated with the indicated concentrations of myricetin and 25 ng/ml TNFα for 15 min. Extracts were separated on 10% SDS-PAGE and analyzed as indicated in Materials and Methods. Similar results were obtained in three independent experiments.

ing the NFκB/IκB system. Moreover, it has been reported that NFκB/IκB system may play a pivotal role in regulating leukocyte adhesion to endothelium cells. Thus, we further examined the effects of myricetin on the adhesion of leukocyte to ECV304 cells. Analysis of monocyte adhesion by [^{3}H] thymidine- labeled HL-60 cells was performed. The results are shown in Figure 6, TNFα induced a significant increase in HL60 adhesion to ECV304 cells, and myricetin inhibited the adhesion of HL60 cells to monolayers in a concentration-dependent manner. The TNFα-induced adhesion was completely inhibited by myricetin at 50 μM concentration. These results suggested that the inhibition of IKK activity by myricetin results in perturbing the NFκB/IκB system and finally myricetin could act as a protector for monocyte adhesion to endothelial cells.

DISCUSSION

The adhesion of circulating leukocytes to the vessel wall is an initiating event in atherogenesis and vascular inflammation [Cybulsky et al., 1991; Davies et al., 1993; Pober et al., 1991]. Under certain conditions the "activated" endothelium expresses cell surface adhesion molecules which mediate specific interactions between the endothelium and circulating leukocytes [Mackay et al., 1993; Smith, 1993]. Recent studies suggest that activation of NFκB is required for the transcriptional induction of endothelial cell adhesion molecules and thereby the NFκB may play a central role in the initiation of atherosclerosis [Collins, 1993; Collins et al., 1995].

Flavonoids occur ubiquitously in the plant kingdom and are common components of the human diet. Flavonoids have been shown to have structurally dependent, highly specific effects on variety enzymes and are able to interfere with numerous cellular processes [Brandi, 1992; Havsteen, 1983]. Several observations suggest that selected flavonoids can affect cell-cell interaction including possible effect on adhesion molecular expression and function [Middleton et al., 1992]. How these effects are modulated is not yet clear but one important mechanism may be the capacity of flavonoids to stimulate or inhibit protein phosphorylation and thereby regulate cell function. Here we found that six flavonoids including apigenin, genistein, kaempferol, myricetin, quercetin, and resveratrol could inhibit the kinase activity of IKK with different potency.

IKK has recently been shown to be an important effector of NFκB/IκB pathway and a key enzyme involved in signal transduction [Maniaties, 1997]. The development of natural IKK inhibitors as vascular protective agents would be helpful in designing analogues that could be used for the treatment of vascular disease. Using ECV304 cells, an immortalized human endothelial cell line that exhibit TNFα-induced IKK activity, the inhibitory effect of myricetin was greatest than that of other flavonoids; res-

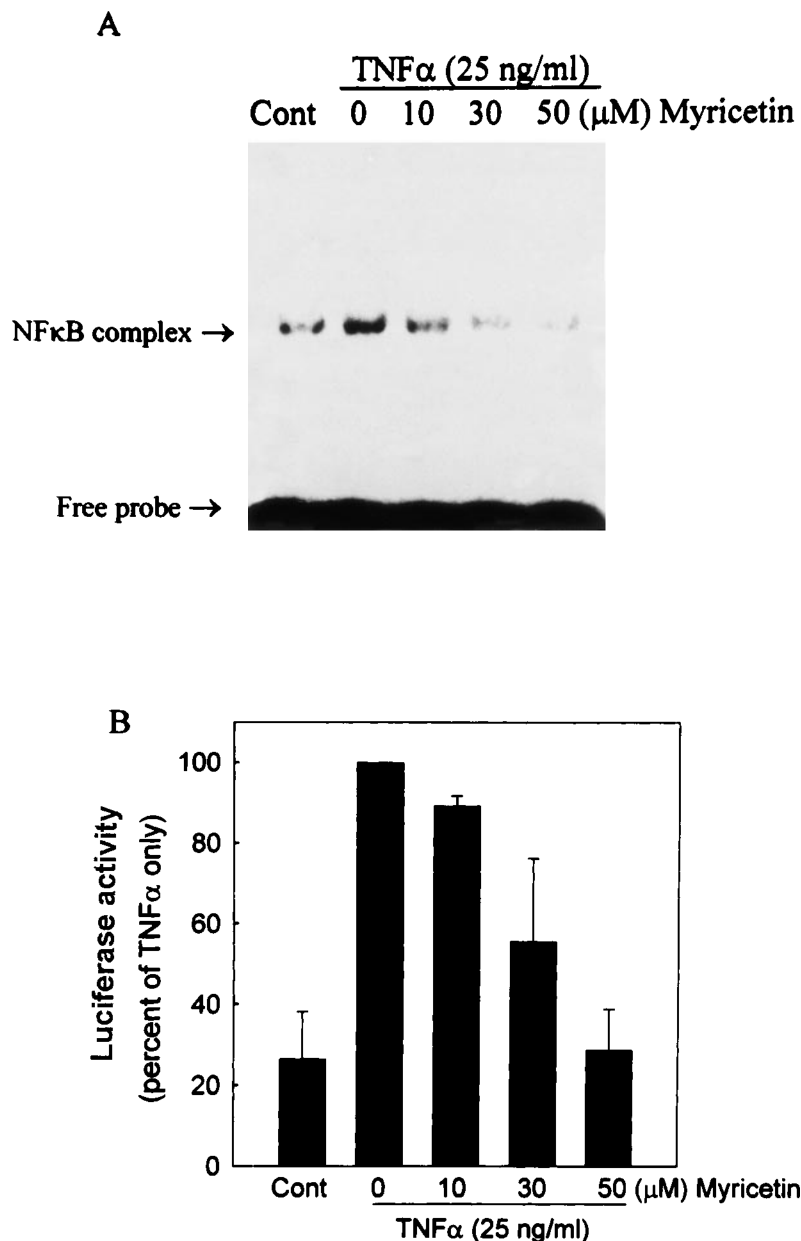

Fig. 5. Inhibition of TNFα-induced activation of NFκB. **A**: Nuclear extracts were prepared from ECV304 cells treated with the indicated concentration of myricetin and 25 ng/ml TNFα for 1 h. Electrophoretic mobility shift assay was carried out as described in Material and Methods, and binding of nuclear extracts to ^{32}P-NFκB DNA oligonucleotide is shown. Position of the NFκB complex with DNA is indicated with an arrow. Similar results were obtained in three independent experiments. **B**: Cells were cotransfected with pNFκB-Luc and pGFPemd-cmv plasmids. After 48 h, cells were treated with the indicated concentration of myricetin and 25 ng/ml TNFα for 4 h. Luciferaseee activities were determined and normalized on the basis of pGFPemd-cmv expression as described in Materials and Methods. Data are expressed as mean ± SE of the percentage of maximal luciferase activity observed with TNFα only as determined by three independent experiments.

veratrol, genistein, and quercetin were moderately with inhibitory activity, furthermore apigenin and kaempferol were almost devoid of inhibitory effect. It is difficult to draw structure-activity relationship from these flavonoids, but it may be noted that myricetin is the one with the most amount of OH groups. In addition, Agullo et al. [1998] has reported that myricetin was the most effective inhibitor of phosphatidylinositol 3-kinase. These suggest that hydroxy groups on benzene ring are important for inhibitory activity of kinase.

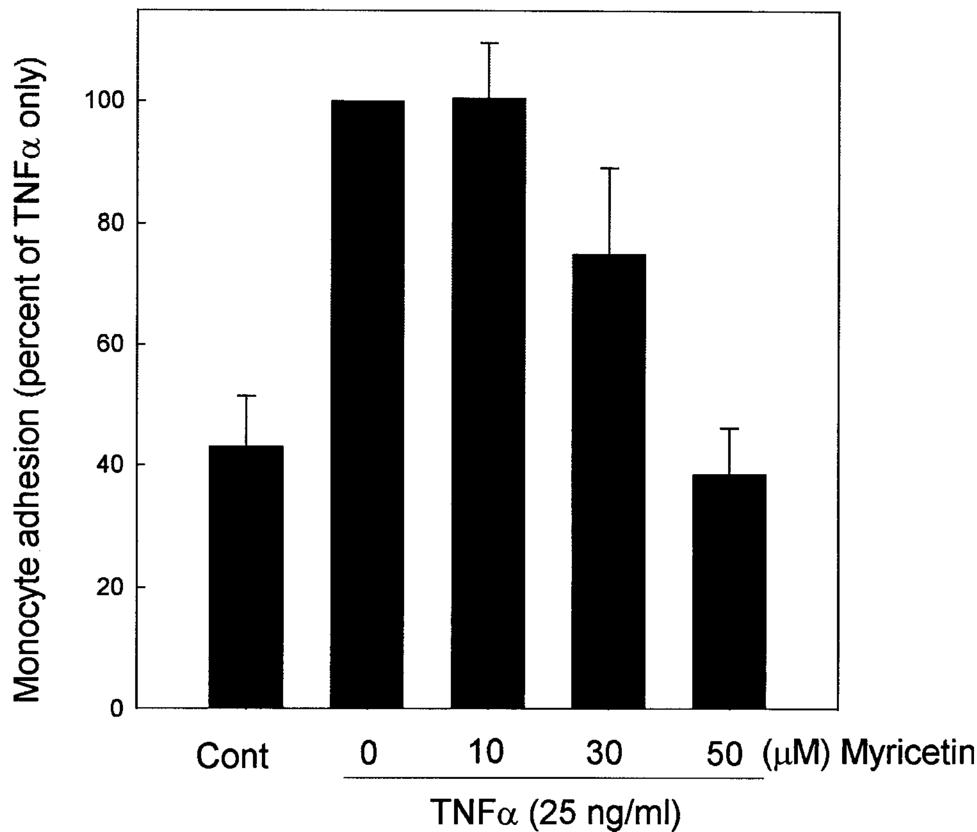

Fig. 6. Effects of myricetin on the adherence of monocyte to ECV304 cell monolayer. ECV304 cells grown to confluence in 24-well plastic culture plates were incubated with the indicated concentration of myricetin and 25 ng/ml TNFα for 12 h at 37°C. At the end of the incubation, ECV304 cells were washed gently with medium and incubated with [^{3}H] thymidine-labeled HL-60 cells for 1 h at 37°C. Nonadherent cells were aspirated, and the monolayers were washed three times with HBSS pH 7.4. The adhesion of HL-60 cells to ECV304 was determined as described in Materials and Methods. Data was normalized (TNFα= 100%) and reported as means ± SD. All determinations were made in triplicate.

To further elucidate the effect of flavonoids, we used myricetin as a model and found that treatment with myricetin resulted in decrease enzyme activity of IKK1 and IKK2. In this study, we also found that myricetin prevented the degradation of IκBα and IκBβ and thereby perturbed the activation of NFκB. Furthermore, the TNFα-induced monocyte adhesion to ECV304 cells was inhibited by myricetin. In endothelial cells, cytokines increase superoxide anion production, and reactive oxygen intermediates may act as an important regulator of NFκB [Matsubara et al., 1986; Collins, 1993a; Weber et al., 1994]. Flavonoids may act as antioxidants by inhibiting the generation of reactive oxygen intermediates, or by scavenging the free radicals to block NFκB activation. In addition, a number of antioxidants have been reported to inhibit cytokine-induced IκBα phosphorylation, degradation, NFκB activation, and NFκB-dependent transcription of vascular adhesion molecules [Schreck et al., 1991; Collins et al., 1993a; Marui et al., 1993; Baeuerle et al., 1994; Finco et al., 1994; Weber et al., 1994; Kumar et al., 1998]. Thus, these flavonoid and antioxidants inhibit NFκB signaling, they would be expected to be the protectors for vascular disease.

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