Quercetin inhibits inducible ICAM-1 expression in human endothelial cells through the JNK pathway

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Kobuchi, Hirotsugu, Sashwati Roy, Chandan K. Sen, Hao G. Nguyen, and Lester Packer. Quercetin inhibits inducible ICAM-1 expression in human endothelial cells through the JNK pathway. *Am. J. Physiol.* 277 (*Cell Physiol.* 46): C403–C411, 1999.—The cell adhesion molecule intercellular adhesion molecule-1 (ICAM-1) plays a pivotal role in inflammatory responses. Quercetin (3,3′,4′,5,7-pentahydroxyflavone), a naturally occurring dietary flavonol, has potent anti-inflammatory properties. The effect of quercetin on ICAM-1 expression induced by agonists phorbol 12-myristate 13-acetate (PMA) and tumor necrosis factor-α (TNF-α) in human endothelial cell line ECV304 (ECV) was investigated. Quercetin treatment downregulated both PMA- and TNF-α-induced surface expression, as well as the ICAM-1 mRNA levels, in ECV cells in a dose-dependent (10–50 μM) manner. Quercetin had no effect on PMA- or TNF-α-induced nuclear factor-κB (NF-κB) activation. However, under similar conditions a remarkable dose-dependent downregulation of activator protein-1 (AP-1) activation was observed. This decrease in AP-1 activation was observed to be associated with the inhibitory effects of quercetin on the c-Jun NH_2-terminal kinase (JNK) pathway. These results suggest that quercetin downregulates both PMA- and TNF-α-induced ICAM-1 expression via inhibiting both AP-1 activation and the JNK pathway.

flavonoids; intercellular adhesion molecule-1; activator protein-1; kinases; inflammation; c-Jun amino-terminal kinase

THE ADHESION OF LEUKOCYTES to the vascular endothelial cells is a critical step in the inflammatory response and involves recruitment and infiltration of leukocytes to the site of tissue injury, infection, or lesion formation. These processes are mediated by a wide variety of adhesion molecules. Intercellular adhesion molecule-1 (ICAM-1, CD54) expressed on endothelial cells is one of the major cell surface glycoproteins that contribute to the cell adhesion processes (7). Although ICAM-1 is constitutively expressed on endothelial cells, it can be significantly induced in response to proinflammatory mediators such as tumor necrosis factor-α (TNF-α) and interleukin-1β (44), as well as phorbol 12-myristate 13-acetate (PMA) (47, 55), oxidants (46), and human immunodeficiency virus-1 tat proteins (19). Elevated levels of ICAM-1 expression have been shown to be critically involved in the development of a variety of autoimmune diseases and pathologic inflammatory disorders, e.g., rheumatoid arthritis, psoriasis, and atherosclerosis (14, 53). Recently, ICAM-1 expression by tumor cells has been reported to be a major contributing factor that facilitates metastatic progression (30). The modulation of ICAM-1 expression is therefore an important therapeutic target, as shown by the beneficial effects of anti-ICAM-1 antibodies and other pharmacological agents on the progression of inflammatory responses in several in vivo studies (1, 5).

Quercetin, a plant flavonoid that occurs naturally, is widely distributed in fruits and vegetables such as apples, berries, and onions. The daily intake of various flavonoids has been estimated to be ~23–34 mg, and quercetin constitutes a major fraction of such intake. It has been estimated that sometimes quercetin may represent as much as 60% of the total flavonoids consumed (18, 26). Quercetin, related flavonoids, and plant extracts from tea and ginkgo have been reported to have various clinically relevant properties such as antioxidant (40, 42, 45, 48), anti-inflammatory (2, 23), and tumoricidal activity (3). Furthermore, beneficial associations between the consumption of quercetin and lower incidences of coronary heart diseases and stroke have been reported (25, 33). Despite much interest in the pharmacological activities of flavonoids as anti-inflammatory agents, only a few studies have investigated the role of flavonoids, especially quercetin, in the regulation of cell adhesion processes.

The ICAM-1 gene contains several transcription factor binding elements within the 5′-flanking region that are recognized by proteins of the nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) families (13). Deletion and point mutation studies have demonstrated that the interaction of transcription factors and these elements is necessary for the induction of ICAM-1 expression by cytokines or phorbol ester (13, 22, 46). Activations of NF-κB and AP-1 are known to occur by distinct pathways both of which are thought to be redox sensitive (4, 31, 50). The inhibitory effect of flavonoids on the expression of adhesion molecules has been suggested to be mediated by downregulation of induced NF-κB activation (23). The role of AP-1 in mediating the inhibitory effect of flavonoids on agonist-induced adhesion molecule expression remains to be elucidated. Quercetin and related flavonoids have been demonstrated to modulate the activities of certain kinases that are critical in the AP-1 signaling pathway (20). Our aim was to investigate the effect of quercetin on agonist (PMA and TNF-α)-induced ICAM-1 expression in ECV304 (ECV) human endothelial cells. Results of this study demonstrate for the first time that at low

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concentrations quercetin downregulates both PMA- and TNF-α-induced ICAM-1 expression in human endothelial cells via inhibition of the c-Jun NH_2-terminal kinase (JNK) pathway.

MATERIALS AND METHODS

Cells and Cell Culture

ECV cells, a spontaneously transformed immortal endothelial cell line established from the vein of an apparently normal human umbilical cord, were obtained from the American Type Culture Collection (Manassas, VA). ECV cells were grown in medium 199 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in a standard culture incubator with humidified air containing 5% CO_2 at 37°C. For extraction of nuclear proteins, cells were made quiescent by being incubated in medium 199 containing 0.2% FCS and antibiotics for 16 h before use. A stock solution of quercetin (Sigma, St. Louis, MO) was prepared fresh in DMSO at concentrations such that the final concentration of the solvent in the cell suspension never exceeded 0.1%. Respective controls were treated with an equal volume of DMSO.

Determination of ICAM-1 Surface Expression

ECV cells were washed twice with Dulbecco's PBS (D-PBS), pH 7.4, and incubated with FITC-labeled ICAM-1 monoclonal antibody (Immunotech, Marseille, France) for 30 min at 4°C. After incubation cells were washed twice with D-PBS and finally resuspended in fresh D-PBS. Expression of ICAM-1 was immediately assayed with the flow cytometer described in the following section. Appropriate isotypic controls were used for background fluorescence in the ICAM-1 assay.

Flow cytometric analysis. The fluorescence and light-scattering properties (forward scatter and side scatter) of the cells were determined by using an EPICS-Elite (Coulter, Miami, FL) flow cytometer. Cells with the FITC-conjugated ICAM-1 antibody were excited with a 488-nm argon ion laser, and emission was recorded at 525 nm. In each sample, at least 10,000 gated viable cells were examined. A logarithmic scale was used to measure both background and endothelial cell fluorescence. Background fluorescence was then subtracted from endothelial cell fluorescence, allowing linear comparisons of ICAM-1 expression among various samples.

RNA Isolation and Analysis

Total RNA was extracted from $2–3 \times 10^6$ ECV cells with guanidinium isothiocyanate by following the method of Chomczynski and Sacchi (12).

Northern blot analysis. RNA samples (10 μg) were subjected to electrophoresis in 1% (wt/vol) formaldehyde-agarose gels and transferred to Hybond-N nylon membranes (Amersham, Piscataway, NJ) overnight in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). The RNA was cross-linked to the nylon membrane by 5 min of UV exposure. Blots were then prehybridized for at least 1 h at 37°C in 50% formamide, 5× SSC, 0.1% SDS, 5× Denhardt's solution (0.1% BSA, Ficoll, and polyvinylpyrrolidine), and 100 μg/ml denatured sperm DNA (Life Technologies). Blots were hybridized at 37°C for 16 h with human ICAM-1 cDNA probes (R & D systems, Minneapolis, MN). cDNA probes were labeled with [γ-^{32}P] ATP by using T4 polynucleotide kinase. Blots were washed twice with a wash solution containing 1× SSC and 0.1% SDS for 10 min at 37°C and twice with one containing 0.2× SSC and 0.1% SDS for 10 min at 60°C. To normalize mRNA content, the blots were stripped and reprobed with a radiolabeled human β-actin cDNA probe (R & D Systems).

RT-PCR. Reverse transcription was performed with an RNA PCR kit (Perkin-Elmer). One microgram of total RNA was reverse transcribed to cDNA by following the manufacturer's procedures. Reverse transcription-generated cDNA containing human c-*fos*, c-*jun*, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified with specific primers (Clontech, Palo Alto, CA) by PCR as described below. The reaction volume was 50 μl and contained (final concentration) 1× PCR buffer, 0.2 mM (each) deoxynucleotide, 2 mM $MgCl_2$, 2 units of *Taq* DNA polymerase, 0.5 μM (each) oligonucleotide primers, and reverse transcription products. After an initial denaturation for 2 min at 95°C, 30 cycles of amplification (95°C for 45 s, 65°C for 45 s, and 72°C for 1 min), followed by a 7-min extension at 72°C, were performed.

An aliquot (10 μl) from each PCR mixture was electrophoresed in a 1.7% agarose gel containing 0.2 μg/ml ethidium bromide. The gel was then photographed under ultraviolet transillumination. For quantification, the c-*jun* and c-*fos* signals were normalized relative to the corresponding GAPDH signal from the same sample with National Institutes of Health (NIH) Image 1.58b29 software.

PKC Activity

Protein kinase C (PKC) activity was determined by ^{32}P incorporation into the PKC-specific peptide substrate (Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys; Sigma) as described previously (47). The ECV cells (0.1×10^6 cells) were incubated with streptolysin-O (0.3 IU), ATP (250 μM), [γ-^{32}P]ATP (300–450 cpm/pmol; DuPont NEN, Boston, MA), and 100 μM peptide substrate in the appropriate reaction buffer. After 10 min of incubation at 37°C, the reaction was stopped by the addition of 25% TCA in 2 M acetic acid, and then the reaction mixture was centrifuged. The supernatants (25 μl each) were spotted on phosphocellulose disc papers (Life Technologies) and washed twice, first with 1% phosphoric acid and then with deionized water. The amount of ^{32}P-incorporated peptide in disc papers was quantified with a liquid scintillation counter. The background phosphorylation in the absence of the substrate peptide was subtracted from all samples. To determine the specificity of the obtained results for PKC, additional measurement of the enzyme activity was done with a pseudosubstrate PKC peptide inhibitor (Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn; Sigma) as reported previously (47).

Nuclear Extraction and EMSA

Nuclear extracts from ECV cells were prepared from 1×10^6 cells. Electrophoretic mobility shift assays (EMSAs) were performed with double-stranded DNA probes (Santa Cruz Biotechnology, Santa Cruz, CA) corresponding to that for AP-1 (sense strand; 5′-CGCTTGATGACTCAGCCGGAA-3′) and NF-κB (sense strand; 5′-AGTTGAGGGGACTTTC-CCAGGC-3′) essentially as described previously (47). Binding reaction mixtures containing 5 μg of protein from nuclear extract, 2 μg of poly(dI-dC) (Pharmacia, Uppsala, Sweden), oligonucleotide probes labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase, 50 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 10% (vol/vol) glycerol, and 4 mM Tris·HCl (pH 7.9) were incubated for 30 min at room temperature. Reaction mixtures were separated by electrophoresis in a native 6% polyacrylamide gel. The NF-κB and AP-1 bands were visualized by autoradiography and quantified with NIH Image 1.58b29 software. For antibody-clearing and

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supershift assays, rabbit affinity-purified antibodies (Santa Cruz Biotechnology) against c-Fos and c-Jun were incubated for 1 h with nuclear extracts at room temperature, before the addition of synthetic oligonucleotides and electrophoretic resolution.

JNK Activity

This assay was performed by following the manufacturer's procedures with a c-Jun kinase assay kit (Stratagene, La Jolla, CA). ECV cells (2×10^6 cells) were incubated in the absence or presence of quercetin for 3 h and then activated with 100 nM PMA or 10 ng/ml TNF-α for the time periods indicated in Fig. 7. The cells were lysed in 0.5 ml of lysis buffer containing 25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride. Lysates were then clarified by centrifugation at 10,000 *g* for 10 min at 4°C. The protein contents of the supernatants were determined by a protein assay (Bio-Rad, Hercules, CA). The supernatants (20 μg protein equivalent vol) were incubated with 5 μg of glutathione *S*-transferase (GST)-c-Jun (1–79) and [γ-^{32}P]ATP (10 μCi) in the kinase buffer for 10 min at 30°C. The reaction was stopped by the addition of Laemmli's sample buffer. Reaction mixtures were subjected to 10% SDS-PAGE. The gel was washed with distilled water three times and dried. Phosphorylated proteins were visualized by autoradiography and quantified with NIH Image 1.58b29 software.

Statistical Analyses

All the results reported are means ± SD of at least three independent experiments. Differences between means of groups were determined by Student's *t*-test and ANOVA. The minimum level of significance was set at $P < 0.05$.

RESULTS

ICAM-1 Expression

To determine whether quercetin modulates PMA- and TNF-α-induced ICAM-1 expression, ECV cells were pretreated with various amounts of quercetin for 3 h and then stimulated with PMA (100 nM) or TNF-α (10 ng/ml) (Fig. 1). The levels of ICAM-1 expression in nonstimulated cells were very low, and treatment with quercetin alone had no effect on the basal expression of ICAM-1 in ECV cells. After activation of ECV cells with TNF-α or PMA for 24 h, the expression of ICAM-1 was markedly upregulated. Pretreatment of ECV cells with quercetin downregulated both TNF-α- and PMA-induced ICAM-1 expression in a dose-dependent manner. Quercetin (50 μM) pretreatment downregulated 55 ± 3.4 and 33 ± 1.3% of the PMA- and TNF-α-induced ICAM-1 expression in ECV cells, respectively (Fig. 1). Suppression of induced ICAM-1 expression by quercetin in cells that were treated with as little as 1 μM flavonol had already been observed. The concentrations of quercetin studied were observed to be not cytotoxic to the ECV cells as determined by a standard trypan blue exclusion test (not shown).

ICAM-1 mRNA Levels

To characterize the molecular mechanisms underlying the inhibitory effects of quercetin on PMA- and TNF-α-induced ICAM-1 expression, the effect of this flavonol on ICAM-1 mRNA levels was investigated by Northern blot analysis. Activation of ECV cells with PMA or TNF-α markedly elevated ICAM-1 mRNA expression, which plateaued in 4 h after activation (not shown). As illustrated in Fig. 2, quercetin pretreatment for 3 h dose dependently inhibited both PMA- and TNF-α-induced ICAM-1 mRNA expression in ECV cells. The effect was already significant in cells pretreated with 1 μM quercetin compared with those stimulated with each agonist alone. These data are consistent with results obtained from the measurement of ICAM-1 expression.

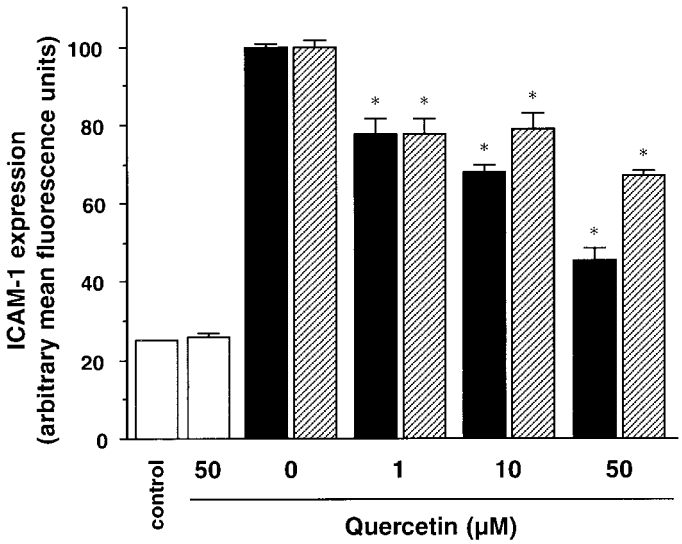

Fig. 1. Quercetin downregulates intercellular adhesion molecule-1 (ICAM-1) expression induced by phorbol 12-myristate 13-acetate (PMA) and tumor necrosis factor-α (TNF-α) in ECV304 (ECV) endothelial cells. ECV cells were incubated with culture medium alone (control) or quercetin (50 μM) alone or were preincubated with different concentrations of quercetin (0–50 μM) for 3 h before stimulation. Cells were then activated with PMA (100 nM; solid bars) or TNF-α (10 ng/ml; hatched bars) for 24 h. Expression of ICAM-1 was assayed by flow cytometry. * Significantly different from PMA- or TNF-α-stimulated cells not treated with quercetin ($P < 0.05$).

PMA-Induced PKC Activity

It has been reported that the activation of PKC in response to PMA, but not TNF-α, treatment may increase the stability of the ICAM-1 mRNA (55). PKC activity increased after treatment of the cell with PMA (100 nM, 1 h) compared with that in cells not treated with PMA (Fig. 3). The PKC inhibitor staurosporine blocked the marked induction of the activity of PKC after PMA treatment. Quercetin (50 μM, 3 h) pretreatment inhibited (~40%) PMA-induced PKC activity in ECV cells (Fig. 3).

ICAM-1 mRNA Stability

To investigate whether the inhibitory effect of quercetin on PKC activity is able to influence the stability of ICAM-1 mRNA in ECV cells, the rate of mRNA degradation was evaluated. The cells were stimulated with PMA alone for 4 h, and then actinomycin D (5 μg/ml)

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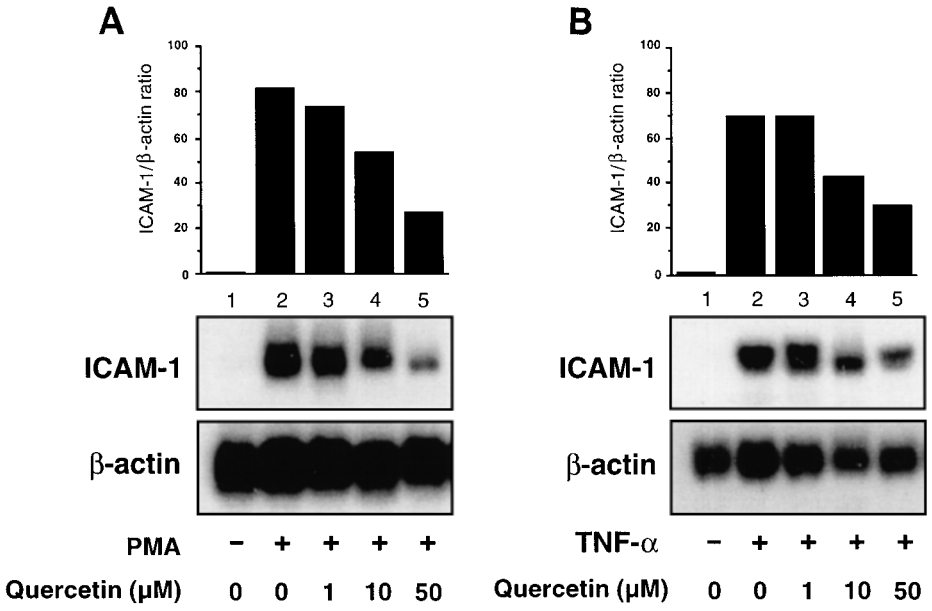

Fig. 2. Quercetin decreases PMA- and TNF-α-induced ICAM-1 mRNA in ECV endothelial cells. ECV cells were pretreated with indicated concentrations of quercetin for 3 h and then activated with PMA (*A*) or TNF-α (*B*) for 4 h. *Lane 1*, control (no treatment); *lanes 2–5*, treatment with 100 nM PMA (*A*) or 10 ng/ml TNF-α (*B*) for 4 h. Levels of mRNA were determined by Northern blot analysis with ICAM-1 or β-actin probes. *A* and *B*, *top*: ICAM-1 mRNA expression is in arbitrary units, and data are normalized to respective amount of β-actin mRNA.

was added to prevent further mRNA synthesis in the absence or presence of quercetin (50 μM). No significant difference in the rates of degradation (half-life ~ 4 h) of ICAM-1 mRNA with or without quercetin was observed (Fig. 4). These results indicate that the inhibitory effect of quercetin on PMA-induced ICAM-1 mRNA expression is not due to the decreased stability of ICAM-1 mRNA.

AP-1 and NF-κB Activation

The binding motifs of AP-1 and NF-κB in the ICAM-1 promoter have been identified and have been shown to be involved in the induction of the ICAM-1 gene. Treatment of ECV cells with 100 nM PMA or 10 ng/ml TNF-α for 1 h increased the activation of AP-1 and NF-κB (Fig. 5). The peak responses of such activation in ECV cells were observed after 1 h of treatment with each agonist (not shown). The specificities of AP-1 and NF-κB bands obtained by EMSA were confirmed by adding excess amounts of the respective unlabeled consensus oligonucleotide probes to the reaction mixtures. Pretreatment of the ECV cells with quercetin for 3 h suppressed both PMA- and TNF-α-induced AP-1 activation in a dose-dependent manner. This inhibitory effect of quercetin on inducible AP-1 activation was observed at a dose range similar to one that was effective in inhibiting induced ICAM-1 mRNA expression. In contrast, at the range of concentrations used, quercetin had no effect on agonist-induced NF-κB activation. c-Fos and c-Jun are known to be major components of the AP-1 complex. Antibody-clearing experiments were performed to verify the presence of these proteins in the activated AP-1 complex. The smallest amount of antibody (1 μg/ml) against c-Fos and c-Jun caused significant reductions in AP-1 DNA binding. These results suggested that c-Fos and c-Jun were some of the major components of the complex.

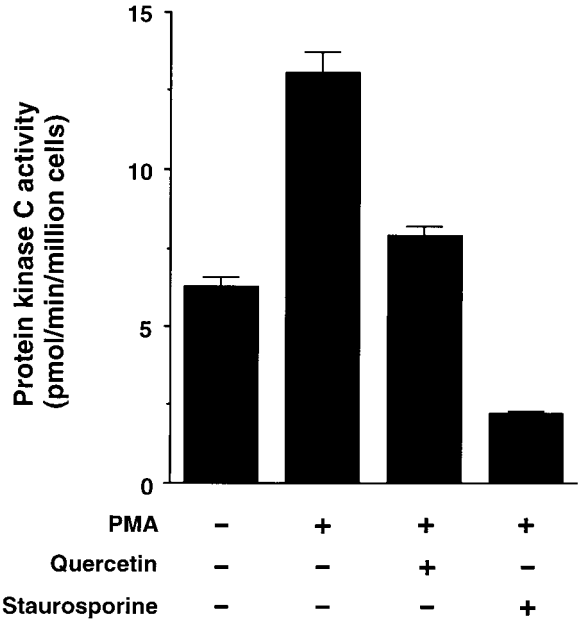

Fig. 3. Effect of quercetin on PMA-induced protein kinase C activity. ECV cells were activated with 100 nM PMA for 60 min in absence or presence of quercetin (50 μM) or staurosporine (50 nM).

Expression of c-jun and c-fos

To elucidate mechanisms by which quercetin inhibits AP-1 activity, the effect of quercetin on the induction of c-*jun* and c-*fos* in ECV cells was investigated (Fig. 6). RT-PCR measurements showed that c-*jun* mRNA was remarkably induced in response to the treatment of cells with PMA and that such an increase in c-*jun* mRNA was prevented in a dose-dependent manner by quercetin pretreatment (Fig. 6). Quercetin did not decrease PMA-induced c-*fos* mRNA expression. These results indicate that quercetin modulates events that are upstream of the induction of c-*jun*.

Inhibition of the Activation of JNK

JNK is a member of the family of mitogen-activated protein (MAP) kinases, and its activation has been

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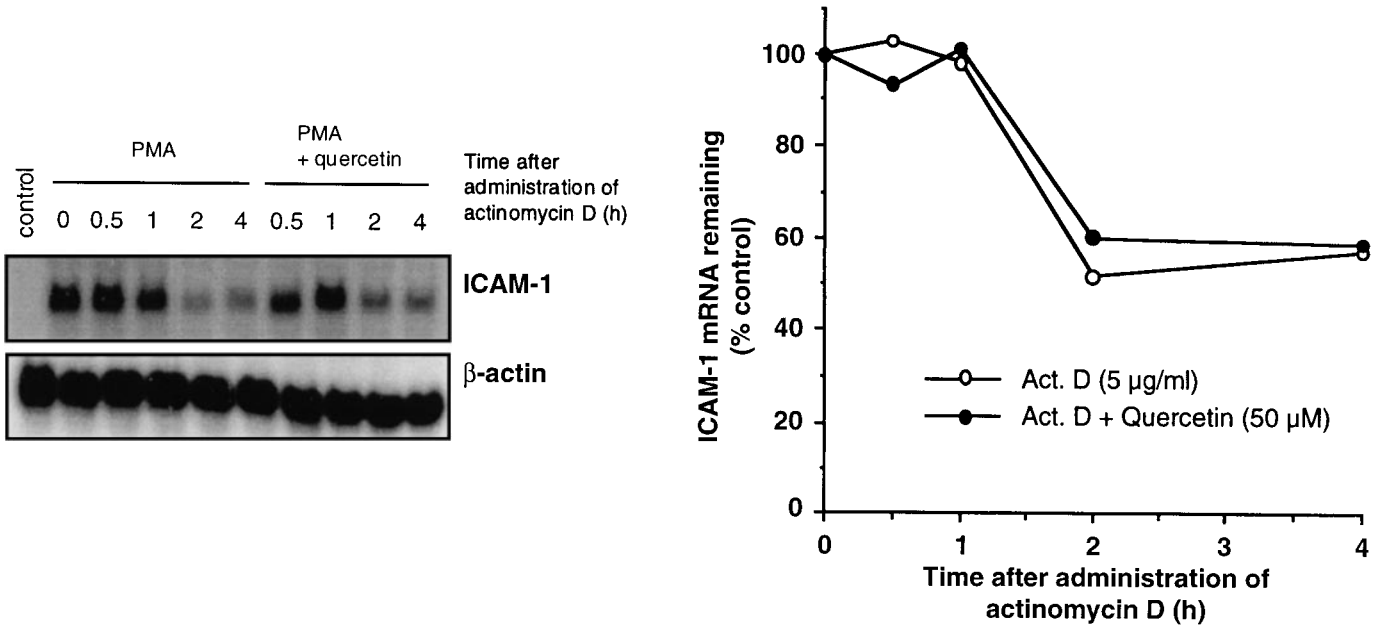

Fig. 4. Effect of quercetin on stability of PMA-induced ICAM-1 mRNA in ECV endothelial cells. ECV cells were treated with PMA (100 nM for 4 h) to achieve peak ICAM-1 mRNA levels, and then cells were treated further with actinomycin D (Act. D; 5 μg/ml), either alone or with quercetin (50 μM) for time periods indicated. Levels of mRNA were determined by Northern blot analysis using ICAM-1 or β-actin probes. Data are relative amounts of ICAM-1 mRNA remaining after addition of actinomycin D and normalization to respective amount of β-actin mRNA.

suggested to regulate the induction of c-*jun* through the phosphorylation of c-Jun (31). The activation of JNK in ECV cells by treatment with PMA or TNF-α was rapid and transient (Fig. 7*A*). Intense bands of phosphorylated GST-c-Jun were observed within 30 or 15 min of PMA or TNF-α treatment, respectively. This phosphorylation of c-Jun gradually decreased and reached almost basal levels after 180 min of both treatments. Thus, to evaluate the effect of quercetin on JNK activity, the activity of this kinase was determined after

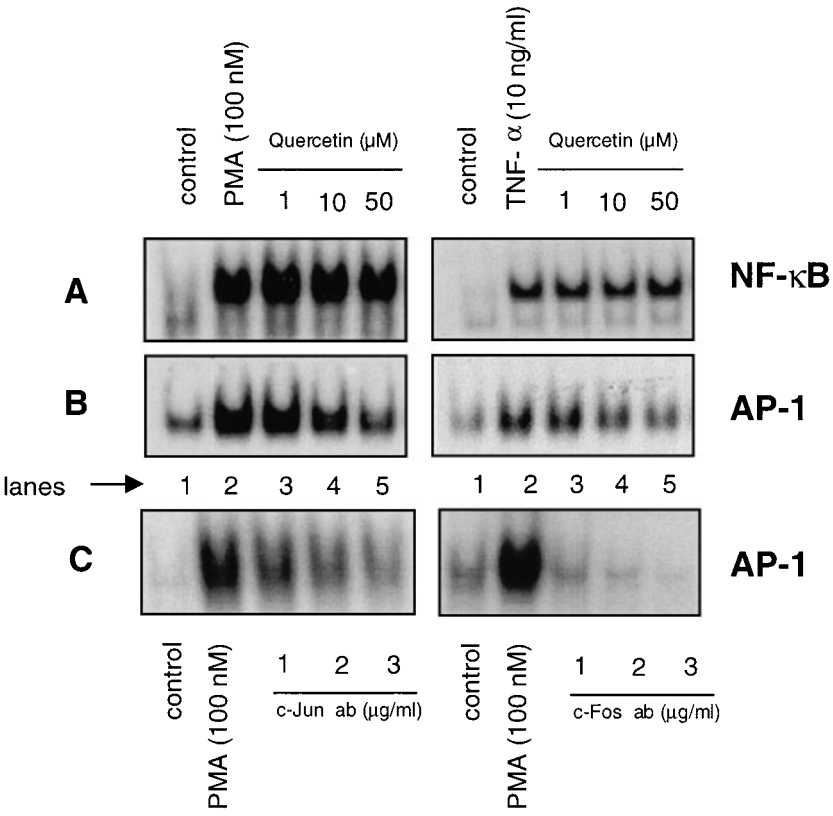

Fig. 5. Effect of quercetin on activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) DNA binding induced by PMA or TNF-α. ECV cells were pretreated with indicated concentrations of quercetin for 3 h and then stimulated with PMA or TNF-α. *A* and *B*: activation of NF-κB and AP-1 was assayed by electrophoretic mobility shift assay. *Lane 1*, no treatment; *lanes 2–5*, treatment with 100 nM PMA or 10 ng/ml TNF-α for 1 h. *C*: presence of c-Fos and c-Jun in PMA-activated AP-1 complex was determined by supershift assay. *Lane 1*, no treatment; *lanes 2–5*, treatment with 100 nM PMA for 1 h; *lanes 3–5*, treatment with indicated amounts of c-Jun antibody (ab) or c-Fos antibody.

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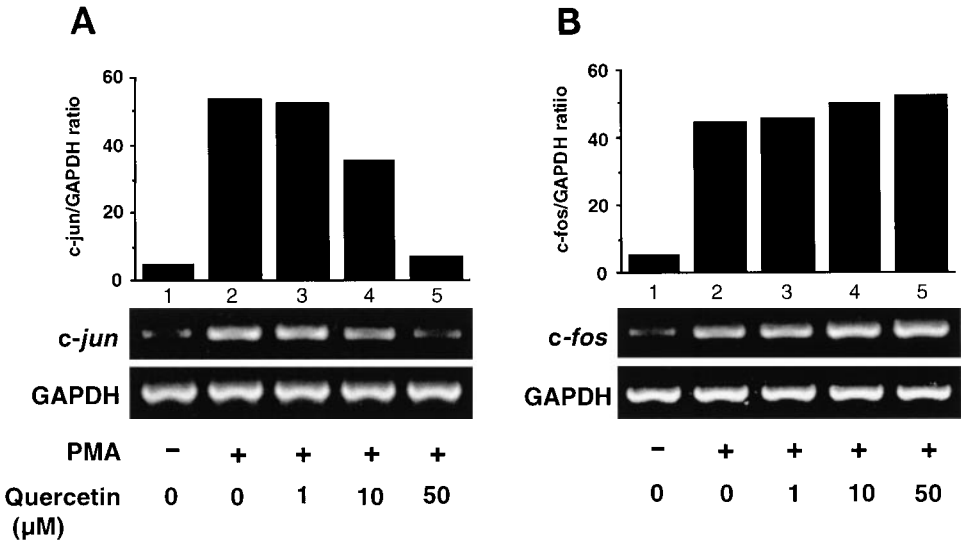

Fig. 6. Effect of quercetin on induction of c-*jun* and c-*fos* activated by PMA. ECV cells were pretreated with different concentrations of quercetin for 3 h and then activated with PMA (100 nM) for 1 h. RT-PCR analysis was performed with primers specific for c-*jun* (*A*) and c-*fos* (*B*). Expression of c-*jun* and c-*fos* mRNA is in arbitrary units, and data are normalized to respective amounts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Results are representative of at least 3 independent experiments.

30 or 15 min of PMA or TNF-α activation. Pretreatment of cells with quercetin dose dependently decreased both PMA- and TNF-α mediated JNK activation (Fig. 7*B*). At 50 μM, quercetin almost completely suppressed PMA- and TNF-α-stimulated JNK activity (Fig. 7*B*).

DISCUSSION

Recently, several in vivo studies have suggested that plant flavonoids possess potent anti-inflammatory (9, 23) and antioxidant properties. Adhesion molecule ICAM-1 is known to play a central role in the regulation of cellular inflammatory responses (7). Regulation of ICAM-1 gene expression has been related to oxidative stress through specific redox-sensitive transcriptional or posttranscriptional mechanisms (29, 38). Various antioxidants, including flavonoids, have been reported to regulate the expression of adhesion molecules on the cell surface (23, 47). Quercetin is known to have beneficial effects in cardiovascular and circulatory disorders (25) and is one of the most potent antioxidants among dietary flavonoids (45). Several studies have shown recovery of dietary quercetin from plasma, suggesting the bioavailability of this flavonoid (18, 37). Furthermore, because of the long half-life of elimination (~24 h), repeated consumption of quercetin-containing foods has been suggested to cause accumulation of quercetin in blood (28). This study shows for the first time that quercetin is an inhibitor of agonist-induced ICAM-1 protein and mRNA expression.

Treatment of human endothelial cells with certain hydroxyflavones and flavonols has been reported to inhibit cytokine-induced ICAM-1, vascular cell adhesion molecule-1, and E-selectin expression in human endothelial cells (23). For example, apigenin is a flavone that inhibits adhesion molecule expression in endothelial cells in a dose- and time-dependent manner. An effect of this flavone at the transcriptional level has been demonstrated (23). Apigenin also inhibits TNF-α-induced ICAM-1 expression in vivo (43). Cell adhesion regulatory effects of flavonoids are also consistently evident from other independent studies. The flavonoid delphinidin chloride (CAS 528–53–0, IdB 1056) inhibited acetylcholine- and sodium nitroprusside-induced adherence of leukocytes to the venular endothelium in diabetic hamsters (6). The flavonoids 5-methoxyflavanone, and more potently 5-methoxyflavone, downregulated indomethacin-induced leukocyte adherence to mesenteric venules (8).

The inhibition of PMA-induced ICAM-1 expression by quercetin correlated with decreases in steady-state mRNA levels. Similar inhibitory patterns of TNF-α-induced ICAM-1 mRNA expression in ECV cells were observed. PKC is known to be involved in PMA-induced cell activation pathways. Upregulation of ICAM-1

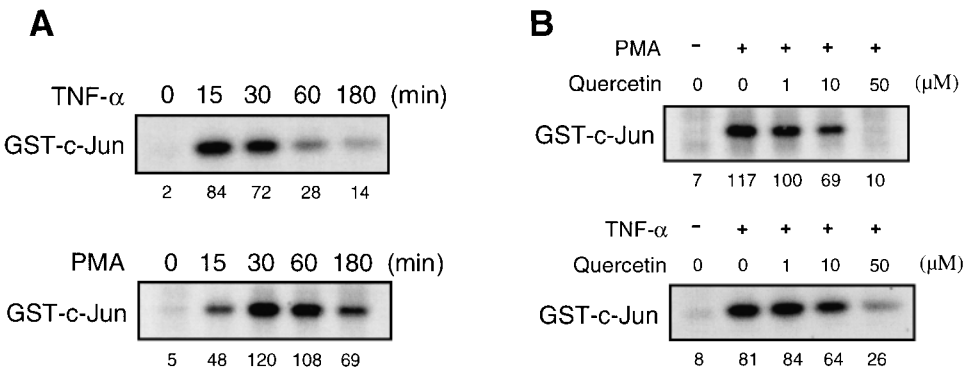

Fig. 7. Effect of quercetin on transient activation of c-Jun NH_2-terminal kinase (JNK) by PMA and TNF-α. *A*: ECV cells were treated with 100 nM PMA or 10 ng/ml TNF-α for indicated time periods and assayed for JNK activity. *B*: ECV cells were pretreated with indicated concentrations of quercetin for 3 h and then activated with PMA (100 nM) or TNF-α (10 ng/ml) for 30 or 15 min, respectively. Position of phosphorylated glutathione *S*-transferase (GST)-c-Jun is indicated. Numbers below autoradiograms represent phosphorylated levels of GST-c-Jun obtained by densitometric analysis.

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mRNA expression by PMA has been reported to be mainly due to an increase in posttranscriptional stabilization of mRNA via PKC activity (55). Studies from other laboratories and our laboratory (not shown) have shown that the PKC inhibitor staurosporine (50 nM) inhibits PMA-induced ICAM-1 expression (10, 15, 24). Quercetin treatment downregulated PMA-induced PKC activity in ECV cells. However, quercetin treatment did not affect the stability of PMA-induced ICAM-1 mRNA. It is not clear whether the modest inhibition of inducible PKC activity by quercetin would require a comparable change in the stability of ICAM-1 mRNA.

Activation of NF-κB is known to be involved in the regulation of inducible ICAM-1 gene expression (16, 51, 54). At concentrations up to 50 μM, quercetin had no effect on PMA- or TNF-α-mediated activation and nuclear translocation of NF-κB. This observation suggests that quercetin does not affect the activities of kinases that are involved in nuclear translocation of NF-κB after PMA or TNF-α activation. AP-1 is another transcription factor that plays an important role in the induction of the ICAM-1 gene (46, 54). Quercetin prevented PMA- or TNF-α-mediated activation of AP-1 in the same dose range that is effective in downregulating the induced ICAM-1 mRNA expression. AP-1 transcription factors consist of homodimers and heterodimers of Jun and Fos and protein products of their related gene family (31). The antibody-clearing and supershift assays confirmed the presence of c-Fos and c-Jun in the activated AP-1 complex. Both PMA and TNF-α are known inducers of c-*jun* and c-*fos* expression (31). The inhibitory effect of quercetin on AP-1 activation may be due to its inhibitory effects on c-*jun* expression. Quercetin has previously been shown to inhibit the expression of c-*jun* mRNA (59).

Several members of the MAP kinase family have been reported to regulate the activation of AP-1 (31). The extracellular signal-regulated kinase (ERK) stimulates AP-1 activity through induction of c-Fos synthesis. Fos-regulating kinase increases AP-1 activity by enhancing the transactivation function of c-Fos. After activation of cells by an appropriate stimulus, JNK phosphorylates c-Jun and activating factor-2, which are involved in the induction of the c-*jun* protooncogene (31). In addition to the activation of c-*jun* expression, JNK also enhances the DNA-binding activity of AP-1 by phosphorylating the activation domain of c-Jun (31). JNK is known to be activated in response to a wide variety of extracellular signals such as TNF-α, interleukin-1β, PMA, H_2O_2, hypertension, shear stress, ischemia-reperfusion, and UV (11, 21, 27, 32, 34, 36, 52, 56, 57). The specific inhibitory effect of quercetin on c-*jun* mRNA and JNK activity observed in this study suggests that quercetin regulates AP-1 activation by modulating JNK activity. No effect of quercetin on c-*fos* mRNA induction suggests that ERK, known to regulate the expression of c-Fos, is not involved in mediating the inhibitory effect of quercetin on ICAM-1 mRNA expression.

The induction of ICAM-1 expression in human endothelial cells by PMA and TNF-α is known to occur via distinct pathways (41, 55). Results of this study show that both TNF-α and PMA activated signaling pathways leading to the activation of NF-κB and AP-1. It is apparent that MAP kinase/ERK kinase kinase-1 (MEKK1), functional upstream of JNK, activates IκB phosphorylation, which is involved in NF-κB activation (35). However, the activities of JNK and ERK are not required for NF-κB translocation (39). A specific inhibitory effect of quercetin on JNK activity suggests that the flavonol regulated JNK activity through either direct interaction with the kinase or downregulation of upstream kinases, such as MKK4/SEK1 and MKK7, which are known to activate JNK directly by catalyzing phosphorylation (17, 49, 58). Further studies are required to elucidate the precise mechanism for the specific JNK-inhibitory action of quercetin.

In summary, this study provides evidence for a novel mechanism of agonist-induced ICAM-1 downregulation by quercetin in a human endothelial cell line. Our results demonstrate that quercetin downregulates ICAM-1 expression induced by either the PKC activator PMA or the proinflammatory cytokine TNF-α. This effect of quercetin in part is due to its inhibitory action on AP-1 transactivation induced by PMA and TNF-α. JNK is involved in such regulation via downregulating the expression of c-*jun* mRNA. This inhibitory effect of quercetin on inducible ICAM-1 expression represents a mechanism that contributes to the anti-inflammatory property of this dietary flavonol.

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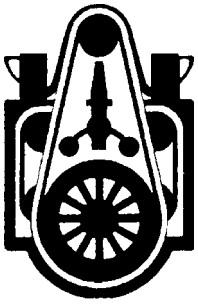

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