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

Kobuchi, Hirotsgu, 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 NH2-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 dis-
concentrations quercetin downregulates both PMA- and TNF-α-induced ICAM-1 expression in human endothelial cells via inhibition of the c-jun NH2-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% CO2 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 × 10⁶ ECV cells with guanidine 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 (Amerham, Piscataway, N.) 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 polyvinylpyrrolidone), 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-32P]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).

PKC Activity

Protein kinase C (PKC) activity was determined by 32P 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 × 10⁶ cells) were incubated with streptolysin-O (0.3 IU), ATP (250 µM), [γ-32P]ATP (300–450 cpnm/µmol; 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 32P-incorporated peptide in disc papers was quantified by 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-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 × 10⁶ cells. Electrophoretic mobility shift assays (EMSA) were performed with double-stranded DNA probes (Santa Cruz Biotechnology, Santa Cruz, CA) corresponding to that for AP-1 (sense strand; 5'-CGCTTGAGTACGACGGGAAA-3') and NF-κB (sense strand; 5'-AGTTGAGGGGACTTTC-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-32P]ATP by using T4 polynucleotide kinase, 50 mM NaCl, 2 mM MgCl₂, 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
and then stimulated with PMA (100 nM) or TNF-α pretreated with various amounts of quercetin for 3 h.

Quercetin downregulated both TNF-α and PMA-induced ICAM-1 expression in ECV cells. After activation of ECV cells with quercetin alone had no effect on the basal expression of ICAM-1 mRNA in nonstimulated cells were very low, and treatment 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.

**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 mRNA 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.

**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)
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.

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
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. 7A). 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...
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. 7B). At 50 µM, quercetin almost completely suppressed PMA- and TNF-α-stimulated JNK activity (Fig. 7B).

**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. Uprogulation of ICAM-1 by 10.220.32.246 on October 13, 2017 http://ajpcell.physiology.org/ Downloaded from
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 Fos and Jun, and protein products of their related gene family (31). The antibody-depleting 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, H₂O₂, hypertension, shear stress, ischemia-reperfusion, and UV (11, 21, 27, 32, 34, 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 IKK 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 transcription induction 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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