2’-Hydroxychalcone Inhibits Nuclear Factor-κB and Blocks Tumor Necrosis Factor-α- and Lipopolysaccharide-Induced Adhesion of Neutrophils to Human Umbilical Vein Endothelial Cells

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ABSTRACT

Inhibition of expression of cell adhesion molecules (CAM), including intercellular CAM-1 (ICAM-1), vascular CAM-1 (VCAM-1), and E-selectin, has been shown to be important in controlling various inflammatory diseases. The cell adhesion proteins are induced by various inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1, and bacterial lipopolysaccharide. The induction process primarily takes place at the level of transcription, where nuclear factor-κB (NF-κB) plays a major role. We demonstrate here that 2’-hydroxychalcone inhibits the adhesion of peripheral neutrophils to the endothelial cell monolayers by inhibiting the expression of ICAM-1, VCAM-1, and E-selectin in a concentration-dependent manner. The inhibition by 2’-hydroxychalcone is reversible. 2’-Hydroxychalcone inhibits the induction of steady-state transcript levels of ICAM-1, VCAM-1, and E-selectin by tumor necrosis factor-α as determined by reverse transcription-polymerase chain reaction, and therefore it may interfere with the transcription of their genes. Because NF-κB is a major transcription factor involved in CAM expression, we studied its status in the 2’-hydroxychalcone treated cells. We demonstrate that 2’-hydroxychalcone inhibits the activation of NF-κB. These results have implications for using NF-κB inhibitors for the treatment of various inflammatory diseases.

The recruitment and subsequent migration of the leukocytes to the site of inflammation is in part regulated by the expression of various cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin (Osborn, 1990; Butcher, 1991; Springer, 1994). These cell adhesion molecules are induced on endothelial cells by various proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) and also by bacterial lipopolysaccharide (LPS; reviewed by Mantovani et al., 1997). These proteins are up-regulated on endothelial cells during various inflammatory diseases (Bochner et al., 1991; Calderon and Lockey, 1992; Gorski, 1994). Therefore, strategies to down-regulate the expression of these molecules might have therapeutic implications. Inhibition of these molecules using specific monoclonal antibodies (mAbs) has been found to be beneficial for controlling various diseases (Gorski, 1994; Weiser et al., 1997). However, because of endotoxin contamination, unpredictable clinical manifestations, such as secondary antibody formation, cellular activation, and other complications (e.g., sensitization leading to serum sickness and anaphylaxis), the practical use of mAbs is limited (Weiser et al., 1997). Also, various small molecules from natural and synthetic sources, such as curcumin, glucocorticoids, pentoxifylline, etc., have been shown to down-regulate the expression of cell adhesion molecules and are effective in controlling various inflammatory diseases (Brojstan et al., 1997; Neuner et al., 1997; Gupta and Ghosh, 1999).

The promoters of ICAM-1, VCAM-1, and E-selectin contain recognition sequences for inducible nuclear transcription factor-κB (NF-κB). NF-κB has been shown to be essential for the expression of cell adhesion molecules as demonstrated by deletion mutagenesis, gel-retardation assays, Western blots, and DNA transfection experiments (Iademarco et al., 1992; Hou et al., 1994; Schindler and Baichwal, 1994; Collins et al., 1995). TNF-α and IL-1β up-regulate ICAM-1, VCAM-1, and

ABBREVIATIONS: ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; mAb, monoclonal antibody; NF-κB, nuclear factor κB; ELISA, enzyme-linked immunosorbent assay; HUVECs, human umbilical vein endothelial cells; RT-PCR, reverse transcription-polymerase chain reaction; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay.
E-selectin on endothelial cells at the transcriptional level through the activation of NF-κB. Identification of inhibitors of NF-κB can thus serve to prevent the up-regulation of adhesion molecules on the surface of endothelial cells.

Chalcones are obtained from natural plant sources and can also be synthesized in the laboratory. Chalcones have been reported to possess a variety of biological properties, including anti-inflammatory, analgesic, antioxidant, antibacterial, antifungal, and antiprotozoal activities (Haraguchi et al., 1998; Hsieh et al., 1998). They are also reported to be gastric protectant, antimutagenic, and antitumorigenic (Makita et al., 1996). Various 2'-substituted chalcones have been shown to possess anti-inflammatory and antioxidant properties (Yu et al., 1995; Wegener et al., 1997). For example, 2',5'-dihydroxychalcone prevents platelet aggregation (Lin et al., 1997), and 2',3-dihydroxychalcone and 2',5'-dihydroxychalcone inhibit polymixin B-induced hind-paw edema (Hsieh et al., 1998). Butein (3,4,2'-tetrahydroxychalcone) prevents antiglomerular basement membrane antibody-associated glomerulonephritis in rats (Hayashi et al., 1996). 2'-Substituted chalcones have also been shown to inhibit production of IL-1β from monocytes stimulated with LPS and also prevent LPS-induced septic shock in mice (Batt et al., 1993). LPS-induced septic shock involves excessive infiltration of neutrophils into the liver because of uncontrolled up-regulation of ICAM-1 expression in the liver (Xu et al., 1994; B. Gupta and B. Ghosh, unpublished observations). As a result, ICAM-1 deficient mice are protected against septic shock (Xu et al., 1994) and inhibitors of ICAM-1 prevent lethality induced by septic shock in mice (B. Gupta and B. Ghosh, unpublished observations). Inhibition of LPS-induced septic shock by 2'-substituted chalcones, therefore, might be caused by the inhibition of infiltration of neutrophils into the liver. Recently, 2'-hydroxychalcone has been shown to be a potent antioxidant, it inhibits lipid peroxidation and is antitumorigenic (Anto et al., 1995). Having the hydroxyl group at the ortho-position on the benzene ring of chalcone increases its antioxidant property compared with other substituted chalcones (Anto et al., 1995). Very little is known in regard to its mechanism of action. The effect of 2'-hydroxychalcone on the expression of cell adhesion molecules has also not been studied so far. Because 2'-hydroxychalcone has been found to be pharmacologically important, we were interested to study its mechanism of action on cell trafficking.

In this study, we show that 2'-hydroxychalcone blocks the adhesion of neutrophils to endothelial monolayers by preventing TNF-α- and LPS-up-regulated cell adhesion molecule expression on endothelial cells. We also show that 2'-hydroxychalcone inhibits TNF-α-induced cell adhesion molecule expression by blocking the activation of NF-κB in endothelial cells.

**Experimental Procedures**

**Materials.** TNF-α, anti-ICAM-1 (BBA3), anti-VCAM-1 (BBA6), and anti-E-selectin (BBA1) antibodies were purchased from R & D Systems (Minneapolis, MN). M199, l-glutamine, penicillin, streptomycin, amphotericin, endothelial cell growth factor, trypsin, Pucks saline, HEPES, o-phenylenediamine dihydrochloride, Ficoll-Hypaque, tetramethyl benzidine, cetrimethyl ammonium bromide, 3-amino-1,2,4 triazole and anti-mouse IgG-horseradish peroxidase was purchased from Sigma Chemical (St. Louis, MO). NF-κB oligonucleotide was purchased from Promega (Madison, WI). The structure of 2'-hydroxychalcone has been shown in Fig. 1. It has been prepared by Claisen-Schmidt condensation between 2-hydroxyacetophenone and benzaldehyde done in ethanol, using partially dehydrated barium oxide as a catalyst and characterized by 1H NMR (Sathyanarayana and Krishnamurthy, 1988). The ICAM-1, VCAM-1, E-selectin, and β-actin primer sets were synthesized by Genset Corp. (Tokyo, Japan). Fetal calf serum was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Anti-mouse-IgG-fluorescein isothiocyanate was purchased from Becton Dickinson (Mountain View, CA). Anti-NF-κB antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cells and Cell Culture.** Primary endothelial cells were isolated from umbilical cord by mild trypsinization. The endothelial cells obtained were grown on gelatin-coated tissue culture flasks in M 199 medium supplemented with 20% heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin, endothelial cell growth factor (50 µg/ml), and heparin (5 U/ml). For subculturing, the cells were dislodged using 0.125% trypsin/0.01 M EDTA solution in Pucks saline and HEPES buffer. The cells were used between passages three to four. The viability of cells was determined by trypsin blue staining and purity of endothelial cells was determined by E-selectin expression.

**Neutrophil Isolation.** Neutrophils were isolated from peripheral blood of healthy individuals as described previously (Clark and Naseef, 1996). Briefly, the peripheral blood was collected in heparin solution (final concentration, 20 U/ml) and erythrocytes were removed by sedimentation with 6% dextran solution. The white-blood-cell-rich plasma layer was collected and layered over Ficoll-Hypaque solution followed by centrifugation at 300g for 20 min at 20°C. The top saline layer and the Ficoll-Hypaque layer were aspirated, leaving the neutrophil/red blood cell pellet. The residual red blood cells were removed by hypotonic lysis. The isolated cells were washed with PBS and resuspended in PBS containing 5 mM glucose, 1 mM CaCl₂, and 1 mM MgCl₂ at a final concentration of 6 × 10⁵ cells/ml. This procedure usually resulted in approximately 95% neutrophils and the cell viability was more than 95% as detected by trypsin blue exclusion test.

**Cell Adherence Assay.** Adhesion of neutrophils to endothelial monolayers was assayed as described previously (Dobrina et al., 1991). Briefly, the endothelial cells were plated in 96-well culture plates at a density of 2 × 10⁴ cells/well and allowed to adhere for 24 h. The cells were incubated without or with 2'-hydroxychalcone for 1 h followed by induction with LPS (1 µg/ml) for 6 h. The endothelial monolayers were washed twice with PBS, and neutrophils (6 × 10⁴/well) were added and allowed to adhere for 1 h at 37°C. Nonadherent neutrophils were removed by washing the wells with PBS thrice. Adherent neutrophils were assayed colorimetrically by adding a substrate solution (100 µl/well) consisting of o-phenylenediamine dihydrochloride (40 mg/100 ml in citrate phosphate buffer, pH 4.5) containing 0.1% cetrimethyl ammonium bromide as perox-
idase solubilizing agent. The interference by the few contaminating eosinophils was abolished by adding a selective eosinophil peroxidase inhibitor, 3-amino-1,2,4 triazole (1 mM) to the substrate solution. After 2 min of incubation, 2N H₂SO₄ (50 μl/well) was added to stop the reaction. The absorbance was determined at 490 nm using an automated microplate reader (Spectramax 190; Molecular Devices, Menlo Park, CA).

**Modified Enzyme-Linked Immunosorbent Assay (ELISA)**

For Measurement of ICAM-1. Expression of ICAM-1 on surface of endothelial cells was quantified using cell-ELISA as described before (Gupta and Ghosh, 1999). Human umbilical vein endothelial cells (HUVECs) were plated to confluency in gelatin-coated, 96-well plates. The cells were then incubated without or with 2'-hydroxychalcone at desired concentrations for desired time periods followed by induction with LPS (1 μg/ml) for 16 h. After incubation, the cells were washed with 1.0% glutaraldehyde and nonspecific binding was blocked using nonfat dry milk (5.0% in PBS). The cells were incubated overnight at 4°C with ICAM-1 mAb or control IgG antibody (0.25 μg/ml, diluted in blocking buffer), followed by washing with PBS and incubation with peroxidase-conjugated goat anti-mouse secondary antibody (1 μg/ml, diluted in PBS). After this, the cells were again washed with PBS and exposed to the peroxidase substrate (o-phenylenediamine dihydrochloride, 4 mg/100 ml in citrate phosphate buffer, pH 4.5). Color development reaction was stopped by the addition of 2 N sulfuric acid. Absorbance was determined at 490 nm by an automated microplate reader (Spectramax 190).

Flow Cytometry. The expression of ICAM-1, VCAM-1, and E-selectin expression on endothelial cells was measured by flow cytometry as described previously (Gupta and Ghosh, 1999). Briefly, the endothelial cells were incubated without or with 2'-hydroxychalcone for 1 h followed by induction with TNF-α (10 ng/ml) for 16 h (for ICAM-1 and VCAM-1) or for 4 h (for E-selectin). After incubation, the cells were dislodged and incubated with anti-ICAM-1, anti-VCAM-1, anti-E-selectin, or control IgG antibody (1.0 μg/10⁶ cells) for 30 min at 4°C. The cells were washed twice with PBS for removing the unbound antibody and then incubated with goat anti-mouse IgG-fluorescein isothiocyanate antibody (1:10 diluted) for 30 min at 4°C. The cells were fixed with 0.1% paraformaldehyde and were analyzed by using a flow cytometer (FACSVantage; Becton Dickinson). For each analysis, 20,000 events were collected and histograms were generated.

Total RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). RNA was isolated according to a modified guanidinium thiocyanate procedure (Chomczynski and Sacchi, 1987). The expression of the transcripts for ICAM-1, VCAM-1, and E-selectin was evaluated by RT-PCR as described previously (Gupta and Ghosh, 1999). The primers were synthesized according to the published cDNA sequences to yield products of length 555, 533, and 479 base pairs, respectively. As a control, β-actin mRNA was also amplified by RT-PCR and a product of 661 base pairs was obtained. The RT-PCR was performed following the manufacturer’s protocol (Access RT-PCR system; Promega). Briefly, 100 ng of the total RNA was reverse transcribed using AMV reverse transcriptase at 48°C for 45 min followed by amplification using T7 polymerase for 25 cycles. The conditions for PCR were as follows: denaturation at 92°C for 1 min, primer annealing at 52°C for 90 s, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR products were analyzed in 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Preparation of Nuclear Extracts. Nuclear extracts were prepared using a modification of previously published methods (Dignam et al., 1983). Primary endothelial cells (2 × 10⁶ cells/ml) were incubated without or with 50 μM 2'-hydroxychalcone for 1 h followed by induction with TNF-α (10 ng/ml) for 30 min. The cells were washed with PBS, dislodged using a cell scraper, and pelleted by centrifugation at 300g. The cells were resuspended in cell lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol (DTT), 0.5% Nonidet P40, 0.1 mM EDTA, and 0.1 mM EDTA) and allowed to swell on ice for 5 min. This was followed by centrifugation at 3300g for 15 min. The supernatant was stored as cytoplasmic extract and the nuclear pellet resuspended in nuclear extraction buffer (20 mM HEPES, 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT) and incubated for 30 min at 4°C. The extracted nuclei were pelleted at 25,000g for 15 min at 4°C and the supernatant was collected as nuclear extract. The protein concentration was estimated by Bradford’s protein estimation method. The nuclear and cytoplasmic extracts were stored at -70°C.

Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed with modifications of a previously published procedure (Marrugo et al., 1996). Briefly, 10 μg of nuclear extract was incubated with 40 to 80 fmol of ³²P-end labeled double-stranded NF-κB oligonucleotide (5'- AGTTGAGGGGACCTTCCCCAGG-3') in binding buffer (12 mM HEPES, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM DTT, and 1.0 μg of poly[dI-dC] for 30 min at RT. The DNA-protein complexes were analyzed by electrophoresis on a 4% native polyacrylamide gel using Tris-glycine buffer, pH 8.5, and visualized by autoradiography.

Western Blot Analysis. Nuclear and cytoplasmic extracts from endothelial cells treated with or without 50 μM 2'-hydroxychalcone were electrophoresed on 10% SDS polyacrylamide gels and transferred to Hybond-C membrane (Amersham, Paisley, UK) in 25 mM Tris, 192 mM glycine, 20% methanol at 15 V overnight. Nonspecific binding sites were blocked by incubating the membrane in 3.0% nonfat dry milk in HEPES-buffered saline (10 mM HEPES, pH 7.4, 100 mM NaCl) at 37°C for 1 h. After being washed twice in HEPES-buffered saline, the membranes were incubated in polyclonal antibody (0.1 μg/ml) overnight at 4°C. After the membranes were washed with HEPES-buffered saline and incubated with anti-rabbit IgG antibody conjugated with horseradish peroxidase for 30 min at 37°C. After extensive washing with HEPES-buffered saline, the blots were exposed to peroxidase substrate (0.25 mg/ml tetramethylbenzidine in 12 mM HEPES, 2 mg/ml diocetyl sodium sulfosuccinate). The color development reactions were stopped by addition of HEPES buffer.

Statistical Analysis. Results are given as mean ± S.D. Independent two-tailed Student’s t test was performed. Differences were considered statistically significant for P < .050. All statistical analysis was performed using the software Microcal Origin (ver 3.0; Microcal Software Inc., Northampton, MA).

Results

**2'-Hydroxychalcone Is Nontoxic to the Endothelial Cells.** To determine any toxic effect of 2'-hydroxychalcone on endothelial cells, the cells grown to confluency in 96-well plates were incubated with varying concentrations of 2'-hydroxychalcone for 24 h. The cell morphology was observed under microscope and the viability was determined by trypan blue exclusion test. We have confirmed that the time of incubation (up to 24 h) and concentration of the drug used (up to 60 μM) in the subsequent experiments had no effect on the viability and morphology of the endothelial cells (data not shown).

**2'-Hydroxychalcone Inhibits Adhesion of Neutrophils to Endothelial Cells.** To determine the effect of 2'-hydroxychalcone on the adhesion of neutrophils to endothelial cells, the cells were incubated without or with 2'-hydroxychalcone at concentrations varying from 10 to 40 μM for 1 h before induction with LPS (1 μg/ml) for 6 h, because both E-selectin and ICAM-1 are expressed at this time point. After this, the endothelial cells were incubated with periph-

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eral blood neutrophils for 1 h. The adhesion of neutrophils to the unstimulated endothelial cells, as detected by adherence assay, was found to be low. There was a 5- to 6-fold up-regulation of neutrophil adhesion to endothelial cells on stimulation with LPS. Although 2′-hydroxychalcone did not affect the adhesion of neutrophils to unstimulated endothelial monolayers, it blocked the neutrophil adhesion to the LPS-stimulated endothelial cells in a concentration-dependent manner (Fig. 2). There was a significant reduction in the adhesion of neutrophils to endothelial cells in the presence of 2′-hydroxychalcone with almost 70% inhibition at a concentration of 40 μM.

2′-Hydroxychalcone Inhibits TNF-α/LPS-Induced ICAM-1, VCAM-1, and E-Selectin Expression on Endothelial Cells in a Concentration- and Time-Dependent Manner. Because cell adhesion molecules are required for adhesion of the neutrophils to the endothelial cells, we studied the effect of 2′-hydroxychalcone on expression of these molecules on endothelial cells. As detected by ELISA, ICAM-1 was expressed at low levels on unstimulated endothelial cells and was induced over 5-fold by LPS stimulation (Fig. 3). 2′-Hydroxychalcone had no effect on the constitutively expressed level of ICAM-1 expression, whereas it led to a significant reduction in the LPS-induced ICAM-1 expression in a concentration-dependent manner. Almost complete inhibition was observed at a concentration of 50 μM.

In addition to LPS-induced ICAM-1 expression, we also tested the effect of 2′-hydroxychalcone on TNF-α-induced ICAM-1 expression. Similar to LPS-induced expression, 2′-hydroxychalcone did not affect the basal level of ICAM-1 expression, whereas it led to a significant reduction in the TNF-α-induced ICAM-1 expression in a concentration-dependent manner, with almost complete inhibition at a concentration of 50 μM (data not shown). This was further confirmed by measuring the expression of ICAM-1 by flow cytometry (Fig. 4). The unstimulated cells expressed low levels of ICAM-1, and on stimulation with TNF-α, there was a substantial increase in ICAM-1 expression. 2′-Hydroxychalcone inhibited TNF-α-induced expression of ICAM-1 up to the basal level.

In addition to ICAM-1, E-selectin is also required for the adhesion of neutrophils to the endothelial cells. We studied the effect of 2′-hydroxychalcone on TNF-α-induced expression of E-selectin. The endothelial cells were incubated with 50 μM 2′-hydroxychalcone for 1 h before induction with TNF-α (10 ng/ml) for 4 h. As analyzed by flow cytometry (Fig. 4), the unstimulated cells expressed undetectable amounts of E-selectin. On stimulation with TNF-α, there was a significant increase in its expression. Treatment of cells with 2′-hydroxychalcone alone did not alter the basal level of E-selectin (data not shown). In the cells treated with 2′-hydroxychalcone before induction with TNF-α, significant reduction in the TNF-α-induced E-selectin expression was observed. This suggests that 2′-hydroxychalcone is effective in blocking adhesion of neutrophils to the endothelial cells by inhibiting the induced level of expression of ICAM-1 and E-selectin.

VCAM-1 is also an important cell adhesion molecule required for the adhesion of eosinophils, lymphocytes, and monocytes to the endothelial cells. To study the effect of 2′-hydroxychalcone on TNF-α-induced expression of VCAM-1, the endothelial cells were incubated with 50 μM 2′-hydroxychalcone for 1 h before induction with TNF-α (10 ng/ml) for 16 h. As analyzed by flow cytometry (Fig. 4), the unstimulated cells expressed undetectable amounts of VCAM-1, and a significant increase in its expression was observed on stimulation with TNF-α. In the cells treated with 2′-hydroxychalcone before induction with TNF-α, significant reduction in the TNF-α-induced VCAM-1 expression was observed.

To determine the effect of time of addition of 2′-hydroxychalcone on the inhibition of ICAM-1 expression, the cells were preincubated with 50 μM 2′-hydroxychalcone for 1

Fig. 2. Inhibition of neutrophil adhesion to endothelial cells. Endothelial cells grown to confluence in 96-well plates were incubated without or with indicated concentrations of 2′-hydroxychalcone for 1 h followed by induction without (hatched bars) or with (closed bars) LPS (1 μg/ml) for 6 h. The cells were then incubated with human peripheral neutrophils for 1 h. The amount of neutrophils adhering to the endothelial cell monolayers was measured by a colorimetric assay as described under Experimental Procedures. The data are representative of two independent experiments. Values shown mean ± S.D. of quadruplicate wells. Statistical significance (P < .0001) is marked with an asterisk.

Fig. 3. Concentration-dependent inhibition of LPS-induced ICAM-1 expression by 2′-hydroxychalcone. Endothelial cells grown to confluence in 96-well plates were incubated without or with indicated concentrations of 2′-hydroxychalcone for 1 h before induction without (hatched bars) or with LPS (1 μg/ml) (closed bars) for 16 h. After this, ICAM-1 level on the cells was measured by ELISA as described under Experimental Procedures. The data presented are representative of four independent experiments. Values shown mean ± S.D. of quadruplicate wells. Statistical significance (P < .0001) is marked with an asterisk.
to 4 h before, simultaneously, or 1 h after induction with LPS for 16 h. It was observed that the inhibition by 2'-hydroxychalcone is time dependent because it inhibits the expression of ICAM-1 when added 1 to 2 h before induction with LPS more effectively than when added simultaneously or after induction with LPS (data not shown). This indicates 2'-hydroxychalcone may be interfering with the early signaling events in response to LPS or TNF-α.

**Inhibition by 2'-Hydroxychalcone Is Reversible.** To study whether 2'-hydroxychalcone causes any permanent change in the endothelial cells, the cells were preincubated with 50 μM 2'-hydroxychalcone for varying time periods ranging from 1 to 4 h, washed, and allowed to recover for 1 h, followed by induction with LPS (1 μg/ml) for 16 h. As detected by ELISA, the effect of 2'-hydroxychalcone was reversible because the cells were fully capable of responding to LPS and no permanent change was observed on treatment with 2'-hydroxychalcone (Fig. 5).

**Transcript levels of ICAM-1, VCAM-1, and E-Selectin Are Decreased Significantly by 2'-Hydroxychalcone.** Because 2'-hydroxychalcone inhibits the induced levels of ICAM-1, VCAM-1, and E-selectin and the activation of their genes occurs at the level of transcription, we examined whether 2'-hydroxychalcone blocks the induction of the steady-state levels of transcripts. For examining the steady-state transcript levels, endothelial cells were preincubated without or with 50 μM 2'-hydroxychalcone 1 h before induction with TNF-α for 4 h. As observed by RT-PCR analysis, there were low levels of ICAM-1 mRNA, and undetectable levels of VCAM-1 and E-selectin mRNA in control cells or cells treated with 2'-hydroxychalcone alone (Fig. 6A, lanes 1, 3, 5 and 7; Fig. 5B, lanes 1 and 3). After stimulation with TNF-α, there was an up-regulation in ICAM-1, VCAM-1, and E-selectin expression (Fig. 6, A, lanes 2 and 6; B, lane 2). However, treatment with 2'-hydroxychalcone for 1 h before addition of TNF-α significantly reduced the transcript levels.
of ICAM-1, VCAM-1, and E-selectin (Fig. 6, A, lanes 4 and 8; B, lane 4). Whereas the levels of β-actin mRNA expressed under these conditions remained the same (Fig. 6B, lanes 5 to 8). These results indicate that 2'-hydroxychalcone may affect the transcription of ICAM-1, VCAM-1, and E-selectin genes.

2'-Hydroxychalcone Inhibits Activation of NF-κB by TNF-α. Previous studies have shown that NF-κB is a key transcription factor for ICAM-1, VCAM-1, and E-selectin expression in TNF-α-induced endothelial cells (Collins et al., 1995). We therefore tested whether 2'-hydroxychalcone affected NF-κB in endothelial cells. For this, we investigated the status of NF-κB by EMSA in 2'-hydroxychalcone-treated cells (Fig. 7A). The endothelial cells were incubated without or with 50 μM 2'-hydroxychalcone followed by induction with TNF-α (10 ng/ml). As shown in Fig. 7A, there was a low level of NF-κB in unstimulated cells (Fig. 7A, lane 2). On stimulation with TNF-α, there was an increased level of NF-κB, thus causing substantial retardation in the mobility of the labeled oligonucleotide (Fig. 7A, lane 3). The specificity of the NF-κB DNA complex induced by TNF-α was confirmed in control experiments. Incubation with an excess unlabeled NF-κB inhibited the formation of the complex, whereas competition with an excess of an irrelevant oligonucleotide, SP1, did not inhibit the complex (Fig. 7A, lanes 7 and 8 with lane 6). 2'-Hydroxychalcone alone had no effect on the basal level of NF-κB (Fig. 7A, lane 4). In contrast, the treatment of cells with 2'-hydroxychalcone before induction with TNF-α caused a substantial decrease in the level of NF-κB at a concentration of 50 μM (Fig. 7A, lane 5).

To test whether 2'-hydroxychalcone interferes with the binding of NF-κB to its DNA, the nuclear extracts prepared from TNF-α treated cells were incubated with varying concentrations of 2'-hydroxychalcone for 15 min followed by incubation with 32P-labeled NF-κB oligonucleotide for 30 min. The products were analyzed by electrophoresis on a 4% polyacrylamide gel. As shown in Fig. 7B, the binding of NF-κB to its DNA was not affected by the presence of even 40 to 50 μM 2'-hydroxychalcone (Fig. 7B, lanes 4 to 6) or the solvent dimethyl sulfoxide (Fig. 7B, lane 7). This demonstrates that 2'-hydroxychalcone does not interfere with the binding of NF-κB to its DNA but inhibits the activation of NF-κB in endothelial cells.

2'-Hydroxychalcone Inhibits the Nuclear Translocation of p65 Subunit of NF-κB. Because the activation of NF-κB requires the translocation of the p65 subunit of NF-κB to the nucleus, we measured the levels of p65 in the cytoplasm and in the nucleus. It was observed that there were low levels of p65 in the nucleus of the control cells or cells treated with 2'-hydroxychalcone alone (Fig. 8A, lanes 1 and 3) although high levels were observed in the cytoplasm (Fig. 8B, lanes 1 and 3). On treatment with TNF-α, the level of p65 in the cytoplasm decreased (Fig. 8B, lane 2), whereas its level increased in the nucleus (Fig. 8A, lane 2). On treatment of the cells with 2'-hydroxychalcone before induction with TNF-α, the level of p65 in the cytoplasm did not decrease (Fig. 8B, lane 4) and there was no concomitant increase in the p65 levels in the nucleus (Fig. 8A, lane 4). These results therefore indicate that 2'-hydroxychalcone prevents the translocation of p65 to the nucleus.

Discussion

Our results demonstrate for the first time that 2'-hydroxychalcone can be used to control cell trafficking by blocking the expression of cell adhesion molecules. 2'-Hydroxychalcone blocked the adhesion of peripheral neutrophils to endothelial cells. It is equally effective in inhibiting either TNF-α- or LPS-induced expression of leukocyte adhesion molecules. The inhibition by 2'-hydroxychalcone was found to be reversible and 2'-hydroxychalcone should be added before or simultaneously with TNF-α or LPS to be effective. Our RT-PCR results demonstrate that 2'-hydroxychalcone significantly blocked cytokine-induced steady-state transcript levels of cell adhesion molecules (Fig. 6), implying that it may be interfering at an early stage of signaling event induced by TNF-α or LPS. We also show that 2'-hydroxychalcone inhibits TNF-α-induced NF-κB levels with the use of gel retardation assays (Fig. 7) and Western blot analyses (Fig. 8). Because NF-κB is essential for the induced expression of ICAM-1, VCAM-1, and E-selectin, these results confirm that 2'-hydroxychalcone inhibits NF-κB dependent transcription of cell adhesion molecule genes. Conceivably, 2'-hydroxychalcone may inhibit other known or unknown transcription factor(s) as well, but at least one of these transcription factors is NF-κB.

Induction of NF-κB activation by TNF-α requires the phosphorylation and degradation of IκB-α (Baldwin, 1996). This process is dependent on the activation of both protein kinase C and protein tyrosine kinase (Meichvle et al., 1990). Recently, a cyclic AMP-independent protein kinase A associated with IκB, and an IκB kinase complex, have also been found to be involved in the activation of NF-κB (Zandi et al., 1997; Zhong et al., 1997). It is possible that 2'-hydroxychalcone may inhibit NF-κB by blocking any of these protein kinases.
This remains to be tested in the future. Butein (3,4,2'-4'-tetrahydroxychalcone), a chalcone derived from Butea frondosa and Dalbergia odorifera, has been shown to be a specific protein tyrosine kinase inhibitor (Yang et al., 1998). It suppresses the accumulation of leukocytes in nephritic glomeruli and prevents glomerular damage in nephritis by down-regulating the expression of ICAM-1 on endothelial cells by about 30% of the induced level (Hayashi et al., 1996). In contrast, 2'-hydroxychalcone under similar conditions of ICAM-1 assay is found to inhibit TNF-α-induced ICAM-1 expression to its basal level (Fig. 3). In addition to ICAM-1, 2'-hydroxychalcone also suppresses TNF-α-induced expression of E-selectin and VCAM-1 (Fig. 4). Butein, however, is unable to inhibit TNF-α-induced expression of E-selectin and VCAM-1 (Hayashi et al., 1996).

Hydroxychalcones, including 2'-hydroxychalcone, have been reported to possess antioxidant and anticarcinogenic activities (Makita et al., 1996; Haraguchi et al., 1998; Hsieh et al., 1998). These antioxidant and anticarcinogenic properties of hydroxychalcones could be caused by various activities, including inhibition of NF-κB and cell adhesion molecules. In our study, 2'-hydroxychalcone at a concentration of 50 μM is found to be a potent inhibitor of NF-κB activation and cell adhesion molecule expression. It is comparatively less than the concentrations (150–200 μM) required for inhibition of superoxide production and lipid peroxidation reported earlier (Anto et al., 1995). Other NF-κB inhibitory compounds are found to work at a wide range of concentrations (10 μM to 1 mM). For example, diclofenac inhibits NF-κB and cell adhesion molecule expression at a concentration of 750 μM (Sakai, 1996; BM and BG, unpublished observations). N-acetyl cysteine and pyrrolidine dithiocarbamate are most effective at concentrations of 100 μM and 1 mM, respectively (Weber et al., 1994). 2'-Hydroxychalcone, therefore, works well as an inhibitor of NF-κB and cell adhesion molecule expression at a comparatively lower range of concentrations.

In various vascular and inflammatory diseases, the adhesive property of the vasculature is primarily altered because of the up-regulation of expression of cell adhesion molecules. Thus, various approaches, such as mAbs specific to cell adhesion molecules and peptides derived from adhesion molecules, have been employed to inhibit the cell adhesion molecules. Also, NF-κB has been used as a target for blocking cell adhesion molecules. A number of small molecules, such as glucocorticoids, curcumin, serine proteinase, and proteasome inhibitors, have been shown to inhibit TNF-α-induced cell adhesion molecule expression by blocking the activation of NF-κB.

**Fig. 7.** Effect of 2'-hydroxychalcone on NF-κB activation and DNA binding in endothelial cells. A, EMSA. The endothelial cells were incubated without or with 50 μM 2'-hydroxychalcone followed by induction with TNF-α (10 ng/ml) for 30 min. The nuclear extracts were prepared, incubated with 32P-labeled oligonucleotide, and analyzed on a 4% polyacrylamide gel electrophoresis as described under Experimental Procedures. B, NF-κB DNA binding. The nuclear extracts from endothelial cells induced with TNF-α for 30 min were incubated without or with indicated concentrations of 2'-hydroxychalcone or dimethyl sulfoxide for 15 min followed by incubation with 32P-labeled NF-κB oligonucleotide and analyzed on 4% polyacrylamide gel as described in A.
NF-κB (Brojstan et al., 1997; Neuner et al., 1997; Gupta and Ghosh, 1999). In this study, 2'-hydroxychalcone is found to be effective in blocking the activation of NF-κB and thus could be employed in conditions where down-regulation of NF-κB is required. 2'-Hydroxychalcone has been tested in the animal model system and it is found to be nontoxic (Batt et al., 1993; Anto et al., 1995). Although the pharmacokinetics and bioavailability of 2'-hydroxychalcone have not been reported yet, its analogs, such as sofalcone and isoliquiritigenin, have been successfully tested in animal models for their antioxidant, anti-inflammatory, and anticarcinogenic properties (Fujioka et al., 1996; Wegener and Nawrath, 1997). 2'-Hydroxychalcone, therefore, offers a novel therapeutic target for controlling various pathological conditions associated with up-regulation of endothelial leukocyte adhesion molecules and NF-κB.

A.

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M: unstimulated control; lane 1, stimulated with TNF-α; lane 2, 2'-hydroxychalcone alone; lane 4, stimulated with TNF-α after 2'-hydroxychalcone pretreatment for 1 h.

B.

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References


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