

# Chinese Herbal Remedy Wogonin Inhibits Monocyte Chemotactic Protein-1 Gene Expression in Human Endothelial Cells

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Received January 4, 2001; accepted May 23, 2001

This paper is available online at <http://molpharm.aspetjournals.org>

## ABSTRACT

Wogonin (Wog), an active component of *Scutellaria baicalensis*, has antioxidant and anti-inflammatory properties. Monocyte chemotactic protein-1 (MCP-1), a potent chemoattractant for monocytes, plays a crucial role in case of early inflammatory responses, including atherosclerosis. In this study, we investigated the effect of Wog on phorbol ester (PMA)-induced MCP-1 expression in human umbilical vein endothelial cells (ECs). The MCP-1 mRNA levels and MCP-1 release in Wog-treated ECs were measured. Wog inhibited PMA-induced MCP-1 mRNA levels and MCP-1 secretion in a dose-dependent manner. The inhibition of MCP-1 induction by Wog is a transcriptional event, as shown by Wog's significant reduction of both MCP-1 promoter and 4× 12-O-tetradecanoylphorbol-13-acetate response element-luciferase reporter activities. By

electrophoretic mobility assay, Wog significantly reduced the AP-1 binding activity induced by PMA. Furthermore, the PMA-induced extracellular signal-regulated kinase 1/2 and c-Jun amino-terminal kinase activities that contributed to AP-1 activity and MCP-1 gene induction were obviously attenuated after pretreating ECs with Wog. The decrease of MCP-1 secretion by Wog pretreatment led to a reduction of monocyte adhesion to ECs. Taken together, our results demonstrate that Wog inhibits MCP-1 induction in ECs; this inhibition is mediated by reducing AP-1 transcriptional activity via the attenuation of ERK1/2 and JNK signal transduction pathways. We conclude that Wog has the potential therapeutic development for use in anti-inflammatory and vascular disorders.

Wogonin (Wog) is an active component isolated from *Scutellaria baicalensis* radix, a Chinese herbal remedy widely used in clinical treatment of inflammatory diseases, including atopic dermatitis, hyperlipemia, and atherosclerosis. It has been reported that Wog has the potential for therapeutic use in the treatment of atherosclerosis and restenosis based upon its antioxidant (Gao et al., 1999), anti-inflammatory (You et al., 1999), antithrombotic (Kimura et al., 1997), and antiproliferative activities (Huang et al., 1994). However, the effect of Wog on the gene expression of monocyte chemotactic protein-1 (MCP-1), a crucial factor for atherogenesis, in endothelial cells (ECs) and the subsequent influence on EC-monocyte interaction has not yet been explored.

MCP-1, an early-response gene expressed by ECs and vascular smooth muscle cells, is a potent chemotactic cytokine for monocytes. In addition to recruiting and accumulating monocyte into the inflamed sites, such as atherosclerotic lesions, MCP-1 also mediates the development of medial

thickening (Koyanagi et al., 2000). MCP-1 expression has been detected in atherosclerotic plaque (Yla-Herttuala et al., 1991; Takeya et al., 1993). A subsequent study using gene-knockout mice demonstrated that mice with deficient MCP-1 receptors were less susceptible to atherosclerosis, with monocytes restricted to accumulation in vascular lesions (Boring et al., 1998). These findings suggest that MCP-1 plays an important role in the development of atherosclerosis.

MCP-1 is induced in ECs by various stimuli, including chemical and hemodynamic forces (Shyy et al., 1990; Hanazawa et al., 1993; Shyy et al., 1994; Wang et al., 1995). The promoter region of the MCP-1 gene containing binding sites for the redox-responsive transcription factors NF- $\kappa$ B and AP-1 has been identified (Shyy et al., 1990; Ueda et al., 1994; Martin et al., 1997). These transcription factors, when induced, are pivotal for the expression of many genes related to inflammatory responses in ECs under oxidative stress. It has been reported that NF- $\kappa$ B and AP-1 consensus binding

**ABBREVIATIONS:** Wog, wogonin; MCP-1, monocyte chemotactic protein-1; EC, endothelial cell; NF- $\kappa$ B, nuclear factor- $\kappa$ B; AP-1, activator protein-1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, 12-O-tetradecanoylphorbol-13-acetate response element; PMA, phorbol-12-myristate-13-acetate; ERK1/2, extracellular signal-regulated kinase; JNK, c-Jun amino terminal kinase; MBP, myelin basic protein; GST, glutathione S-transferase; NAC, N-acetyl-cysteine; EMSA, electrophoretic mobility shift assay; PKC, protein kinase C; MAPK, mitogen-activated protein kinase.

sites are required for maximal up-regulation of TNF- $\alpha$ -induced MCP-1 expression (Martin et al., 1997). Although the increased MCP-1 expression by hemodynamic forces in ECs was mainly mediated via the increase of AP-1 activity, a phenomenon similar to those cells under phorbol ester (TPA) treatment (Shyy et al., 1994; Wung et al., 1997). This TPA-responsive element (TRE) corresponding to AP-1 binding site in the MCP-1 promoter region is crucial for gene induction. Antioxidant pretreatment of ECs inhibits this redox-sensitive MCP-1 induction by attenuating NF- $\kappa$ B and/or AP-1 activation (Sato et al., 1997; Wung et al., 1997). Because Wog has been shown to possess both antioxidant and anti-inflammatory activities, the inhibitory effect of Wog on MCP-1 gene induction in ECs is worthy of investigation.

In the present study, we investigated the inhibition mechanism of Wog on MCP-1 induction by PMA in ECs. We found that Wog reduced MCP-1 gene expression and secretion. Our results showed that the decrease in MCP-1 expression by Wog was a transcriptional event mediated via the reduction of AP-1 activity. Attenuation of the ERK1/2 and JNK signaling pathways contributed to this inhibited AP-1 activity. Decreasing MCP-1 secretion by Wog leads to a decrease of monocyte adhesion to ECs. Thus, our results provide a basis for the potential development of Wog in treating patients with atherosclerosis and vascular disorders.

## Materials and Methods

**Reagents.** A 0.6-kb fragment of MCP-1 cDNA isolated from a human aortic endothelium cDNA library was used as a probe in Northern blot analysis. The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (Manassas, VA). Wogonin was purchased from Nacalai Tsque (Kyoto, Japan). Actinomycin D and other chemicals of reagent grade were obtained from Sigma Chemical (St. Louis, MO).

**Endothelial Cell Cultures.** Human umbilical vein ECs were isolated from human umbilical cord with collagenase. After 3 days of growth in medium 199 (Invitrogen, Carlsbad, CA) containing 20% fetal calf serum, ECs ( $2.0 \times 10^5$  cells per well) were seeded on a culture well and grown for 3 more days until the monolayer became confluent. The medium for the cultured ECs was then changed to the same medium containing only 2% fetal calf serum, and the cells were incubated overnight before the experiment.

**Enzyme-Linked Immunosorbent Assay for MCP-1.** ECs were plated on 12-well tissue culture dishes and incubated at 37°C until confluent. The cells were pretreated with Wog at different doses for 3 h and then incubated with PMA for another 8 h. The supernatants were collected and analyzed for MCP-1 protein concentration with a MCP-1 Quantikine kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

**RNA Isolation and Northern Hybridization.** Total cellular RNA was obtained with guanidine thiocyanate and separated by electrophoresis on a 1.2% agarose formaldehyde gel (10  $\mu$ g of RNA per lane), then transferred onto a membrane by a vacuum blotting system (VacuGene XL; Amersham Pharmacia Biotech). After hybridizing with the  $^{32}$ P-labeled MCP-1 cDNA probes, the membrane was washed with  $1\times$  standard saline citrate containing 1% SDS at room temperature for 15 min and was then exposed to X-ray film (X-Omat-R; Kodak, Rochester, NY) at  $-70^\circ\text{C}$ . Autoradiographic results were scanned and analyzed using a densitometer (Computing Densitometer 300S; Molecular Dynamics, Sunnyvale, CA).

**Kinase Activity Assay.** ERK1/2 and JNK activities were assayed as described previously with minor modifications (Li et al., 1996; Wung et al., 1999). ECs were lysed in a kinase lysis buffer containing 25 mM HEPES, pH7.4, 0.5 M NaCl, 1% Nonidet P-40, 0.5% sodium

deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 50 mM NaF, 10 mM  $\text{Na}_3\text{VO}_4$ , and 2 mM  $\beta$ -glycerophosphate. ERK1/2 or JNK was immunoprecipitated with an anti-ERK1/2 or anti-JNK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A/G agarose beads. After centrifugation and washing, the agarose-bound immune complex was incubated with kinase reaction buffer containing myelin basic protein (MBP) or glutathione *S*-transferase (GST)-c-Jun-(1-79) fusion protein. For the ERK assay, the kinase reaction buffer contained 0.3 g/l MBP, 50  $\mu$ M ATP, and 1  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP. For JNK activity, 2  $\mu$ g of GST-c-Jun, 25  $\mu$ M ATP, and 10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP were contained in 30  $\mu$ l of a kinase reaction buffer. The kinase reaction was carried out for 20 min at 30°C and stopped by adding an equal volume of sample buffer containing SDS and boiling for 3 min. The samples were electrophoresed on a 10% polyacrylamide gel and the gel was exposed to X-ray film after drying.

**Reporter Gene Construct, Transfection, and Luciferase Assay.** A MCP-1 promoter construct (P540-Luc) containing 540 base pairs of the MCP-1 promoter region with a canonical TRE site, and a chimeric gene construct (4xTRE-Luc) containing four copies of the TRE consensus sequence (TGACTCA), both followed by the luciferase reporter gene (Luc), were used in the luciferase assay (Shyy et al., 1995). DNA plasmids, purified by a Wizard Maxipreps DNA purification system (Promega, Madison, WI), were transfected into bovine aortic endothelial cells at 60% confluence using the LipofectAMINE method (Invitrogen). The pSV- $\beta$ -galactosidase gene driven by the simian virus 40 promoter and enhancer was cotransfected to normalize the transfection efficiency. After transfection, cells were incubated overnight with 10% fetal bovine serum-Dulbecco's modified Eagle's medium to reach confluence. The transfected cells were treated in a manner analogous to that described for ELISA. The cell extract was prepared and assayed for luciferase activity using the Promega Biotec assay system. To normalize the transfection efficiency for individual samples, the  $\beta$ -galactosidase activity was assayed by adding the substrate, *o*-nitrophenyl- $\beta$ -D-galactopyranoside, to 20  $\mu$ L of cell lysate and incubating at 37°C before recording at 420 nm.

**Electrophoretic Mobility Shift Assay (EMSA).** Extracts of nuclear protein were prepared according to the procedures described previously (Wung et al., 1997). ECs were washed with cold PBS and immediately removed by scraping in PBS. After centrifugation of the cell suspension at 2000 rpm, the cell pellets were resuspended in cold buffer A (10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) for 15 min. The cells were lysed by adding 10% Nonidet P-40 and then centrifuged at 6000 rpm to obtain a pellet of nuclei. The nucleic pellets were resuspended in cold buffer B (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), vigorously agitated from time to time, and then centrifuged. The supernatant containing the nuclear proteins was used for the EMSA or stored at  $-70^\circ\text{C}$  for later used.

AP-1 (c-jun) oligonucleotide, 5'-CGCTTGATGAGTCAGCCG-GAA-3' (Promega), and a consensus NF- $\kappa$ B oligonucleotide, 5'-AGT-TGAGGGGATTTTCCCAGGC-3' (Promega), were end-labeled with [ $\gamma$ - $^{32}$ P]ATP and T4 polynucleotide kinase. Nuclear extract (10  $\mu$ g) was incubated with 0.1 ng  $^{32}$ P-labeled DNA for 15 min at room temperature in a final volume of binding buffer of 25  $\mu$ l containing 1  $\mu$ g of poly(dI-dC). The mixtures were electrophoresed on 6% nondeaturing polyacrylamide gels under high ionic strength. Gels were dried and imaged by autoradiography.

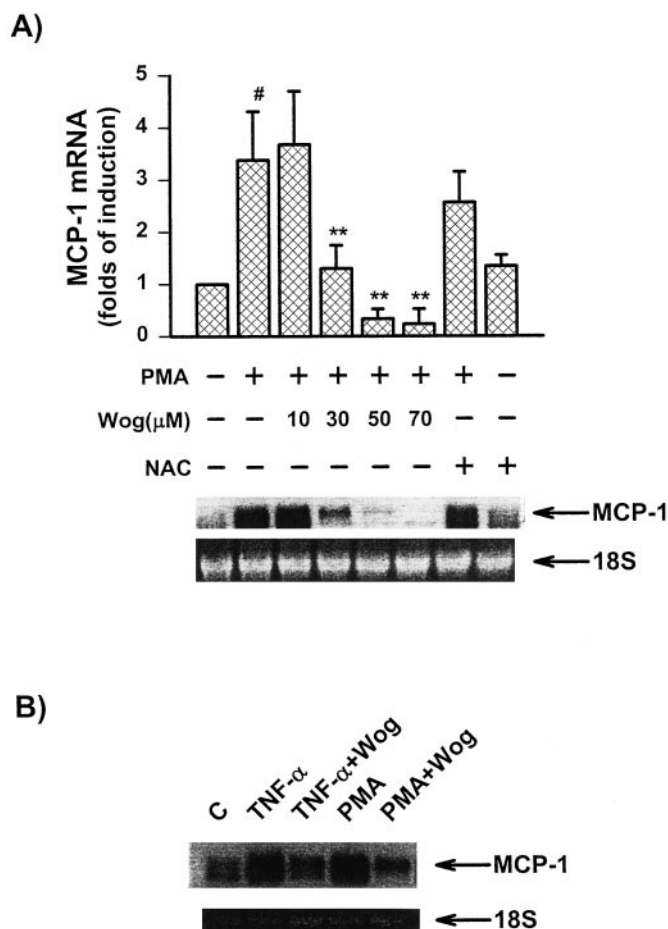
**Cell Adherence Measurements.** THP-1 cells suspended in RPMI-1640 medium containing 0.1% fetal bovine serum were labeled with 1  $\mu$ Ci of [ $^3\text{H}$ ]thymidine overnight (specific activity, 23 Ci/mmol; Amersham Pharmacia Biotech). Cells were washed three times in fresh RPMI-1640 medium. THP-1 cells ( $3 \times 10^5$ ) were then added to each well containing ECs and incubated for 1 h. Nonadherent THP-1 cells were removed by washing with medium 199. ECs with adherent

THP-1 cells were lysed with lysis buffer, and radioactivity was counted by a scintillation counter.

**Statistical Analysis.** Statistical analyses were performed using Student's *t* test for experiments consisting of only two groups and by analysis of variance for experiments consisting of more than two groups. Data were presented as mean  $\pm$  S.E.M. Statistical significance was defined as  $P < 0.05$ .

## Results

**Wog Inhibits PMA and TNF-Induced MCP-1 Gene Expression.** Human umbilical vein endothelial cells (ECs) were under oxidative stress when cells were exposed to PMA for 2 h. *N*-acetyl-cysteine (NAC), an antioxidant, was used to illustrate the oxidant stress of ECs under PMA treatment. ECs were pretreated with various dosages of Wog for 3 h followed with a treatment of PMA for another 2 h. As shown in Fig. 1A, Wog treatment of ECs markedly inhibited the increment of PMA-induced MCP-1 mRNA in a dose-dependent manner, whereas NAC pretreatment showed only partial inhibition. To determine whether this inhibitory effect of

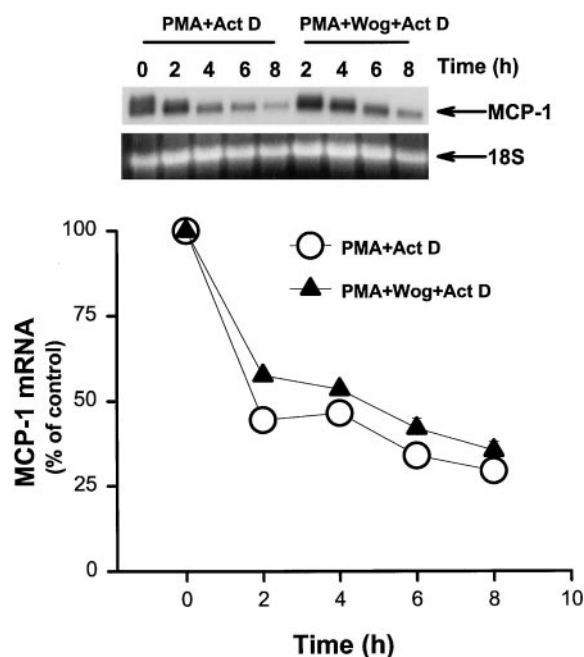


**Fig. 1.** Inhibitory effect of Wogonin on MCP-1 gene induction in ECs. A, after incubating ECs with either different concentrations of Wog for 3 h or NAC for 1 h, as indicated, PMA (100 ng/ml) was added and incubated for another 2 h. B, ECs were treated with TNF- $\alpha$  (100 U/ml) or PMA (100 ng/ml) in the presence or absence of 30  $\mu$ M Wog. Total cellular RNAs were isolated for Northern blot analysis using a  $^{32}$ P-labeled gene-specific probe. Autoradiographic results from three separate experiments were analyzed by densitometry. The level of MCP-1 mRNA in each lane is normalized to 18S RNA and data are presented as fold change of control groups in bar graphs. Each bar is shown as mean  $\pm$  S.E.M. <sup>#</sup> $P < 0.05$  versus controls; <sup>\*\*</sup> $P < 0.01$  versus PMA.

Wog was restricted to PMA, we measured the effect of Wog on TNF- $\alpha$ -induced MCP-1 gene expression. As shown in Fig. 1B, Wog (30  $\mu$ M) treatment of ECs decreased TNF- $\alpha$ -induced MCP-1 mRNA by 76%, which is comparable with the 83% reduction in PMA-stimulated MCP-1 expression. These results indicate that Wog inhibits PMA- and TNF- $\alpha$ -induced MCP-1 gene expression.

**Wog Treatment Does Not Affect MCP-1 mRNA Stability.** MCP-1 mRNA expression can be regulated at the transcriptional or post-transcriptional level. To study whether the inhibitory effect on MCP-1 gene expression by Wog was caused by a decrease in mRNA stability, ECs were pretreated with PMA for 4 h followed with an incubation of an inhibitor to transcription, actinomycin D, to prevent further synthesis of MCP-1 mRNA. As indicated in Fig. 2, there was no discrepancy regarding the MCP-1 mRNA degradation rate (half-life  $\sim$ 5 h) in actinomycin D-treated ECs in the presence or absence of Wog (30  $\mu$ M). This indicates that the inhibitory effect of Wog on MCP-1 gene induction was not caused by the decreasing stability of MCP-1 mRNA.

**Wog Inhibiting MCP-1 Promoter Activity Is Mediated via the Decrease of AP-1/TRE Binding Activity.** We previously demonstrated that the AP-1 binding site (i.e., the TRE) in the MCP-1 promoter region is crucial for MCP-1 gene induction (Wung et al., 1997). To determine whether the TRE consensus sequence contributes to the inhibitory effect of Wog on MCP-1 expression, the promoter activities of MCP-1 and 4xTRE were determined. As shown in Fig. 3, there was a  $3.49 \pm 0.52$ -fold induction in the promoter activity of MCP-1 (Fig. 3A) and a  $4.98 \pm 0.27$ -fold induction in the



**Fig. 2.** Wogonin has no effect on the stability of MCP-1 mRNA in PMA-activated ECs. ECs were treated with 100 ng/ml PMA for 4 h, then actinomycin D (Act D; 10  $\mu$ g/ml) was added with or without 30  $\mu$ M Wog. After cells were harvested at the indicated times, total cellular RNAs were isolated and analyzed by Northern blot. Data are presented as a percentage change in the amounts of MCP-1 mRNA remaining after actinomycin D treatment compared with the amounts of MCP-1 mRNA without actinomycin D treatment as indicated at zero time point. The mRNA levels of MCP-1 were quantified by scanning densitometry. Autoradiographic results were similar in two independent experiments.

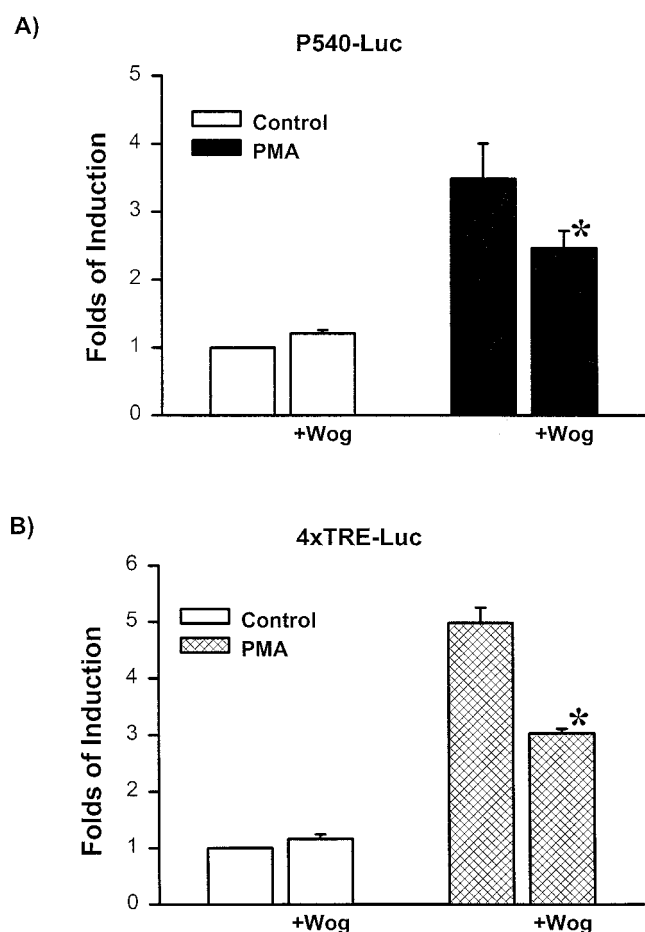


promoter activity of 4xTRE (Fig. 3B) from PMA-treated ECs compared with control cells. In contrast to control cells, PMA-induced MCP-1 promoter and 4xTRE-luciferase reporter activities were inhibited significantly by Wog pretreatment of ECs.

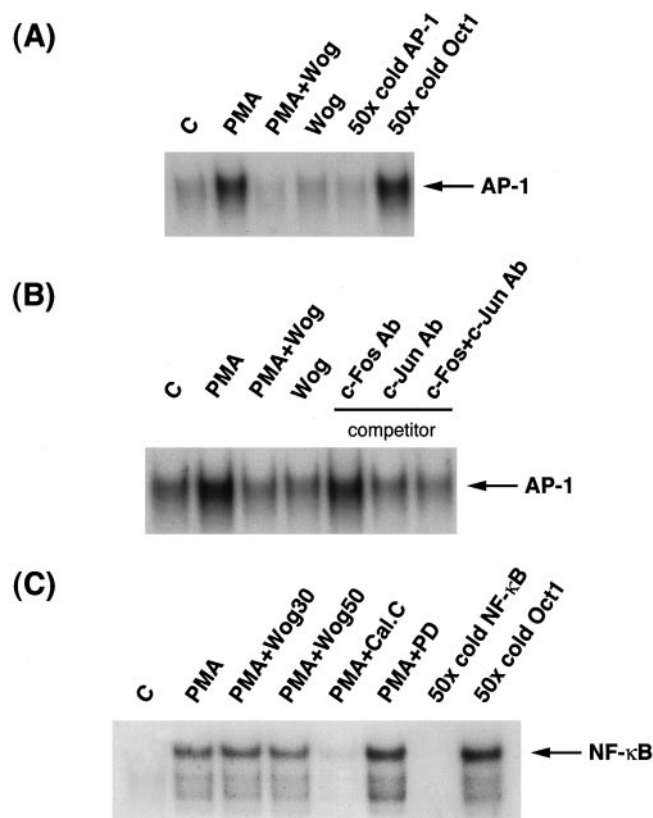
The *trans*-activation of MCP-1 gene is driven by AP-1 binding to the TRE site in the 5' promoter region. To further confirm that MCP-1 expression inhibited by Wog is a transcriptional event mediated via a decrease in AP-1 binding activities, nuclear extracts from PMA-treated ECs in the presence or absence of Wog were analyzed for their AP-1 activity by electrophoretic mobility shift assay (EMSA). AP-1 binding activity induced by PMA was significantly inhibited by Wog (30  $\mu$ M) (Fig. 4, A and B, lane 3). The specificity of this AP-1 binding activity was verified by coinubation of nuclear extracts with 50-fold molar excess of unlabeled AP-1 probe. As shown in Fig. 4A, lane 5, an inhibition of the retarded AP-1 band was observed. In contrast, PMA-treated nuclear extracts coinubated with scramble oligonucleotides

did not affect the band density (Fig. 4, lane 6). These results suggest that inhibition of PMA-induced MCP-1 expression by Wog is a transcriptional event. EMSA was also performed to identify the presence of AP-1 components in PMA-activated AP-1 activity by coinubations of nuclear extracts with antibodies against c-Jun and/or c-Fos. As shown in Fig. 4B, lane 7, antibodies against c-Fos and c-Jun clearly decreased the density of retarded AP-1 band, suggesting that these proteins were the major components responsible for PMA-induced AP-1 activity. Furthermore, the PMA-induced AP-1 activities were greatly inhibited if nuclear extracts were coinubated with c-Jun antibody (lane 6). From this finding, we postulated that inhibition by Wog of AP-1 binding activity is mediated predominantly via c-Jun expression.

Because the MCP-1 promoter region contains NF- $\kappa$ B binding sites, we determined whether Wog treatment affects the NF- $\kappa$ B activity induced by PMA. As shown in Fig. 4C, lanes 3, 4, and 6, cells treated with either Wog or a MAPK kinase inhibitor (PD098059) had no inhibitory effects on PMA-in-



**Fig. 3.** Wog's inhibition of MCP-1 expression is a transcriptional event. BAECs were transiently transfected with P540-Luc (A) or 4xTRE-Luc and pSV- $\beta$ -gal plasmids (B). P540-Luc is a construct in which the 540-base-pair MCP-1 promoter drives the luciferase reporter. 4xTRE-Luc is a construct in which the rat prolactin promoter conjugated to the luciferase reporter is driven by four copies of the TRE consensus sequence. Before 8-h PMA treatment, transfected BAECs were incubated with 30  $\mu$ M Wog for 3 h. Luciferase activities determined by chemiluminescence assays are expressed as the fold induction of unstimulated control cells. Results are shown as mean  $\pm$  S.E.M. from three separate experiments. The induction of luciferase activities is the significant difference between PMA-treated groups and unstimulated controls ( $P < 0.05$ ). \* $P < 0.05$  versus PMA.



**Fig. 4.** Wogonin inhibits PMA-induced activation of transcription factor AP-1. Endothelial cells were treated with 30  $\mu$ M Wog for 3 h before PMA (100 ng/ml) was added and were then coinubated for another 2 h. Total nuclear extracts were prepared and analyzed by EMSA using a  $^{32}$ P-labeled oligonucleotide probe containing a canonical TRE site. The specificity of the retarded complex (AP-1) was assessed by preincubation of the nuclear extracts with competitors including unlabeled TRE-containing oligonucleotides (50 $\times$  unlabeled AP-1) or unrelated oligonucleotides (50 $\times$  unlabeled Oct-1) (A) or with antibodies (1  $\mu$ g) to c-Fos and/or c-Jun (B). C, Wog has no inhibitory effect on NF- $\kappa$ B activity. ECs were treated with various doses of Wog [30  $\mu$ M (Wog30) and 50  $\mu$ M (Wog50)] for 3 h before PMA (100 ng/ml) was added. After incubation with PMA for 2 h, nuclear proteins were isolated and binding reactions were performed with a consensus NF- $\kappa$ B oligonucleotide labeled with [ $\gamma$ - $^{32}$ P]ATP. In some experiments, ECs were treated with calphostin C (100 nM, Cal C) for 30 min or PD098059 (20  $\mu$ M, PD) for 1 h before adding PMA. The figure represents one of three different experiments, with similar results.

duced NF- $\kappa$ B binding activity (lane 5). In contrast, cells treated with a PKC inhibitor (calphostin C) prevented this PMA-induced NF- $\kappa$ B activation. These results are consistent with those of previous studies indicating that PKC can contribute to NF- $\kappa$ B activation (Sen and Baltimore, 1986). However, this PKC-induced NF- $\kappa$ B activation is not mediated via the classical mitogenic kinase cascade (Baumann et al., 2000). Wog treatment consistently failed to inhibit TNF- $\alpha$ -induced NF- $\kappa$ B activation (data not shown). Collectively, these data support the idea that the inhibition of MCP-1 gene expression by Wog treatment is predominantly mediated via the reduction of AP-1/DNA binding and *trans*-activation.

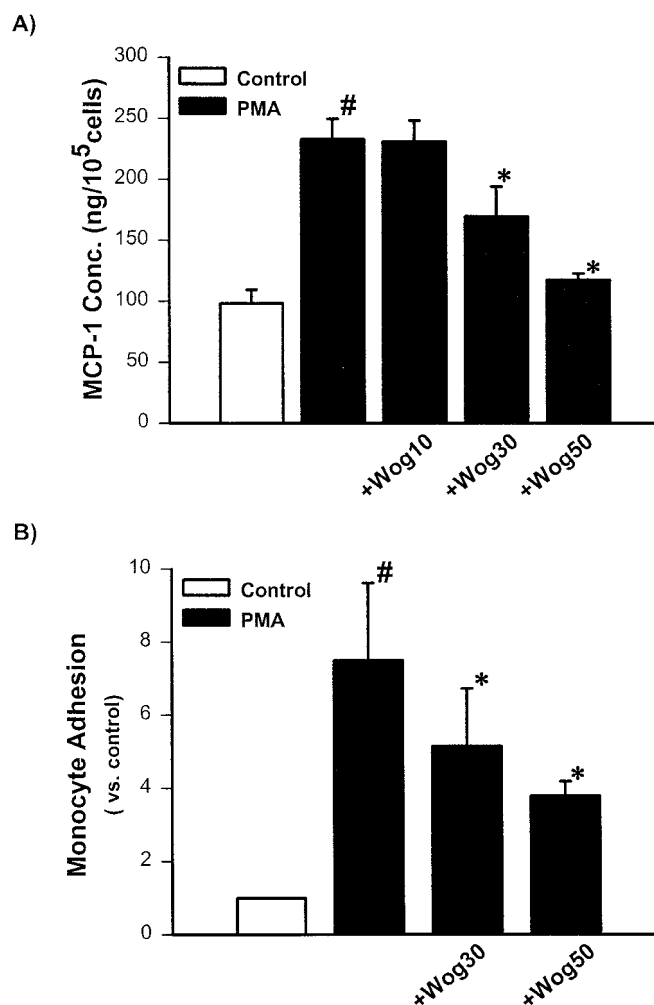
**Wog Treatment Inhibits the ERK and JNK Signaling Pathways.** ERK1/2 and JNK are the upstream signaling regulators of AP-1 activation that regulate the distinct AP-1 components c-Fos and c-Jun, respectively (Karin, 1995). To investigate the signaling pathway involved in the suppression of MCP-1 expression by Wog, ERK1/2 and JNK kinase activities were determined. As indicated in Fig. 5, cells treated with Wog inhibited ERK1/2 and JNK activities. Consistently, pretreatment of cells with PD098059 completely abrogated PMA-induced ERK1/2 activity in ECs without affecting JNK activity (data not shown). Cells treated with 30  $\mu$ M Wog significantly inhibited the JNK activity ( $\sim 40\%$ ) but had less effect on ERK1/2 activity ( $\sim 13\%$ ). However, cells treated with 50  $\mu$ M Wog showed similar inhibitory effects on both kinase activities. This result suggests that JNK activity is more sensitive to Wog treatment. Because c-Jun is regulated by JNK phosphorylation, our data also consistent with the aforementioned observation that c-jun is the predominantly transcriptional factor for the MCP-1 induction by PMA (Fig. 4B).

**Wog Treatment Reduces PMA-Induced MCP-1 Protein Secretion and Monocyte Adhesion to ECs.** Upon the activation of ECs, MCP-1 is released and triggers rolling monocytes to adhere to ECs (Gerszten et al., 1999). MCP-1 protein secretion is associated with the expression of MCP-1 mRNA levels. Because Wog was shown to inhibit MCP-1 gene expression, we next studied whether Wog treatment affects MCP-1 protein secretion and monocyte adhesion to activated ECs. ECs were pretreated with different concentrations of Wog for 3 h and were then immediately followed by PMA exposure. As shown in Fig. 6A, Wog treatment of ECs dose-dependently reduced MCP-1 protein secretion, which conse-

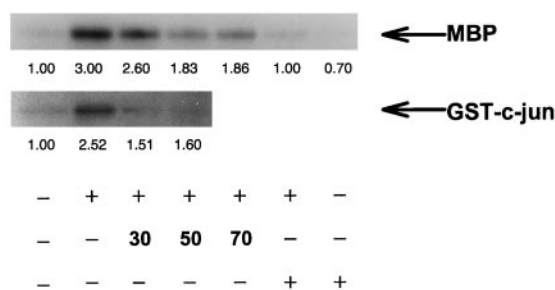
quently led to a decrease in monocyte adhesion to ECs (Fig. 6B). Our results clearly indicate that Wog treatment of ECs reduces MCP-1 mRNA levels and decreases MCP-1 secretion. Consequently, these events contribute to a reduction in monocyte adhesion to ECs.

## Discussion

Endothelial cells play a key role in the maintenance of vascular homeostasis by performing many biological processes, including regulation of the permeability of plasma lipoproteins, adhesion of leukocytes, and release of prothrombotic and antithrombotic factors, growth factors, and vasoactive substances (Rubanyi, 1993). Once these biological processes of ECs are impaired, the pathogenesis of atherosclerosis is induced (Libby, 2000). However, in the various ECs pathological processes, from inflammation to atherosclerosis, transcriptional factor AP-1 is thought to be



**Fig. 6.** Wogonin inhibits MCP-1 protein secretion and monocyte adhesion in PMA-activated ECs. ECs pretreated with Wog of various concentration for 3 h were then incubated with PMA (100 ng/ml) for another 8 h. A, the culture media were collected and the MCP-1 protein levels were determined by enzyme-linked immunoassay, or (B) <sup>3</sup>H-labeled monocytic THP-1 cells were added and incubated for 30 min after PMA incubation. After removal of nonadherent THP-1 cells, ECs were lysed and total radioactivity was counted by liquid scintillation. Data are presented as fold induction of the control group. Each column represents mean  $\pm$  S.E.M. from five to six separate experiments. <sup>\*</sup>*P* < 0.05 versus control cells; <sup>#</sup>*P* < 0.05 versus PMA.



**Fig. 5.** Wogonin treatment inhibits ERK and JNK signaling pathways. ECs were treated with various concentrations of Wog for 3 h as indicated and then incubated with PMA for 30 min. ECs were lysed and the lysates were immunoprecipitated with anti-ERK1/2 or anti-JNK antibody. Immunocomplex kinase assays were performed by adding [ $\gamma$ -<sup>32</sup>P]ATP and MBP or GST-c-Jun (1–79) to the immunoprecipitates. In some experiments, ECs were preincubated with a MAPK kinase inhibitor, PD098059, for 1 h followed by PMA treatment. The figure under each lane represents the absorbance of each band.

an important gene regulator (Wang et al., 1999). Wog has already been reported to inhibit the proliferation of vascular smooth muscle cells (Huang et al., 1994) and the elevation of trypsin-induced plasminogen activator inhibitor-1 production in ECs (Kimura et al., 1997). In this study, we further demonstrated that Wog inhibits MCP-1 expression by reducing AP-1 activation, supporting the previous opinion of its developmental potential in treating atherosclerosis.

Our results demonstrated that Wog inhibits monocyte adhesion to ECs by reducing MCP-1 gene expression and protein secretion in a dose-dependent manner. This inhibitory effect by Wog is exerted at the transcriptional level. Several lines of evidence support this notion. First, Wog treatment inhibited MCP-1 gene expression without affecting mRNA stability. Second, ECs exposed to Wog reduced AP-1 transcriptional activation. Third, Wog treatment of ECs attenuated MCP-1 promoter activities. Furthermore, Wog's inhibition of MCP-1 gene expression via reducing AP-1 activity was further substantiated by its attenuation of the promoter activity of TRE, an AP-1 binding site in the 5' promoter region.

Wog is a 5,7-dihydroxy-8-methoxyflavone that possesses antioxidant activity by inhibiting NAD(P)H: quinone acceptor oxidoreductase (Liu et al., 1990), NADPH-dependent cytochrome P-450 reductase (Sato et al., 1992), and xanthine oxidase (Chang et al., 1993). These various enzymes have been demonstrated to generate free radicals in human umbilical vein endothelial cells (for review, see Palmer and Paulson, 1997). Recent studies, including ours, have indicated that reactive oxygen species act as second messengers (Schreck and Baeuerle, 1991; Wung et al., 1997) and are responsible for MCP-1 expression induced by various stimuli (Satriano et al., 1993; Wung et al., 1997). In the present study, Wog was found to be more potent than the antioxidant NAC in the inhibition of MCP-1 expression. The inhibition of Wog on MCP-1 expression may be attributed, in part, to its antioxidant activity by reducing reactive oxygen species levels.

Both NF- $\kappa$ B and AP-1, redox-sensitive transcriptional factors, are known to be involved in the regulation of MCP-1 expression. Our result showed that the inhibition of Wog on PMA-induced MCP-1 expression is mediated mainly via the reduction of AP-1 activity, not NF- $\kappa$ B activation. The inhibitory effect of Wog on PMA-induced MCP-1 gene expression is mediated mainly at the transcriptional level by reducing AP-1 activity. This inhibition is unlikely to be mediated via the direct inhibition of protein kinase C activity because Wog does not inhibit PMA activation of NF- $\kappa$ B, which can be translocated into nucleus by PKC activation (Sen and Baltimore, 1986). Furthermore, Wog treatment did not inhibit PMA-induced PKC $\alpha$  activation (data not shown), which was reported to activate Raf, and subsequently mediated transcriptional activation (Kolch et al., 1993; Baumann et al., 2000).

Previous studies, including ours, have confirmed that the TRE, an AP-1 binding site, in the 5' promoter region of MCP-1 is responsible for PMA- and hemodynamic forces-induced MCP-1 expression (Shyy et al., 1995; Wung et al., 1997). In this study, we observed a similar inhibition effect of Wog on MCP-1 promoter activity as well as on TRE-luciferase reporter activity. However, Wog treatment also reduced the basal level of MCP-1 RNA. Thus, in addition to the inhibition of AP-1 activity, Wog might affect other transcrip-

tion factors, such as Sp-1, which was reported to regulate the basal level of MCP-1 expression (Ueda et al., 1994).

The activity of AP-1 is regulated by those that increase the abundance of AP-1 components c-Jun and c-Fos and those that stimulate their activity (Karin, 1995; Gómez del Arco et al., 1997). There are two major signaling pathways of MAPKs, one through ERK1/2 and the other through JNK, to regulate AP-1 activity in response to a spectrum of stimuli, such as cytokine, mitogen, and stress stimuli (Karin, 1995). The activation of ERK results in an increase in AP-1 activity via *c-fos* induction, whereas JNK activation leads to c-Jun phosphorylation (Karin, 1995; Whitmarsh and Davis, 1996). In a gel shift assay, we found that both c-Jun and c-Fos contributed to PMA-induced AP-1 activity. In fact, in addition to inhibiting c-Fos activity, Wog greatly attenuated the c-Jun pathway and abolished AP-1 activation. This was further confirmed by a MAPK activity assay that showed Wog treatment not only reduced ERK1/2 kinase activity, but also significantly inhibited JNK kinase activity. Wog, at 30  $\mu$ M, showed almost the same magnitude of inhibitory effects on MCP-1 gene expression, AP-1 DNA binding activity, and JNK activity. Taken together, these results suggest that Wog predominantly blocks the PMA-induced MCP-1 expression through the inhibition of JNK activity, thereby reducing AP-1 activation.

In summary, this study suggests that inhibition by Wog of PMA-induced MCP-1 gene expression in human vascular endothelial cells is mediated via the down-regulation of JNK and ERK activities, which lead to decreases in AP-1 activity. Our results provide molecular evidence for a novel mechanism of Wog acting at the transcriptional level and exerting its anti-inflammatory activity by abrogating MCP-1 gene expression. This study suggests that Wog may be a worthy template for therapeutic development to attenuate inflammatory responses during endothelial dysfunction in such conditions as atherosclerosis.

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