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Ochnaflavone Inhibits TNF-α-Induced Human VSMC Proliferation via Regulation of Cell Cycle, ERK1/2, and MMP-9

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Abstract Ochnaflavone (c-3 of apigenin-0-c-4 of apigenin; OC), a biflavonoid present in the human diet, is known to inhibit angiotensin II-induced hypertrophy and serum-induced smooth muscle cell proliferation. OC is known to have anti-fungal and anti-inflammatory activities. However, it is not known whether OC exerts similar cardioprotective effects in cells treated with tumor necrosis factor (TNF)-α. In this study, we isolated OC from *Lonicera japonica* and studied its effect on matrix metalloproteinase-9 (*MMP-9*) gene expression in human aortic smooth muscle cells (HASMC). Furthermore, we investigated whether OC exerts the multiple suppressive effects on cytokine TNF-α-induced HASMC. Treatment of OC showed its potent inhibitory effects on DNA synthesis of cultured HASMC in the presence of TNF-α. These inhibitory effects were associated with reduced extracellular signal-regulated kinase 1/2 (ERK1/2) activity and G1 cell cycle arrest. Treatment of OC, which induced a cell cycle block in G1-phase, induced downregulation of cyclins and CDKs and upregulation of the CDK inhibitor $p21^{waf1}$ expression, whereas upregulation of p27 or p53 by OC was not observed. Because anti-atherogenic effects need not be limited to anti-proliferation, we decided to examine whether OC exerts inhibitory effects on MMP-9 activity in TNF-α-induced HASMC. OC inhibited TNF-α-induced MMP-9 secretion on HASMC in a dose-dependent manner. This inhibition was characterized by downregulation of MMP-9, which was transcriptionally regulated at nuclear factor (NF)-κB site and activation protein (AP)-1 site in the MMP-9 promoter. These findings indicate the efficacy of OC in inhibiting cell proliferation, G1 to S-phase cell cycle progress, and MMP-9 expression through the transcription factors NF-κB and AP-1 on TNF-α-induced HASMC. The findings of the present study may provide a potential mechanism that explains the anti-atherogenic activity of OC. J. Cell. Biochem. 99: 1298–1307, 2006. © 2006 Wiley-Liss, Inc.

Key words: ochnaflavone; matrix metalloproteinase-9; human aortic smooth muscle cells; extracellular signal-regulated kinase1/2; atherosclerosis; TNF-α; G1 cell cycle; NF-κB; AP-1

Abbreviations used: OC, ochnaflavone (c-3 of apigenin-0-c-4 of apigenin); MMP-9, matrix metalloproteinase-9; HASMC, human aortic smooth muscle cells; TNF-α, tumor necrosis factor-α; ERK, extracellular signal-regulated kinase; NF, nuclear factor; AP, activation protein; VSMC, vascular smooth muscle cells; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; FBS, fetal bovine serum; GST, glutathione *S*-transferase.

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The proliferation of vascular smooth muscle cells (VSMC) is a key event in the development of advanced lesions associated with atherosclerosis [Ross, 1993]. The abnormal growth of VSMC and extracellular matrix proteins are prominent features of vascular disease, including atherosclerosis, restenosis after angioplasty [Ross, 1993]. Vascular lesions form during several pathological processes, which involve accumulation of inflammatory cells and the release of cytokines [Abedi and Zachary, 1995]. Tumor necrosis factor (TNF)-α is a cytokine secreted by VSMC in the neointima after balloon-injury as well as by macrophages in atherosclerotic lesions [Jovinge et al., 1997]. TNF-α induces the activation of extra cellular signal-regulated kinase 1/2 (ERK1/2), a key transducer of extracellular signals that promote cell growth and movement, which are critical for the initiation and progression of vascular lesions [Goetze et al., 1999, 2001]. Little is known, however, about the cell cycle regulation in TNF-α-induced VSMC proliferation.

Increased proteolytic activity in the vessel wall mediates the degradation of the extra cellular matrix surrounding smooth muscle cells in response to injury [Newby and Zaltsman, 2000], a necessary step to allow medial smooth muscle cells to migrate into the intimal space. One class of matrix metalloproteinases (MMPs) that has been implicated as mediator of lesion development in response to vascular injury is the gelatinases, MMP-2 (72 kDa), and MMP-9 (92 kDa). In most cases, MMP-2 is constitutively present in tissues in the form of a 72-kDa proenzyme. In contrast, MMP-9, like most other MMPs, is expressed and secreted only upon demand and regulation occurs at the gene transcription level. In a rat arterial injury model, MMP-9 is expressed within 6 h after injury in rat carotid arteries and continues to be expressed up to 6 days, whereas MMP-2 activity is markedly increased after 4 days of injury [Bendeck et al., 1994]. The basal levels of MMP-9 are usually low, and its expression can be induced by treatment of cells with TNF-α, but not platelet-derived growth factor [Galis et al., 1994; Fabunmi et al., 1996]. The promoter of MMP-9 possesses several functional enhancer element-binding sites including three activation protein (AP)-1 sites, a non-consensus nuclear factor (NF)-κB, an Ets site, an SP-1 site, and a retinoblastoma element [Sato and Seiki, 1993; Sato et al., 1993]. Accumulated data demonstrated that TNF-α may activate or induce MMP-9 expression through pathways leading to activation of NF-κB and AP-1 in several tumor lines [Sato and Seiki, 1993; Sato et al., 1993].

In the arterial media, VSMCs are normally quiescent, proliferate at low indices (<0.05%), and remain in the G_0/G_1-phase of the cell cycle [Gordon et al., 1990]. After vessel injury, VSMC migrate into the intimal layer of the arterial wall, where they leave their quiescent state and reenter the cell cycle [Ross, 1993]. In many cells, transit through G1 of the cell cycle and entry into the S-phase require the binding and activation of cyclin/CDK complexes, predominantly cyclin D1/CDK4 and cyclin E/CDK2 [Sherr, 1994; Sherr, 1996]. The kinase activities of the cyclin/CDK complexes are negatively regulated by CDK inhibitors, such as $p21^{waf1/cip1}$ and $p27^{kip1}$ [Xiong et al., 1993; Toyoshima and Hunter, 1994].

Flavonoid present in the human diet has been studied widely and its biological properties are consistent with its protective role in the cardiovascular system. Biflavonoid is one of the classes of naturally occurring bioflavonoid [Beretz et al., 1979; Ruckstuhl et al., 1979; Iwu et al., 1990; Lee et al., 1995; Kim et al., 1998; Oliveira et al., 2005]. Certain biflavonoids, such as ochnaflavone (OC), amentoflavone, isoginkgetin, sciadopitysin, 4′-*O*-methylochnaflavone, cryptomerin B, isocryptomerin bilobetin, ginkgetin, and morelloflavone were reported to have anti-inflammatory activity [Amella et al., 1985; Bronner and Landry, 1985; Lin et al., 1989; Son et al., 1992], mast cell histamine release-inhibitory activity [Amella et al., 1985; Bronner and Landry, 1985], anti-tumor activity [Lin et al., 1989], and lymphocyte anti-proliferating activity. In a recent study [Chang et al., 1994], we reported the inhibitory mechanism of group II phospholipase A2 by OC.

One of the major challenges of anti-atherosclerotic chemoprevention is the development of new effective drugs that have little or no effect on normal cells. The present result provides a possibility for OC to develop as a new therapeutic or chemopreventive agent against chronic and acute atherosclerosis in which MMP-9 induction involves in their pathogenesis. The findings of the present study showed the inhibition of TNF-α-induced ERK1/2 activation and repression of cyclin D1/CDK4 and cyclin

E/CDK2 activities by the increased expression of p21waf1 may be a part of main mechanisms by which OC inhibits VSMC proliferation in cultured human aortic smooth muscle cells (HASMC). We also report here for the first time that TNF-α-induced MMP-9 expression was abolished by OC in HASMC via inhibition of transcription factors NF-κB and AP-1 binding activities.

MATERIALS AND METHODS

Materials

TNF-α was obtained from R&D systems. Polyclonal antibody to cyclin D1, cyclin E, CDK2, CDK4, p21waf1, p53, p27, EER1/2, and phospho-ERK1/2 were obtained from New England Biolabs. Polyclonal MMP-9 antibody was obtained from Chemicon.

Extraction and Isolation of Ochaflavone

OC was isolated from *Lonicera japonica.* Dried aerial part of *L. japonica* was extracted with hot MeOH for 6 h (3 times). MeOH extract was partitioned with *n*-hexane, chloroform, and ethylacetate, successively. The ethylacetate soluble fractions were separately subjected to column chromatography over silica gel (70–230 mesh) using chloroform–MeOH gradients. The isolated compound was chemically and structurally identified according to the previous reports [Son et al., 1992]. Its chemical structure is shown in Figure 1A. OC used in this study showed a single spot on thin layer chromatography and was prepared by dissolving in dimethyl sulfoxide (DMSO) and diluted with phosphate-buffered saline (PBS). Final concentrations of DMSO were adjusted to 0.1% (v/v) in culture media.

Cell Cultures

HASMC were purchased from Bio-Whittaker (San Diego, CA) and cultured in smooth muscle cell growth medium-2 containing 10% fetal bovine serum (FBS), 2 ng/ml human basic fibroblast growth factor, 0.5 ng/ml human epidermal growth factor, 50 μg/ml gentamicin, 50 μg/ml amphotericin-B, and 5 μg/ml bovine insulin. For all experiments, early passage HASMCs were grown to 80–90% confluence

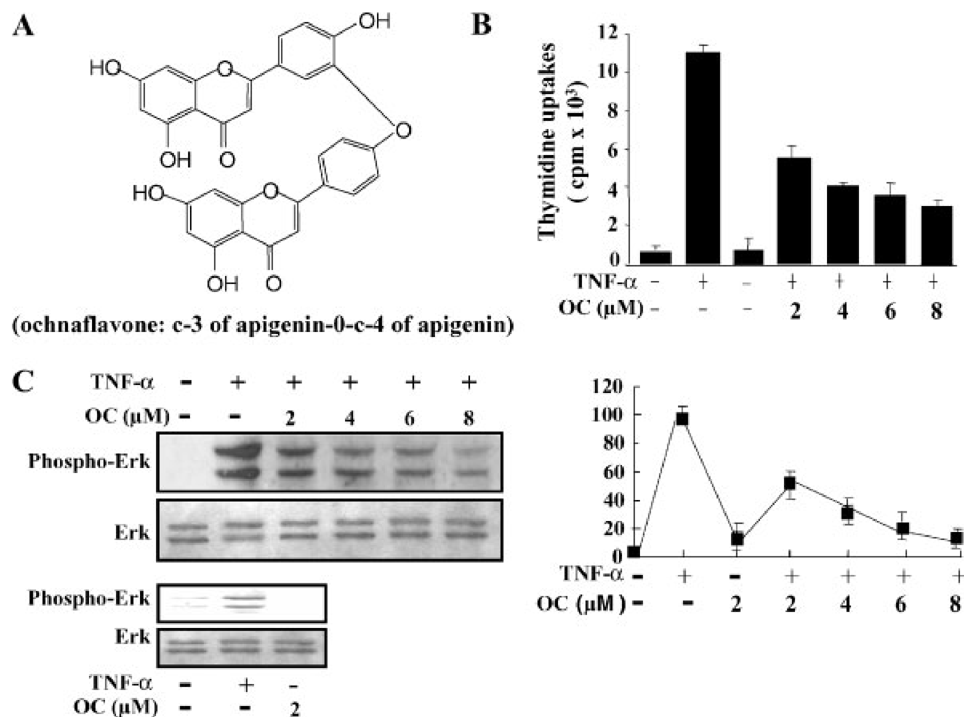


Fig. 1. Structure (**A**) and effects of OC on DNA synthesis and ERK1/2 activity. **B**: Measurement of DNA replication by thymidine uptake as a marker for cell proliferation. **C**: The phosphorylation or protein level of ERK1/2 was detected by immunoblot analysis using phspho-specific or specific for ERK1/2. The quantification of the phosphorylated ERK1/2 is shown in the lower panel, and indicated values are means of three triplicate experiments.

and made quiescent by serum starvation (0.1% FBS) for at least 24 h.

Cell Counts, [^{3}H]thymidine Incorporation, and Apoptosis Assays

For cell counts, HASMCs were plated overnight at equivalent densities (5×10^5 cells/plate) in 100-mm plates. At intervals after plating, cells were trypsinized, and cell numbers were determined using a Coulter Counter. For [^{3}H]thymidine-uptake experiments, cells were incubated for an additional 24 h and labeled with [methyl-^{3}H]thymidine (New England Nuclear, Boston, MA) at 1 μCi/ml during the last 18 h of this time period. Incorporated [^{3}H]thymidine was extracted in 0.2 M NaOH and measured in a liquid scintillation counter. Values were expressed as means from six wells from triplicate experiments.

Cell Cycle Analysis

Cells were harvested and fixed in 70% ethanol and stored at −20°C. Cells were then washed twice with ice-cold PBS and incubated with RNase and DNA intercalating dye propidium iodide, and cell cycle phase analysis was performed by flow cytometry using a Becton Dickinson Facstar flow cytometer and Becton Dickinson cell fit software.

Immunoprecipitation, MAPK Activity Assay, Western Blotting, Immune Complex Kinase Assays, and Zymography

Growth-arrested HASMC were treated with TNF-α in the presence or absence of OC for the specified time periods at 37°C. Cell lysates were prepared, and immunoprecipitation, ERK1/2 activity assay, Western blotting, and zymography were performed as described previously [Kim and Moon, 2005]. Immune complex kinase assay was also performed using glutathione *S*-transferase (GST)−pRb C-terminal (pRb amino acids 769−921) fusion protein (Santa Cruz Biotechnology) or histone H_1 (Life Technologies, Inc.), and 5 μCi of [γ-^{32}P]ATP (4500 Ci/mmol; ICN), as described [Kim and Moon]. Phosphorylated pRb and histone H_1 were visualized and quantified with a BAS 2000 bioimaging analyzer.

MMP-9 Promoter Activity

A 0.7 kb segment at the 5′-flanking region of the human *MMP-9* gene was amplified by PCR using specific primers from the human *MMP-9* gene (Accession No. D10051): 5′-ACATTTGCCCGAGCTCCTGAAG (forward/*Sac*I) and 5′-AGGGGCTGCCAGAAGCTTAT GGT (reverse/*Hind*III). The pGL2-Basic vector containing a polyadenylation signal upstream from the luciferase gene was used to construct expression vectors by subcloning PCR-amplified DNA of MMP-9 promoter into the *Sac*I/*Hind*III site of the pGL2-Basic vector. MMP-9 promoter plasmid was transfected into HASMC using the Lipofectamine reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. MMP-9 promoter luciferases were tested using the luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Firefly luciferase activities were standardized for β-galactosidase activity.

Nuclear Extracts and Electrophoretic Mobility Shift Assay

Nuclear extract was prepared essentially as described elsewhere [Yan et al., 2001]. Cultured cells were collected by centrifugation, washed, and suspended in a buffer containing 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF. After 15 min on ice, the cells were vortexed in the presence of 0.5% Nonidet NP-40. The nuclear pellet was then collected by centrifugation and extracted in a buffer containing 20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF for 15 min at 4°C. The nuclear extract (2 μg) was preincubated at 4°C for 30 min with a 100-fold excess of an unlabeled oligonucleotide spanning the −79 MMP-9 cis element of interest. The sequences were as follows: AP-1, CTGACCCCTGAGTCAGCACTT; NF-κB, CAGTGGAATTCCCCA GCC. After this time, the reaction mixture was incubated at 4°C for 20 min in a buffer (25 mM Hepes buffer pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl, and 2.5% glycerol) with 2 μg of poly dI/dC and 5 fmol (2×10^4 cpm) of a Klenow end-labeled (^{32}P-ATP) 30-mer oligonucleotide, which spans the DNA binding site in the MMP-9 promoter. The reaction mixture was electrophoresed at 4°C in a 6% polyacrylamide gel using a TBE (89 mM Tris, 89 mM boric acid, and 1 mM EDTA) running buffer. The gel was rinsed with water, dried, and exposed to X-ray film overnight.

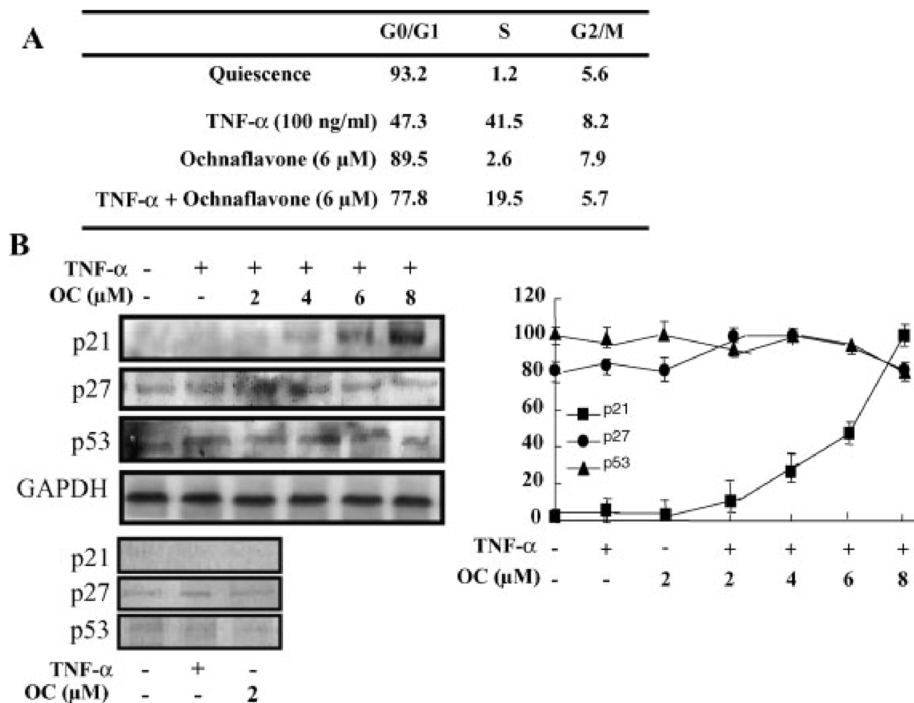
RESULTS

Structure of OC (c-3 of Apigenin-0-c-4 of Apigenin) and OC Inhibits HASMC Proliferation

We investigated the effect of OC on HASMC proliferation in response to a 100 ng/ml of TNF-α. TNF-α stimulated the proliferation of HASMC measured as DNA synthesis, which was significantly inhibited by OC in a dose-dependent manner. The OC inhibited thymidine incorporation into HASMC 49, 64, 75, and 80% of control at final concentrations of 2.0, 4.0, 6.0, and 8.0 μM, respectively (Fig. 1B). We then examined the effect of OC on the early signal-transduction pathway by TNF-α stimulation using phospho-ERK1/2-specific antibody. The cells were pretreated with OC for 40 min before the addition of TNF-α (100 ng/ml) for 10 min. TNF-α-induced ERK1/2 activation was inhibited by OC in a concentration-dependent manner (2.0–8.0 μM) (Fig. 1C).

OC Induced G1 Cell Cycle Arrest Is Mediated via an Induction in $p21^{waf1}$ and Consequent Inhibition in Cyclins and CDKs on HASMC in Response to TNF-α

Next, flow cytometric analysis was performed to determine whether the OC-induced cell growth inhibition is due to an arrest in a specific point of the cell cycle. OC markedly increased G_0/G_1-phase of the cell cycle (Fig. 2A). Because OC treatment of TNF-α-induced HASMC results in a G1-phase cell cycle arrest, we examined the effect of OC on cell cycle regulatory molecules operative in the G1-phase of the cell cycle. We assessed the effect of OC on the induction of $p21^{waf1}$, which is known to regulate the entry of cells at the G1 to S-phase transition checkpoint. Immunoblot analysis revealed that OC treatment of the TNF-α-induced HASMC resulted in a significant dose-dependent induction of $p21^{waf1}$ compared with the basal levels (Fig. 2B). However, under similar experimental conditions, the expression levels of another



A

	G0/G1	S	G2/M
Quiescence	93.2	1.2	5.6
TNF-α (100 ng/ml)	47.3	41.5	8.2
Ochnaflavone (6 μM)	89.5	2.6	7.9
TNF-α + Ochnaflavone (6 μM)	77.8	19.5	5.7

Fig. 2. G1 cell cycle arrest and $p21^{waf1}$ induction on treatment of OC in TNF-α-induced HASMC. **A**: Effect of OC on cell cycle distribution. HASMC were incubated in serum-free medium for 2 days to induce quiescence, followed by addition of TNF-α (100 ng/ml) or TNF-α plus 6 μM of OC, and cultured for 24 h, and FACS analysis. **B**: Effect of OC on $p21^{waf1}$, p27, and p53 expression. HASMC were stimulated with TNF-α in the presence or absence of indicated concentration of OC at 24 h, and then harvested. The cell lysates were immunobloted with antibodies specific for $p21^{waf1}$, p27, and p53. Indicated values are means of three triplicate experiments.

cyclin-dependent kinases inhibitor p27 protein and p53 tumor suppressor protein, were not changed, suggesting that p27 and p53 are unlikely to be involved in the cell cycle arrest induced by OC. Using immunoblot analysis, we analyzed the protein expressions of the cyclins and CDKs, which are known to regulate by $p21^{waf1}$, following treatment with OC. OC treatment of the HASMC at 24 h resulted in a dose-dependent decrease in protein expressions of cyclin D1 and cyclin E as well as CDK2 and CDK4 (Fig. 3A).

OC Treatment of TNF-α-Induced HASMC Results in a Decrease in the Kinase Activities Associated With CDKs

Kinase activities associated with the CDKs are the driving force for progression of the cell cycle through the transition checkpoints because they activate the cyclins, the essential component of cyclin-CDK complexes; therefore, we assessed the effect of OC treatment on the kinase activities associated with CDK2 and CDK4. CDK complexes were immunoprecipitated using specific anti-CDKs antibodies, and the levels of CDK-associated kinase activity were measured against Rb protein or histone H1 as substrate (Fig. 3B). Treatment of HASMC with TNF-α resulted in an increase of CDK2 and CDK4 activities. OC inhibited both CDK2 and CDK4 kinase activities in a dose-dependent manner (Fig. 3B).

OC Inhibits TNF-α Induced Induction of MMP-9 Enzyme Activity

To determine the efficacy of OC in blocking the regulation of MMM-9 expression by TNF-α, treatment of HASMC with TNF-α (100 ng/ml) were cultured in serum-free media in the absence or presence of varying concentrations of OC. After 24 h, conditioned medium and cell lysates were harvested and MMP-9 activity was determined by zymography (Fig. 4A,C). Media from control smooth muscle cells did not demonstrate proteolytic activity at 92 kDa, corresponding to MMP-9. In contrast, treatment with a 100 ng/ml of TNF-α induced the expression of a band of proteolytic MMP-9 activity. This induction of MMP-9 activity by TNF-α was inhibited in the presence of OC in a

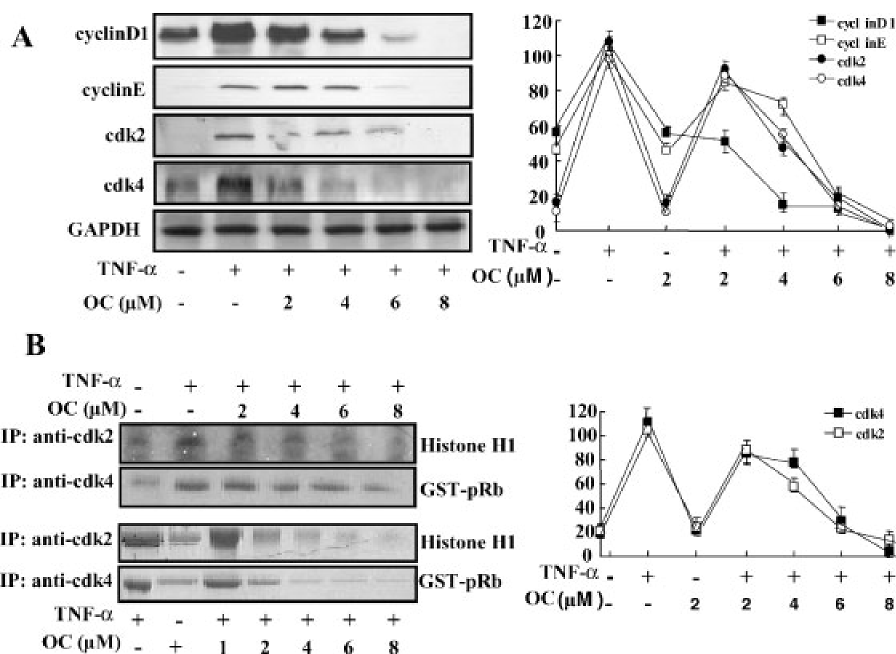


Fig. 3. Effect of OC on G1 cell cycle regulator cyclin D1, cyclin E, CDK2, and CDK4. **A**: HASMC were stimulated with TNF-α (100 ng/ml) in the presence or absence of indicated concentration of OC and Western blot analysis was performed with antibodies specific for cyclin D1, cyclin E, CDK2, and CDK4. Results from representative experiments were normalized to GAPDH expression by densitometry. **B**: HASMC were stimulated with TNF-α in the presence or absence of indicated concentration of OC at 24 h, and then harvested. Total cell lysates were then immunoprecipitated with anti-CDK2 and anti-CDK4 antibodies. The kinase reaction was performed using histone H1 (for CDK2) or GST-Rb (for CDK4) as substrate. Indicated values are means of three triplicate experiments.

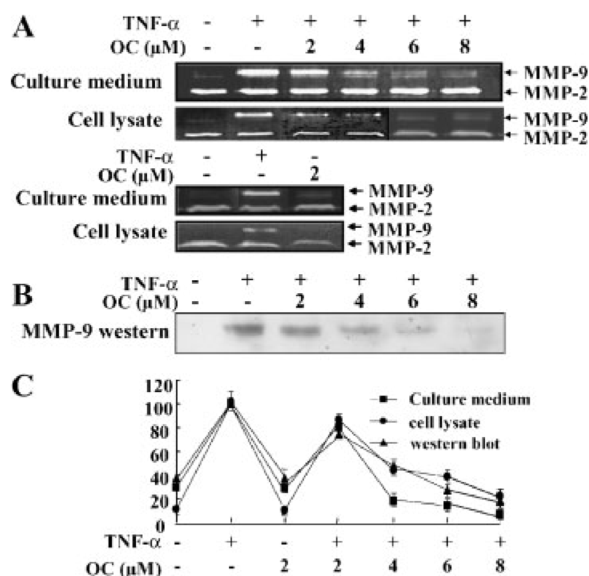


Fig. 4. Effect of OC on TNF-α-induced MMP-9 expression in HASMC. HASMC were grown to 70% confluence in DMEM supplemented with 10% FBS and the medium was changed to serum-free medium. The HASMC were stimulated with TNF-α (100 ng/ml) in the presence or absence of indicated concentration of OC and then culture supernatants and cell lysates (**A**) were analyzed zymographically for the MMP activities. **B**: Similarly, Western blot analysis using the cell lysates was performed with antibodies specific for MMP-9. **C**: Densitometric analysis of the MMP-9 activities. Indicated values are means of three triplicate experiments.

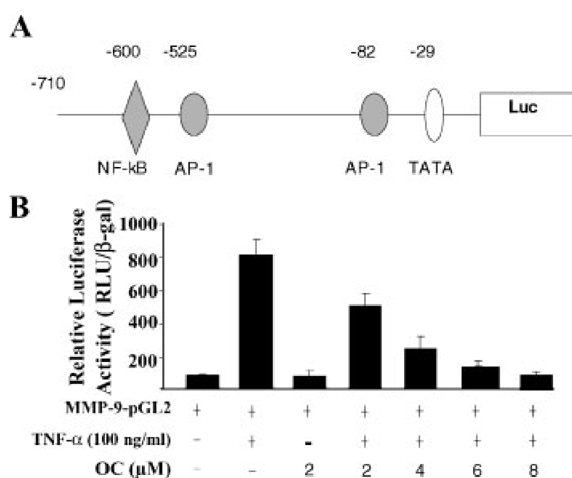


Fig. 5. OC inhibition of the TNF-α-induced MMP-9 promoter activity. **A**: Schematic map of the MMP-9 promoter showing the *cis*-regulatory elements. **B**: HASMC were transiently transfected with MMP-9-pGL2, which contains 710 bp of 5′-promoter of the *MMP-9* gene and then cultured with TNF-α (100 ng/ml) in the presence or absence of indicated concentration of OC. Luciferase activity was determined from cell lysates. Indicated values are means of three triplicate experiments.

dose-dependent manner. Similar results were found in immunoblot results (Fig. 4B). These data indicate that OC inhibits TNF-α-stimulated increase in MMP-9 activity. However, under similar experimental conditions, the expression level of MMP-2 was not significantly changed.

OC Inhibits Activation of the MMP-9 Promoter by TNF-α

Because the effects of OC were pronounced on MMP-9 expression in TNF-α-stimulated HASMC, we examined the MMP-9 promoter to see if OC regulates MMP-9 expression transcriptionally. HASMC were transiently transfected with a plasmid containing a luciferase reporter gene driven by a segment of 710 bp from the 5′-promoter region of *MMP-9* gene (Fig. 5A) and subsequently treated with TNF-α in the absence, or presence of OC. TNF-α strongly increased reporter activity, which was attributed to the MMP-9 promoter sequence (Fig. 5B). And also, this TNF-α-stimulated MMP-9 promoter activity was reduced to over 80% following OC treatment of HASMC, suggesting that the repressive effect of OC is due, at least in part, to reduced transcription of the *MMP-9* gene (Fig. 5B).

OC Inhibits the MMP-9 Promoter by Decreasing the NF-κB Binding Activity

The reporter assay experiments have shown that the response elements for TNF-α stimulation are located within −710 bp upstream of the transcription start site. To examine if TNF-α-induced MMP-9 expression was associated with an increase in the quantity of NF-κB and AP-1 in the nucleus, EMSA was performed with the nuclear extracts of HASMC treated with TNF-α (100 ng/ml). Nuclear extracts were incubated with a radiolabeled double-stranded oligonucleotide probe with the consensus sequence for NF-κB and AP-1 binding site, respectively. An oligonucleotide derived from the MMP-9 promoter sequence spanning this motif was bound specifically with NFs derived from TNF-α-stimulated HASMC (Fig. 6A,B). Nuclear extract from HASMC treated with TNF-α showed increased binding to the NF-κB and AP-1 motifs (Fig. 6A,B). Next, we examined whether the repressive effect of OC on MMP-9 expression was mediated through these two kinds of motifs. OC effectively suppressed the increased NF-κB

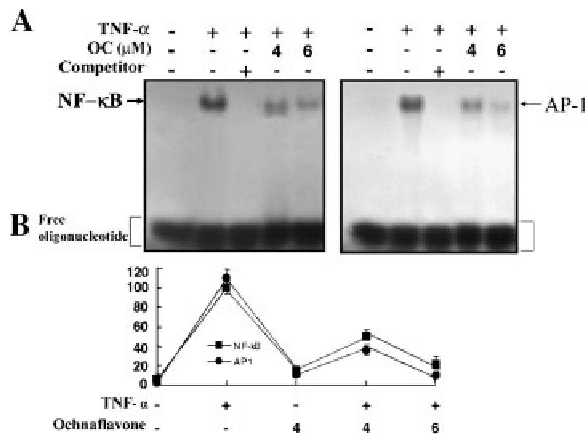


Fig. 6. Effect of OC on the TNF-α-induced DNA binding activities of MMP-9, NF-κB, and AP-1 motif in HASMC. Cells were pretreated with indicated OC for 40 min in serum-free medium, were incubated with TNF-α (100 ng/ml) for 24 h. Nuclear extracts were analyzed by EMSA for the activated NF-κB (**A**) and AP-1 (**B**) using radiolabeled oligonucleotide probes, respectively. Indicated values are means of three triplicate experiments.

and AP-1 binding activities (Fig. 6A,B). These data may suggest that OC blocks MMP-9 expression at least in part by decreasing DNA binding of transcription factors NF-κB and AP-1.

DISCUSSION

Our aim was to investigate the mechanism of anti-atherogenic response of OC against treatment of HASMC with TNF-α because TNF-α is known to function as an autocrine and paracrine mediator in the pathogenesis of vascular lesions, such as atherosclerosis [Jovinge et al., 1996]. The major findings of the present study are as follows: (1) TNF-α-induced DNA synthesis and ERK1/2 activation on HASMC is inhibited by OC in a concentration-dependent manner (1.0–8.0 μM), (2) OC inhibits cyclin D1/CDK4 and cyclin E/CDK2 activities by the increased expression of p21 on HASMC following TNF-α, (3) OC inhibits the production of pro-MMP-9 in a dose-dependent manner and the activation of MMP-9 promoter on treatment of HASMC with TNF-α, (4) OC represses TNF-α-induced MMP-9 promoter activity by reducing NF-κB and AP-1 binding activity. These findings are, for the first time, show that OC inhibits TNF-α-induced HASMC proliferation through the inhibition of ERK1/2 activity and cell cycle dysregulation. We also demonstrate, for the first time, that OC inhibits MMP-9 expression on TNF-α-stimulated HASMC and the MMP-9 inhibition can be ascribed to the repression of MMP-9 promoter activity by decreased NF-κB and AP-1 binding activities.

OC treatment induced a rapid growth arrest in TNF-α-stimulated HASMC at 24 h. The result of thymidine uptakes as an index of DNA synthesis in VSMC after OC treatment indicated that there is a cessation of DNA synthesis. Our studies have demonstrated that OC treatment of HASMC results in a G1-phase arrest of the cell cycle (Fig. 2A), and therefore, we further examined the effect of OC on cell cycle regulatory molecules operative in the G1-phase of cell cycle. $p21^{waf1}$ protein was increased in response to TNF-α (Fig. 2B). Our data demonstrate a significant upregulation of the $p21^{waf}$, CKI, during G1-phase arrest of HASMC by OC. Many studies have shown that regulation of G1 cell cycle arrest has been attributed to a number of cellular proteins, including p53 and other CDK inhibitor, p27 [Toyoshima and Hunter, 1994; Macleod et al., 1995]. But, OC had no effect on p53 or p27 protein levels in HASMC, suggesting that OC-induced accumulation of $p21^{waf1}$ could also be responsible for G1-phase arrest. Because $p21^{waf}$ is regarded as a universal inhibitor of cyclin-CDK complexes [Xiong et al., 1993], we assessed the effect of OC treatment on the cyclins and CDKs operative in the G1-phase of the cell cycle, specifically cyclin D1, cyclin E, CDK2, and CDK4. Our experiment indicated that OC treatment of HASMC results in significant downmodulation of all these regulatory molecules, although to a different extent. OC treatment results in a dose-dependent inhibition in the kinase activities associated with the CDKs examined. We suggest the series of events by which OC results in an imposition of an artificial checkpoint at G_1 to S-phase transition, thereby resulting in an arrest of VSMC in the G_1-phase of the cell cycle.

Recently, it was reported that the MAPK kinase superfamily plays a crucial role in cell growth, differentiation, or even programmed cell death in response to diverse extracellular stimuli in eukaryotic cells [Nishida and Gotoh, 1993]. We then examined the effect of OC on the early signal-transduction pathway (ERK1/2) by TNF-α stimulation. In consistent with cell proliferation study (Fig. 1B,C), OC treatment inhibited ERK signaling pathway in

TNF-α-stimulated VSMC. These results suggest that OC may have an anti-proliferative effect on VSMC through the inhibition of TNF-α-induced ERK1/2 activation.

VSMC proliferation and migration plays an important role in the pathogenesis of atherosclerosis and restenosis after vascular injury [Ross, 1995]. VSMC migration requires breakdown of extracellular matrix [Mason et al., 1999]. One possible mechanism by which VSMC breakdown extracellular matrix is secreted MMPs [Dollery et al., 1995]. In the past several years, a number of studies have demonstrated that MMPs, specifically MMP-2 and MMP-9, are important for smooth muscle cell proliferation and migration into the intima [Newby and Zaltsman, 2000]. Of considerable interest in this study was the marked decrease in the secretion of the MMP-9 activity from TNF-α-stimulated VSMC in response to OC. Also, the study herein clearly revealed that the ability of OC to reduce MMP-9 expression in VSMC is achieved via reduced NF-κB and AP-1 binding as well as diminished trans-activation of the MMP-9 promoter. OC anti-atherogenic effects seem to be mediated through transcriptional downregulation of MMP-9, this effector molecule implicated in regulating the progression of plaque rupture [Newby and Zaltsman, 2000]. In fact, our study with the MMP-9 promoter revealed that OC regulates this transcription at NF-κB site and AP-1 site. These findings suggest that OC may have an anti-atherogenic effect on VSMC through the inhibition of MMP-9 expression, which has been linked to progression of plaque rupture and intimal formation in arterial lesions. Excessive proinflammatory cytokine and MMP-9 production through NF-κB and AP-1 activation play an important role in atherosclerosis, and the inhibition of proinflammatory cytokines and *MMP-9* gene expression in HASMC would be beneficial to suppress excessive inflammatory reaction. We have found that OC inhibits this transcription at NF-κB site and AP-1 site in cells treated with TNF-α.

In conclusion, we showed that OC induces TNF-α-stimulated HASMC proliferation through several mechanisms; by inhibiting ERK activation, by arresting G1-phase, by reducing cyclin D1/CDK4 and cyclin E/CDK2 activities through $p21^{waf1}$ expression. Furthermore, we demonstrated that OC potently inhibits NF-κB and AP-1 binding activities in repressing TNF-α-induced MMP-9 expression. The findings of the present study may provide a potential mechanism that explains the anti-atherogenic activity of OC.

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