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ARTICLE

Polyphenol-Rich Extract from Mulberry Leaf Inhibits Vascular Smooth Muscle Cell Proliferation Involving Upregulation of p53 and Inhibition of Cyclin-Dependent Kinase

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This study was carried out to investigate the impact of polyphenol-rich extract from mulberry leaf on the proliferation of vascular smooth muscle cell (VSMC) and verify its mechanism *in vitro*. VSMC proliferation is an important pathophysiological process in the development of atherosclerosis, which is the major cause of coronary artery disease (CAD). Polyphenol-rich foods, such as mulberry leaf, have been reported to reduce the risk of CAD. The effect of mulberry leaf extract (MLE) on cell growth was measured by a growth curve assay, on distribution of cells in the cell cycle by flow cytometry, and on cyclin-dependent kinase (CDK) activity and cell-cycle regulatory proteins by Western blot, immunoblotting, and immunoprecipitation analyses. The results showed that MLE induced phosphorylation of p53, promoted expression of p21 and p27, decreased CDK2/4 activity, inhibited phosphorylation of Rb, and thereby blocked the G1 to S transition in the cell cycle.

KEYWORDS: Mulberry leaf extract; vascular smooth muscle cell proliferation; cell cycle; atherosclerosis

INTRODUCTION

Atherosclerosis is a chronic inflammatory process that involves many soluble mediators, monocytes, endothelial cells, and smooth muscle cells. Plaques with a thin fibrous cap and a large core of lipids and inflammatory cells have a high risk of rupture, leading to acute coronary syndrome. This risk does not appear to be dependent upon the size of the plaque. The stimuli that induce fibrous cap formation probably act by inducing smooth muscle proliferation. Cell migration and proliferation will ensue, together with collagen and proteoglycans, synthesis (*1*). The proliferation of vascular smooth muscle cells (VSMCs) plays an important pathophysiological role in the development and progression of atherosclerosis and restenosis after percutaneous coronary intervention (PCI) (*2*−*4*). VSMCs normally remain in a quiescent and contractile condition, but upon vascular injury, they transform into a more synthetic phenotype and activate, proliferate, and migrate to the intimal layer of the artery wall. Intimal hyperplasia of VSMC also occurs in restenosis after PCI and is controlled by growth factors. The subsequent migration and continued proliferation depend upon stimulation by the platelet-derived growth factor (PDGF), which is released from adherent, activated platelets on the vascular surface, from infiltrating monocytes, and at certain stages, also from vascular endothelial and smooth muscle cells themselves (*5*−*9*). Therefore, drugs and biochemical components of healthy foods capable of inhibiting VSMC proliferation will contribute to the prevention of atherosclerosis and restenosis after PCI.

Dietary mulberry and mulberry leaf have been reported to have anti-diabetic (*10*, *11*), anti-hyperlipidemic (*12*) and antioxidative effects (*13*−*16*). Polyphenols were reported to have many biochemical activities, including inhibition of VSMC proliferation and migration (*17*−*20*). Our previous study showed that mulberry leaf extract (MLE) was rich in polyphenols and effectively inhibited VSMC migration by suppressing small GTPase and Akt/NF-κB signals (*21*). Mulberry (*Morus alba* L.) leaves are rich in many nutritional components, and nine flavonoids were isolated from leaves of *M. alba* thus far. Most of them are quercetin and its derivatives (*22*). Flavonoids, with variable phenolic structures, are found in tea, wine, grains, fruit, vegetables, bark, flowers, stems, and roots (*23*). There are four main groups of flavonoids, including flavones, flavanones, catechins, and anthocyanins. Some *in vitro* studies revealed that flavonoids had anti-inflammatory, antitumor, antithrombogenic, antiallergic, antiosteoporotic, and antiviral properties. However, the best-described characteristic of flavonoids is their antioxidative effect, thus to prevent atherosclerosis (*24*). Previous studies suggest a protective role of dietary flavonoids against coronary artery disease (CAD) and a reduction of mortality (*25*, *26*). However, the impact of flavonoids on VSMC was still not clear. In the

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current study, we investigated the impact of polyphenol-rich MLE on VSMC proliferation and attempted to verify its mechanism *in vitro*.

MATERIALS AND METHODS

Cell Culture. Cell line A7r5, a rat thoracic aorta smooth muscle cell line, was obtained from the American Type Culture Collection (ATCC) (ATCC number CRL-1444, Manassas, VA). A7r5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 1% penicillin−streptomycin, and 1.5 g/L sodium bicarbonate (all from Gibco/BRL, Gaithersburg, MD). All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Before treatment, the cells were precultured in 0.5% FBS medium for 48 h.

Aqueous Extraction from Mulberry Leaves. Fresh mulberry leaves (100 g) were harvested, dried at 50 °C, and heated in 1500 mL of deionized water. After filtration, we removed the residue. The suspension was stored at −80 °C overnight and then freeze-dried (Labconco Corp., Kansas City, MO) to a powder, which was the aqueous fraction of MLE. The components of MLE were determined by high-performance liquid chromatography (HPLC) analysis using a Hewlett-Packard Vectra 436/33N system with a diode array detector. The HPLC method employed a 5 μm RP-18 column (4.6 × 150 mm inner diameter). The MLE was filtered through a 0.22 μm filter disk, and then 25 mg/mL MLE was injected into the column. Chromatography was monitored at 280 nm, and UV spectra were collected to confirm peak purity. The mobile phase contained two solvents: A, 2% acetic acid/water; B, 0.5% acetic acid in water/acetonitrile. The total phenolic compound content in each extract was spectrophotometrically determined in accordance with the Folin−Ciocalteu procedure by reading the absorbance at 725 nm against a methanol blank. Briefly, samples (20 μL, add water to 1.6 mL) were introduced into test tubes, and then 100 μL of Folin−Ciocalteu reagent and 300 μL of sodium carbonate (20%) were added. The contents of the tubes were mixed and incubated at 40 °C for 40 min. Absorption at 725 nm was measured. The total phenolic contents were expressed as milligrams per gram of MLE for gallic acid (GA), quercetin, rutin, caffeic acid (CA), and gallocatechin gallate (GCG). Briefly, in accordance with our previous study (*21*), the yield of MLE was 38.63% and the major components of MLE were polyphenols (44.82%, quercetin as the standard; 23.60%, gallic acid as the standard), polysaccharide (27.73%), protein (2.33%), and lipid (8.4%). The polyphenol and phenolic acids separated from MLE included gallic acid (7.64%), caffeic acid (1.02%), protocatechuic acid (4.69%), catechin (1.2%), gallocatechin gallate (5.88%), epicatechin (0.8%), rutin (1.87%), quercetin (1.24%), and naringenin (2.67%).

Growth Curve. A7r5 cells were seeded into 6-well culture plates at a density of 5×10^5 cells/well. After reaching about 80% confluence, the cells were transferred to 0.5% FBS medium for 48 h. Then, MLE (0.5, 1.0, 1.5, and 2.0 mg/mL) was added to the culture medium. The cell numbers were counted using a cell-counting plate each day for 7 days. On the basis of the mean number of cells in these wells, growth curves were generated. Results are representative of at least three independent experiments.

Cell-Cycle Analysis (Flow Cytometric Analysis). Cells synchronized at G0 phase by serum starvation for 24 h were incubated in fresh serum-containing medium to allow for cell-cycle progression. At various time points after release from G0 arrest, cells were analyzed by flow cytometry to determine cell-cycle distribution. Cells were seeded in 10 cm dishes at a density of 10^7 cells/dish and cultured in normal or high glucose DMEM. Cells cultured in high glucose were treated with MLE at various concentrations (0.5, 1.0, 1.5, and 2.0 mg/mL) at 37 °C for 48 h. At the end of treatment, they were collected, fixed in 1 mL of ice-cold 70% ethanol, incubated at −20 °C for at least 24 h, and centrifuged at 380*g* for 5 min at room temperature. Cell pellets were treated with 1 mL of cold staining solution containing 20 μg/mL propidium iodide (PI), 20 μg/mL RNase A, and 1% Triton X-100 and incubated for 15 min in darkness at room temperature. Subsequently, the samples were analyzed in a FACSCalibur system (version 2.0, BD Biosciences, Franklin Lakes, NJ) using CellQuest software. Results are representative of at least three independent experiments.

Western Blot Analysis. Specific antibodies were used to evaluate the expression of p27 (F-8), p21 (F-5) (all from Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma, St. Louis, MO). After MLE treatment, equal amounts of cell lysate (50 μg of protein) were separated by electrophoresis on 8−12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred to nitrocellulose membranes (Millipore, Bedford, MA). The membranes were incubated with Tris-buffered saline (TBS) containing 1% (w/v) nonfat milk and 0.1% (v/v) Tween-20 (TBST) for 1 h to block non-specific binding, washed with TBST for 30 min, incubated with the appropriate primary antibody for 2 h, incubated with horseradish-peroxidase-conjugated second antibody (Sigma, St. Louis, MO) for 1 h, and developed by enhanced chemiluminescence (ECL reagent, Millipore, Bedford, MA). Protein was measured by densitometry using AlphaImager Series 2200 software (Alpha Innotech, San Leandro, CA). Results are representative of at least three independent experiments.

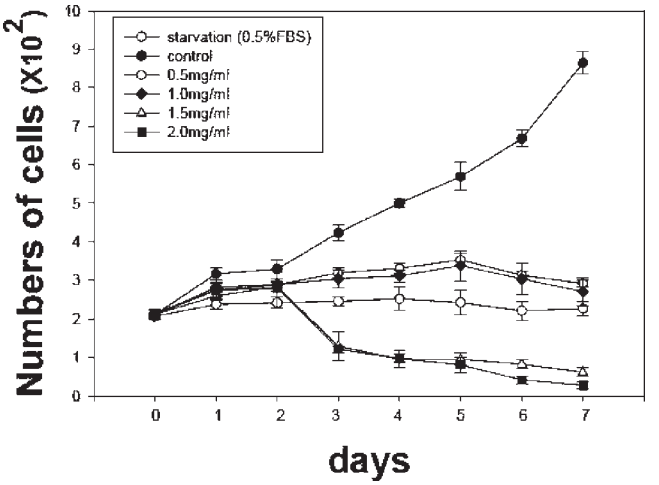

Figure 1. Impact of MLE on the growth of A7r5 cells. A7r5 cells were incubated with DMEM and treated with 0.5, 1.0, 1.5, and 2.0 mg/mL MLE. Each 6-well plate was seeded with 5×10^5 cells. The cell numbers were counted every day. A7r5 cells were photographed at a magnification of about 40×. Growth curves of 7 days were constructed. Data are reported as means ± SD of three independent experiments.

Immunoblotting and Immunoprecipitation Analysis. After treatment with MLE, the A7r5 cells were lysed. For immunoprecipitation, 500 μg of cell lysate protein was precleared with protein-A-conjugated Sepharose beads (Amersham Bioscience, Piscataway, NJ) and immunoprecipitated using monoclonal anti-CDK4 (DCS-35), anti-CDK2 (D-12), anti-E2F (H-137) (all from Santa Cruz Biotechnology, Santa Cruz, CA), and anti-p53 (BD Biosciences, Franklin Lakes, NJ) antibodies. Immune complexes were harvested with protein-A-conjugated Sepharose beads. Immunoprecipitated proteins were analyzed by SDS−PAGE and immunoblotting with antibodies against cyclin D1 (HD-11), cyclin A (H-432), cyclin E (M-20) (all from Santa Cruz Biotechnology, Santa Cruz, CA), phospho-retinoblastoma (Rb) protein (serine 807/811) (Cell Signaling Technology, Beverly, MA), Rb (BD Biosciences, Franklin Lakes, NJ), and Mdm2 (Sigma, St. Louis, MO). Results are representative of at least three independent experiments.

Statistical Analysis. Results are reported as means ± standard deviation (SD) of three independent experiments, and statistical comparisons were evaluated by one-way analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

MLE Inhibited VSMC Proliferation. Previous studies have shown that proliferation and migration of VSMC contributed to the development of atherosclerosis. To evaluate the effects of MLE on the proliferation of VSMCs, a growth curve assay was performed. As shown in **Figure 1**, we treated A7r5 cells with 0.5, 1.0, 1.5, and 2.0 mg/mL MLE for 7 days. After 3 days of treatment, MLE had a significant inhibitory effect on VSMC growth in a dose-dependent manner following 24 and 48 h of incubation with 0.5−2.0 mg/mL MLE. This result indicated that MLE effectively inhibited the growth of VSMC.

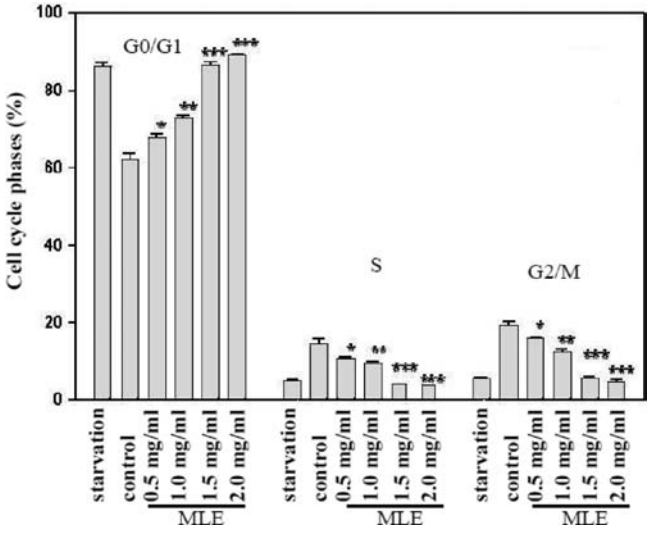

Figure 2. Impact of MLE on cell-cycle arrest. A7r5 cells were treated with 0.5−2.0 mg/mL MLE for 24 h. The change in cell-cycle phase distribution was analyzed by flow cytometry. Data are reported as means ± SD of three independent experiments. (*) $p < 0.05$, (**) $p < 0.005$, and (***) $p < 0.001$ compared to the control group.

MLE Inhibited VSMC Proliferation by Causing Cell-Cycle Arrest at G0/G1 Phase. To examine the relationship between MLE-induced inhibition of VSMC proliferation and cell cycle, flow cytometric analysis was performed to determine the fraction of A7r5 cells (treated with 0.5−2.0 mg/mL MLE for 24 h) in each phase of the cell cycle. As shown in **Figure 2**, MLE dose-dependently induced apparent accumulation of cells at G0/G1 phase (6−28%), while cell numbers at S and G2/M phases were significantly decreased (4−12 and 4−15%, respectively). These results indicated that MLE treatment induced cell-cycle arrest at G0/G1 phase, thereby inhibiting VSMC proliferation.

MLE Inhibited Phosphorylation of Rb Protein by Inducing Phosphorylation of p53 and Promoting the Expression of CDK Inhibitors. To identify the pathway involved in the MLE-induced cell-cycle arrest in A7r5 cells, a series of studies were performed in which the expression of CDKs and cyclins was measured upon MLE treatment. As shown in **Figure 3A**, the protein levels of cyclin D1 and CDK4 but not cyclin D3 were increased significantly (up to 1.8- and 1.3-fold as compared to the control group, respectively) in a dose-dependent manner after treatment with MLE (0.5−2.0 mg/mL for 24 h) and increased significantly (up to 1.35- and 1.75-fold as compared to the control group, respectively) in a time-dependent manner after treatment with MLE (1.5 mg/mL) (data shown in the Supporting Information). Nevertheless, the amount of immunoprecipitated cyclin D1/CDK4 complex was decreased significantly (up to 0.49- and 0.91-fold as compared to the control group, respectively) in a dose-dependent manner (**Figure 3B**). These results indicated that the action of MLE effectively inhibits CDK4 activity by decreasing the amount of cyclin D1/CDK4 complex formation but not the levels of cyclin D1 and CDK4 proteins. The protein levels of cyclin A, cyclin E, and CDK2 were decreased significantly (up to 0.53-, 0.49-, and 0.36-fold as compared to the control group, respectively) in a dose-dependent manner after treatment with MLE (0.5−2.0 mg/mL for 24 h; **Figure 4A**) and in a time-dependent manner (up to 0.9-, 0.5-, and 0.74-fold as compared to the control group, respectively) after treatment with MLE (1.5 mg/mL) (data shown in the Supporting Information). Moreover, the amounts of cyclin A/CDK2 and cyclin E/CKD2 complexes were significantly decreased (up to 0.5- and 0.48-fold as compared to the control group, respectively)

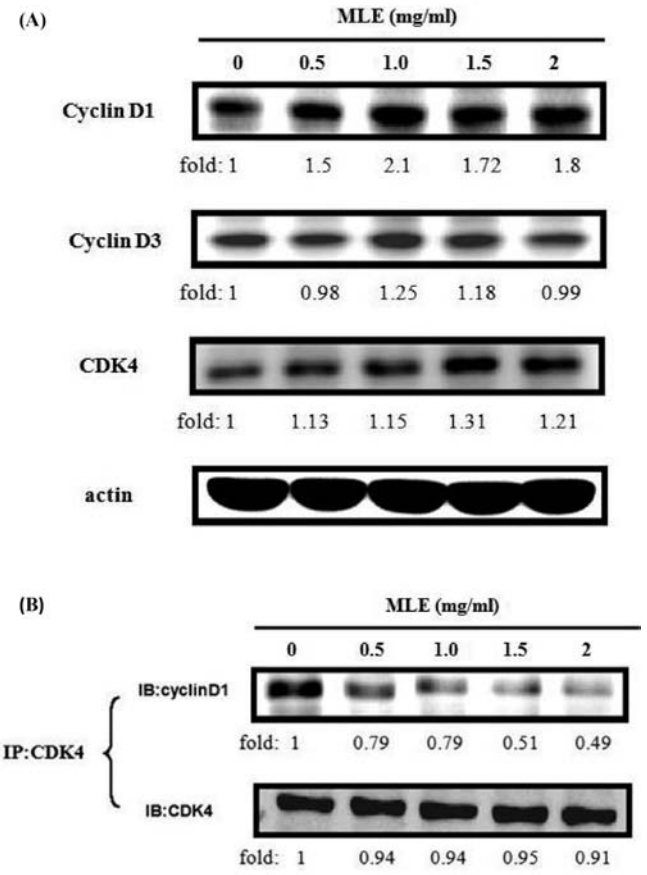

Figure 3. Impact of MLE on cyclin D, CDK4, and cyclin D/CDK4 complex. (A) Protein levels of cyclin D1, cyclin D3, and CDK4 were assessed by a Western blot assay after treatment with MLE (0.5−2.0 mg/mL) for 24 h. (B) Cell extracts [prepared from A7r5 cells following treatment with different concentrations of MLE (0.5−2.0 mg/mL)] were immunoprecipitated with anti-CDK4. The presence of cyclin D1 in the immunoprecipitates was examined by immunoblotting with cyclin D1 antibody. Results from three replicate experiments were similar.

in a dose-dependent manner (**Figure 4B**). Therefore, MLE effectively inhibited CDK2 activity by either decreasing the formation of cyclin A/CDK2 and cyclin E/CDK2 complexes, decreasing the protein levels of cyclin A, cyclin E, and CDK2, or both.

As shown in **Figure 5A**, following treatment with 0.5−2 mg/mL MLE, immunoblotting and immunoprecipitation analyses revealed that the phosphorylation of Rb and the expression of E2F were decreased (up to 0.5-fold as compared to the control group) in a dose-dependent manner upon MLE treatment, while Rb−E2F complex formation was apparently increased (up to 1.42-fold as compared to the control group) (**Figure 5B**). These results indicate that MLE inhibits the phosphorylation of Rb and release of E2F, thereby blocking the G1 to S transition. The phosphorylation of p53 was increased significantly (up to 2.5-fold as compared to the control group) in a dose-dependent manner upon MLE treatment (**Figure 6A**) and in a time-dependent manner after 1.5 mg/mL MLE treatment (up to 2.3-fold as compared to the control group) (data shown in the Supporting Information). MLE treatment decreased p53/Mdm2 complex formation (up to 0.75-fold as compared to the control group) in a dose-dependent manner (**Figure 6B**). These results indicated that MLE treatment induces phosphorylation of p53 in A7r5 cells.

Upregulation of p21 and p27 was known to be involved in cell-cycle arrest. Thus, by Western blotting, we examined the effect of MLE on p21 and p27 protein levels. MLE treatment markedly

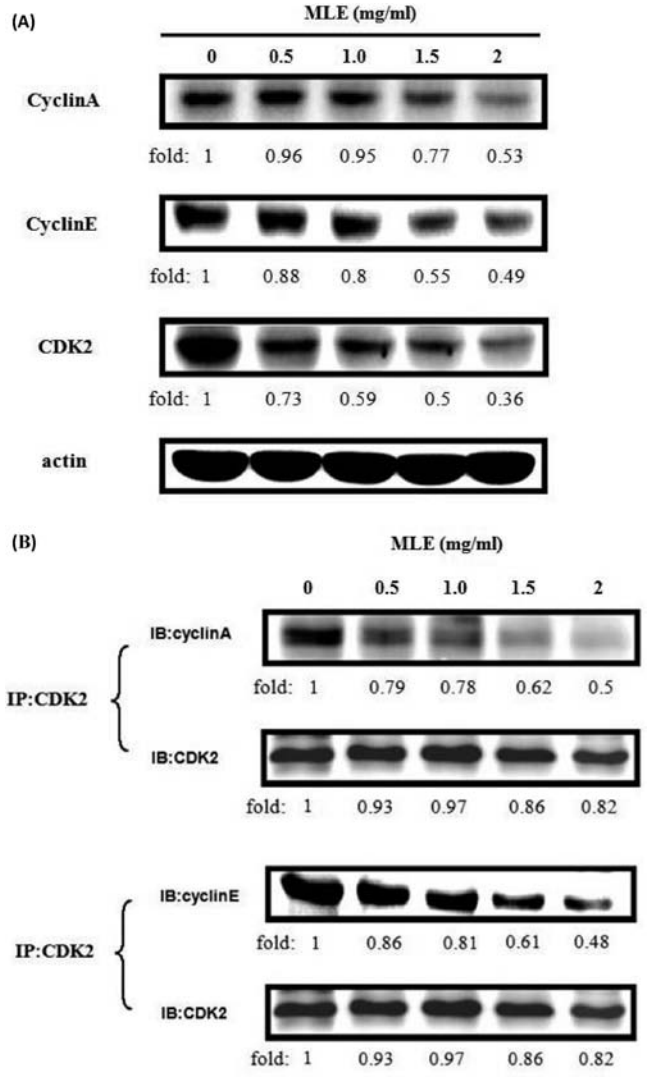

Figure 4. Impact of MLE on CDK2, cyclin A, cyclin E, cyclin A/CDK2, and cyclin E/CDK2 complexes. (A) Protein levels of cyclin A, cyclin E, and CDK2 were assessed by a Western blot assay after treatment with MLE (0.5−2.0 mg/mL) for 24 h. (B) Cell extracts [prepared from A7r5 cells following treatment with MLE (0.5−2.0 mg/mL)] were immunoprecipitated with anti-CDK2. The presence of cyclin A and cyclin E in the immunoprecipitates were examined by immunoblotting with cyclin A and cyclin E antibodies. Results from three replicate experiments were similar.

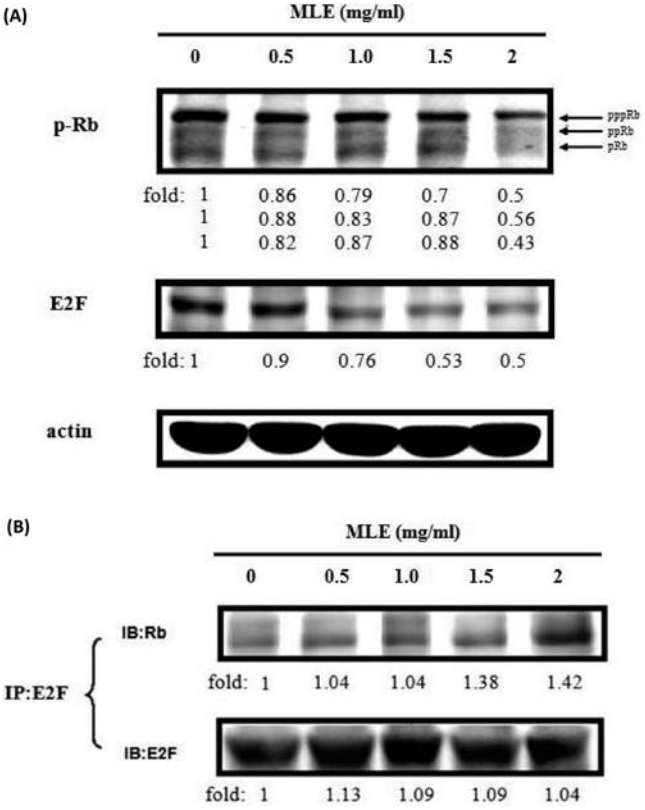

Figure 5. Impact of MLE on Rb phosphorylation. (A) Phosphorylation of Rb and expression of E2F were assessed by Western blot analysis after treatment with MLE (0.5−2.0 mg/mL) for 24 h. (B) Cell extracts following treatment with MLE (0.5−2.0 mg/mL) were immunoprecipitated with E2F. The precipitated complexes were analyzed by immunoblotting using Rb antibody. The expression of Rb was assessed after treatment with MLE (0.5−2.0 mg/mL) for 24 h. Results from three replicate experiments were similar.

increased the expression of p21 and p27 in a dose-dependent manner (up to 2.5-fold as compared to the control group) (**Figure 7A**) and in a time-dependent manner (up to 1.52- and 2.37-fold as compared to the control group, respectively) (**Figure 7B**). These results indicated that the activities of the CDK inhibitors (p21 and p27) are inhibited by MLE treatment in a dose- and time-dependent manner.

Cyclin-dependent kinases (CDK2, CDK4/6, and Cdc2), working in conjunction with their activating subunits (cyclins A, B, D, and E), provide the driving force for cell-cycle transitions (*27*, *28*). Upon vascular injury, VSMCs begin to divide in response to mitogens, exit the G1 phase, and then enter the S phase. Cyclin D1/CDK4 and cyclin E/CDK2 act predominantly and are required during the G1/S transition (*29*). The kinase activity of these cyclin/CDK complexes are regulated by cyclin-dependent kinase inhibitors, including Ink4 proteins (p16, p15, p18, and p19) and Cip/Kip proteins (p21, p27, and p57) (*30*). Retinoblastoma (Rb) protein, the product of the retinoblastoma tumor suppressor gene, is the main negative regulator of cell-cycle progression, and hyperphosphorylation of the Rb protein is a hallmark of the G1 to S transition. Unphosphorylated Rb protein can bind to the E2F transcription factor, and phosphorylation of pRb will release E2F, allowing the cell to enter S phase. Cyclin E−CDK2 and cyclin D−CDK4/CDK6 complexes participate in the phosphorylation of pRb (*31*, *32*). Acting through its effector (p21), the gatekeeper of the mammalian cell cycle, p53, plays an important role in controlling G0/G1 arrest of the cell cycle (*27*). Mdm2 (the product of an oncogene) is transcriptionally activated by p53. Mdm2 also inactivates p53-mediated transcription and, therefore, forms a p53−Mdm2 autoregulatory loop (*28*). Besides, p53 has been reported to mediate density-dependent growth arrest, and p16, p21, and p27 have been implicated in density-dependent inhibition (*33*).

Endothelial dysfunction (induced by hyperglycemia, dyslipidemia, smoking, hypertension, and free radicals) is the initial step of atherosclerosis. MLEs were reported to improve endothelial function by inhibiting TNF (tumor necrosis factor)-α-induced nuclear factor κB (NF-κB) activation and lectin-like oxidized LDL receptor-1 (LOX-1) expression in vascular endothelial cells (*34*). In addition, mulberry leaf was also reported to improve metabolic syndrome by increasing the expression of adiponectin and decreasing the expression of TNF-α, MCP-1, macrophage markers, and NADPH oxidase (*35*).

Polyphenols have been reported to inhibit proliferation and migration of VSMCs (*17*−*20*). Our previous study revealed that

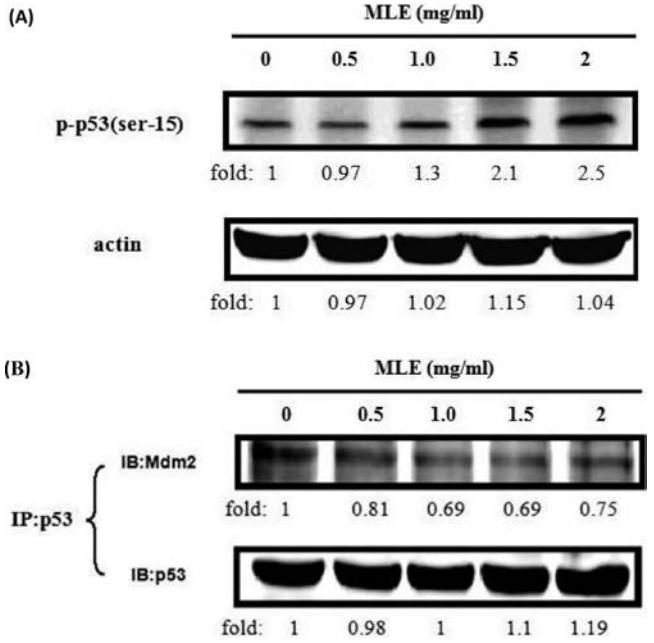

Figure 6. Impact of MLE on the expression of p53. (A) Cultured cells were treated with MLE (0.5−2.0 mg/mL) for 24 h. The protein level of p-p53 was analyzed by Western blotting. (B) Cell extracts following treatment with MLE (0.5−2.0 mg/mL) were immunoprecipitated with p53. The precipitated complexes were examined by immunoblotting using Mdm2 antibody. Results from three replicate experiments were similar.

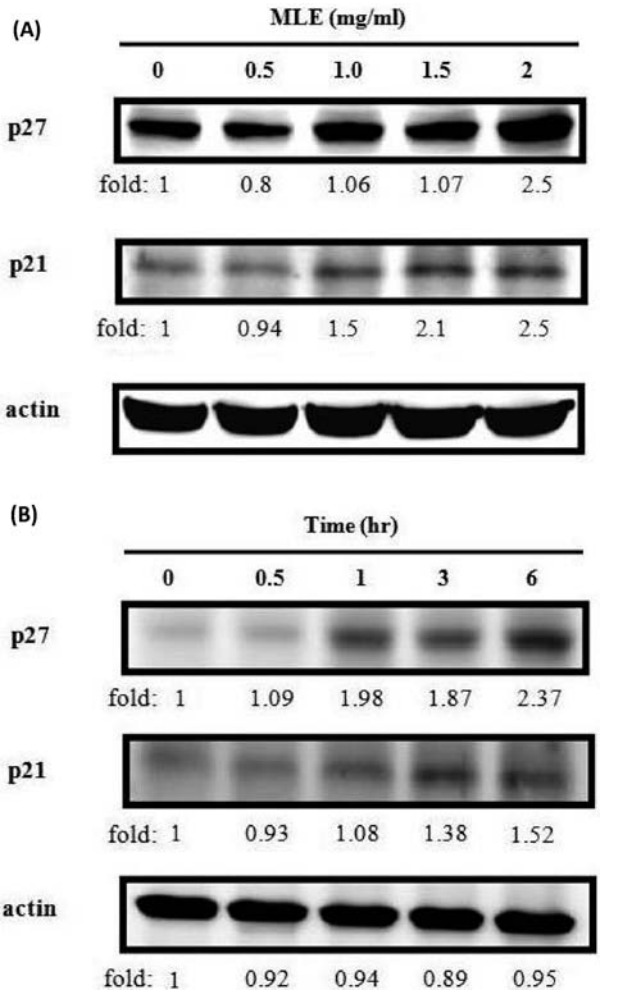

Figure 7. Impact of MLE on the expression of CKI proteins. (A) Cultured cells were treated with MLE (0.5−2.0 mg/mL) for 24 h. The protein levels of p21 and p27 were analyzed by Western blotting. (B) Cell lysates were prepared at the times indicated following treatment with MLE (1.5 mg/mL). The protein levels of p21 and p27 were analyzed by Western blotting. Results from three replicate experiments were similar.

MLE effectively inhibits VSMCs migration by blocking small GTPase and Akt/NF-κB signals, and this effect was due to its polyphenol component (*21*). However, the mechanism by which polyphenols inhibit VSMC proliferation is still unknown. The present study revealed that MLE can effectively inhibit VSMC proliferation. Treatment with MLE induced a G0/G1 phase growth arrest by inducing phosphorylation of p53, increasing expression of the CDK inhibitory proteins (p21 and p27), decreasing CDK2/4 activities, and thereby inhibiting phosphorylation of Rb.

Our study revealed that MLE was rich in phenolic acids and flavonoids, which include gallic acid, caffeic acid, protocatechuic acid, catechin, gallocatechin gallate, epicatechin, rutin, quercetin, and naringenin. Gallic acid, an abundant red wine polyphenol and natural antioxidant, was reported to have a potential hypolipidemic effect on mice fed a high-fat diet (*36*). It was also shown that gallic acid can intervene in platelet−leukocyte interactions by binding and antagonizing P-selectin (*37*), thus preventing atherosclerosis. Protocatechuic acid, a virgin olive oil biophenol, was also reported to have an anti-inflammatory effect by inhibiting oxidation of low-density lipoprotein (LDL) (*38*,*39*). The flavones and catechins are the most powerful flavonoids to protect the body against free radicals (*24*). Quercetin and silibin inhibit the activity of xanthine oxidase, thereby reducing oxidative injury. Similarly, catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate also inhibited free radical production by inhibiting xanthine oxidase (*40*−*43*). Some clinical studies have revealed that flavonoid intakes can protect CAD and reduce the risk of death from CAD in elderly men (*25*,*26*). Besides, a Japanese study reported a beneficial effect of flavonoid intake for the reduction of the total plasma cholesterol concentration (*44*). It was also reported that the intake of flavonoids can reduce the risk of dementia (*45*). Quercetin can inhibit the activities of cyclooxygenase and lipoxygenase, thus diminishing the formation of inflammatory metabolites (*46*). It was stated that flavonoids can inhibit angiogenesis, such as proliferation and migration of endothelial cells (*47*). A large clinical study suggested the inverse correlation between quercetin and the incidence of lung cancer (*48*). Quercetin and apigenin were reported to inhibit melanoma growth in mice (*49*). Some flavonoids, such as quercetin, kaempferol, and myricetin, were revealed to inhibit platelet aggregation in dogs and monkeys (*50*). On the basis of the limited data, approximately 150 mg of flavonoids is needed to observe an acute antioxidant effect and 500 mg is needed to observe a chronic antioxidant effect (*51*,*52*). MLE was reported to exhibit potential hypoglycemic and hypolipidemic effects in patients with type II diabetes, which amounts to a dose of 3 g/day (*53*). However, there is a dearth of information about the amount of MLE that is needed to inhibit VSMC proliferation effectively, thus preventing atherosclerosis.

In summary, these *in vitro* studies demonstrated the molecular mechanisms by which MLE effectively inhibits proliferation of VSMCs. MLE inhibits VSMC proliferation, inducing G0/G1 phase growth arrest by acting on multiple cell-cycle regulatory components upstream of Rb protein, including p53, p21, p27, and CDK2/4, thereby inhibiting phosphorylation of Rb. Additional verification of these *in vitro* results in a long-term clinical study is

necessary to understand the long-term effects of mulberry therapy in preventing CAD and atherosclerosis.

Supporting Information Available: A7r5 cells incubated with DMEM and treated with 0.5, 1.0, 1.5, and 2.0 mg/mL MLE (Supplement 1) and cell lysates prepared from A7r5 cells at the times indicated following treatment with 1.5 mg/mL MLE (Supplements 2−4). This material is available free of charge via the Internet at http://pubs.acs.org.

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