

Full Paper

Elucidating the Inhibitory Mechanisms of Magnolol on Rat Smooth Muscle Cell Proliferation

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Abstract. The pathological mechanism of percutaneous transluminal coronary angioplasty-induced restenosis has been attributed to outgrowth of vascular smooth muscle cells. Pretreatment with antioxidants has been shown to reduce restenosis. Magnolol, an active compound of *Magnolia officinalis*, has exhibited approximately 1,000 times more potent antioxidant effects than alpha-tocopherol. In this study, we demonstrate, using cytometric analysis, an approximate 61% reduction of smooth muscle cells progressing to the S-phase by 0.05 mg/ml of magnolol. A BrdU incorporation assay also showed a significant reduction (73%) of DNA synthesis using 0.05 mg/ml of magnolol. The protein level of the proliferating cell nuclear antigen was suppressed by approximately 48% using 0.05 mg/ml of magnolol. This was in agreement with the promoter activity of nuclear factor-kappa B, which was also attenuated by 0.05 mg/ml of magnolol. Since receptor interacting protein and caspase-3 protein expression levels were both increased by magnolol in a dose-dependent manner, the apoptotic pathway may mediate the inhibition of cell growth. Our finding that malondialdehyde formation was significantly inhibited by 0.05 mg/ml of magnolol further supported the antioxidant effect of magnolol. These studies suggest that magnolol might be a potential pharmacological reagent in preventing balloon injury-induced restenosis.

Keywords: percutaneous transluminal coronary angioplasty, restenosis, magnolol, neointima formation

Introduction

Percutaneous transluminal coronary angioplasty (PTCA) has been routinely used in many patients with stable and unstable angina and acute myocardial infarction (1). More than 300,000 cases of coronary angioplasty are performed in the United States each year (2). Approximately 30% to 50% of the patients receiving a successful PTCA were affected by restenosis within six months (3–6). Restenosis remains the major restraint on the long-term success of coronary angioplasty (PTCA). Stents are used to decrease restenosis; however, 20% to 30% of the patients are still affected by restenosis after coronary stenting (6).

Many gene encoding proteins responsible for vascular

relaxation and free radical scavenging have been studied to prevent restenosis such as vascular endothelium growth factor (VEGF) (7–9), superoxide dismutase (SOD), catalase (10), cyclooxygenase (COX) (11), and extracellular nitric oxide synthase (eNOS) (12). A remarkable 20-fold increase in superoxide anion production occurs immediately after balloon injury and continues for up to 10 min when compared to uninjured arteries (13). Pretreatment of porcine with antioxidants was found to reduce coronary stenosis (14–16).

Chinese herbalists have used *Magnolia* (*Hou Po*), *Magnolia officinalis*, medicinally since the first century. *Magnolia* is a commonly prescribed Chinese medicine for many treatments, including as an anti-biotic and for blood circulation. It has been shown to possess antioxidant activity approximately 1,000 times greater than α -tocopherol (17). Earlier studies have shown that

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pretreatment with antioxidants can significantly reduce balloon injury-induced neointima formation (18, 19). Therefore, it is expected that magnolol, the active compound of *Magnolia officinalis*, might play an important role in preventing balloon injury-induced restenosis. This hypothesis has been confirmed by Chen et al. who showed that magnolol can attenuate intimal thickening after balloon injury in hyperlipidemic rabbits (20). However, the molecular and cellular mechanisms of magnolol on neointimal formation were not fully elucidated.

Restenosis emerges from the migration of smooth muscle cells from the arterial media to the intima, where they change to a synthetic phenotype, produce extracellular matrix, and proliferate, thereby resulting in a stenosis within the vessel lumen (21, 22). There has been a consensus that inhibition of smooth muscle cell proliferation can reduce intimal hyperplasia after angioplasty (23, 24). Proliferating cell nuclear antigen (PCNA), a cofactor for DNA polymerase delta (25, 26), is required for DNA synthesis and S-phase progression and therefore, cell proliferation (26). This protein combines with other key cell-cycle control proteins, such as the cyclins and cyclin-dependent kinases (27). Wei and his colleagues showed that PCNA was markedly induced after balloon injury using a rat carotid-injury model (28).

In addition to PCNA activation, nuclear factor-kappa B (NF- κ B), receptor interacting protein (RIP), and caspase-3 also play important roles in cell proliferation. It has been reported that NF- κ B, a potent transcription factor, was significantly increased in the aortic smooth muscle cells followed by balloon injury (29). Receptor interacting protein, a death domain associated protein possessing serine/threonine kinase activity, and caspase-3, a member of the cysteine protease family, are two important apoptotic components leading to cell death (30). Apoptosis resulting in cell death may regulate abnormal smooth muscle cell proliferation.

To test the hypothesis that magnolol can be an effective therapeutic intervention for PTCA, the molecular and cellular mechanisms of magnolol in preventing abnormal cell proliferation were evaluated. The protein levels of PCNA, RIP, and caspase-3, as well as the effect on lipid oxidation by different concentrations of magnolol, were evaluated to explore its inhibitory mechanism on neointimal formation. The inhibitory effect of magnolol on the promoter activity of NF- κ B leading to cell proliferation was also studied. The present study provides a general insight into the pharmacological mechanism of magnolol in preventing outgrowth of smooth muscle cells, which is a potential remedy for clinical PTCA-induced restenosis.

Materials and Methods

Cell culture

A10 vascular smooth muscle cells (VSMCs) derived from rat thoracic aorta were obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured in 10-cm dishes containing DMEM supplemented with 10% FBS, 3.7 g/l NaHCO₃, 1.028 g/l *N*-acetyl L-alanyl-L-glutamine, 1% Na-pyruvate, 4.5 g/l D-glucose, 100 units/ml penicillin G, and 100 μ g/ml streptomycin sulfates. The culture medium was replaced every two days and the cells were passaged every week. In the last passage, the same numbers of cells were subcultured to 6-well plates. The cells that became at least 80% confluent were starved for 24 h in DMEM containing 0.5% FBS followed by a treatment with 0.001, 0.01, or 0.05 mg/ml of magnolol in DMEM containing 15% FBS for 24 h.

Western blotting

A10 cells cultured in 6-well plates were incubated with 0.001 mg/ml, 0.01 mg/ml, or 0.05 mg/ml of magnolol in DMEM containing 15% FBS for 24 h. Magnolol was purchased from Vercotech, Inc. (Lot number: MYB0528; Taipei, Taiwan). The purity of magnolol was confirmed by HPLC showing that the peak absorbance at wave length of 280 nm was observed at 6.473 min (data provided by the manufacturer). The cells were then lysed in a buffer containing 2% SDS, 50 mM DTT, 62.5 mM Tris-HCl, pH 6.8, followed by incubation at 95°C for 5 min. Samples were separated using SDS-PAGE, transferred to PVDF membranes, blocked with 5% nonfat dry milk in PBS-Tween for 1 h, and then probed with the desired antibody (anti-PCNA, anti-RIP, and anti-caspase-3; Novus Biologicals, Littleton, CO, USA) overnight at 4°C. The blots were then incubated with horseradish peroxidase-linked secondary antibody for 1 h followed by development with the ECL reagent and exposure to Hyperfilm (Amersham, Arlington Height, IL, USA).

DNA synthesis assay

A10 cells were treated in the same manner as described in the Western blotting section. The cells were then subjected to 10 mM 5'-bromo-2'-deoxyuridine (BrdU) incubation for 3 h. BrdU incorporation into DNA was measured by utilizing a colorimetric reaction with peroxidase-linked anti-BrdU antibody using a cell proliferation ELISA kit according to the manufacturer's instructions (Boehringer Mannheim, Germany).

Cell cycle analysis

The DNA content of the treated cells was assessed

using flow cytometry following propidium iodide (PI) staining. Cells were harvested with trypsin-EDTA, washed twice with 10 ml ice-cold PBS, fixed in 70% ethanol, and kept at 4°C prior to FACS analysis. For DNA content analysis, cells were centrifuged and resuspended in 0.3 ml of DNA staining solution [100 Ag/ml PI, 0.2% Nonidet P-40, and 1 mg/ml RNase A (DNase-free) in PBS lacking Ca^{2+} and Mg^{2+} ; at a 1:1:1 ratio by volume]. The cell suspension was stored on ice in a dark room for a minimum of 30 min and analyzed within 2 h. Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). PI fluorescence was linearly amplified and both the area and width of the fluorescence pulse were measured. Ten thousand events were acquired, and the percentages of hypodiploid (apoptotic, sub-G1) events and percentages of cells in G0/G1, S-, and G2-M phases were determined using the DNA analysis software ModFitLT, version 2.0 (Verity Software, Topsham, ME, USA).

Promoter analysis

A10 cells were transfected with 1 μg NF- κB promoter-luciferase construct using a DMRIE-C kit (Gibco, Carlsbad, CA, USA) according to the manufacturer's protocol. The full-length NF- κB promoter sequence was ligated to a luciferase reporter gene in a pGL2-basic vector (Promega, Madison, WI, USA). The beta-galactosidase gene was co-transfected with a luciferase construct to determine the transfection efficiency. Briefly, about 3.5×10^5 cells were seeded in each well of the 6-well plates and co-incubated with lipid DNA containing 1 μg each for NF- κB promoter-luciferase and β -galactosidase constructs for 6 h in a serum-free condition. The cells were then cultured in the regular medium containing 10% FBS for 12 h followed by the addition of 0.001, 0.01, or 0.05 mg/ml of magnolol in 15% FBS culture medium for 24 h of incubation. The cells were then subjected to total protein extraction, from which 30 μg of protein was allowed to react with 50 μl of luciferase reagent and subjected to luminometer analysis (Optocomp I; MGM Instrument, Inc., Hamden, CT, USA) to determine the effects of magnolol on luciferase activity. Equal amount of protein extract from each cell group was processed for β -galactosidase activity by reacting with ONPG (Sigma, St. Louis, MO, USA) followed by spectrophotometer analysis at 540 nm. The data from β -galactosidase activity was used to normalize the luciferase data to smooth out the differential transfection efficiency between each cell group.

MDA (malondialdehyde) assay to determine the antioxidant effect of magnolol

A 3-ml aliquot of blood was withdrawn from the tail vein of SD rats and centrifuged at 13,000 rpm for 15 min for serum separation. A 10- μl sample of serum was mixed with 10 μl of 10 mM CuSO_4 and either 15 μl of distilled H_2O (control group) or 0.001, 0.01, or 0.05 mg/ml of magnolol (experimental groups) to make a final volume of 50 μl with an additional 15 μl distilled H_2O . The sample mixture was incubated at 37°C for 4 h followed by the addition of 350 μl of 20% TCA and 350 μl of 0.67% of TBA (dissolved in NaOH). The whole mixture was incubated at 69°C for 30 min. After centrifugation at 10,000 rpm for 2.5 min, the supernatant was measured at 540 nm to determine the MDA amount.

Genomic DNA extraction for DNA laddering assay

The genomic DNA extracted from smooth muscle cells was prepared according to the protocol provided by the Genomic DNA Isolation kit (BioVision Inc., Mountain View, CA, USA). After the cells cultured with various concentrations of magnolol for 24 h, about 1.85 ml of cell suspension solution was added with 50 μl RNase mix and 100 μl cell lysis/denaturing solution in a 55°C water bath for 30 min, followed by addition of 25 μl of protease mix in the 55°C water bath for 60 min. The mixture was then gently mixed and centrifuged at $10,000 \times g$ for 10 min. The supernatant was added with 2 ml of TE buffer and 8 ml of absolute ethanol at $1,000 \times g$ for 10 min. After pouring out the supernatant, the pellet was then air dried followed by re-suspension in 40–90 μl of TE buffer. The DNA-containing solution was then subjected to electrophoresis. This experiment was repeated three times.

Statistics

Data are expressed as the mean $\pm$ S.E.M. ANOVA was performed for statistical analysis of continuous variables followed by the Newman-Keuls test. A P value <0.05 was considered statistically significant.

Results

Effects of magnolol on serum-induced DNA synthesis

Serum-stimulated A10 cells were treated with 0.001, 0.01, and 0.05 mg/ml of magnolol for 24 h to evaluate their effects on DNA synthesis. Analysis of DNA synthesis was evaluated using BrdU incorporation into the deoxynucleotide backbone. Figure 1 shows that 0.05 mg/ml magnolol significantly decreased ($P<0.01$) serum-induced BrdU incorporation by 76.8% as compared to the serum control, whereas 0.001 and

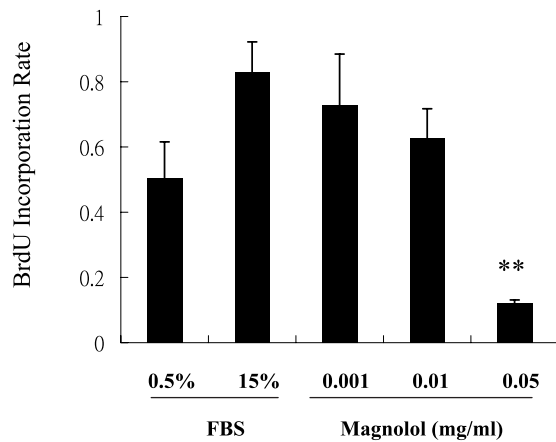


Fig. 1. Effects of magnolol on BrdU incorporation of rat smooth muscle cells. Only 0.05 mg/ml of magnolol significantly reduced the BrdU incorporation by 76.8%. ** $P < 0.01$, $n = 6$.

0.01 mg/ml of magnolol did not affect DNA synthesis.

Effect of magnolol on cell cycle

Cellular total DNA contents were measured using flow cytometry in serum-stimulated A10 cells treated with 0.001, 0.01, and 0.05 mg/ml of magnolol for 24 h. Figure 2 shows that 0.05 mg/ml of magnolol significantly reduces cell entry into the S-phase by 61% as compared to the control group. This resulted in a 2.8-fold increase of cells remaining in the G1 phase using 0.05 mg/ml of magnolol. This result is similar to the BrdU incorporation assays, where only 0.05 mg/ml, and not 0.001 or 0.01 mg/ml of magnolol, reduced cell proliferation.

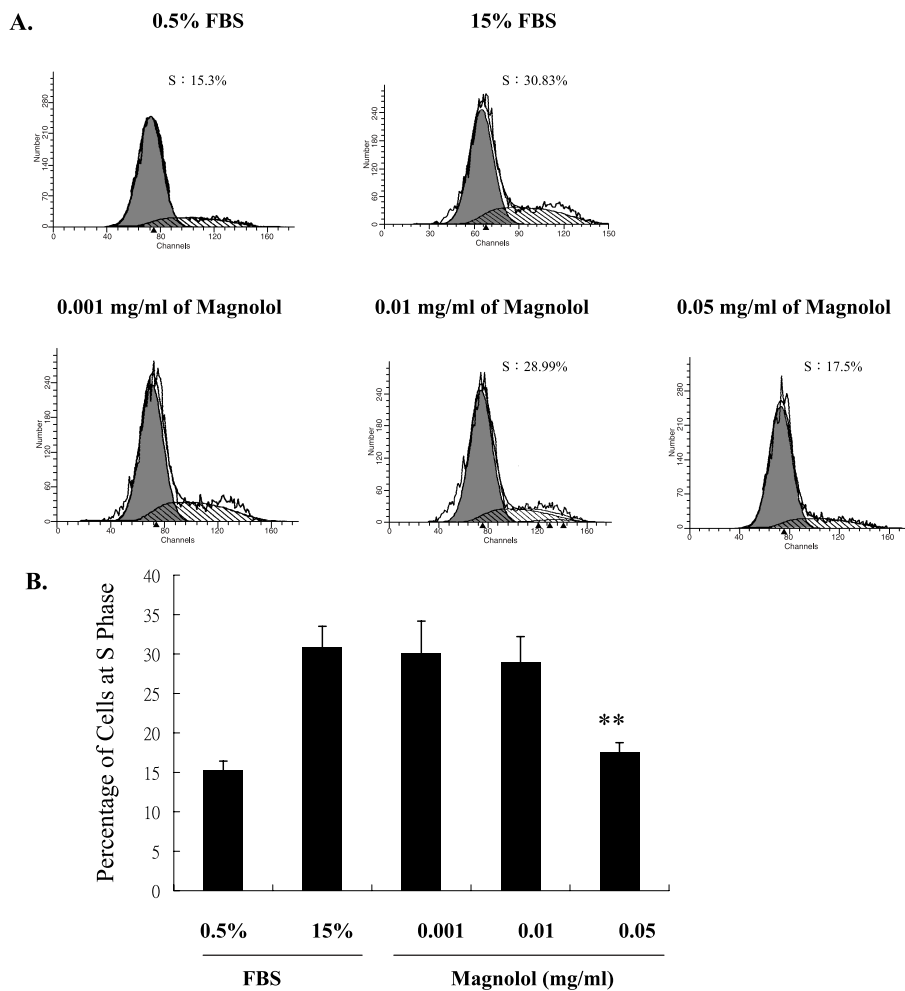


Fig. 2. Flowcytometric analysis of the inhibitory effects of magnolol on the cell cycle of rat smooth muscle cells. All the cells were cultured in 15% FBS with addition of 0.001, 0.01, or 0.05 mg/ml of magnolol. A: Each phase (G₀/G₁, S, and G₂/M) of the cell cycle was determined at various doses of magnolol. Value on the X-axis represents the DNA content, while the shaded area indicates the % of cells at the S phase. B: Effect of magnolol on the SMC arrested at S phase. Each value represents the mean $\pm$ S.D. ** $P < 0.01$, as compared to 15% FBS, $n = 6$.

Effects of magnolol on protein expression levels of PCNA, RIP, and caspase-3

Serum-stimulated A10 cells were treated with 0.001, 0.01, and 0.05 mg/ml of magnolol for 24 h. Total proteins were extracted from cells and subjected to Western blot analysis with antibodies against PCNA, RIP, and caspase-3. Figure 3 shows that 0.05 mg/ml of magnolol significantly reduces ($P<0.01$) the protein expression level of PCNA by 48%. The data represent the ratio of PCNA protein expression level normalized by a 15% serum control. Figure 3 also indicates that the apoptotic protein, RIP, was significantly increased by magnolol in a dose-dependent manner. The increase in RIP protein expression level by 0.001, 0.01, and 0.05 mg/ml of magnolol was 112%, 129%, and 141%, respectively. Similarly, caspase-3, a direct enzyme to induce apoptosis, was also activated by magnolol (Fig. 3). The increase in the caspase-3 protein level by 0.001, 0.01, and 0.05 mg/ml of magnolol was 80%, 117%, and 113%, respectively.

Inhibition of NF- κ B promoter activity by magnolol

Promoter activity of NF- κ B is evaluated in the smooth muscle cells transfected with a plasmid construct containing NF- κ B promoter conjugated with luciferase reporter. These transfected cells were treated with 0.001, 0.01, and 0.05 mg/ml of magnolol in the presence of 15% FBS as a mitogen. Since transfection efficiency for each cell group may be different, cells were co-transfected with the β -galactosidase gene to normalize the NF- κ B/luciferase activity. Our results showed that the serum-stimulated activation of NF- κ B promoter manifested as luciferase activity was significantly reduced by 77% (Fig. 4).

Inhibition of magnolol on MDA formation

MDA formation, representing the lipid oxidation, was used to evaluate the anti-oxidation effects of magnolol. The serum withdrawn from the rat tail vein was oxidized with CuSO_4 in the presence of 0.001, 0.01, and 0.05 mg/ml of magnolol. The MDA formation was significantly inhibited (62%) using 0.05 mg/ml of magnolol; however, 0.001 and 0.01 mg/ml of magnolol did not affect the MDA value (Fig. 5).

DNA laddering induced by magnolol in a dose-dependent manner

DNA laddering provides another evidence for apoptosis. As seen in Fig. 6, we found that vascular smooth muscle cells can be induced for DNA laddering only at higher concentrations of 0.05 mg/ml but not at 0.001 and 0.01 mg/ml of magnolol. This finding suggests that magnolol-induced apoptosis on vascular

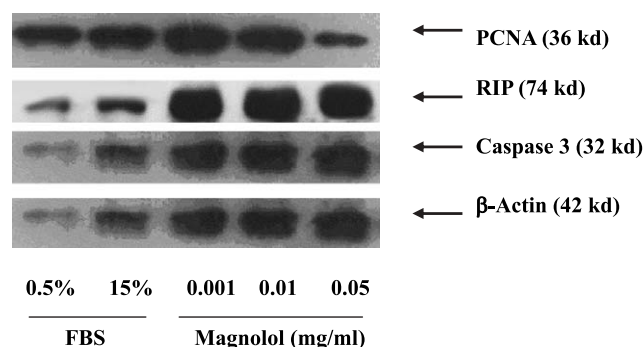


Fig. 3. Effects of magnolol on protein expression level of PCNA, RIP, and caspases-3 in rat smooth muscle cells. Each Western blot result represents the protein expression level affected by 0.001, 0.01, or 0.05 mg/ml of magnolol.

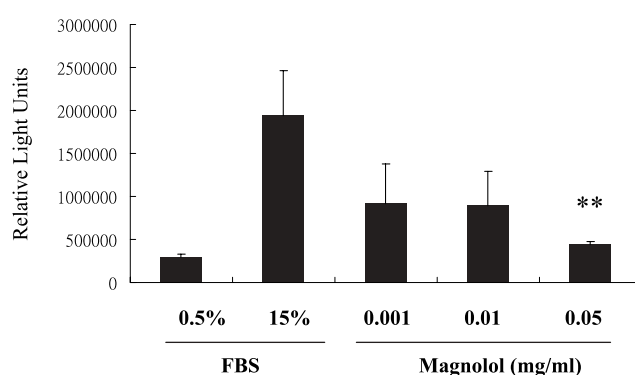


Fig. 4. Inhibitory effects of magnolol on NF- κ B activity in rat smooth muscle cells. The activation of NF- κ B activity was significantly suppressed by 0.05 mg/ml of magnolol. Cells receiving 15% FBS served as the control, while 0.5% FBS served as the basal level. Each value represents the mean $\pm$ S.D. ** $P<0.01$, as compared to the control, $n = 6$.

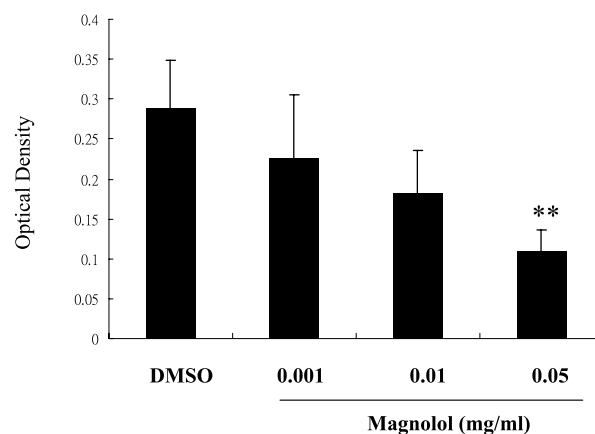


Fig. 5. Antioxidation effects of magnolol on MDA formation in rat serum. The oxidation effects of 2 mM CuSO_4 on rat serum was significantly suppressed by 0.05 mg/ml of magnolol. DMSO added to serum served as the control. Each value represents the mean $\pm$ S.D. ** $P<0.01$, as compared to the control, $n = 6$.

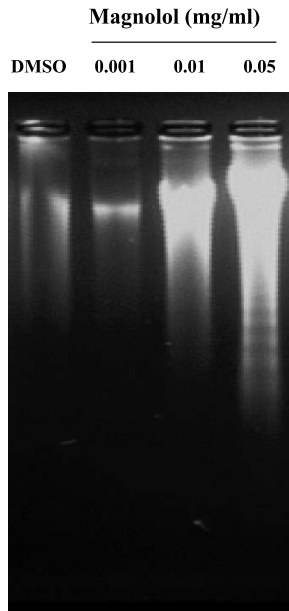


Fig. 6. The DNA laddering demonstrates the apoptotic effects of magnolol on smooth muscle cells. All the cells were cultured in 15% FBS with addition of 0.001, 0.01, and 0.05 mg/ml of magnolol. DMSO added to serum served as the control. The DNA laddering was only observed at the highest concentration of 0.05 mg/ml of magnolol but not at 0.001 and 0.01 mg/ml. $n = 3$.

smooth muscle cells is dose-dependent.

Discussion

In animal studies, a significant role of ROS in intimal hyperplasia results in restenosis after arterial balloon angioplasty (31). Evidence has been presented that ROS contributes to initial VSMC apoptosis, as well as remodeling, proliferation, and migration of medial VSMCs or adventitial myofibroblasts. A remarkable increase in superoxide anion production occurs immediately after balloon injury, and levels, for up to 10 min, remain 20-fold higher than those in control uninjured arteries (13). An approximate 60% drop of glutathione levels within 30 min after injury, coinciding with medial cell apoptosis, suggests that this early step of balloon injury is associated with severe oxidant stress. This observation is supported by the finding that the administration of antioxidants prevents glutathione loss and SMC apoptosis (18). Proliferation of neointimal cells has also been linked to the ROS reaction. Superoxide anion production and lipid peroxidation increased and glutathione peroxidase activity decreased when comparing uninjured arteries 10 to 14 days after balloon injury on pig coronary arteries and rat aorta with substantial neointima formation (18, 32). Furthermore, treatment with a variety of antioxidants (probucol,

vitamin C and E, butylated dihydroxytoluene, and L-cysteine) reduces neointimal proliferation and promotes vessel remodeling in several animal models (15, 33).

Magnolol, a commonly prescribed Chinese medicine, was found to possess an antioxidant effect about 1,000 times higher than that of α -tocopherol (17). Since several genes responsible for free radical scavenging, such as SOD and catalase (10), have been regarded as effective in preventing balloon injury-induced neointima formation, it is therefore expected that magnolol might be beneficial in reducing smooth muscle cell proliferation. In fact, it has been reported that magnolol was effective in preventing balloon injury-induced restenosis (34). However, the mechanistic insights into the inhibitory effects of mangolol on the cellular levels were not fully elucidated.

In the present study, we first demonstrated that magnolol exerted potent inhibitory effects on the growth of VSMCs. The antiproliferative effects were supported by the inhibition of BrdU incorporation and flow cytometric analysis of cell cycle progression, indicating that magnolol blocked the entry of VSMCs into the S-phase of the cell cycle. To further characterize the molecular mechanism of magnolol in suppressing smooth muscle cell proliferation, we investigated the effect of magnolol on the protein expression level of PCNA, which functions as a cofactor of polymerase- δ essential for DNA replication, and is an excellent marker for cell proliferation (26). Our results demonstrated a significant reduction of 48% on PCNA protein expression using 0.05 mg/ml of magnolol, which is consistent with the BrdU incorporation and flow cytometric analysis.

Apoptosis is another mechanism, which prevents cells from abnormal outgrowth. RIP is a death domain associated protein possessing serine/threonine kinase activity. It has been reported that RIP complexes with a death receptor, CD95 (Fas), with subsequent activation of the proenzymatic caspase-2 leading to a caspase cascade (30). As a member of the cysteine protease family, caspase-3 is one of the most important components in this cascade. Upon activation, two caspase-3 molecules dimerize to induce an irreversible apoptotic process and cause cell death (35). In our present study, the protein expression levels of RIP and caspase-3 were increased by magnolol, suggesting that the inhibitory effects of magnolol on cell proliferation may be mediated through the apoptotic mechanism. This observation is in agreement with a previous report showing the induction of apoptosis in vascular smooth muscle cells in response to magnolol (34).

Considering the dosage difference of magnolol on cell proliferation and pro-apoptotic protein expression

(0.05 vs 0.001 mg/ml, respectively), a tentative explanation can be outlined as follows: RIP and caspases are responsible for cell apoptosis and thus may contribute to reducing cell proliferation. However, translational activation of RIP and caspases by magnolol at 0.001 mg/ml may not be potent enough to suppress cell proliferation. This is supported by our current finding that PCNA, an index for cell proliferation, is also inhibited only by 0.05 mg/ml but not 0.001 mg/ml of magnolol. In addition, we have performed a DNA laddering experiment showing that DNA laddering only occurred at 0.05 mg/ml but not 0.001 mg/ml of magnolol.

NF- κ B is a key transcription element closely related to cell proliferation. It has been reported that NF- κ B is responsible for smooth muscle cell proliferation induced by serum and tumor necrosis factor- α (36, 37). It was also noted that NF- κ B expression was considerably increased in aortic smooth muscle cells following balloon injury (29). In addition, the NF- κ B expression level was substantially higher in atherosclerotic smooth muscle cells as compared to those in normal vessels (34). Therefore, suppression of NF- κ B can be expected to result in cell proliferation arrest. This was supported by our finding that the promoter activity of NF- κ B was significantly inhibited by 0.05 mg/ml of magnolol (Fig. 4) and the SMC proliferation was suppressed at this same dose of magnolol (Fig. 1).

There have been quite a few reports stating the antioxidant effects of magnolol. Copper-induced LDL oxidation can be inhibited by magnolol treatment in cholesterol-fed rabbits (38). Phorbol-12-myristate-13-acetate (PMA) induced rapid intracellular accumulation of superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) in neutrophils was also found to be attenuated by magnolol (39). Furthermore, magnolol has been used as a lead compound to derive a potent antioxidant based on an in vitro assay using enhanced chemiluminescence and flow cytometric analysis (40). In the present study, we further confirm that lipid oxidization manifested as MDA formation was significantly inhibited by magnolol in a dose-dependent manner. The effective antioxidant concentration of magnolol was also found to inhibit cell proliferation and cell cycle progression (Figs. 1 and 2). This finding is in agreement with the previous reports that pretreatment of swine with antioxidants was found to reduce coronary stenosis (14–16).

The specific aim of this study was to explore the mechanism of magnolol in preventing neointimal formation induced by balloon injury. It is well known that excessive oxidation and abnormal cell proliferation contributed largely to this pathological process (15, 18, 32). Our observations suggest that an appropriate dosage of magnolol exerts both anti-oxidant and anti-prolifera-

tion effects. This was evident by inhibition of MDA formation and cell cycle arrest as well as down-regulation of PCNA protein level and promoter activity of NF- κ B by magnolol. Apoptotic activation was also another mechanism of magnolol to suppress outgrowth of smooth muscle cells. This was supported by the up-regulation of several pro-apoptotic proteins such as RIP and caspases-3 (Fig. 3). DNA laddering induced by magnolol provides another evidence of programmed cell death (Fig. 6). Taken together, all these results merit further clinical investigations regarding the therapeutic significance of magnolol on vascular disorders with outgrowth of smooth muscle cells, including restenosis and atherosclerosis.

Acknowledgments

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