Liuwei Dihuang, a traditional Chinese medicinal formula, inhibits proliferation and migration of vascular smooth muscle cells via modulation of estrogen receptors

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Received January 25, 2016; Accepted November 10, 2017

DOI: 10.3892/ijmm.2018.3622

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Key words: Liuwei Dihuang formula, vascular smooth muscle cell, proliferation, migration, phenotypic switching, estrogen receptor, angiotensin II

Abstract. The phenotypic modulation of vascular smooth muscle cells (VSMCs) serves an important role in atherosclerosis-induced vascular alterations, including vascular remodeling. However, the precise mechanisms underlying VSMC phenotypic modulation remain to be elucidated. Our previous study demonstrated that Liuwei Dihuang formula (LWDHF) could improve menopausal atherosclerosis by upregulating the expression of estrogen receptors (ERs). The present study examined the role of ERs in the effects of LWDHF on VSMC phenotypic modulation. VSMC proliferation and cell cycle progression were examined by MTT assay and flow cytometry, respectively. The expression levels of α-smooth muscle actin, osteopontin and ERs were determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. Cell ultrastructure was observed under an electron microscope. F-actin polymerization was detected by fluorescein isothiocyanate-phalloidin staining using fluorescence microscopy. A modified Boyden chamber assay was employed to assess VSMCs migration. Small interfering (si)RNA technology was used to examine the role of ERα in the effects of LWDHF on VSMC phenotypic modulation. The results indicated that LWDHF (3–12 µg/ml) inhibited proliferation and induced a cell cycle arrest in VSMCs treated with angiotensin II (Ang II; 100 nM) in a concentration-dependent manner. In addition, Ang II-stimulated migration of VSMCs and reorganization of actin were markedly inhibited by treatment with 12 µg/ml LWDHF. Results of RT-qPCR and western blotting demonstrated that LWDHF markedly stimulated transcription and expression of ERα and ERβ, and inhibited VSMC synthetic phenotype. Furthermore, LWDHF-induced inhibition of phenotypic switching was partially suppressed by tamoxifen, and transfection with ERα siRNA markedly abolished the effects of LWDHF on VSMC phenotypic switching. In conclusion, these results revealed that ERα served an important role in LWDHF-induced regulation of the VSMC phenotype, including proliferation and migration.

Introduction

Abnormal proliferation of vascular smooth muscle cells (VSMCs) is believed to serve an important role in the formation of atherosclerotic plaques and restenosis following percutaneous coronary intervention (1,2). Vascular remodeling has recently garnered increasing attention due to its critical role in the progression of vascular occlusion diseases (3). During vascular lesion formation, phenotypic switching of VSMCs from the physiological contractile phenotype to the pathological synthetic phenotype occurs, and VSMCs migrate to the intima (4). Under normal conditions, VSMCs constitute the major structural component of the vasculature, and are crucial for maintaining vessel tone, blood pressure and blood flow. In the media layer of mature blood vessels, VSMCs exhibit a differentiated and contractile phenotype, characterized by the expression of contractile proteins, including α-smooth muscle actin (α-SMA), calponin and smooth muscle heavy chain (5). In various vascular pathologies, VSMCs undergo a rapid and reversible alteration from a quiescent contractile phenotype to a proliferative and secretory phenotype, which is characterized by increased proliferation, migration and extracellular matrix (ECM) production (6), and osteopontin (OPN) is the main protein marker of synthetic phenotypes. The synthetic phenotype of VSMCs acts as a critical factor in various cardiovascular diseases, including atherosclerosis, restenosis after angioplasty or bypass, and hypertension (7,8).

It is well known that numerous cytokines and growth factors are released to stimulate VSMC proliferation during vascular injury repair (9,10). Angiotensin II (Ang II), which has previously been reported to serve an important role in normal vascular physiology and cardiovascular disease, is a...
modulating the phenotypic modulation of VSmcs, which was inhibits proliferation and migration may be associated with in addition, the molecular mechanism by which lWdHf the expression of estrogen receptors (ERs) following vascular in vitro endothelial cell injury (19-21). Therefore, the present study aimed to investigate the effects of lWdHf on Ang II-induced VSmc proliferation by the nanjing university of Chinese medicine committee on laboratory animal care and all animals received humane care according to the national Institutes of Health guidelines. The animals were housed under diurnal lighting conditions (12:12) and had access to food and water ad libitum. Female Sprague-Dawley rats (weighting 200±30g) were provided by Zhejiang Experimental Animal Center (Nanjing, China).

Materials and methods

Reagents and antibodies. Human Ang II, tamoxifen and cell proliferation reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Antibodies used to detect the protein expression levels of α-SMA (ab124964), OPN (ab8448), ERβ (ab92306), β-actin and β-tubulin were obtained from Abcam (Cambridge, MA, USA). Anti-ERα antibody was purchased from Cell Signaling Technology, Inc.(Danvers, MA, USA). ERs small interfering (si)RNA and control siRNA were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Preparation of LWDH. The method of LWDH preparation was reported by Yang et al (22). Briefly, Rehmannia glutinosa Libosc. (Scrophulariaceae family), Cornus officinalis Sieb. (Cornaceae family), Dioscorea opposita Thunb. (Dioscoreaceae family), Alisma orientale (G. Samuelsson) Juz. (Alismataceae family), Poria cocos (Schw.) Wolf (Polyporaceae family) and Paeonia suffruticosa Andrews (Paeoniaceae family) were mixed at a ratio of 8:4:3:3:3. The mixture was twice decocted in distilled water for 30 min. The water extracts were concentrated to 2 g/ml for further use. High-performance liquid chromatography was used to analyze the constituents of LWDH (22). Five major constituents, including gallic acid, paeonoside, verbascoside, loganin and paeoniflorin, were identified in LWDH (Fig. 1). Their contents were 2.74, 0.05, 0.06, 0.74 and 0.43 mg/g, respectively. The standard samples of gallic acid, paeonoside, verbascoside, loganin and paeoniflorin were purchased from Beijing Beina Chuanglian Biotechnology Research Institute (Beijing, China).

Cell culture. Primary VSMCs were isolated from thoracic aortas of 7-week-old male Sprague-Dawley rats by explant technique, and were then cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 KU/l benzylpenicillin and 100 mg/l streptomycin at 37°C in a humidified chamber containing 5% CO2 (23). The identification of VSMCs was performed by α-SMA immunostaining; >90% of cells were α-SMA-positive and exhibited a spindle-shaped appearance. VSMCs were passaged by trypsinization, and cells at passages 3-7 were used for subsequent experiments to ensure genetic stability of the culture. All animal experimental protocols were approved by the Nanjing University of Chinese Medicine Committee on Laboratory Animal Care and all animals received humane care according to the National Institutes of Health guidelines.

Cell proliferation assay. Cell proliferation was analyzed using the MTT assay. VSMCs (1x10^4/well) were seeded in a 96-well microplate and were cultured with 200 µl DMEM supplemented with 10% FBS. Once the cells had reached 60% confluence they were serum-starved for 16 h at 37°C in a humidified chamber containing 5% CO2. VSMCs were then treated with 100 nM Ang II or 100 nM Ang II + LWDH (3, 6 and 12 µg/ml) for 24 h; cells were incubated with MTT (5 mg/ml) for the last 4 h at 37°C in a humidified chamber containing 5% CO2 and then dissolved into 150 µl DMSO. Untreated
Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from VSMCs was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA concentration was quantified by measuring the absorbance at 260 nm using a spectrophotometer, and RNA purity was assessed according to the manufacturer's protocol. RNA (5 µg) was then reverse transcribed into cDNA using a SuperScript II First-Strand Synthesis Super mix (Thermo Fisher Scientific, Inc.) with the manufacturer's protocol. RNA (5 µg) was then reverse transcribed into cDNA using a First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.); the obtained cDNA was used to determine the mRNA expression levels of α-SMA, OPN, ERα and ERβ by qPCR using a Thermo Fisher Scientific ABI7500 instrument (Thermo Fisher Scientific, Inc.) with SuperScript II First-Strand Synthesis SuperMix (Thermo Fisher Scientific, Inc.), and relative target gene expression was normalized to GAPDH or β-tubulin. The primer sequences were designed by Biogotte Technology Co., Ltd. (Nanjing, China) and are presented in Table 1. RT-qPCR was performed using AceQ qPCR SYBR Green Master Mix. Amplification was initiated at 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec, and was finally terminated at 60°C for 40 sec and analysed using the ΔΔCt method on an ABI 7500 Thermal Cycler (both from Invitrogen; Thermo Fisher Scientific, Inc.). The quantification cycle (Cq) value of the gene of interest was normalized to GAPDH or β-tubulin (ΔCq), after which the value was further normalized to the control (ΔΔCq). The fold-change of each target gene was calculated using the 2^−ΔΔCq method (24).

Transmission electron microscopy. The cells were fixed in 2% glutaraldehyde in neutral phosphate buffer, post-fixed in osmium tetroxide, and embedded in Epon. Sections were cut at 80 nm and examined under a Philips Tecnai 10 electron microscope.

Flow cytometry. Primary VSMCs were seeded in 6-well plates at a density of 2x10^5 cells and were incubated at 37°C for 24 h. After VSMCs were treated with Ang II and/or LWbHf at 37°C for 24 h, they were trypsinized, collected and washed twice with cold PBS at 4°C for 5 min. Precipitated cells were fixed in 1 ml ice-cold 70% ethanol overnight at 4°C. The fixed cells were then washed in PBS, treated with RNase A (10 µg/ml), and DNA was stained with propidium iodide (50 µg/ml) for 30 min at 37°C in the dark. Cells (1x10^6) were finally analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Wound-healing assay. Primary VSMCs were seeded in 6-well plates at a density of 2x10^5 cells for 24 h to ensure a single cell distribution. Confluent cells were scratched along the edge of the attached cells to create a double-sided wound using a 10 µl plastic pipette tip and were rinsed twice with PBS to remove cell debris. Cells were then grown in serum-free medium supplemented with Ang II and/or LWbHf for 24 h. Cell gaps were observed under a Zeiss microscope (CarlZeiss, Hallbergnoos, Germany) and images were captured of the wound sites. VSMC migration was assessed on the basis of the injured area covered by cells counted from the wound borders.

Transwell migration assay. The effects of LWbHf on VSMC migration were examined by Transwell chamber assay. Primary VSMCs cells (1x10^5 cells/well) at passages 3-7 were seeded in a 36 mm culture dish for 24 h. Cells were then trypsinized, resuspended in 0.5% FBS medium in the upper chamber and incubated with LWbHf (12 µg/ml), DMEM medium containing Ang II (100 nM) was added to the lower chamber. The cells were incubated at 37°C in an atmosphere containing 5% CO₂ for 24 h and were allowed to migrate through the micropores to the bottom side of the Transwell apparatus. The remaining cells in the upper chamber were removed with a cotton swab, the cell membrane surface was wiped, and the lower side of the filter harboring the migrated VSMCs was fixed with 4% paraformaldehyde for 30 min. The migrating cells were then stained with 0.5% Coomassie Brilliant Blue for 10 min. Stained cells in five random visual fields from each of the Transwell filters were selected and images were captured under a Zeiss microscope (x200 magnification; CarlZeiss).

Analysis of F-actin cytoskeleton by fluorescence microscopy. Primary VSMCs were cultured on glass coverslips treated with indicated agents at 37°C for 24 h. Cells were then fixed with pre-cooled 4% paraformaldehyde and rinsed three times with PBS at room temperature. Cells were permeabilized in 0.1% Triton X-100 and incubated with 1% bovine serum albumin (10735108001; Biosharp, Hefei, China)/PBS to block nonspecific binding, after which they were incubated with phallolidin-flourescin isothiocyanate for 60 min at room temperature in the dark. After three washes with PBS at 4°C for 5 min, images were captured and were analyzed using the ZEN 2011 imaging software on a Zeiss inverted microscope (Carl Zeiss AG, Oberkochen, Germany) under 400-fold magnification.
Transfection of siRNA. Human VSMCs were seeded in a 6-well culture plate at a density of 2x10^5 cells in 2 ml antibiotic-free normal growth medium supplemented with 10% FBS. The cells were incubated at 37°C in a CO₂ incubator until the cells reached 60% confluence. Cells were then transfected with a transfection mixture composed of ERα siRNA (sc-29305) or control siRNA (sc-37007) and siRNA transfection reagent (sc-29528) (all Santa Cruz Biotechnology, Inc.) according to the manufacturer's protocols. After 6 h, cells were washed and cultured for 18 h in complete medium, and were treated with Ang II and/or LWDHF for 24 h. The cells were subsequently lysed and collected for western blot analyses.

Statistical analysis. SPSS 10.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. All data are presented as the mean ± standard deviation of at least three independent experiments. Differences in the results between two groups were evaluated using either two-tailed Student's t-test or one-way ANOVA followed by Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

LWDHF inhibits Ang II-induced VSMC proliferation and induces cell cycle arrest. The present study evaluated the effects of LWDHF on normal primary VSMC proliferation using the MTT assay. Growth-arrested cells were treated with LWDHF (0.1-100 g/l) in the presence of 10% FBS; the results indicated that no significant difference in cell viability was observed following treatment with ≤10 g/l LWDHF (Fig. 2A), suggesting that ≤10 g/l LWDHF did not exert significant cytotoxicity to normal cells. However, treatment with Ang II (100 nM) induced a 1.45-fold increase in VSMC proliferation, whereas treatment with LWDHF attenuated the effects of Ang II on VSMC proliferation in a dose-dependent manner (Fig. 2B); treatment with the highest concentration of LWDHF (12 µg/ml) significantly inhibited Ang II-induced VSMC proliferation. In addition, flow cytometric analysis was performed to observe the effects of LWDHF on cycle progression of VSMCs. As shown in Table II, the percentage of cells at G0/G1, S and G2/M phases in the Ang II-stimulated group was 58.85±5.01, 21.82±2.19 and 19.34±2.86, respectively. LWDHF, at concentrations of 3, 6 and 12 µg/ml, effectively increased the proportion of cells in the G0/G1 phase and simultaneously decreased the S and G2/M phase cell populations. These results indicated that LWDHF may exert suppressive effects on Ang II-induced VSMC proliferation; these effects may be associated with the induction of cell cycle arrest at G0/G1 phase.

LWDHF suppresses Ang II-stimulated VSMC migration. The effects of LWDHF on Ang II-stimulated VSMC migration were observed using a wound-healing assay. Equal numbers of confluent VSMCs were scratched to create a double-sided wound using a 10 µl pipette tip. The cells were then treated with Ang II (100 nM) or Ang II (100 nM) + LWDHF (12 µg/ml). As presented in Fig. 3A, Ang II promoted VSMCs to migrate into the wound, whereas cell migration was reduced in the LWDHF-treated group compared with in the Ang II-stimulated group, thus suggesting that LWDHF may reduce VSMC migration. In addition, a Transwell chamber assay was conducted to verify the effects of LWDHF on Ang II-induced VSMC migration. As shown in Fig. 3B, LWDHF treatment markedly decreased migration of VSMCs to the bottom chamber in response to Ang II compared with those treated with Ang II alone. Taken together, these results indicated that LWDHF may reduce Ang II-induced VSMC migration.

Effects of LWDHF on Ang II-induced F-actin organization in VSMCs. The present study demonstrated that LWDHF

### Table I. Primer sequences used for polymerase chain reaction.

| Gene       | Sense primer | Antisense primer |
|------------|--------------|------------------|
| β-tubulin  | 5-CGCAAGCTGACGTGATACCAAT-3 | 5-CTGCTCACCCATGCTTCGTC-3 |
| α-SMA      | 5-CATCATCGTGCTGGACTTGG-3 | 5-CCAGGGAGAGGAGAAGGAAGCA-3 |
| OPN        | 5-AGCCATAGCTAAGCTCAAGCT-3 | 5-ACTCGCCGCTAAGCTGATG-3 |
| GAPDH      | 5-CCTCATGACCAACAGACCTG-3 | 5-CTGCTTTGATGATACCCACATC-3 |
| ERα        | 5-TGGCTAAGTGCCTGGTGTGA-3 | 5-GCTTTCATGACCCACATT-3 |
| ERβ        | 5-TGACGACCTTGGATCCAGAG-3 | 5-AGTCCACATTAGCACCTC-3 |

α-SMA, α-smooth muscle actin; ER, estrogen receptor; OPN, osteopontin.

### Table II. Effects of LWDHF on Ang II-induced cell cycle progression.

| Group     | Dose    | Phases |         |         |
|-----------|---------|--------|---------|---------|
|           |         | G0/G1  | S       | G2/M    |
| Control   |         | 70.81±0.39a | 16.24±0.53a | 12.96±0.44a |
| Ang II    | 100 nM  | 58.85±5.01 | 21.82±2.19 | 19.34±2.86 |
| LWDHF     | 3 µg/ml  | 67.01±0.27b | 19.20±1.03b | 13.79±0.77a |
|           | 6 µg/ml  | 67.90±0.47b | 18.27±0.59b | 13.84±0.37a |
|           | 12 µg/ml | 68.73±0.29a | 17.68±0.42b | 13.59±0.61a |

Data are presented as the mean ± standard deviation, n=4. *P<0.01, †P<0.05 vs. the Ang II-stimulated group. Ang II, angiotensin II; LWDHF, Liuwei Dihuang formula.
inhibited the migration of Ang II-stimulated VSMCs. Therefore, in order to determine whether LWDHF decreased cell migration by influencing reorganization of the cytoskeleton, F-actin was visualized by phalloidin staining, 24 h after Ang II (100 nM)-stimulated VSMC spreading. Treatment with Ang II resulted in a substantial increase in the number of stress fibers, as well as the rearrangement of these structures into ordered parallel arrays, in cultured VSMCs. Conversely, treatment with LWDHF (12 µg/ml) abolished reorganization of the actin cytoskeleton induced by Ang II (fig. 3c).

**LWDHF inhibits Ang II-induced VSMC phenotypic switching.**

VSMCs transform from a differentiated contractile phenotype into a synthetic phenotype, which is associated with low expression of contractile proteins and high expression of rough endoplasmic reticulum (RER) and ECM proteins (25). The ultrastructure of VSMCs was observed under transmission electron microscopy (Fig. 4). The cytoplasm in normal VSMCs was filled with myofilaments, while homogeneous distribution of chromatin in nucleus. In cells treated with Ang II for 24 h, the muscle filament in the cytoplasm obviously decreased compared to that in the control group, but the Golgi body obviously increased, and the endoplasmic reticulum was obviously dilated. These ultrastructural alterations are characteristics of the synthetic phenotype, thus suggesting that Ang II induced phenotypic modulation of VSMCs. However, after treatment with LWDHF (12 µg/ml), the myocytes in the cytoplasm were still rich, but the organelles of the Golgi bodies were decreased. These ultrastructural observations indicated that LWDHF reversed Ang II-induced VSMC phenotypic switching. Furthermore, the present study measured alterations in the expression of molecular markers associated with contractile

Figure 1. Representative high-performance liquid chromatograms of Liuwei Dihuang formula. (A) Gallic acid; (B) loganin; (C) paeoniflorin; (D) verbascoside; and (E) paeonoside.

Figure 2. Effects of LWDHF on Ang II-induced VSMC proliferation. (A) MTT assays were performed to determine the effects of LWDHF on the proliferation of normal VSMCs stimulated by 10% fetal bovine serum. Relative proliferation rate was displayed using control cells as a standard (n=6). *P<0.05 vs. the control group. (B) MTT assays were performed to determine the rate of VSMC proliferation in each group. Relative proliferation rate was displayed using Ang II-stimulated cells as a standard (n=6). *P<0.05, **P<0.01 vs. the Ang II-stimulated group. Ang II, angiotensin II; LWDHF, Liuwei Dihuang formula; OD, optical density; VSMC, vascular smooth muscle cell.
Figure 3. Effects of LWDHF (12 µg/ml) on VSMC migration in response to Ang II (100 nM). (A) Cells were cultured in the presence of 10% FBS. After generation of a single scratch, medium was replaced with medium containing 2% FBS and cells were treated with Ang II with or without LWDHF. Cell wounds were observed under a microscope (x40 magnification). (B) Primary VSMCs were seeded in the upper chamber of a modified Boyden chamber and were treated with Ang II (100 nM) with or without LWDHF for 24 h. Untreated cells were used as a control. Images of five random visual fields from the bottom chamber of each group were captured under a microscope (x200 magnification). (C) Inhibitory effects of LWDHF (12 µg/ml) on reorganization of the actin cytoskeleton following treatment with Ang II (100 nM) for 24 h. The cells were fixed, stained with fluorescein isothiocyanate-labeled phalloidin and were examined by fluorescence microscopy. Untreated cells were used as a control. Images of five random visual fields from each group were captured under a microscope (x200 magnification).

Ang II, angiotensin II; FBS, fetal bovine serum; LWDHF, Liuwei Dihuang formula; VSMCs, vascular smooth muscle cells.

Figure 4. Ultrastructural alterations of VSMCs observed by transmission electron microscopy. (A and D) Representative images of the cytoplasm from normal VSMCs; (B and E) VSMCs treated with Ang II (100 nM) for 24 h. The numbers of rough endoplasmic reticulum and Golgi apparatus were increased in the cytoplasm, and the nuclei were larger compared with in untreated cells. (C and F) Following treatment with LWDHF, the nuclear shape became regular, and the numbers of rough endoplasmic reticulum and Golgi apparatus were decreased in the cytoplasm (A-C) x5,000 magnification; (D-F) x30,000 magnification.

Ang II, angiotensin II; LWDHF, Liuwei Dihuang formula; VSMCs, vascular smooth muscle cells.
and synthetic VSMC phenotypes. RT-qPCR and western blot analysis were used to measure the relative mRNA and protein abundance of α-SMA and OPN, respectively. The results indicated that exposure of VSMCs to Ang II for 24 h resulted in a 75% decrease in the mRNA expression levels of α-SMA and a robust upregulation of OPN (5-fold) (Fig. 5A). In addition, a decline in the protein expression levels of α-SMA and an increase in OPN protein expression were determined by western blotting (Fig. 5B and C). Conversely, addition of LWDHF dose-dependently rescued the downregulation of α-SMA and abolished the upregulation of OPN in Ang II-treated VSMCs. These results confirmed that the aforementioned ultrastructural alterations were due to LWDHF-induced inhibition of VSMC phenotypic transition caused by Ang II, and indicated that LWDHF suppressed VSMC proliferation and migration via inhibiting VSMC phenotypic switching.

**LWDHF regulates expression of ERs in VSMCs.** Our previous studies demonstrated that LWDHF exerts estrogen-like effects on the prevention of cardiovascular diseases in experimental rat models (15,16). The present study investigated whether LWDHF could affect the expression of ERs in VSMCs. As shown in Fig. 6, the mRNA expression levels of ERα and ERβ were significantly downregulated in VSMCs stimulated with Ang II (100 nM) compared with in untreated cells. Conversely, VSMCs treated with LWDHF and exposed to Ang II for 24 h exhibited a significant upregulation of ERα; in addition, ERβ exhibited higher expression in LWDHF-treated cells compared with in cells treated with Ang II alone. The results of western blotting were consistent those of RT-qPCR.

**Role of ERs in Ang II-induced phenotypic modulation of VSMCs.** To examine whether ERs were involved in the inhibitory effects of LWDHF on the phenotypic modulation of VSMCs, tamoxifen, a nonselective estrogen antagonist, was used to characterize the role of ER in LWDHF-mediated effects. As shown in Fig. 7A, part suppression of ER signaling by tamoxifen markedly attenuated the inhibitory effects of LWDHF on VSMC phenotypic switching; the ability of LWDHF to increase α-SMA protein expression and decrease OPN protein expression was significantly abrogated by tamoxifen. Furthermore, human aortic VSMCs (HAVSMCs) were transfected with ERα siRNA to observe the role of ERα in LWDHF-mediated inhibition of VSMC phenotypic switching. When 0.24 nM ERα siRNA was transfected into HAVSMCs, the constitutive expression of ERα was significantly reduced after 6 h (Fig. 7B), indicating that HAVSMCs were successfully transfected with this siRNA. HAVSMCs were transfected with ERα siRNA for 6 h and were then stimulated with Ang II (100 nM) for 24 h; these cells exhibited a significant reduction in α-SMA expression and a significant increase in OPN expression compared with in cells transfected with control siRNA and treated with Ang II. In addition, HAVSMCs transfected with ERα siRNA and stimulated with Ang II in the presence of LWDHF for 24 h exhibited weakened upregulated SMA expression, while

![Figure 5. Effects of LWDHF on Ang II-induced contractile-to-synthetic phenotypic switching.](image-url)

(A) mRNA expression levels of α-SMA and OPN were measured by reverse transcription-quantitative polymerase chain reaction. GAPDH was used as an internal control (n=3). (B) Western blot analysis was performed to determine the expression levels of contractile and synthetic proteins. β-tubulin was used as an internal control (n=3). (C) Histograms represent relative protein expression levels. Data are presented as the mean ± standard error of the mean. *P<0.01, **P<0.05 vs. Ang II (100 nM)-stimulated group. α-SMA, α-smooth muscle actin; Ang II, angiotensin II; LWDHF, Liuwei Dihuang formula; OPN, osteopontin; VSMC, vascular smooth muscle cell.

![Figure 6. Mice carrying the OPN transgene under the control of the SM22α promoter.](image-url)

![Figure 7. Effects of LWDHF on Ang II-induced phenotypic switching.](image-url)

(A) mRNA expression levels of α-SMA and OPN were measured by reverse transcription-quantitative polymerase chain reaction. GAPDH was used as an internal control (n=3). (B) Western blot analysis was performed to determine the expression levels of contractile and synthetic proteins. β-tubulin was used as an internal control (n=3). (C) Histograms represent relative protein expression levels. Data are presented as the mean ± standard error of the mean. *P<0.01, **P<0.05 vs. Ang II (100 nM)-stimulated group. α-SMA, α-smooth muscle actin; Ang II, angiotensin II; LWDHF, Liuwei Dihuang formula; OPN, osteopontin; VSMC, vascular smooth muscle cell.
the expression of OPN was not downregulated. The results showed that the expression of SMA and OPN was related to ERα, and the change of SMA and OPN expression were mediated by ERα (Fig. 8). Taken together, these results indicated that ERα knockdown abolished the inhibitory effects of LWDHF on Ang II-induced VSMC phenotypic switching,
and ERα may be involved in LWDHF-mediated upregulation of α-SMA.

Discussion

Our previous study investigated the effects of LWDHF on ovariectomized rats with atherosclerosis and demonstrated that LWDHF improved lipid metabolism in the serum and reduced Ang II levels through regulating ER expression, thereby producing therapeutic effects (15). In addition, our in vitro study demonstrated that LWDHF-mediated serum protected HUVECs against H2O2-induced apoptosis via ERα (16). Therefore, it may be hypothesized that LWDHF exerts preventive and therapeutic effects against atherosclerosis in menopausal women, and that ERs may mediate the effects of LWDHF. The present study provided further evidence supporting the antiatherosclerotic effects of LWDHF, indicating that LWDHF inhibited the Ang II-induced phenotypic modulation and migration of VSMCs, and that the effects of LWDHF were associated with ERs, particularly ERα.

The development of atherosclerosis- and restenosis-associated advanced lesions is highly dependent on VSMC proliferation. Therefore, inhibition of VSMC proliferation is considered a potential strategy in the prevention of atherosclerosis (7). LWDH is a commonly used traditional Chinese medicine. Therefore, inhibition of VSMC proliferation is involved in the effects of LWDHF on VSMC phenotype. In general, α-SMA is considered a biomarker of VSMCs with a contractile phenotype, whereas OPN is a biomarker of VSMCs with a synthetic phenotype (28,29). VSMC phenotypic switching is characterized by markedly increased expression of synthetic phenotype markers, alongside increased VSMC proliferation and migration. Consistent with previous observations, treatment of VSMCs with Ang II resulted in downregulation of α-SMA and OPN; these effects were suppressed by LWDHF in a concentration-dependent manner. These results confirmed the hypothesis that LWDHF may inhibit proliferation and migration of VSMCs via modulating the VSMC phenotype. In general, α-SMA is considered a biomarker of VSMCs with a contractile phenotype, whereas OPN is a biomarker of VSMCs with a synthetic phenotype (28,29). VSMC phenotypic switching is characterized by markedly increased expression of synthetic phenotype markers, alongside increased VSMC proliferation and migration. Consistent with the previous observations, treatment of VSMCs with Ang II resulted in downregulation of α-SMA and OPN; these effects were suppressed by LWDHF in a concentration-dependent manner. These results confirmed the hypothesis that LWDHF may inhibit proliferation and migration of VSMCs via modulating the VSMC phenotype. In general, α-SMA is considered a biomarker of VSMCs with a contractile phenotype, whereas OPN is a biomarker of VSMCs with a synthetic phenotype (28,29). 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Consistent with the previous observations, treatment of VSMC
exerted anti-apoptotic effects, mainly via an ERα-mediated estrogen signaling pathway (16). Therefore, to determine whether ERα mediated the inhibitory effects of LWDHF on phenotypic modulation of VSMCs, siRNA technology was used to reduce the expression of ERα in HUVSMCs, which led to a significant increase in the Ang II-induced synthetic phenotype. In addition, ERα knockdown in HUVSMCs significantly attenuated the effects of LWDHF on phenotypic modulation. LWDHF-elevated α-SMA expression was markedly reduced in HUVSMCs treated with ERα siRNA, whereas OPN expression was not markedly altered in ERα siRNA-transfected cells treated with Ang II and LWDHF compared with in control siRNA-transfected cells. These data suggested that LWDHF may upregulate α-SMA expression predominantly via ERα-mediated signaling pathways; however, another ER subtype may be responsible for LWDHF-induced downregulation of OPN.

In conclusion, the present study suggested that LWDHF possesses estrogenic properties and may mimic E2 to suppress altered in ERα and migration of VSmcs requires further research.

**References**

The authors declare that they have no competing interests.

**Acknowledgements**

The present study was supported by the National Natural Science Foundation of China (grant no. 81774029).

**Competing interests**

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**References**

1. Rivard A and Andrés V: Vascular smooth muscle cell proliferation in the pathogenesis of atherosclerotic cardiovascular diseases, Histol Histopathol 15: 557-571, 2000.

2. Lacolley P, Regnaut V, Nicoletti A, Li Z and Michel JB: The vascular smooth muscle cell in arterial pathology: a cell that can take on multiple roles. Cardiovasc Res 95: 194-204, 2012.

3. Deatrick KB, Eliasion JL, Lynch EM, Moore AJ, Dewyer NA, Varma MA, Pearce CG, Upchurch GR Jr, Wakefield TW and Henke PK: Vein wall remodeling after deep vein thrombosis involves matrix metalloproteinases and late fibrosis in a mouse model. J Vasc Surg 42: 140-148, 2005.

4. Owens GK, Kumar MS and Wamhoff BR: Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 84: 767-801, 2004.

5. Renssen SS, Doevendans PA and van Eys GJ: Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. Neth Heart J 15: 100-108, 2007.

6. Schwartz SM: Perspectives series: cell adhesion in vascular biology. Smooth muscle migration in atherosclerosis and restenosis. J Clin Invest 99: 2814-2816, 1997.

7. Doran AC, Meller N and McNamara CA: Role of smooth muscle cells in the initiation and early progression of atherosclerosis. Arterioscler Thromb Vase Biol 28: 812-819, 2008.

8. Hao H, Gabbiani G and Bochaton-Piallat ML: Arterial smooth muscle cell heterogeneity: implications for atherosclerosis and restenosis development. Arterioscler Thromb Vase Biol 23: 1510-1520, 2003.

9. Babapulle MN and Eisenberg MJ: Coated stents for the prevention of restenosis: Part I. Circulation 106: 2734-2740, 2002.

10. Zhao Y, Liu YX, Xie SL, Deng QG, Wang JF and Nie Q: Increased expression of granulocyte colony-stimulating factor mediates mesenchymal stem cells recruitment after vascular injury. Chin Med J (Engl) 124: 4286-4292, 2011.

11. Watanabe T, Pakala R, Katagiri T and Benedict CR: Synergistic effect of ursodeoxycholic acid on the liver by modulation of glucocorticoid receptor in human hepatocytes. Life Sci 80: 800-812, 2007.

12. Intengan HD and Schiffrin EL: Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis. Hypertension 38: 581-587, 2001.

13. Liu JF, Feng L, Zhang MH, Ma DY, Wang SY, Gu J, Fu Q, Qu R and Ma SP: Neuroprotective effect of Liwei Dihuang decoction on cognition deficits of diabetic encephalopathy in streptozotocin-induced diabetic rat. J Ethnopharmacol 150: 371-381, 2013.

14. Kalu DN: The ovarioctomized rat model of postmenopausal bone loss. Bone Miner 15: 175-191, 1991.

15. Wu Y, L Y and Zhang QC: The effect of LWDHF on menopausal atherosclerosis in ovarioctomized rats. Chinese Traditional Patent Medicine 34: 553-556, 2012 (In Chinese).

16. Yin QY, Guo J and Meng QH: Effects of Liuweidihuang formula mediated serum on H2O2 -injured human umbilical vascular endothelial cells. Chin Pharmacol Bull 29: 1753-1757, 2013 (In Chinese).

17. Mendelssohn ME and Karas RH: Molecular and cellular basis of cardiovascular gender differences. Science 308: 1583-1587, 2005.

18. Farhat MY, Lavigne MC and Ramwell PW: The vascular protective effects of estrogen. FASEB J 10: 615-624, 1996.

19. Kawagoe J, Ohmichi M, Tsutsumi S, Ohta T, Takahashi K and Kurachi H: Mechanism of the divergent effects of estrogen on the cell proliferation of human umbilical endothelial versus aortic smooth muscle cells. Endocrinology 148: 6092-6099, 2007.

20. Sivritas D, Becher MU, Ebrahimian T, Arla O, Sapp S, Bohner A, Mueller CF, Umemura T, Wassmann S, Nickenig G, et al: Antiproliferative effect of estrogen in vascular smooth muscle cells is mediated by Kruppel-like factor-4 and manganese superoxide dismutase. Basic Res Cardiol 106: 563-575, 2011.

21. Liu HM, Zhao XF, Guo L, Yan B and Wang TH: Effects of caveolin-1 on the 17β-estradiol-mediated inhibition of VSMC proliferation induced by vascular injury. Life Sci 80: 800-812, 2007.

22. Yang S, Zhou W, Zhang Y, Yan C and Zhao Y: Effects of Liuwei Dihuang decoction on ion channels and synaptic transmission in cultured hippocampal neuron of rat. J Ethnopharmacol 106: 194-204, 2006.

23. Rodríguez A, Fortuño A, Gómez-Ambrosi J, Balza G, Diez J and Frühbeck G: The inhibitory effect of leptin on angiotensin II-induced vasoconstriction in vascular smooth muscle cells is mediated via a nitric oxide-dependent mechanism. Endocrinology 148: 524-527, 2007.

24. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCt Method. Methods 25: 402-408, 2001.

25. Hedin U and Thiberg J: Plasma fibronectin promotes modulation of arterial smooth-muscle cells from contractile to synthetic phenotype. Differentiation 33: 239-246, 1987.

26. Rousseau S, Houle F, Région G and Huot J: p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. Circ Res 95: 2169-2177, 1999.

27. Gunst SJ and Zhang W: Actin cytoskeletal dynamics in smooth muscle: a new paradigm for the regulation of smooth muscle contraction. Am J Physiol Cell Physiol 295: C576-C587, 2008.

28. Shannahann CM, Weissberg PL and Metcalf JC: Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells. Circ Res 73: 193-204, 1993.

29. Hultgårdh-Nilsson A, Lövdahl C, Blomgren K, Kallin B and Hultgårdh-Nilsson A: Vascular remodeling in atherosclerosis and restenosis development. Arterioscler Thromb Vase Biol 34: 418-430, 1997.

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