JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Mulberry 1-Deoxynojirimycin Pleiotropically Inhibits Glucose-Stimulated Vascular Smooth Muscle Cell Migration by Activation of AMPK/RhoB and Down-regulation of FAK

Kuei-Chuan Chan,[†,§,∥] Ming-Cheng Lin,[†,§,∥] Chien-Ning Huang,[†,§] Wen-Chun Chang,[‡] and Chau-Jong Wang[#,‡,⊥]

[†]Department of Internal Medicine, Chung-Shan Medical University Hospital, No. 110, Sec. 1, Jianguo N. Road, Taichung 402, Taiwan

[‡]Institute of Biochemistry and Biotechnology, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Road, Taichung 402, Taiwan

[§]School of Medicine, Institute of Medicine, Chung-Shan Medical University, No. 110, Sec. 1, Jianguo N. Road, Taichung 402, Taiwan

[⊥]Department of Medical Research, Chung Shan Medical University Hospital, No. 110, Sec. 1, Jianguo N. Road, Taichung 402, Taiwan

ABSTRACT: Mulberry 1-deoxynojirimycin (DNJ), an inhibitor of α-glucosidase, has been reported to help prevent diabetes mellitus and suppress lipid accumulation. The aim of this study was to determine whether mulberry DNJ has pleiotropic effects on the development of atherosclerosis. The mechanisms by which mulberry DNJ might inhibit migration of A7r5 vascular smooth muscle cells (VSMCs) under hyperglycemic conditions mimicking diabetes were investigated. The antimigratory effects of DNJ on VSMCs were assessed by Western blot analysis of migration-related proteins and by electric cell-substrate impedance sensing (ECIS) and visualization of F-actin cytoskeleton. Two pathways of DNJ-mediated inhibition of VSMC migration were identified. The first involved AMPK activation to inhibit fatty acid synthase (FASN) and Akt activity and then RhoB activation to inhibit nuclear factor-κB (NF-κB) and matrix metalloproteinase-2 (MMP) activity. The second involved inhibition of focal adhesion kinase (FAK), Ras, and RhoA activity leading to inhibition of F-actin activity.

KEYWORDS: *DNJ, VSMC, migration, atherosclerosis, AMP activated protein kinase*

INTRODUCTION

Compared to the general population, diabetic patients are 2−4 times more likely to develop atherosclerosis. Many risk factors, such as hyperglycemia, hypertension, hyperlipidemia, and smoking, may cause endothelial dysfunction, which is the first step in the atherosclerotic process. Subsequently, chemotactic cytokines and adhesion molecules may attract monocytes and cause their migration into the intimal layer of the vascular wall, where they become macrophages. Thereafter, macrophages take up oxidized low-density lipoprotein (LDL) and then transition into foam cells, a key component of atherosclerotic plaque. Monocyte-derived cytokines and growth factors will further stimulate vascular smooth muscle cell (VSMC) proliferation and migration. Monocytes may also secrete matrix metalloproteinases (MMPs) to induce plaque instability and then rupture. In atherosclerosis, the VSMC contributes to vascular wall inflammation, lipoprotein retention, and fibrous cap formation (which stabilizes plaque). Upon vascular injury, VSMCs transform into a more synthetic phenotype from their normally quiescent and contractile condition, then proliferate and migrate into the intimal layer of the artery wall to induce intimal hyperplasia, which is the mechanism of restenosis after percutaneous coronary intervention (PCI).[1−4] Understanding the mechanisms underlying VSMC proliferation and migration is important for evaluating the involvement of these processes in the pathophysiology of atherosclerosis and restenosis after PCI.

Mulberry leaf and dietary mulberry leaf have been reported to have hypoglycemic, hypolipidemic, and antioxidative effects and to prevent coronary artery disease.[5−11] Mulberry 1-deoxynojirimycin (DNJ) has been reported to be the main component of mulberry leaf that prevents diabetes mellitus (DM) by inhibiting α-glucosidase in the small intestine and in postprandial hyperglycemia.[5,6,12] Intake of DNJ also suppresses lipid accumulation.[12,13] Our previous studies have shown that mulberry leaf extract can effectively inhibit proliferation and migration of VSMCs and thereby atherosclerosis by up-regulating p53, inhibiting cyclin-dependent kinase, and blocking small GTPase, Akt/NF-κB signals.[3,14] Our aim in this study was to examine whether the pleiotropic effect of mulberry DNJ on the development of atherosclerosis goes beyond antidiabetes and antihyperlipidemia effects. Thus, we investigated mechanisms underlying mulberry DNJ inhibition of VSMC migration under hyperglycemic conditions mimicking diabetes.

MATERIALS AND METHODS

Cell Culture. The cell line A7r5, a smooth muscle cell line derived from rat thoracic aorta, was obtained from the American Type Culture Collection (ATCC no. CRL-1444; Manassas, VA, USA). A7r5 cells

Received: August 16, 2013
Revised: September 19, 2013
Accepted: September 19, 2013
Published: September 19, 2013

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, USA). All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Before treatment, the cells were precultured in 0.5% FBS medium for 48 h.

Reagents. DNJ was 1-deoxynojirimycin hydrochloride (D9305; Sigma, St. Louis, MO, USA). The DNJ stock solutions were made in deionized distilled water and later diluted in media or phosphate-buffered saline (PBS) prior to use in culture. Compound C, an AMPK inhibitor, was purchased from Calbiochem (catalog no. 171260; Billerica, MA, USA). The cells were incubated in low-glucose (5.5 mM) and high-glucose (25 mM) media. High-glucose-cultured cells were treated first with 5 μM AMPK inhibitor for 30 min and then with 1.5 mM DNJ for 48 h.

Cell Viability Analysis. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[15] Cells were seeded in 24-well culture plates at a density of 2×10^4 cells/well, incubated for 48 h, treated with DNJ at various concentrations (1.0, 1.5, and 2.0 mM) for 48 h to evaluate any dose-dependent effects of DNJ on VSMC growth and viability, cultured with 0.5 mg/mL MTT at 37 °C in a humidified atmosphere of 5% CO_2 for another 4 h, and solubilized with isopropanol. The viable cell number was directly proportional to the production of formazan measured spectrophotometrically at 563 nm.

MMP Gelatin Zymography. The activities of MMP-2 and MMP-9 were assayed by gelatin zymography as described previously.[16] A7r5 cells were plated onto 6-well culture plates (5×10^5/well), incubated for 48 h, treated with DNJ at various concentrations (1.0, 1.5, and 2.0 mM) at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h, washed twice with PBS, and incubated with 1 mL of 0.1% FBS DMEM for 24 h. The culture medium was collected, centrifuged at 12000 rpm (Kubota, 2420) for 5 min at 4 °C to remove cell debris, and subjected to electrophoresis on 8% sodium dodecyl sulfate (SDS)–polyacrylamide gels containing 0.1% gelatin. Following electrophoresis, the gels were washed twice with 2.5% Triton X-100 on a gyrating shaker for 30 min at room temperature to remove SDS, incubated in 50 mL of reaction buffer (40 mM Tris-HCl, 10 mM $CaCl_2$, 0.01% NaN_3) at 37 °C overnight on a rotary shaker, stained with Coomassie Brilliant blue R-250, and destained with methanol/acetic acid/water (50:75:875, v/v/v). Gelatinolytic activities were detected as horizontal white bands on a blue background.

Wound Healing. A7r5 cells were seeded at a density of 1×10^6 mL in 6-well culture plates and incubated for 48 h. A sterile 100 μL pipet tip was used to make a straight scratch in the cell monolayer in each well.[17] The nonadhering cells were washed out with PBS, and the remaining cells were treated with DNJ (1.0, 1.5, and 2.0 mM) at 37 °C in a humidified atmosphere of 5% CO_2. Under a 40× lens, images of the linear wound were taken of nine fields per well at 0, 24, and 48 h. Migrated cells were counted per well, and the counts were averaged.

Western Blot Analysis. Western blot analysis[18] was used to assess the expressions and/or activities of these migration-related proteins and thereby the mechanisms underlying the antimigratory effects of DNJ on VSMCs. Specific antibodies were used to evaluate the expressions of Akt, Cdc42, RhoA, RhoB, Ras, FASN, NOS, p-AMPK, p-FAK, p-IκB (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), PI3-K (Becton-Dickinson, San Jose, CA, USA), and p-Akt, β-actin (Sigma, St. Louis, MO, USA). After the indicated treatment with DNJ (1.0, 1.5, or 2.0 mM) for 48 h, the cells were lysed and equal amounts of cell lysates (50 μg of protein) were separated by electrophoresis on 8–12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). 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 nonspecific binding, washed with TBST for 30 min, incubated with the appropriate primary antibody for 2 h, incubated with horseradish peroxidase-conjugated second antibody (Sigma) for 1 h, developed using ECL chemiluminescence (Millipore), and analyzed by densitometry using AlphaImager series 2200 software. Results are representative of at least three independent experiments.

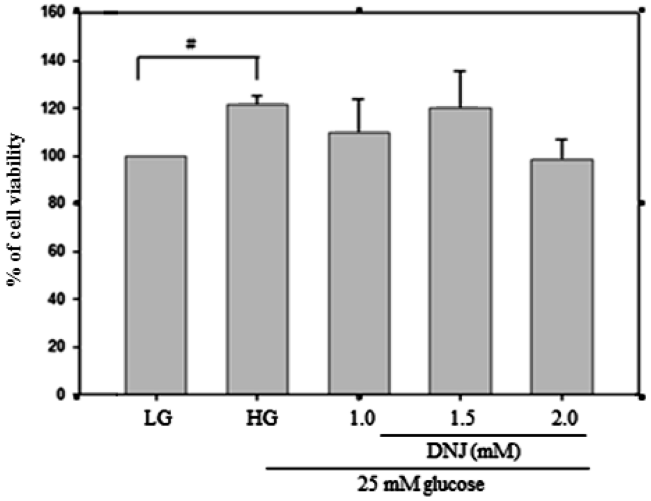

Figure 1. Effect of DNJ on viability of A7r5 VSMCs as assessed by the MTT assay. A7r5 cells were treated with different concentrations (1.0–2.0 mM) of DNJ for 48 h and then assayed using MTT. LG, low glucose (5.5 mM glucose); HG, high glucose (25 mM glucose). The data are expressed as the mean ± SD from four samples for each group (#, $p < 0.05$, as compared with the low-glucose group).

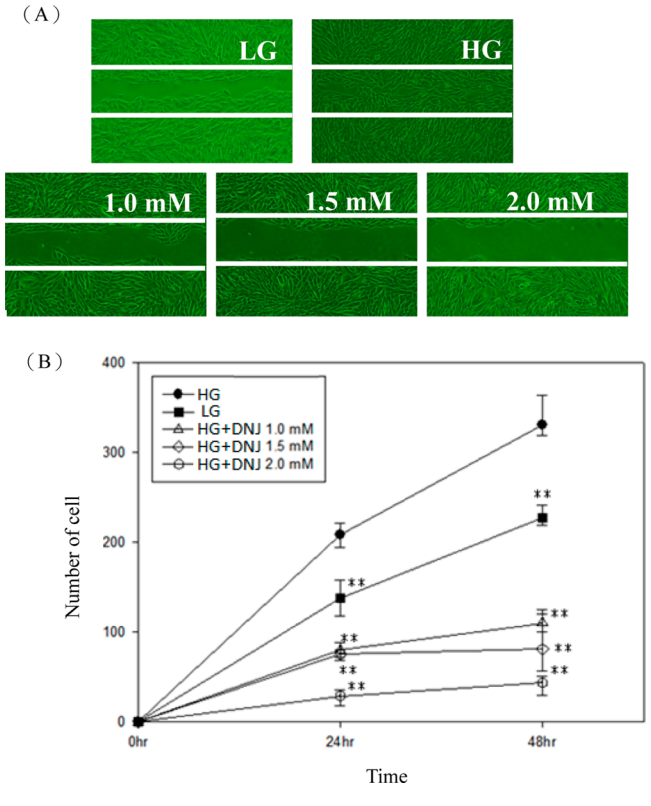

Figure 2. Effect of DNJ on migration of A7r5 cells as assessed by the wound-healing assay. The wound-healing assay was performed on cells treated with DNJ (1.0–2.0 mM). (A) Cell number in the denuded zone (i.e., wound) decreased after DNJ was added. White lines indicate the wound edge. Treatment with DNJ decreased the migration of A7r5 cells cultured in HG. (B) The mean number of cells was determined at 48 h in the denuded zone and represents the average of three independent experiments ± SD ($p < 0.005$, as compared with the control group; **, $p < 0.05$, as compared with the low-glucose group). LG, low glucose (5.5 mM glucose); HG, high glucose (25 mM glucose).

dx.doi.org/10.1021/jf403636z | J. Agric. Food Chem. 2013, 61, 9867–9875

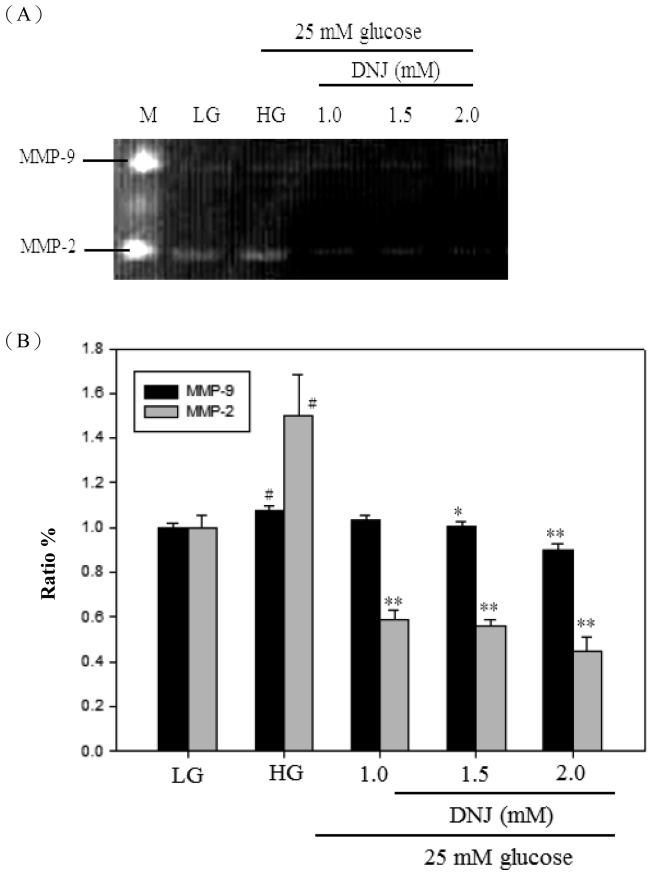

Figure 3. Effect of DNJ on MMP-9 and MMP-2 activities in A7r5 cells as assessed by the gelatin zymography assay. (A) The activities of MMP-2 and MMP-9 were inhibited by DNJ in a dose-dependent manner, but the DNJ-induced inhibitory effect of MMP-2 is more obvious. (B) Each mean value is the average of three independent experiments ± SD (#, $p < 0.05$, as compared with the low-glucose group; *, $p < 0.05$; **, $p < 0.005$, as compared with the high-glucose group). M, marker; LG, low glucose (5.5 mM glucose); HG, high glucose (25 mM glucose).

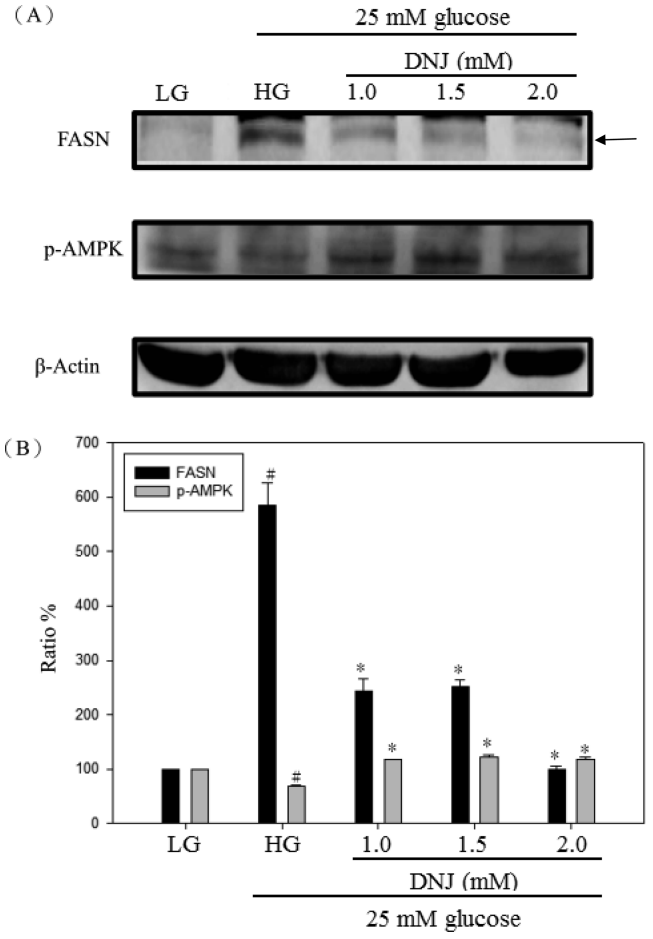

Figure 4. Effect of DNJ on the expression of FASN and phosphorylated AMPK (p-AMPK). Cultured A7r5 cells were treated with DNJ (1.0–2.0 mM) for 48 h. (A) Cell lysates (50 μg) were prepared and subjected to Western blot analysis. Proteins were detected by specific antibodies (FASN, p-AMPK), and β-actin was used as a loading control. (B) Each mean value is the average of three independent experiments ± SD (#, $p < 0.05$, as compared with the low-glucose group; *, $p < 0.005$, as compared with the control group). LG, low glucose (5.5 mM glucose); HG, high glucose (25 mM glucose).

Electric Cell-Substrate Impedance Sensing (ECIS). A commercial ECIS system (Applied Biophysics, Troy, NY, USA) was used to measure the resistance resulting from the migration[19] of A7r5 cells. In our experimental settings for cell growth modeling analysis, the cells were seeded (1×10^5/well) and cultured in eight separate wells of an ECIS 8W1E sensing chip, each well filled with culture medium. Because the interface impedance of the active electrode (for the standard electrode, ≈10 times that of the 1000 ohm series resistance) is inversely related to the electrode area, the current was proportionally reduced, causing approximately the same voltage drop across the cell layer. A7r5 monolayers plated in ECIS plates were subjected to increased voltage pulses of 64 kHz frequency, 1.25 V amplitude, and 20 s duration to create a wound. Then we changed the medium to normal (low)-glucose (5.5 mM) or high-glucose (25 mM) medium. High-glucose-cultured cells were treated with 5 μM AMPK inhibitor for 30 min and then with 1.5 mM DNJ for 55 h. A logarithmic graph of normalized resistance and normalized capacitance measurements was obtained through ECIS. The normalized values were determined by dividing the cell-covered and cell-free measurements for the corresponding frequency.

Visualization of the F-Actin Cytoskeleton. A7r5 cells were seeded in 12-well plates at a density 5×10^3/well and cultured in culture medium for 24 h, treated with 5 μM AMPK inhibitor for 30 min, treated with 1.5 mM DNJ for 48 h, fixed in 4.6% formaldehyde in PBS for 10 min, permeabilized in 0.2% Triton X-100/PBS for 5 min, stained with phalloidin-FITC (A12379; Invitrogen, Grand Island, NY, USA) to visualize polymerized F-actin microfilaments under a Nikon upright fluorescence microscope (×100 objective),[20] extensively washed with PBS, briefly counterstained with 1 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei, and photographed.

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

RESULTS

Effect of DNJ on VSMC Viability. As shown in Figure 1, A7r5 cells were seeded in low-glucose medium, cultured for 48 h, and switched to fresh low- or high-glucose medium, respectively. Thereafter, cells in high-glucose medium were treated with different DNJ concentrations (1.0, 1.5, and 2.0 mM) for 48 h, and their viability was assessed using MTT. Because DNJ (1.0, 1.5, and 2.0 mM) was not toxic, these three concentrations were used to test the effect of DNJ on A7r5 cell migration.

Effect of DNJ on Migration of A7r5 Cells. The wound-healing assay (Figure 2) and gelatin zymography assay (Figure 3) showed, respectively, that DNJ dose-dependently inhibits migration of A7r5 VSMC cells and both the MMP-2 and MMP-9

dx.doi.org/10.1021/jf403636z | J. Agric. Food Chem. 2013, 61, 9867–9875

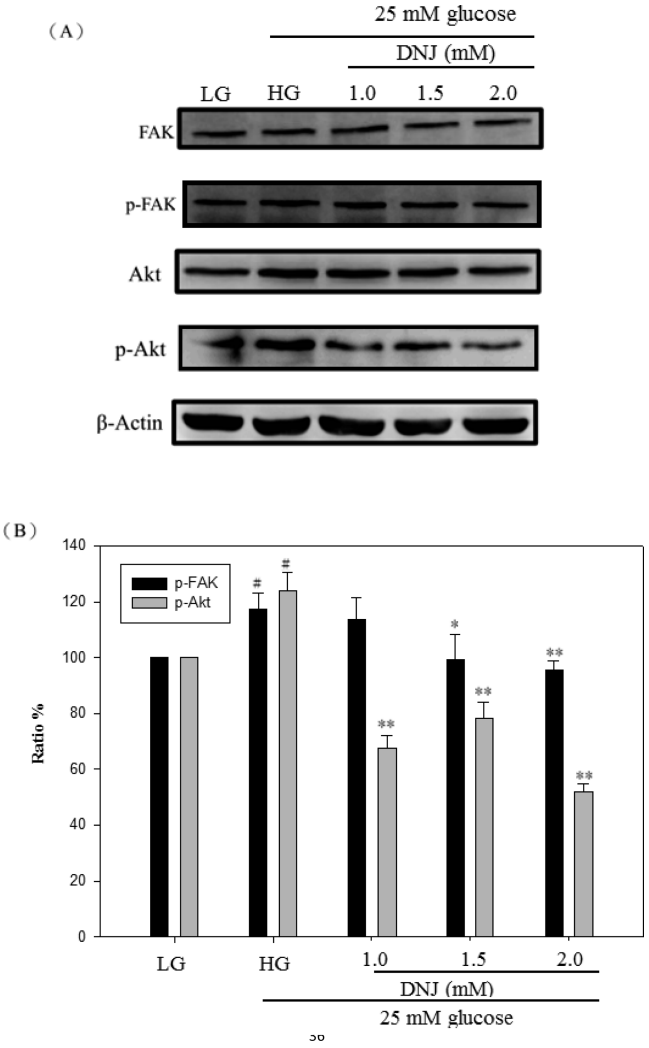

Figure 5. Effect of DNJ on the expression of phosphorylated FAK (p-FAK) and Akt in A7r5 cells. Cultured A7r5 cells were treated with DNJ (1.0–2.0 mM) for 48 h. (A) Cell lysates (50 μg) were prepared and subjected to Western blot analysis. Proteins were detected by specific antibodies (FAK, p-FAK, Akt, p-Akt), and β-actin was used as a loading control. (B) Each mean value is the average of three independent experiments ± SD (#, $p < 0.05$, as compared with the low-glucose group; ∗, $p < 0.05$; ∗∗, $p < 0.005$, as compared with the control group). LG, low glucose (5.5 mM glucose); HG, high glucose (25 mM glucose).

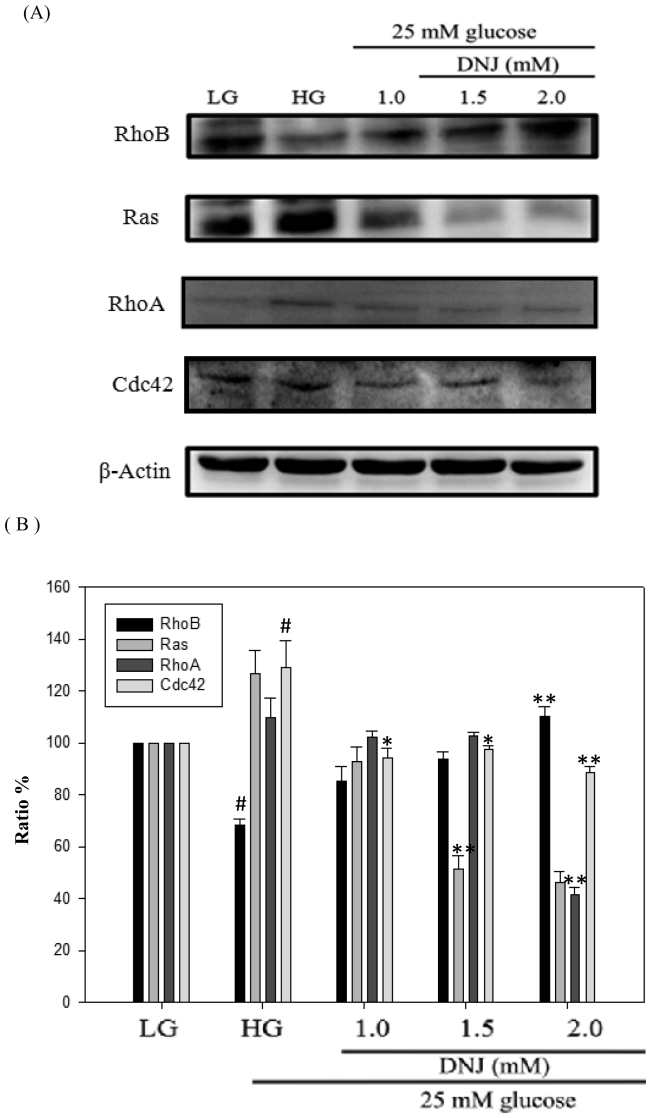

Figure 6. Effect of DNJ on the expression of small G proteins in A7r5 cells. Cultured A7r5 cells were treated with DNJ (1.0–2.0 mM) for 48 h. (A) Cell lysates (50 μg) were prepared and subjected to Western blot analysis. Proteins were detected by specific antibodies (RhoB, Ras, RhoA, Cdc42), and β-actin was used as a loading control. (B) Each mean value is the average of three independent experiments ± SD (#, $p < 0.05$, as compared with the low-glucose group; ∗, $p < 0.05$; ∗∗, $p < 0.005$, as compared with the control group). LG, low glucose (5.5 mM glucose); HG, high glucose (25 mM glucose).

activities in these cells. However, the inhibitory effect of DNJ on MMP-2 activity was more obvious. Our results therefore suggest that DNJ may inhibit MMP-2 activity ($p < 0.01$) and thereby VSMC migration.

Effect of DNJ on the Expression of FASN and Phosphorylation of AMPK, FAK, and Akt. Western blot analysis (Figure 4) revealed that DNJ can dose-dependently inhibit the expression of FASN ($p < 0.05$) and enhance the expression of p-AMPK ($p < 0.05$) in A7r5 cells, indicating that the inhibition of VSMC migration is mediated via increasing AMPK activity and decreasing FASN activity. Western blot analysis (Figure 5) showed that DNJ dose-dependently inhibits the expression of p-FAK and p-Akt in A7r5 cells, suggesting that inhibition of migration may be mediated by inhibition of FAK and Akt phosphorylation ($p < 0.05$).

Effect of DNJ on the Expression of Small G Proteins. DNJ treatment dose-dependently reduced the expression of small G proteins, including Ras, RhoA, and Cdc42, but increased the expression of RhoB (Figure 6), suggesting that treatment with DNJ may inhibit migration of VSMCs by activation of RhoB ($p < 0.05$) and inhibition of Ras, RhoA, and Cdc42 ($p < 0.05$).

Effect of AMPK Inhibitor on Signal Transduction Proteins Related to Migration of A7r5 VSMCs. The inhibition of p-AMPK and RhoB expression by treatment with AMPK inhibitor (Figure 7a,e) and the enhancement of FASN, p-Akt, and p-IκB expression by treatment with AMPK inhibitor (Figure 7b,d,g) were restored to normal levels by treatment with DNJ. As shown in Figure 7c, treatment with AMPK inhibitor did not enhance p-FAK expression, but DNJ treatment of cells treated with AMPK inhibitor significantly reduced p-FAK expression. Figure 7f shows that treatment with AMPK

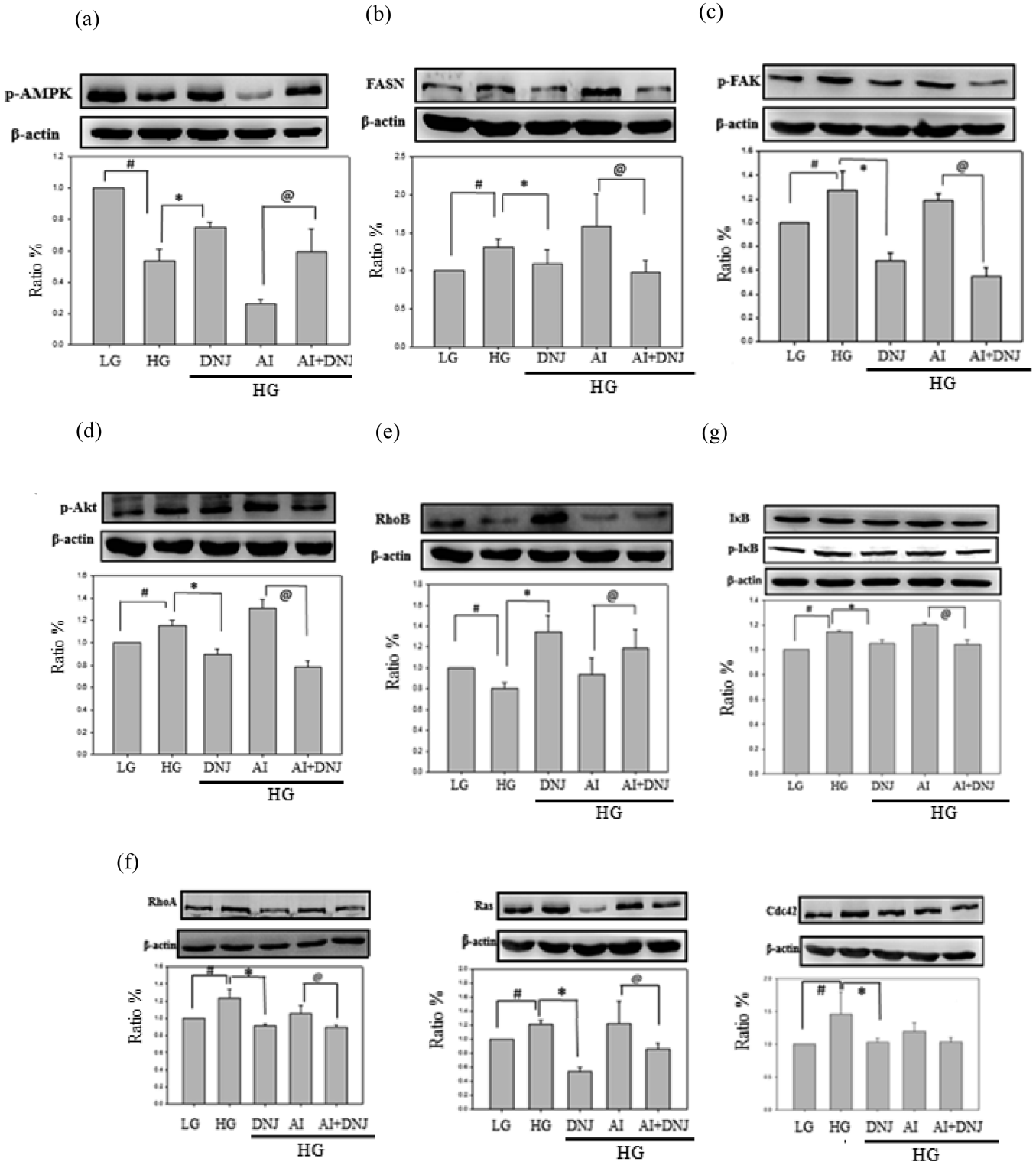

Figure 7. Effect of AMPK inhibitor on the expression of p-AMPK, FASN, p-FAK, small GTP proteins, IκB, and p-IκB in A7r5 cells. Cultured A7r5 cells were treated with AMPK inhibitor (5 μM) and DNJ (1.5 mM) for 48 h. Cell lysates (50 μg) were prepared and subjected to Western blot analysis. Proteins were detected by specific antibodies (p-AMPK, FASN, p-FAK, RhoB, RhoA, Ras, Cdc42, IκB, and p-IκB), and β-actin was used as a loading control. Each mean value is the average of three independent experiments ± SD (#, $p < 0.05$, as compared with the low-glucose group; ∗, $p < 0.05$; ∗∗, $p < 0.005$, as compared with the control group). LG, low glucose (5.5 mM glucose); HG, high glucose (25 mM glucose).

inhibitor did not enhance RhA, Ras, and Cdc42 expression, but DNJ treatment of AMPK inhibitor-treated cells inhibited RhoA and Ras, but not Cdc42 expression.

Effect of DNJ and AMPK Inhibitor on Migration of A7r5 Cells Using the ECIS Assay. Treatment with DNJ inhibited VSMC migration under low-glucose conditions as compared with hyperglycemic conditions (Figure 8). The migration of A7r5 cells was enhanced by inhibition of AMPK. Retreatment with DNJ of AMPK inhibitor-treated cells did not inhibit their migration, indicating that once AMPK has been

dx.doi.org/10.1021/jf403636z | J. Agric. Food Chem. 2013, 61, 9867−9875

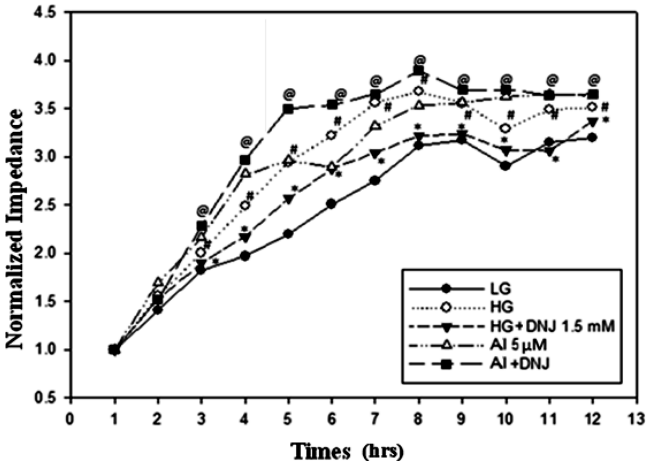

Figure 8. Effect of DNJ and AMPK inhibitor on migration of A7r5 cells. A7r5 monolayers plated in ECIS plates were subjected to increased voltage pulses of 60 kHz frequency, 4.5 V amplitude, and 20 s duration to create a wound. AMPKI (5 μM) or DNJ (1.5 mM) was added. The wound was then allowed to heal, and then resistance was measured every few seconds for 12 h (#, $p < 0.05$, as compared with the LG-treated cells; ∗, $p < 0.05$, as compared with the HG-treated cells; @, $p < 0.05$, as compared with the AI-treated cells). LG, low glucose (5.5 mM glucose); HG, high glucose (25 mM glucose); AI, AMPK inhibitor.

inhibited by AMPK inhibitor, retreatment with DNJ can no longer activate AMPK to inhibit migration of VSMCs.

Effect of DNJ on Actin Organization. Immunofluorescence microcopy of phalloidin (F-actin)- and DAPI (nuclei)-stained A7r5 cells revealed a marked increase in phalloidin staining after treatment under hyperglycemic conditions and a decrease in phalloidin staining after DNJ treatment (Figure 9). After treatment with AMPK inhibitor, phalloidin staining was increased and partially inhibited again after retreatment with DNJ. Taken together with the above results, this result demonstrates that DNJ is a positive regulator of actin organization.

DISCUSSION

VSMC migration is affected by many signal transduction proteins including AMPK, FASN, FAK, Akt/PI3K (phosphoinositide-3-kinase), the Rho family of small guanosine triphosphatases (GTPases), IκB, and MMPs. AMPK, which is a key regulator of cellular metabolism, possesses vasoprotective effects. Recent studies have shown that activation of AMPK reduces adhesion of inflammatory cells to vascular endothelium and lipid accumulation and improves blood glucose homeostasis and blood pressure, thus making this protein kinase a therapeutic target in the treatment of atherosclerosis and type 2 diabetes mellitus.[21,22] FASN is a multifunctional enzyme that regulates the synthesis of fatty acid. Because fatty acid is generally supplied by dietary fatty acid, FASN expression levels are relatively low in normal cells. In contrast, FASN is expressed at significantly higher levels in a variety of human cancers and has been shown to correlate with tumor progression and metastasis.[23] It is reported that FASN regulates proliferation and migration of colorectal cancer cells via the HER2-PI3K/Akt signaling pathway.[24] Activation of AMPK was reported to modify the mRNA expression of FASN and thereby inhibit fatty acid synthesis.[25] From our study, we find that DNJ treatment of AMPK inhibitor-treated cells resulted in restoration of p-AMPK expression inhibited by AMPK inhibitor, which means the effect of DNJ on migration of VSMCs is mediated through the AMPK pathway. DNJ treatment of AMPK inhibitor-treated cells was found to restore inhibition of the AMPK inhibitor-induced enhancement of FASN expression, which is compatible with the fact that AMPK regulates FASN. Focal adhesion kinase (FAK) plays a role in integrin signaling, which induces many intracellular events, including rearrangement of the actin cytoskeleton and cell migration. Increasing FAK expression will stimulate cell migration.[26,27] PI3K signaling was found to mediate cellular proliferation and migration.[28] The Akt kinase, an important component of postsurvival signaling pathways, is activated via the PI3K pathway.[29] In our study, treatment with AMPK inhibitor did not enhance p-FAK expression, which means AMPK is not a regulator of FAK. DNJ treatment of AMPK inhibitor-treated cells significantly reduced p-FAK expression, which means DNJ can directly inhibit FAK expression without acting on AMPK. DNJ treatment of AMPK inhibitor-treated cells restored the inhibition of the AMPK inhibitor-induced increase in p-Akt expression, which means AMPK acts upstream of Akt. Rho-family small GTPases (including Rho, Rac, and Cdc42) act as molecular switches to regulate the actin cytoskeleton. RhoA was reported to have a central role in cytoskeletal regulation. Rho and Rho kinase were also found to mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration.[30−32] Increased expression of RhoB and a block of the Ras/Akt signal were reported to be related to VSMC migration.[2] The Rho family of GTPases is related to the regulation of NF-κB-dependent transcription. NF-κB is activated by RhoA and Ras-related small GTPases (Rac, Cdc42) and inhibited by RhoB.[33,34]

In our study, treatment with DNJ restored the expression of RhoB inhibited by AMPK inhibitor and the expressions of RhoA, Ras, and Cdc42 are not enhanced by treatment with AMPK inhibitor, which means that AMPK is not a regulator of RhoA, Ras, and Cdc42 expression. DNJ treatment of AMPK inhibitor-treated cells inhibited the expressions of RhoA and Ras, but not Cdc42, which means DNJ inhibits VSMC migration by inhibiting the expression of RhoA and Ras. Our study also demonstrates that DNJ increases the activity of AMPK activity, thereby regulating the activity of RhoB, but not RhoA or Ras, which in turn inhibits VSMC migration. DNJ can also inhibit VSMC migration by inhibiting the activities of RhoA and Ras. The increase in p-IκB expression caused by AMPK inhibitor is reversed by DNJ treatment, which means AMPK acts upstream of IκB. VSMC migration after vascular injury in vivo may be related to increase in MMP-2 activity, and antisera to MMP-2 have been found to inhibit in vitro migration of VSMCs.[35] NF-κB plays a central role in inflammation, which is related to atherosclerosis and restenosis after PCI, as well as angiogenesis.[36] NF-κB regulates angiogenesis via the induction of MMPs.[37] The transcription factor NF-κB is controlled by inhibitory IκB proteins. NF-κB is retained in the cytoplasm by its inhibitor IκB. Upon cellular stimulation, IκB is functionally inactivated and NF-κB translocates to the nucleus to trigger transcription of genes.[38,39] According to the results of previous and our present studies, the mechanism of DNJ to inhibit VSMC migration is related to inhibition of MMP-2 by way of activation of AMPK and inhibition of NF-κB activity.

Clinically, we often use acarbose, an inhibitor of α-glucosidase, to control postprandial hyperglycemia in diabetes, which is an important cardiovascular risk factor. It was reported that acarbose could rapidly stabilize carotid plaque within 1 month of therapy in patients with acute coronary syndrome and type 2 DM. Acarbose might reduce oxidative stress by reducing postprandial

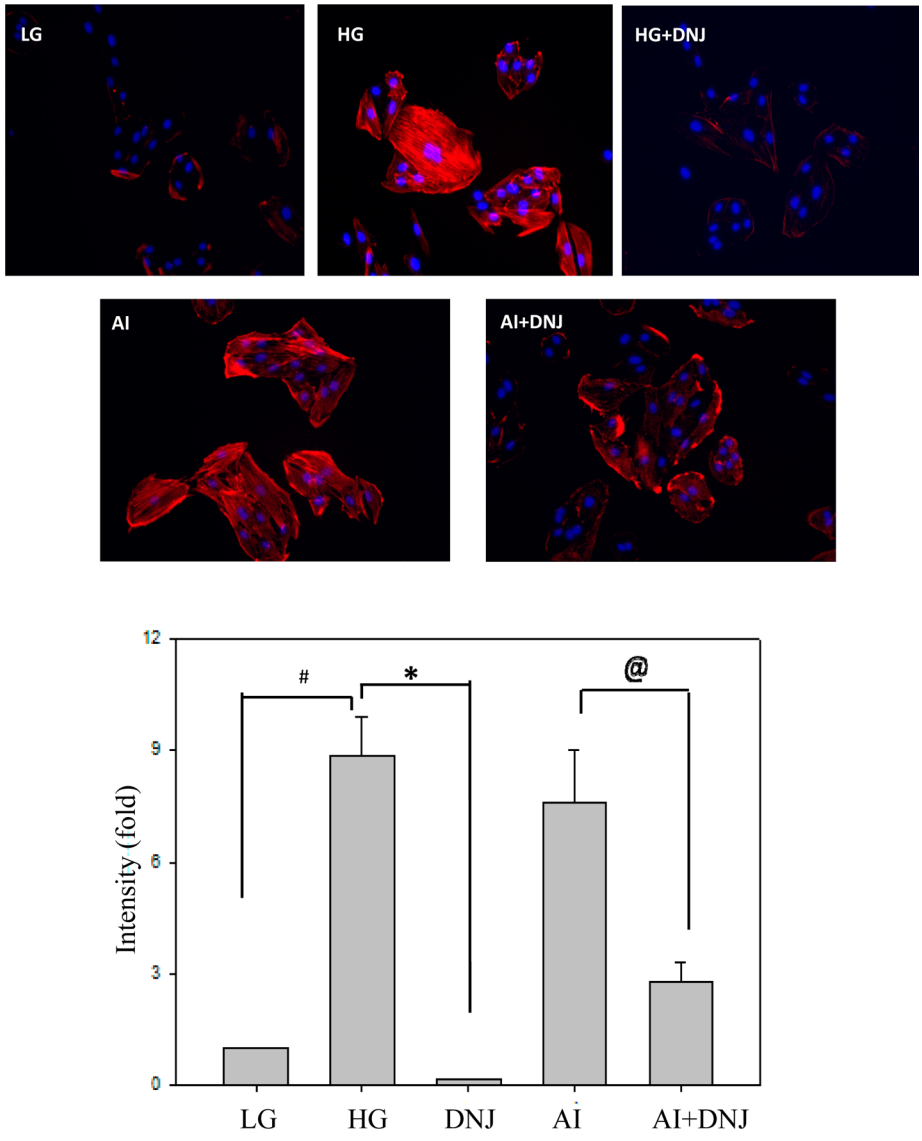

Figure 9. Effect of DNJ on actin organization. Immunofluorescence microscopy of A7r5 cells stained with phalloidin (F-actin) and DAPI (nuclei) showed a marked increase in phalloidin staining after treatment with high glucose (25 mM) and a decrease in phalloidin staining after treatment with DNJ (#, $p < 0.05$, as compared with the LG-treated cells; *, $p < 0.05$, as compared with the DNJ-treated cells; @, $p < 0.05$, as compared with the AMPK inhibitor-treated cells). LG, low glucose (5.5 mM glucose); HG, high glucose (25 mM glucose).

hyperglycemia.[40] Our previous studies have shown that mulberry leaf extract can effectively inhibit proliferation and migration of VSMCs, thereby preventing atherosclerosis. Our present study is the first to demonstrate that DNJ pleiotropically inhibits VSMC migration. This effect is unrelated to the antidiabetic effect of DNJ. Previous and our present studies indicate that DNJ inhibits VSMC migration via two pathways. DNJ can inhibit VSMC migration by activating AMPK (which in turn inhibits FASN and Akt activities, then activates RhoB, and finally inhibits NF-κB and MMP-2 activities). Another pathway is through inhibition of F-actin by way of inhibition of FAK, Ras, and RhoA activities. Besides, our previous studies have shown that polyphenol-rich mulberry leaf extract can effectively inhibit proliferation and migration of VSMCs, thus preventing atherosclerosis. This time, we find that mulberry DNJ also has inhibitory effect on migration of VSMCs. Therefore, the antiatherosclerotic effect of mulberry leaf may come from both polyphenol and DNJ.

In summary, this in vitro study has demonstrated the molecular mechanisms by which mulberry leaf DNJ effectively inhibits migration of VSMCs, including activation of AMPK/RhoB and down-regulation of FAK. Further in vivo and clinical studies may be needed to prove this pleiotropic effect, and such an in vivo study is ongoing in our laboratory.

AUTHOR INFORMATION

Corresponding Author

#(C.-J.W.) Mailing address: Institute of Biochemistry and Biotechnology, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Road, Taichung 402, Taiwan. Phone: 886-4-24730022, ext. 11670. Fax: 886-4-23248167. E-mail: wcj@csmu.edu.tw.

Author Contributions

‖K.-C.C. and M.-C.L. contributed equally to this work and therefore share first authorship.

Funding

This work was supported by a research fund from Chung Shan Medical University Hospital, Taichung, Taiwan (CSH-2013-C-005), and the National Science Council, Taiwan (NSC 102-2313-B-040-003-MY3).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AMPK, AMP-activated protein kinase; DM, diabetes mellitus; DMEM, Dulbecco's modified Eagle's medium; DNJ, 1-deoxynojirimycin; DAPI, 4′,6-diamidino-2-phenylindole; ECIS, electric cell-substrate impedance sensing; FASN, fatty acid synthase; FAK, focal adhesion kinase; FBS, fetal bovine serum; GTPase, guanosine triphosphatase; LDL, low-density lipoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PCI, percutaneous coronary intervention; PI3K, phosphoinositide-3-kinase; TBS, tris-buffered saline; VSMC, vascular smooth muscle cell

■ REFERENCES

(1) Beckman, J. A.; Creager, M. A.; Libby, P. Diabetes and atherosclerosis. *JAMA: J. Am. Med. Assoc.* **2002**, *287*, 2570−2581.

(2) Chan, K. C.; Wu, C. H.; Huang, C. N.; Lan, K. P.; Chang, W. C.; Wang, C. J. Simvastatin inhibits glucose-stimulated vascular smooth muscle cell migration involving increased expression of RhoB and a block of Ras/Akt signal. *Cardiovasc. Ther.* **2012**, *30*, 75−84.

(3) Chan, K.-C.; Ho, H.-H.; Peng, C.-H.; Lan, K.-P.; Lin, M.-C.; Chen, H.-M.; Wang, C.-J. Polyphenol-rich extract from mulberry leaf inhibits vascular smooth muscle cell proliferation involving upregulation of p53 and inhibition of cyclin-dependent kinase. *J. Agric. Food Chem.* **2010**, *58*, 2536−2542.

(4) Dzau, V. J.; Braun-Dullaeus, R. C.; Sedding, D. G. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat. Med.* **2002**, *8*, 1249−1256.

(5) Asano, N.; Yamashita, T.; Yasuda, K.; Ikeda, K.; Kizu, H.; Kameda, Y.; Kato, A.; Nash, R. J.; Lee, H. S.; Ryu, K. S. Polyhydroxylated alkaloids isolated from mulberry trees (*Morus alba* L.) and silkworms (*Bombyx mori* L.). *J. Agric. Food Chem.* **2001**, *49*, 4208−4213.

(6) Kimura, T.; Nakagawa, K.; Kubota, H.; Kojima, Y.; Goto, Y.; Yamagishi, K.; Oita, S.; Oikawa, S.; Miyazawa, T. Food-grade mulberry powder enriched with 1-deoxynojirimycin suppresses the elevation of postprandial blood glucose in humans. *J. Agric. Food Chem.* **2007**, *55*, 5869−5874.

(7) El-Beshbishy, H. A.; Singab, A. N. B.; Sinkkonen, J.; Pihlaja, K. Hypolipidemic and antioxidant effects of *Morus alba* L. (Egyptian mulberry) root bark fractions supplementation in cholesterol-fed rats. *Life Sci.* **2006**, *78*, 2724−2733.

(8) Isabelle, M.; Lee, B. L.; Ong, C. N.; Liu, X.; Huang, D. Peroxyl radical scavenging capacity, polyphenolics, and lipophilic antioxidant profiles of mulberry fruits cultivated in southern China. *J. Agric. Food Chem.* **2008**, *56*, 9410−9416.

(9) Kojima, T.; Fujimoto, Y. Mulberry leaf extract inhibits the oxidative modification of rabbit and human low density lipoprotein. *Biol. Pharm. Bull.* **2000**, *23*, 1066−1071.

(10) Andallu, B.; Suryakantham, V.; Lakshmi Srikanthi, B.; Kesava Reddy, G. Effect of mulberry (*Morus indica* L.) therapy on plasma and erythrocyte membrane lipids in patients with type 2 diabetes. *Clin. Chim. Acta* **2001**, *314*, 47−53.

(11) Andallu, B.; Varadacharyulu, N. C. Antioxidant role of mulberry (*Morus indica* L. cv. Anantha) leaves in streptozotocin-diabetic rats. *Clin. Chim. Acta* **2003**, *338*, 3−10.

(12) Kojima, Y.; Kimura, T.; Nakagawa, K.; Asai, A.; Hasumi, K.; Oikawa, S.; Miyazawa, T. Effects of mulberry leaf extract rich in 1-deoxynojirimycin on blood lipid profiles in humans. *J. Clin. Biochem. Nutr.* **2010**, *47*, 155.

(13) Tsuduki, T.; Nakamura, Y.; Honma, T.; Nakagawa, K.; Kimura, T.; Ikeda, I.; Miyazawa, T. Intake of 1-deoxynojirimycin suppresses lipid accumulation through activation of the β-oxidation system in rat liver. *J. Agric. Food Chem.* **2009**, *57*, 11024−11029.

(14) Chan, K.-C.; Ho, H.-H.; Huang, C.-N.; Lin, M.-C.; Chen, H.-M.; Wang, C.-J. Mulberry leaf extract inhibits vascular smooth muscle cell migration involving a block of small GTPase and Akt/NF-κB signals. *J. Agric. Food Chem.* **2009**, *57*, 9147−9153.

(15) Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.* **1988**, *48*, 4827−4833.

(16) Chu, S. C.; Chiou, H. L.; Chen, P. N.; Yang, S. F.; Hsieh, Y. S. Silibinin inhibits the invasion of human lung cancer cells via decreased productions of urokinase-plasminogen activator and matrix metalloproteinase-2. *Mol. Carcinog.* **2004**, *40*, 143−149.

(17) Winter, G. Epidermal regeneration studied in the domestic pig. *Epidermal Wound Healing* **1972**, 71−112.

(18) Burnette, W. N. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **1981**, *112*, 195−203.

(19) Wegener, J.; Keese, C. R.; Giaever, I. Electric cell−substrate impedance sensing (ECIS) as a noninvasive means to monitor the kinetics of cell spreading to artificial surfaces. *Exp. Cell Res.* **2000**, *259*, 158−166.

(20) Bubb, M. R.; Senderowicz, A.; Sausville, E. A.; Duncan, K.; Korn, E. D. Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J. Biol. Chem.* **1994**, *269*, 14869−14871.

(21) Ewart, M.-A.; Kennedy, S. AMPK and vasculoprotection. *Pharmacol. Ther.* **2011**, *131*, 242−253.

(22) Viollet, B.; Lantier, L.; Devin-Leclerc, J.; Hébrard, S.; Amouyal, C.; Mounier, R.; Foretz, M.; Andreelli, F. Targeting the AMPK pathway for the treatment of type 2 diabetes. *Front. Biosci.* **2009**, *14*, 3380.

(23) Okawa, Y.; Hideshima, T.; Ikeda, H.; Raje, N.; Vallet, S.; Kiziltepe, T.; Yasui, H.; Enatsu, S.; Pozzi, S.; Breitkreutz, I. Retraction: Fatty acid synthase is a novel therapeutic target in multiple myeloma. *Br. J. Haematol.* **2008**, *141*, 659−671.

(24) Li, N.; Bu, X.; Tian, X.; Wu, P.; Yang, L.; Huang, P. Fatty acid synthase regulates proliferation and migration of colorectal cancer cells via HER2-PI3K/Akt signaling pathway. *Nutr. Cancer* **2012**, *64*, 864−870.

(25) McFadden, J. W.; Corl, B. A. Activation of AMP-activated protein kinase (AMPK) inhibits fatty acid synthesis in bovine mammary epithelial cells. *Biochem. Biophys. Res. Commun.* **2009**, *390*, 388−393.

(26) Cary, L.; Han, D.; Guan, J. Integrin-mediated signal transduction pathways. *Histol. Histopathol.* **1999**, *14*, 1001−1009.

(27) Cary, L. A.; Chang, J. F.; Guan, J.-L. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *J. Cell Sci.* **1996**, *109*, 1787−1794.

(28) Goncharova, E. A.; Ammit, A. J.; Irani, C.; Carroll, R. G.; Eszterhas, A. J.; Panettieri, R. A.; Krymskaya, V. P. PI3K is required for proliferation and migration of human pulmonary vascular smooth muscle cells. *Am. J. Physiol.−Lung Cell. Mol. Physiol.* **2002**, *283*, L354−L363.

(29) Khwaja, A. Apoptosis: Akt is more than just a Bad kinase. *Nature* **1999**, *401*, 33−34.

(30) Takai, Y.; Sasaki, T.; Tanaka, K.; Nakanishi, H. Rho as a regulator of the cytoskeleton. *Trends Biochem. Sci.* **1995**, *20*, 227−231.

(31) Van Aelst, L.; D'Souza-Schorey, C. Rho GTPases and signaling networks. *Genes Dev.* **1997**, *11*, 2295−2322.

(32) Seasholtz, T. M.; Majumdar, M.; Kaplan, D. D.; Brown, J. H. Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. *Circ. Res.* **1999**, *84*, 1186−1193.
(33) Perona, R.; Montaner, S.; Saniger, L.; Sanchez-Perez, I.; Bravo, R.; Lacal, J. C. Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. *Genes Dev.* **1997**, *11*, 463−475.
(34) Fritz, G.; Kaina, B. Ras-related GTPase RhoB represses NF-κB signaling. *J. Biol. Chem.* **2001**, *276*, 3115−3122.
(35) Van Leeuwen, R. Extracellular proteolysis and the migrating vascular smooth muscle cell. *Fibrinolysis* **1996**, *10*, 59−74.
(36) de Martin, R.; Hoeth, M.; Hofer-Warbinek, R.; Schmid, J. A. The transcription factor NF-κB and the regulation of vascular cell function. *Arteriosclerosis, Thrombosis, Vasc. Biol.* **2000**, *20*, e83−e88.
(37) Bond, M.; Fabunmi, R. P.; Baker, A. H.; Newby, A. C. Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-κB. *FEBS Lett.* **1998**, *435*, 29−34.
(38) Beg, A. A.; Sha, W. C.; Bronson, R. T.; Baltimore, D. Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. *Genes Dev.* **1995**, *9*, 2736−2746.
(39) Lin, Y.-C.; Brown, K.; Siebenlist, U. Activation of NF-kappa B requires proteolysis of the inhibitor I kappa B-alpha: signal-induced phosphorylation of I kappa B-alpha alone does not release active NF-kappa B. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 552−556.
(40) Hirano, M.; Nakamura, T.; Obata, J. E.; Fujioka, D.; Saito, Y.; Kawabata, K.; Watanabe, K.; Watanabe, Y.; Kugiyama, K. Early improvement in carotid plaque echogenicity by acarbose in patients with acute coronary syndromes. *Circ. J.* **2012**, *76*, 1452−1460.