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Basic Science

Inhibition of Notch Signaling Alleviated Diabetic Macrovasculopathy in an In Vitro Model

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Background: Interactions between endothelial cells and vascular smooth muscle cells (VSMCs) through the Notch signal pathway causing diabetic microvasculopathy have been reported.

Objectives: The purpose of this study was to investigate whether the effect of high glucose on VSMCs through the Notch-2 signaling pathway could induce extracellular matrix (ECM) accumulation, VSMC proliferation and migration and thus directly mediate diabetic macrovasculopathy.

Methods: Rat smooth muscle cells (SV40LT-SMC Clone HEP-SA cells) were cultured in different concentrations of D-glucose to evaluate the impact of high glucose on ECM accumulation including fibronectin and collagen I measured by Western blot analysis, and on VSMC proliferation and migration evaluated by MTT assay and wound healing assay. The expression of Notch-2 intra-cellular domain (Notch-2 ICD) protein was also checked in high glucose-stressed VSMCs. N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT), an inhibitor of γ-secretase, was used to modulate the Notch-2 signaling pathway.

Results: High glucose (D-glucose 25 mM) induced fibronectin and collagen I expressions in VSMCs, promoted VSMC proliferation/migration, and enhanced the expression of Notch-2 ICD. DAPT inhibited Notch-2 signal to abolish the expressions of fibronectin and collagen I in VSMCs, and also prevented the proliferation/migration of VSMCs under high glucose (D-glucose 25 mM) stress.

Conclusions: Our study suggests that high glucose can enhance the Notch-2 signaling pathway thereby directly mediating diabetic macrovasculopathy. Blocking the Notch-2 signaling pathway decreased fibronectin and collagen I expressions secreted by VSMCs, and reduced the proliferation and migration of VSMCs under high glucose stress. Inhibition of Notch-2 signaling represents a promising target for treating diabetic macrovasculopathy.

Key Words: Diabetic mellitus • High glucose • Macrovasculopathy • Notch • Vascular smooth muscle cell

INTRODUCTION

Diabetic vasculopathy includes macrovasculopathy and microvasculopathy. Diabetic microvasculopathy mediates aberrant angiogenesis and affects capillary basement membrane including arterioles in the retina, glomeruli and myocardium. In contrast, the major pathologic changes in diabetic macrovasculopathy are vascular smooth muscle cell (VSMC) proliferation and migration combined with extracellular matrix (ECM) accumulation. Consequently, individuals with diabetes mellitus (DM) have a high prevalence of atherosclerosis, multivessel coronary artery disease (CAD), stroke, myocardial

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infarction (MI), and peripheral artery obstructive disease (PAOD) compared with the general population.[1,2] According to previous clinical studies, patients with DM had a 2- to 4-fold greater risk of developing CAD[3] and 2- to 4-fold increased risk of PAOD.[4] The Copenhagen Heart Study also reported a 2- to 3-fold increased risk of MI or stroke in patients with DM and a 2-fold higher risk of death independently of other risk factors for cardiovascular disease. Clearly, DM and its macrovascular complications impose a considerable socio-economic burden. Therefore, preventative strategies are of paramount importance in reducing the incidence of macrovasculopathy in patients with DM.

VSMCs play important roles in determining the caliber of blood vessels, regulating blood pressure and local tissue perfusion. They retain plasticity throughout life, enabling marked transitions away from contractile behavior to motility, invasion and proliferation in the pathogenesis of vascular diseases. The determination of VSMC fate (growth, migration, differentiation, and apoptosis) is fundamental to the pathogenesis of vascular diseases. The pathogenesis of macrovascular diseases involves abnormal migration and proliferation of VSMCs,[5-7] and ECM proteins secreted by VSMC accumulation.[8] An in vitro study showed that photodynamic therapy using indocyanine green could prevent VSMC proliferation to avoid macrovasculopathy.[9]

The pathologic molecular mechanisms of VSMCs in diabetic macrovasculopathy are unclear, and new therapeutic strategies via manipulation of VSMC fate to ameliorate the outcome of progressive diabetic macrovasculopathy have yet to be elucidated. Better understanding the pathological changes of VSMCs under high glucose may lead to new therapeutic strategies for diabetic macrovasculopathy.

The Notch receptors are membrane-bound receptors which regulate binary cell fate determination and embryonic development.[10] Notch signaling has been shown to determine proliferation, differentiation, and more recently, apoptosis in several mammalian cell types including VSMCs. Notch-1, Notch-2, and Notch-3 signaling pathways have been shown in VSMCs.[11] Jagged-1, a Notch ligand, on the cell surface interacts with Notch receptors in adjacent cells resulting in a series of sequential proteolytic cleavage events of the Notch receptor by proteases, metalloproteases, and γ-secretase. The resulting Notch intracellular domain migrates into the nucleus and binds to signal sequence-binding protein leading to transcriptional activation and cell fate determination.[12,13] The Notch signaling pathway has been implicated in the pathogenesis of vascular diseases through interactions between Jagged-1 and Notch receptors, as well as in the embryonic development of the vasculature. Recent in vitro studies have suggested that the Notch signaling pathway may play an important role in regulating VSMC proliferation[7] and dedifferentiation.[14] A vascular injury model indicated that Notch signaling mediates the proliferation of VSMCs and neointimal formation following vascular injury, thereby mediating interactions between endothelial cells (ECs) and VSMCs.[15] In addition, a high glucose microvasculopathy model showed aberrant angiogenesis through Jagged-1 ligated Notch receptors between ECs and VSMCs.[16] In vitro studies of both primary VSMCs cultured from the thoracic aorta of normal Sprague-Dawley (SD) rats[17,18] and A-10 VSMCs derived from the thoracic aorta of Rattus norvegicus[9] have shown that high glucose can induce the proliferation and migration of VSMCs. However, whether high glucose induces macrovasculopathy directly through the Notch signaling pathway has not yet been reported. The aims of the present study were to investigate the role of the Notch signaling pathway in diabetic macrovasculopathy, and whether or not modulating Notch signaling could reduce diabetic macrovasculopathy.

METHODS

Materials

Dulbecco's Modified Eagle's Medium (DMEM), 10% bovine calf serum, Trypsin ethylenediaminetetraacetic acid (EDTA) solution, and phosphate-buffered saline (PBS) were purchased from GIBCO (Gaithersburg, MD, USA). Extraction buffer (Cellytic M mammalian cell lysis extraction reagent), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and umol/l N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenyl-glycine t-butyl ester (DAPT) were purchased from Sigma (St. Louis, MO, USA). Antibodies specific for Notch-2 ICD and Actin were purchased from Cell Signaling (Beverly, MA, USA). Antibodies specific for fibronectin and collagen I were purchased from Abcam (Cambridge, UK).

Vascular smooth muscle cell culture

Rat smooth muscle cells (SV40LT-SMC Clone HEP-SA cells) which were cultured from the thoracic aorta of SD rats and had maintained defined VSMCs characteristics[19] were purchased from FIRDI (Food Industry Research and Development Institute, Taiwan) and cultured in DMEM medium containing 10% bovine calf serum, 1% penicillin/geneticin and 4 mM L-glutamine. Cells were growth at 37 °C in a humidified atmosphere of 5% carbon dioxide. The growth medium was changed every 2-3 days until the cells had reached confluence. Then the growth medium was removed, and the monolayer of cells was rinsed with PBS. A trypsin-EDTA solution was added, and the monolayer was incubated at 37 °C for 2.5 minutes until the cells had detached. The cells were removed with 10 ml of DMEM and centrifuged at 1200 rpm for 7 min. The pellets were resuspended in DMEM in culture dishes. For each experiment, an equal amount of cells were cultured in each culture dish. Prior to any treatment, the cells were starved by changing the medium containing 1% bovine calf serum and left overnight.

Protein extraction and Western blot analysis

Cytosolic extracts were harvested by lysing cells with buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.05% IGEPAL CA630 (Sigma) for 10 min at 4 °C and centrifuging at 200 × g for 5 min at 4 °C, and then supernatants were harvested. The pellets were further lysed with buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 1% sodium dodecyl sulfate (SDS) and sonicated to break deoxyribonucleic acid (DNA). Protein concentrations in cytosolic and nuclear extracts were determined using a thermo protein assay kit.

Lysates from each sample were mixed with 2x sample buffer (0.125 M Tris HCl, 4% w/v SDS, 20% v/v glycerol, 10% 2-mercaptoethanol, 0.004% w/v bromophenol blue, pH 6.8) and heated to 95 °C for 5 min. Protein was separated by electrophoresis and transferred onto poly-vinylidene difluoride membranes for fibronectin, collagen I, Notch-2 full length, Notch-2 ICD, and actin. The membranes were then blocked with 5% non-fat milk in TBS-0.1% Tween 20, and sequentially incubated with primary antibodies and HRP-conjugated secondary antibodies, followed by enhanced chemiluminescence (ECL) detection (Advansta). For quantitative densitometric analysis, BIO-PROFIL Bio 1D light analytical software (Vilber Lourmat, Marue La Vallee, France) was used. Image J software was used to quantify the intensity of bands and to decrease the background noise of the Western blot results according to the manufacturer's instructions. The data of specific protein levels were presented as relative multiples in relation to the control.

MTT assay

The details have been described previously.[20] Briefly, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-disphenyl tetrazolium bromide) assay was used to measure the proliferation of VSMCs at 24 h, 48 h and 72 h of incubation with high glucose concentrations (D-glucose; 15 mM, 25 mM and 35 mM) and in a control group (D-glucose 5 mM). Initially, VSMCs were cultured in 1% bovine calf serum-DMEM (Gibco) and seeded on 96-well plates (5×10^3 cell/well) for 24 h. Then, the VSMCs were treated with different D-glucose concentrations (15 mM, 25 mM, 35 mM) for 48 h. In the DAPT study, the VSMCs were also cultured in 1% bovine calf serum-DMEM (Gibco) and seeded on 96-well plates (5×10^3 cell/well) for 24 h. Different concentrations of DAPT, an inhibitor of γ-secretase (0.01 μM, 0.05 μM, 0.1 μM; Calbiochem, Gibbstown, NJ) were then used to treat the cells for 2 h before high D-glucose treatment. After aspiration of supernatants, the VSMCs were treated with the indicated concentrations of high D-glucose (25 mM) for 48 h.

Finally, to measure the proliferation of VSMCs, the cells were seeded in 96-well plates and incubated with 10 μl of 5 mg/ml MTT at 37 °C for 4 h, washed with 100 μl cold PBS, and lysed with 80 μl of DMSO (Sigma). After the formazan crystals had been completely dissolved, the optical density of each well was immediately measured at 570 nm using an automatic micro-plate reader (Thermo / LabSystems Multiskan RC Microplate Reader).

Wound healing assay

Cell migration was measured by wound healing assay.[21] VSMCs were grown in culture-inserts (Ibidi, Munich, Germany) attached on 6-well plates with DMEM supplemented with 10% bovine calf serum. After the VSMCs reached confluence, the culture-inserts were gently removed and subsequently induced by high glucose (25 mM D-glucose) in the absence or presence of 0.5 μm DAPT with DMEM supplemented with 0.3% bovine calf serum. Photographs were taken at 0, 24, 36

and 48 h after wound healing.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 statistical software (SPSS, Chicago, IL, USA). All values were expressed as mean ± standard deviation from at least three independent experiments. Wilcoxon's test was used to evaluate differences between the sample and its respective control. p values < 0.05 were considered to be significant.

RESULTS

High glucose induced fibronectin and collagen I expression in cultured vascular smooth muscle cells

To determine the effect of high glucose on fibronectin and collagen I expressions of rat VSMCs, the cells were cultured with different high concentrations of D-glucose (15, 25, 35 mM). In normal control cells, 5 mM D-glucose was maintained in the culture medium to provide the basal requirement for cell growth. Additionally, mannitol served as an osmotic control for high glucose. After culturing for 48 h, the cell lysate was prepared and subjected to Western immunoblotting using anti-fibronectin and anti-collagen I antibodies. As illustrated in Figure 1(A), the expressions of fibronectin and collagen I in rat VSMCs cultured with high glucose (D-glucose 25 mM) were higher than in normal control rat VSMCs. To determine the effect of time of high glucose treatment on fibronectin and collagen I expressions in rat VSMCs, the cells were cultured with high glucose (D-glucose 25 mM) for 24 h and 48 h. In normal control cells group, the cells were also cultured for 24 h and 48 h. As illustrated in Figure 1(B), the expressions of fibronectin and collagen I in rat VSMCs cultured with high glucose (D-glucose 25 mM) at 48 h were higher than at 24 h. These results indicated high expressions of fibronectin and collagen I in the rat VSMCs cultured with high glucose (D-glucose 25 mM).

High glucose induced proliferation and migration of cultured vascular smooth muscle cells

An MTT assay was used to determine the effect of high glucose on the proliferation of rat VSMCs. Cell number of rat VSMCs cultured with D-glucose 25 mM for 48 h were higher than the rat VSMCs growth arrest at 72 h (Figure S1). The cells were also cultured in medium containing different high concentrations of D-glucose (15, 25, 35 mM). In the control study group, the culture me-

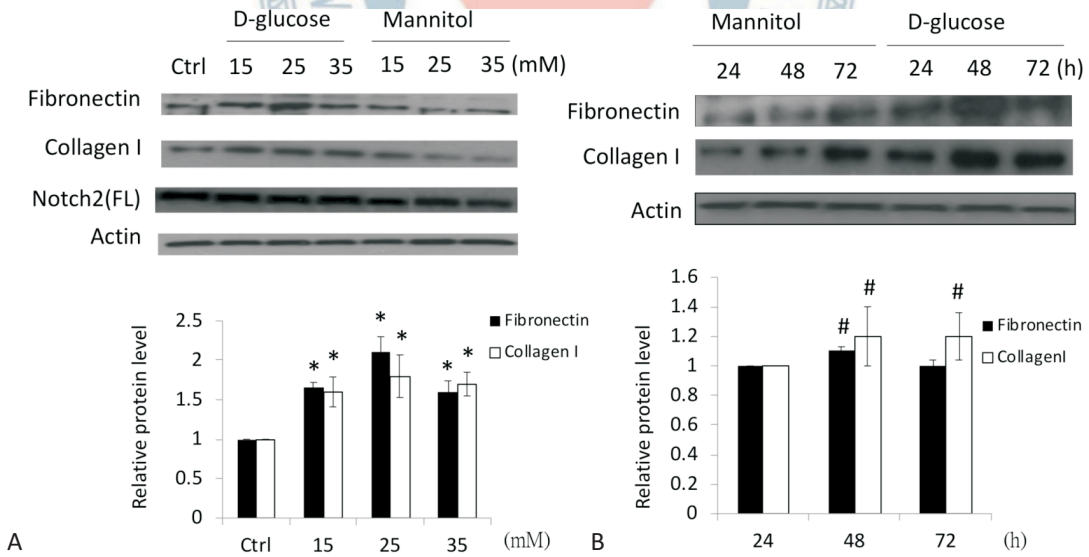

Figure 1. *High glucose induced fibronectin and collagen I expression in cultured rat vascular smooth muscle cells (VSMCs). Rat VSMCs were cultured in different concentration of D-glucose (15 mM, 25 mM, 35 mM), The control group (Ctrl) represented rat VSMCs cultured in culture medium contained D-glucose 5 mM. And the rat VSMCs treated with different concentration of Mannitol (15 mM, 25 mM, 35 mM) served as osmotic control group. (A) the expression of fibronectin and collagen I in the rat VSMCs cultured in high glucose group was higher than in Ctrl group. (B) the expression of fibronectin and collagen I in rat VSMCs cultured in high glucose (D-glucose 25 mM) in the period of 48 h was higher than in the period of 24 h. Notch2 (FL); Notch-2 full length, * p < 0.05 versus Ctrl group, # p < 0.05 versus 24 h group.*

dium contained D-glucose 5 mM to provide the basal requirement for cell growth. Additionally, mannitol was used in parallel experiments as an osmotic control for high glucose. Figure 2 shows that the proliferation of rat VSMCs increased in high glucose conditions.

We also used a wound healing assay to determine the effect of high glucose (D-glucose 25 mM) on the migration of rat VSMCs, in which the cells were cultured in medium with and without high glucose (D-glucose 25 mM). For the control study group, 5 mM D-glucose was maintained in the culture medium to provide the basal requirement for cell growth. Figure 3 shows that high glucose (D-glucose 25 mM) enhanced rat VSMC migration at 48 h.

High glucose enhanced the Notch-2 signaling pathway in cultured vascular smooth muscle cells

To determine the effect of high glucose on Notch-2 expression of rat VSMCs, the cells were cultured with different high concentration of D-glucose (15, 25, 35 mM). In the control study group, the culture medium contained D-glucose 5 mM to provide the basal requirement for cell growth. Additionally, mannitol was used in parallel experiments as an osmotic control for high glucose. After culturing for 48 h, the cell lysate was prepared and subjected to Western immunoblotting using anti-Notch-2 ICD antibodies. As illustrated in Figure 4(A), the expression of Notch-2 ICD in rat VSMCs in the high glucose group (D-glucose 25 mM and 35 mM) was higher than in the normal control group. Besides, as illustrated in Figure 4(B), the expression of Notch-2 ICD in rat VSMCs cultured with D-glucose 25 mM at 48 h was higher than at 24 h. These results indicated a high expression of Notch-2 signaling in rat VSMCs cultured with high glucose.

Inhibitors of Notch-2 signaling abolished the effect of high glucose on fibronectin and collagen I expression in cultured vascular smooth muscle cells

To examine the relationship between Notch-2 signaling and ECM proteins including fibronectin and collagen I in high glucose (D-glucose 25 mM)-treated rat VSMCs, DAPT, a γ-secretase inhibitor, was used to inhibit Notch-2 signaling. Rat VSMCs were pretreated with DAPT at different concentrations (0.01 μM, 0.05 μM and 0.1 μM) for 2 h, and then continual DAPT treatment proceeded in the presence or absence of high glucose (D-glucose 25 mM). After culturing for 48 h, the cell lysate was prepared and subjected to Western immunoblotting using anti-fibronectin, anti-collagen I and anti-

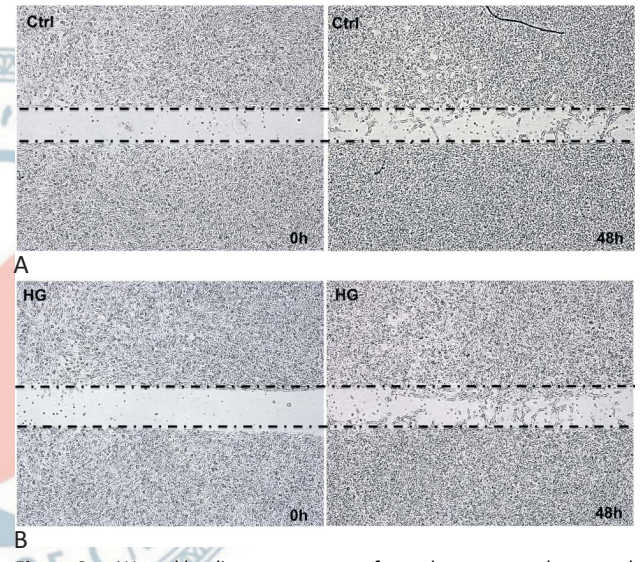

Figure 3. *Wound healing assay was performed on rat vascular smooth muscle cells (VSMCs) in (A) culture medium containing D-glucose 5 mM served as control (Ctrl) group; in (B) 25 mM D-glucose served as high glucose (HG) group for 48 h. High glucose (D-glucose 25 mM) enhanced rat VSMCs migration in the period of 48 h.*

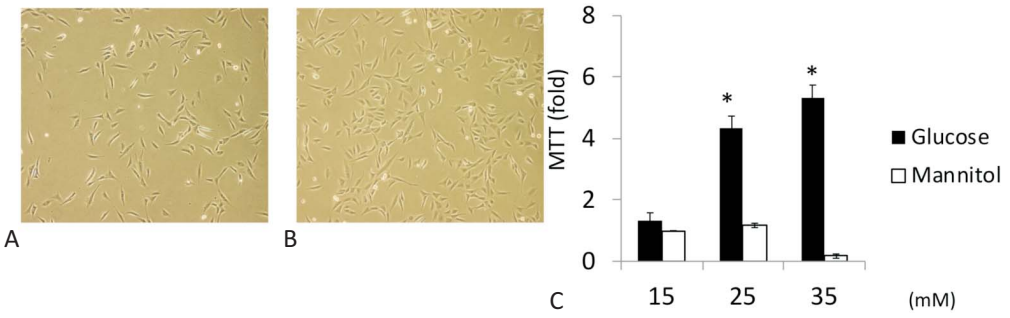

Figure 2. *Rat vascular smooth muscle cells (VSMCs) was cultured in (A) D-glucose 15 mM; in (B) D-glucose 25 mM for 48 h showed in 100X microscopic view. (C) VSMCs proliferation was measured by MTT assay in rat VSMCs cultured in different concentration of D-glucose (15 mM, 25 mM and 35 mM). High glucose enhanced proliferation of rat VSMCs. * $p < 0.05$ versus 15 mM Mannitol group.*

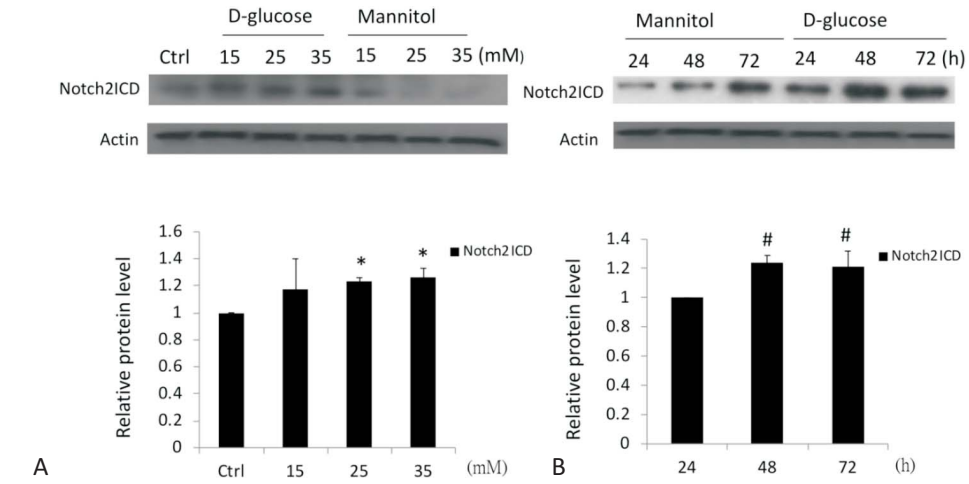

Figure 4. *High glucose induced Notch-2 signaling expression in cultured rat vascular smooth muscle cells (VSMCs). Rat VSMCs were cultured in different concentration of D-glucose (15 mM, 25 mM, 35 mM). The control group (Ctrl) represented rat VSMCs cultured in culture medium contained D-glucose 5 mM. And the rat VSMCs treated with different concentration of Mannitol (15 mM, 25 mM, 35 mM) served as osmotic control group. (A) The expression of Notch-2 ICD in rat VSMCs treated with high concentration of D-glucose (25 mM, 35 mM) was higher than in Ctrl group. (B) The expression of Notch-2 ICD in VSMCs cultured with D-glucose 25 mM in the period of 48 h was higher than in the period of 24 h. * $p < 0.05$ versus Ctrl group, # $p < 0.05$ versus 24 h group.*

Notch-2 ICD antibodies. As illustrated in Figure 5, treatment with DAPT (0.05 μM; 0.1 μM) markedly abolished the expressions of fibronectin, collagen I and Notch-2 ICD in high glucose (D-glucose 25 mM)-cultured rat VSMCs. These results indicated that DAPT, an inhibitor of γ-secretase, abolished the expressions of ECM proteins including fibronectin and collagen I in high glucose (D-glucose 25 mM)-cultured VSMCs through blocking the Notch-2 signaling pathway.

Inhibitors of Notch-2 signaling abolished the proliferation and migration of cultured vascular smooth muscle cells under high glucose stress

An MTT assay was used to measure the proliferation of rat VSMCs treated with DAPT in high glucose (D-glucose 25 mM) conditions. The rat VSMCs were pretreated with DAPT at different concentrations (0.01 μM, 0.05 μM and 0.1 μM) for 2 h, and then continual DAPT treatment proceeded in the presence of high glucose (D-glucose 25 mM). As illustrated in Figure 6(A), DAPT (0.01 μM; 0.05 μM; 0.1 μM) markedly inhibited the proliferation of rat VSMCs under high glucose (D-glucose 25 mM) stress.

We also used a wound healing assay to evaluate the migration of rat VSMCs treated with DAPT in high glucose (D-glucose 25 mM) conditions. The rat VSMCs were pretreated with 0.5 μM DAPT for 2 h. Next, continual DAPT treatment proceeded in the presence of high glu-

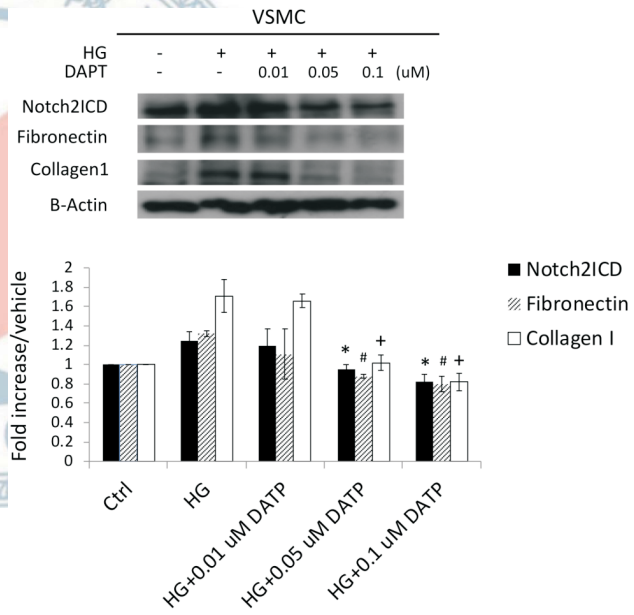

Figure 5. *Effect of N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) on Notch-2 signaling in high glucose (D-glucose 25 mM)-treated (HG) rat vascular smooth muscle cells (VSMCs). Rat vascular smooth muscle cells (VSMCs) were cultured in culture medium contained D-glucose 5 mM served as control group (Ctrl); cultured in D-glucose 25 mM served as high glucose group (HG). Additionally, the rat VSMCs cultured in HG were treated with different concentration of DATP (0.01 μM, 0.05 μM, 0.1 μM) and were served as HG + 0.01 μM DAPT group; HG + 0.05 μM DAPT group and HG + 0.1 μM DAPT group. DAPT (0.05 μM; 0.1 μM) markedly abolished the expression of fibronectin, collagen I and Notch-2 ICD in high glucose (D-glucose 25 mM)-cultured (HG) rat VSMCs. * $p < 0.05$ versus Notch-2 ICD in HG group; # $p < 0.05$ versus fibronectin in HG group; + $p < 0.05$ versus collagen I in HG group.*

cose (D-glucose 25 mM). Figure 6(B) shows that 0.5 μM DAPT abolished the migration of rat VSMCs under high glucose (D-glucose 25 mM) stress. These results showed that DAPT decreased the proliferation and migration of cultured rat VSMCs under high glucose (D-glucose 25 mM) stress through blocking the Notch-2 signaling pathway.

DISCUSSION

The main findings of this study were that (1) High glucose (D-glucose 25 mM) induced the accumulation of ECM proteins (fibronectin and collagen I) secreted by cultured VSMCs and enhanced the proliferation and migration of cultured VSMCs through the Notch-2 signaling pathway directly. (2) Inhibition of the Notch-2 signaling pathway using DAPT, an inhibitor of γ-secretase, reduced the synthesis of ECM proteins and the proliferation and migration of cultured VSMCs under high glucose stress.

VSMCs are located in the medial layer of arteries, and their major physiological function is contraction to maintain vascular tone and regulate blood pressure.[22] Atherosclerosis, the formation of fibrofatty lesions in the artery wall, represents a series of specific cellular and molecular responses. Initially, EC-induced injury increases oxidized lipid permeability, macrophage recruitment, foamy cell formation, and recruitment of inflammatory cells. The ECs, platelets and inflammatory cells then release many mediators which induce the VSMCs to switch from a quiescent "contractile" phenotype to the active "synthetic" phenotype that can migrate and proliferate from the media to the intima accompanied by accumulation of new ECM. This results in vessel neointima formation and atherosclerotic change, causing the artery to become narrow due to atheroma plaque formation contributing to macrovascular diseases including MI, PAOD and stroke.[23] The in vitro and in vivo studies of Keuylian et al.[24] showed that Jagged-1 ligated with the Notch-3-HRT3 and/or HRT1 signaling pathways leading to VSMC differentiation and the overexpression of HRT3 and/or HRT1 in mice VSMCs to contribute to neointima formation. Activating Notch signaling counteracted the transdifferentiation of the VSMCs. In the process of atheroma formation, VSMCs migrate into the neointima and secrete ECM proteins to stabilize the plaque.[25]

The major vessel complications of DM include microvasculopathy and macrovasculopathy. The mechanisms of DM macrovasculopathy are complex and not completely understood. It is known that under medical stress, the contractile type of VSMCs may dedifferentiate to the synthetic type of VSMCs which secrete ECM proteins, and that this is mainly responsible for intimal plaque formation, proliferation or migration.[26,27] Some

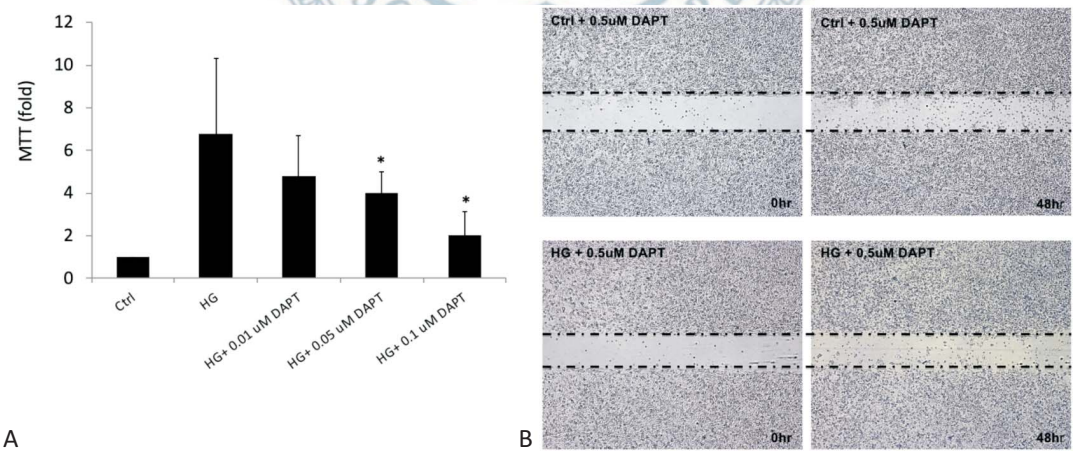

Figure 6. *(A) Cell proliferation was measured by an MTT assay. Progressive high concentration of N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) inhibited proliferation of rat vascular smooth muscle cells (VSMCs) under high glucose (D-glucose 25 mM) stress (HG). Rat VSMCs cultured in culture medium contained D-glucose 5 mM served as control group (Ctrl). (B) Cell migration was measured by a wound healing assay. Rat VSMCs were treated with 0.5 μM DAPT in culture medium containing D-glucose 5 mM served as control group (Ctrl) and in D-glucose 25 mM served as high glucose group (HG) for 48 h. 0.5 μM DAPT abolished the migration of VSMCs under high glucose (D-glucose 25 mM) stress. * $p < 0.05$ versus HG group.*

studies have shown that high glucose can enhance the proliferation of VSMCs through different pathways.[28] The overexpression of interferon regulatory factor-1 has been shown to promote ERK-1 activation to induce VSMC proliferation under high glucose conditions.[29] Connective tissue growth factor (CTGF) has also been shown to mediate cardiac remodeling after myocardial infarction.[30] In addition, high glucose has been shown to increase CTGF protein and promote VSMC proliferation.[31] In addition, high glucose has also been shown to stimulate ECM protein synthesis and accumulation through activation of transforming growth factor-beta (TGF-β).[32,33] An in vivo study showed that high glucose could induce the proliferation of VSMCs through activation of STAT3/Pim 1 signaling.[34]

In DM microvasculopathy, our previous study showed that activated Notch signaling in high glucose-treated renal podocytes induced vascular endothelial growth factor (VEGF) expression and subsequent nephrin repression and apoptosis which mediated microvasculopathy and nephropathy.[35]

Notch signaling is known to be a regulator of VSMC phenotypes, and the functions of Notch signaling in VSMCs include anti-differentiation[36] and pro-differentiation[37-39] under different pathophysiological conditions. However, there are conflicting data with regards to its role in VSMC performance under high glucose stress which contributes to macrovasculopathy including VSMC proliferation and ECM accumulation secreted by VSMCs in a secretory status.

In this study, we demonstrated that high glucose enhanced the production of ECM proteins including fibronectin and collagen I (Figure 1), and promoted VSMC proliferation (Figure 2). VSMCs migrated from the medial layer of arteries to form neointima in response to vascular injury. It has also been shown that hyperglycemia promotes the filopodia formation of VSMCs and enhanced migration of VSMCs.[40] We also found that the VSMCs proliferated and migrated under high glucose stress (Figure 2 and 3).

Notch signaling is an important signaling pathway for communication between ECs and VSMC during the embryologic process and in pathophysiologic conditions.[15,16] Interactions between ECs and VSMCs contribute to vessel development and lead to VSMC differentiation in embryonic growth. In pathologic vessel remodeling, Jagged-1, a Notch ligand, on the surface of ECs interacts with Notch receptors in adjacent VSMCs resulting in migration of the Notch intracellular domain into the nucleus where it binds to signal sequence-binding protein which activates target genes to product ECM and induce vessel neointima formation. In a vessel injury model, the overexpression of Notch-1 signaling was shown to induce the proliferation of VSMCs and the formation of neointima through Jagged-1 ligated Notch receptor between ECs and VSMCs.[15] In our in vitro study, unlike the pathophysiology of vessel injury, Notch-2 signaling was directly overexpressed in high glucose-treated VSMCs (Figure 3). Furthermore, repression of Notch-2 signaling by DAPT, an inhibitor of γ-secretase, in high glucose-stressed VSMCs decreased the production of fibronectin and collagen I and inhibited the proliferation and migration of VSMCs (Figure 5 and 6). We also found that high glucose promoted the dedifferentiation of the contractile type of VSMCs to the synthetic type of VSMCs, and consequently to proliferate, migrate and secrete ECM proteins including fibronectin and collagen I through the Notch-2 signaling pathway. Inhibition of Notch-2 signaling may be useful to prevent macrovasculopathy in diabetic vessels.

Limitations

The present study also has several limitations. In the in vitro study, we only used the SV40LT-SMC Clone HEP-SA cell line to show that inhibition of the Notch-2 signaling pathway reduced ECM protein synthesis, and the proliferation and migration of cultured VSMCs under high glucose stress. Further studies of the expression of the Notch signaling pathway in different primary VSMC cultures or VSMC lines under high glucose stress are warranted. Besides, the atherosclerotic process is complex and includes vessel endothelial dysfunction, proliferation and migration of VSMCs, and inflammation reactions. Further in vivo studies are necessary to clarify the role of the Notch-2 signaling pathway in diabetic macrovasculopathy.

CONCLUSIONS

Unlike high glucose-mediated microvasculopathy which leads to aberrant angiogenesis through Jagged-1

ligated Notch receptors between ECs and VSMCs, our data suggested that high glucose can induce the accumulation of ECM proteins including fibronectin and collagen I secreted by VSMCs, and enhance the proliferation and migration of VSMCs through the Notch-2 signaling pathway to mediate macrovasculopathy directly. Inhibition of the Notch-2 signaling pathway decreased the expressions of fibronectin and collagen I and reduced the proliferation and migration of VSMCs under high glucose stress. Inhibition of Notch-2 signaling represents a promising target for treating diabetic macrovasculopathy (Figure S2).

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

All the authors declare no conflicts of interest.

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SUPPLEMENT

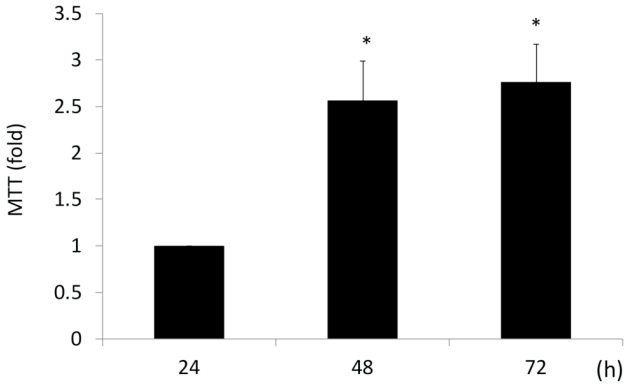

Figure S1. *Rat vascular smooth muscle cells (VSMCs) proliferation was measured by MTT assay in rat VSMCs cultured with 25 mM D-glucose in different period of time (24 h, 48 h, and 72 h). * p < 0.05 versus 24 h group.*

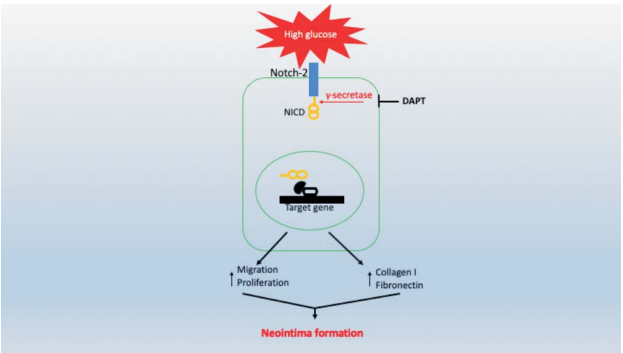

Figure S2. *Role of Notch-2 signaling pathway in pathophysiology of diabetic macrovasculopathy.*