Insulin enhances neointimal hyperplasia following arterial injury through the PI3K/Akt pathway in type 1 diabetic rats

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Abstract. Although insulin is known to affect neointimal hyperplasia via distinct signaling pathways, how neointimal hyperplasia is affected in insulin-deficient type 1 diabetes remains unknown. The aim of the current study was to investigate two major signaling branches of insulin action regulating neointimal hyperplasia following arterial injury in type 1 diabetes with or without exogenous insulin administration. Rats were treated with vehicle (control group), streptozotocin (STZ) alone (STZ group; uncontrolled type 1 diabetes) or STZ followed by insulin (STZ + I group; controlled type 1 diabetes). Subsequently, a type 1 diabetic rat model of carotid artery balloon injury was established. Following this, the intima-to-media area ratios were examined for evidence of neointimal hyperplasia in the carotid arteries of the rats by performing hematoxylin-eosin staining. Furthermore, the protein expression of extracellular signal-regulated kinase (ERK), phosphorylated (p-) ERK, protein kinase B (Akt) and p-Akt in the carotid arteries of the rats was determined via immunoblotting. Moreover, an *in vitro* model of type 1 diabetes was induced by incubation of primary vascular smooth muscle cells (VSMCs) with glucose and/or insulin. Cellular proliferation and signaling protein expression levels in VSMCs were determined by measuring the incorporation of tritiated thymidine and performing immunoblotting, respectively. The results demonstrated that compared with that in control rats, neointimal hyperplasia and expression of p-Akt in uncontrolled type 1 diabetic rats were significantly decreased. This decrease was recovered in controlled type 1 diabetes with insulin therapy. Furthermore, the difference in the expression of p-ERK between groups was not significant. Additionally, the results of the cell experiments were consistent with those from the animal studies. In conclusion, the preferential signaling along the phosphatidylinositol 3-kinase/Akt pathway of insulin action in response to insulin restoration may contribute to neointimal hyperplasia. The present study provides a novel approach for the further treatment of neointimal hyperplasia in type 1 diabetes.

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Abbreviations: Akt, protein kinase B; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; VSMC, vascular smooth muscle cell

Key words: neointimal hyperplasia, type 1 diabetes, stenosis, proliferation

Introduction

Diabetes is one of the most prevalent and costly chronic diseases worldwide, and has increased the morbidity of cardiovascular, cerebrovascular and peripheral arterial diseases (1,2). The prevalence of adults with diabetes around the world in 2014 was 8.5% and in 2016, diabetes results in 1.6 million deaths (3,4). Due to the increasingly aggressive and accelerated course of atherosclerosis in diabetes, patients have a greater probability of having strokes. In 2010, stroke was the second leading cause of death in patients >60 years old and the fifth leading cause of death in people aged 15-59 years worldwide (5,6). As a result, diabetic patients with ischemic stroke undergo more revascularization procedures compared with the general population (7). Vascular interventions have several advantages, including microtrauma, short procedural duration and quick recovery, compared with carotid endarterectomy surgery, making them an essential treatment for carotid artery stenosis or occlusion (7). However, neointimal hyperplasia following the procedure, including balloon angioplasty and stenting is a common problem in patients with diabetes (8-10).

Insulin affects neointimal hyperplasia via distinct signaling pathways (10-12). In vascular tissues, insulin stimulates two major signaling pathways: The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways (13). PI3K activation is essential for insulin-mediated glucose uptake, cell survival and nitric oxide (NO) production, while MAPK activation stimulates cellular proliferation and migration, and prothrombotic and proinflammatory responses (8). In the state of insulin resistance following balloon or stent injury, excess insulin stimulates signaling from the PI3K/Akt pathway to the MAPK/ERK pathway, and is involved in the endothelial production of NO and vascular smooth muscle cell (VSMC) proliferation and migration (11,14). Furthermore, a higher ratio of phosphorylated (p-)ERK/total ERK to p-Akt/total Akt was associated with increased neointimal hyperplasia following vascular injury (11). Thus, it is evident that insulin is, at least in part, responsible for enhanced neointimal hyperplasia in insulin-resistant or type 2 diabetic models secondary to MAPK activation and/or PI3K impairment. However, given the different metabolic environments exhibited by patients with type 1 vs. type 2 diabetes, the detailed signaling pathways regulating neointimal hyperplasia in type 1 diabetes remain unclear.

Thus, we hypothesized that the PI3K/Akt or MAPK/ERK pathway regulated neointimal hyperplasia following arterial injury in type 1 diabetes, with or without insulin therapy. The preferential signaling along the PI3K/Akt pathway of insulin action in response to insulin deficiency may be involved. The current study performed *in vitro* cellular experiments and constructed an *in vivo* rat model of neointimal hyperplasia in type 1 diabetes, in which the roles of the PI3K/Akt and MAPK/ERK pathways were investigated. The present study provided a novel approach for the reduction of neointimal hyperplasia in type 1 diabetes.

Materials and methods

Animal model. The rats used in the current study were from the same strain as those used in our previous studies on type 1 diabetes (15,16). A total of 30 male Sprague-Dawley (age, 11 weeks; weight, ~300 g) rats were maintained at the Animal Centre of Jinling Hospital (Nanjing, China). Rats were housed at room temperature with 12-h light/dark cycles, 60±5% relative humidity, and free access to food and water in a pathogen-free animal facility. Rats were randomly selected for a single intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich; Merck KGaA; 60 mg/kg dissolved in pH 4.2 citrate buffer; n=22) or citrate buffer alone (control group; n=8) (15). A total of 19 STZ-treated rats (19/22) with a fasting blood glucose >16.67 mmol/l (300 mg/dl), which typically exhibits within 5 days of STZ injection, were considered as type 1 diabetic rats (8,15). Rats (n=3) with a low blood glucose (<16.67 mmol/l) within 7 days of STZ injection were excluded from the subsequent experiments. A subset of the type 1 diabetic rats (9/19; at random) received insulin glargine (Sanofi SA; 3 units; STZ + I group; controlled type 1 diabetes; n=9) daily via subcutaneous injection once hyperglycemia was detected. This treatment was continued daily for days prior to the establishment of the rat carotid injury model (8,10). Insulin therapy in this group was continued for the remaining 2 weeks following surgery, with each rat receiving daily insulin administration for a total of 21 days. The other subset of the type 1 diabetic rats (10/19) without insulin administration were considered as uncontrolled type 1 diabetes (STZ group; n=10).

Animal surgery. At ~day 14 following STZ injections, all 27 rats underwent surgery for the carotid artery balloon injury model. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH; publication no. 85-23; 1996) and approved by the Institutional Animal Care and Use Committee of Nanjing University (Nanjing, China). Rats were anesthetized with inhaled isoflurane (2-3% induction; 0.5-1.5% maintenance) (8,17). Atropine was administered subcutaneously (0.1 mg/kg) to decrease airway secretions. The neck was shaved and prepped with betadine and alcohol (75%). Following a midline neck incision, the rat carotid artery balloon injury model was performed using a 2F Fogarty catheter (Edwards Lifesciences), as previously described (8,12,18). Following injury and restoration of blood flow, the neck incision was closed. A total of 5 rats (1 in the control group and 2 in the STZ and STZ + I groups) died of cardiopulmonary arrest during surgery. Additionally, 4 rats (1 in the control group, 2 in the STZ group and 1 in the STZ + I group) died of systemic embolism or serious infection during the follow-up period. A total of 18 surviving rats were euthanized at day 14 post-surgery by exposure to CO_2 for 5 min (displacement rate, 20% of home cage volume/minute). Following this, cervical dislocation (rats weighing <200 g) or decapitation (>200 g) were performed under CO_2 anesthesia. Presumed death was confirmed based on unambiguous signs of death, including cardiopulmonary arrest and/or fixed dilated pupils.

Morphometric analysis. Carotid arteries harvested at 2 weeks post-surgery were examined histologically for evidence of neointimal hyperplasia using routine hematoxylin-eosin staining. Briefly, the vessels were fixed with 4% paraformaldehyde at 4˚C overnight. Then, the fixed vessels were embedded in paraffin, and 5-μm thick sections were cut and mounted on slides. The paraffin sections were stained with hematoxylin and eosin as previously described (15). Representative images of the aorta from the rats were observed under a light microscope (magnifications, x10 and x20). Both the intimal and medial areas were measured using ImageJ software (version 1.46r; National Institutes of Health) with uniform arbitrary units for subsequent calculation of the intima-to-media area (I/M) ratios.

Proliferation assay. VSMCs, isolated from rat carotid arteries as previously described (19), were characterized by smooth muscle cell morphology (multilayer sheets; 'hills and valleys') and smooth muscle α-actin expression (19). A VSMC proliferation assay was conducted according to previous studies (19,20). Briefly, primary VSMCs were plated in 12-well plates ($5x10^4$ cells/well) and cultured at 37˚C for 24 h in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The cells

Table I. Metabolic parameters of rats (n=6).

Characteristic	Control	STZ	STZ + I
Initial age, weeks	11	11	11
Initial weight, g	313±10	315±8	310±8
Final weight, g	415±21	289±9[a]	413±16
Glucose, 72 h after STZ treatment, mM	6.1±0.6	20.8±3.0[a]	20.4±3.3[a]
Glucose, 2 weeks after surgery, mM	6.6±0.4	21.1±2.2[a]	7.3±0.5
Insulin, 2 weeks after surgery, ng/ml	4.32±0.47	0.83±0.15[a]	3.93±0.19

Values are presented as mean ± standard deviation (n=6). [a]P<0.05 vs. control group. STZ, streptozotocin-injected rats without insulin therapy; STZ + I, streptozotocin-injected rats with insulin therapy.

were then exposed to serum-free media containing tritiated (^{3}H) thymidine (1 μCi/ml; China Institute of Atomic Energy,), glucose and/or bovine insulin for an additional 24 h. Exposing VSMCs to normal or high glucose (5 or 25 mM) and/or normal insulin (100 nM) concentrations for 24 h mimicked starved, normal, uncontrolled or controlled type 1 diabetes, respectively, as per previous studies (8,21) (Table SI). [^{3}H]thymidine incorporation into trichloroacetic acid-precipitated DNA was quantified by scintillation counting using a liquid scintillation counter (Beckman LS6500; Beckman Coulter, Inc.). Sorbitol (25 mM) was used as an osmotic control for all experiments.

Immunoblotting. Immunoblotting was performed as previously described (22,23). Briefly, total proteins were extracted from the carotid arteries or VSMCs treated without [^{3}H thymidine using RIPA Lysis Buffer (Beyotime Institute of Biotechnology). Protein concentrations were determined using the BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Samples containing 50 μg protein or 10 μl pre-stained molecular weight marker (cat. no. P0076; Beyotime Institute of Biotechnology) were separated via 10% SDS-PAGE and electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked overnight with 5% nonfat dry milk in PBS-T [0.05% Tween-20 in 10 mmol/l PBS] at 4˚C with constant shaking. The blots were probed with primary monoclonal rabbit anti-rat ERK (1:800; cat. no. 4695), Akt (1:800; cat. no. 4685), p-ERK (1:600; cat. no. 4370), p-Akt (1:600; cat. no. 4060) or GAPDH (1:1,000; cat. no. 5174) antibodies (all Cell Signaling Technology) overnight at 4˚C. Subsequently, the membranes were incubated with polyclonal IRDye® 800CW-labeled goat anti-rabbit IgG secondary antibodies (1:10,000; cat. no. 102673-300; LI-COR Biosciences) for 1 h at room temperature in dark. Proteins were visualized using an infrared imaging system (LI-COR Biosciences). The density of each sample was calculated using Odyssey software (version 3.0; LI-COR Biosciences). The ratio of p-ERK/ERK to p-Akt/Akt in the carotid arteries was also calculated.

Blood chemistry assay. Blood glucose levels were screened on alternate days for 1 week following injection of STZ and twice weekly thereafter. Blood glucose was measured with a standardized, portable glucometer (Johnson & Johnson) via puncture of the tail vein. Serum samples were collected from non-fasted animals at death and frozen at -20˚C until assay. Insulin levels were determined by radioimmunoassay with an antibody (cat. no. SRI-13K; 1:1; Linco; EMD Millipore) made specifically against rat insulin. The rat insulin antibody had 100% cross-reactivity with human insulin.

Statistical analysis. Statistical analysis was performed using SPSS software (version 25.0; IBM Corp.). Data are expressed as the mean ± standard deviation. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Duncan's and Tukey's (>3 groups) post-hoc tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Metabolic parameters of SD rats. A total of 18, or two-thirds, of the rats survived until euthanasia, which was consistent with a published model of arterial injury in diabetic rats (10). The type 1 diabetic rats without exogenous insulin administration exhibited significant weight loss, increased blood glucose and decreased insulin. Blood glucose was also significantly increased at 72 h after STZ treatment in the STZ + I group compared with the control group. The sample size, age, weight, blood glucose and insulin levels in each group are presented in Table I.

Enhanced neointimal hyperplasia in STZ + I rats. The representative images of balloon-injured carotid artery in SD rats treated with controls, STZ and STZ + I stained with hematoxylin-eosin at day 14 post-surgery are presented in Fig. 1A. The upper and lower images were observed under a microscope at magnifications of x10 and x20, respectively, exhibiting the neointimal hyperplasia following arterial injury (Fig. 1A). The results demonstrated that the I/M ratios were significantly decreased in the carotid arteries of the STZ rats compared with those in the controls (Fig. 1B). These levels were recovered and slightly increased in STZ + I rats (Fig. 1B); however, this increase was not significant.

Effect of insulin on Akt expression in balloon-injured carotid arteries. To elucidate the effect of insulin on the PI3K/Akt pathway *in vivo*, immunoblotting was used to measure the levels of p-Akt and total Akt in the carotid arteries of the rats (Fig. 2A). The results demonstrated that p-Akt/total Akt in the carotid arteries of STZ rats was significantly decreased

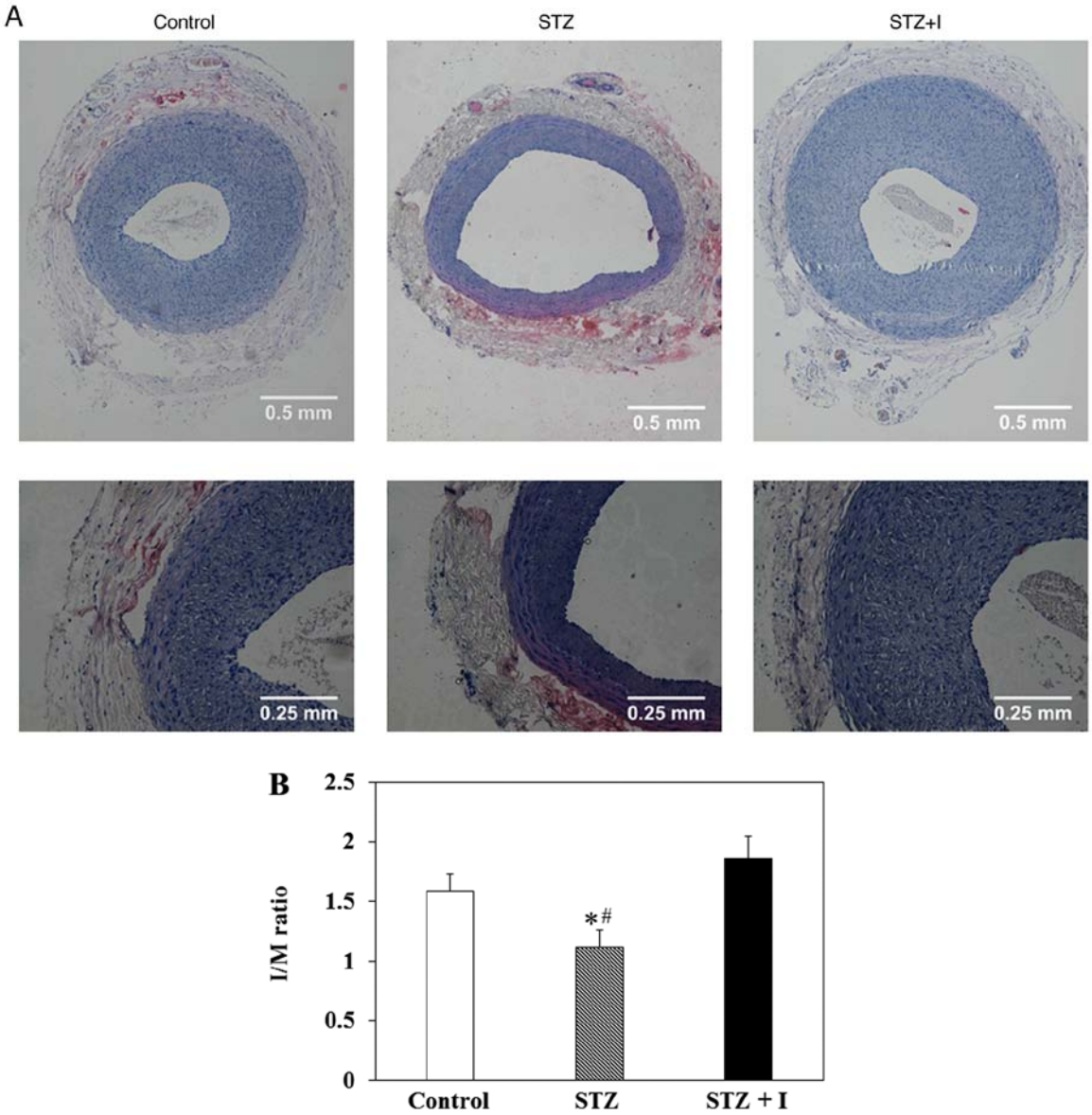

Figure 1. Effect of insulin on neointimal hyperplasia in balloon-injured carotid arteries. (A) Representative images of hematoxylin-eosin-stained carotid arteries from the rats treated with control, STZ or STZ followed by insulin at 14 days post-vascular balloon injury. The upper and lower images were observed under a microscope at magnifications of x10 and x20, respectively. (B) Bar graph demonstrating I/M ratios in the carotid arteries of rats. Values are presented as mean ± standard deviation (n=6). *P<0.05 vs. control group; #P<0.05 vs. STZ + I group. STZ, streptozotocin; I, insulin; I/M, intima-to-media area.

compared with that in the control (Fig. 2B). This effect was ameliorated by insulin in the STZ + I group (Fig. 2B).

Effect of insulin on ERK expression in balloon-injured carotid arteries. To elucidate the influence of insulin on the MAPK/ERK pathway *in vivo*, immunoblotting was performed to measure the levels of p-ERK and total ERK in the carotid arteries of the rats (Fig. 3A). The results revealed that p-ERK/ERK in the carotid arteries of SD rats were not significantly different between groups (Fig. 3B).

Representative migration patterns of the pre-stained molecular weight marker and target proteins, including Akt, p-Akt, ERK, p-ERK and GAPDH, as determined by immunoblotting, are presented in Fig. S1.

Association between the effect of insulin and the ratio of p-ERK/ERK to p-Akt/Akt. To further clarify the effect of insulin on the MAPK/ERK and/or PI3K/Akt pathway, the ratio of p-ERK/ERK to p-Akt/Akt in the carotid arteries was also calculated. The results demonstrated that the ratio of p-ERK/ERK to p-Akt/Akt was significantly increased in the STZ group compared with that in the control group (Fig. S2).

Cell proliferation and signaling proteins in VSMCs. To investigate VSMC proliferation in the state of uncontrolled or controlled type 1 diabetes, an *in vitro* assay was conducted using [^{3}H]thymidine incorporation to serve as a surrogate for cell proliferation. Primary VSMCs exposed to the high glucose (25 mM) in the uncontrolled type 1 diabetes group demonstrated a significant reduction in proliferation compared with the low glucose (5 mM) in the control or controlled type 1 diabetes groups (Fig. 4). Furthermore, the results demonstrated that the p-Akt/total Akt in the uncontrolled type 1 diabetes group was significantly decreased compared with the control or controlled

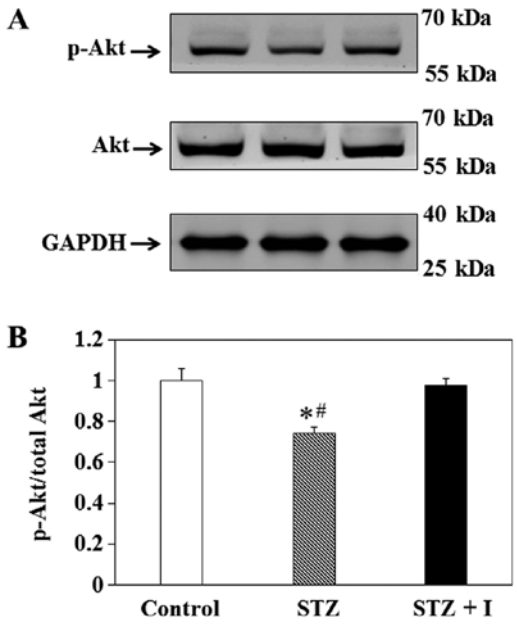

Figure 2. Akt expression in balloon-injured carotid arteries. (A) Immunoblotting with p-Akt (Ser473), total Akt and GAPDH in carotid arteries in the control, STZ and STZ + I rats 2 weeks post-surgery. Values are presented as relative to control. (B) Bar graph demonstrating p-Akt/total Akt expression in the carotid arteries of rats. Values are presented as the mean ± standard deviation (n=6). *P<0.05 vs. control group; #P<0.05 vs. STZ + I group. p-, phosphorylated; STZ, streptozotocin; I, insulin; Akt, protein kinase B.

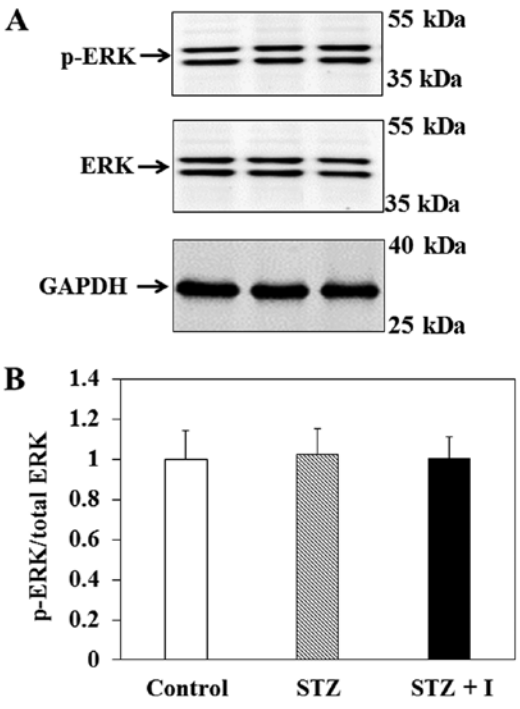

Figure 3. ERK expression in balloon-injured carotid artery. (A) Immunoblotting with p-ERK (Thr202/Tyr204), total ERK and GAPDH in the carotid arteries of control, STZ and STZ + I rats 2 weeks post-surgery. Values are presented as relative to control. (B) Bar graph demonstrating p-ERK/total ERK expression in the carotid arteries. Values are presented mean ± standard deviation (n=6). There was no significant difference between groups. p-, phosphorylated; ERK, extracellular signal-regulated kinase.

type 1 diabetes groups (Fig. 5). However, the p-ERK/total ERK in VSMCs was not significantly different between groups.

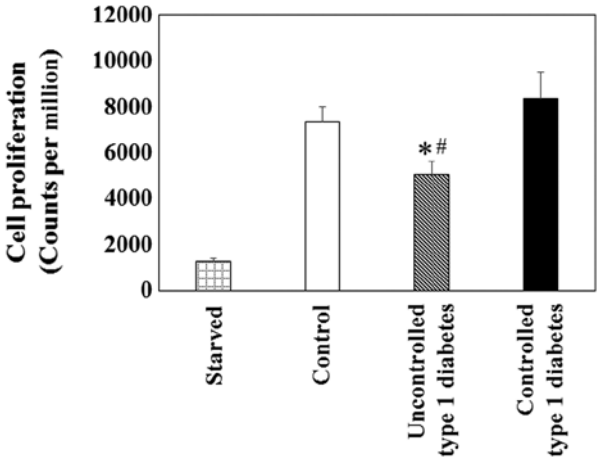

Figure 4. VSMC proliferation was assessed by [^{3}H]thymidine incorporation. Primary VSMCs harvested from the carotid arteries rats were exposed to varying concentrations of glucose (5 or 25 mM) and/or insulin (100 nM), which mimicked normal, uncontrolled or controlled type 1 diabetes. Values are presented as the mean ± SD (n=4). *P<0.05 vs. control group; #P<0.05 vs. controlled type 1 diabetes group. VSMC, vascular smooth muscle cell.

Discussion

The results of the current study demonstrated that, compared with controls, in uncontrolled type 1 diabetes both the neointimal hyperplasia and expression of p-Akt were significantly decreased, but were recovered following exogenous insulin administration in controlled type 1 diabetes. However, the difference in the expression of p-ERK was not significant between groups. Furthermore, the cellular results of the *in vitro* experiments were consistent with those from the *in vitro* animal experiments. These findings indicated that high glucose may inhibit VSMC proliferation by inhibiting the PI3K/Akt signaling pathway in type 1 diabetes with low insulin levels, which can be improved by exogenous insulin supplementation.

Consistent with previous studies, the results of the current study demonstrated that PI3K/Akt signaling was impaired in uncontrolled type 1 diabetes, compared with controls, most likely due to low insulin and subsequent high glucose (11,14,24). The present study indicated that once the insulin and glucose levels normalized, the impairment was recovered. Unexpectedly, neointimal hyperplasia was slightly increased, but not significant, in controlled type 1 diabetes compared with the controls. A possible explanation is that Akt activation by abundant insulin in VSMCs may be necessary for maintaining a quiescent, fully differentiated phenotype rather than a migratory, proliferative one (14,21). Furthermore, endothelial production of NO induced by Akt activation may result in vascular protective effects (25,26). However, in the setting of insulin resistance, the balance that normally regulates VSMC proliferation/migration is disrupted by differentially shunted signaling from Akt to ERK activation (11). Furthermore, Akt inhibition coupled with excess insulin may lead to endothelial reduced production of NO and increased expression of cellular adhesion molecules, such as CD11a or ICAM-1, with increased monocyte rolling and arrest (27).

Additionally, the results of the present study reported that MAPK/ERK signaling was not significantly different in uncontrolled or controlled type 1 diabetes, with low or normal

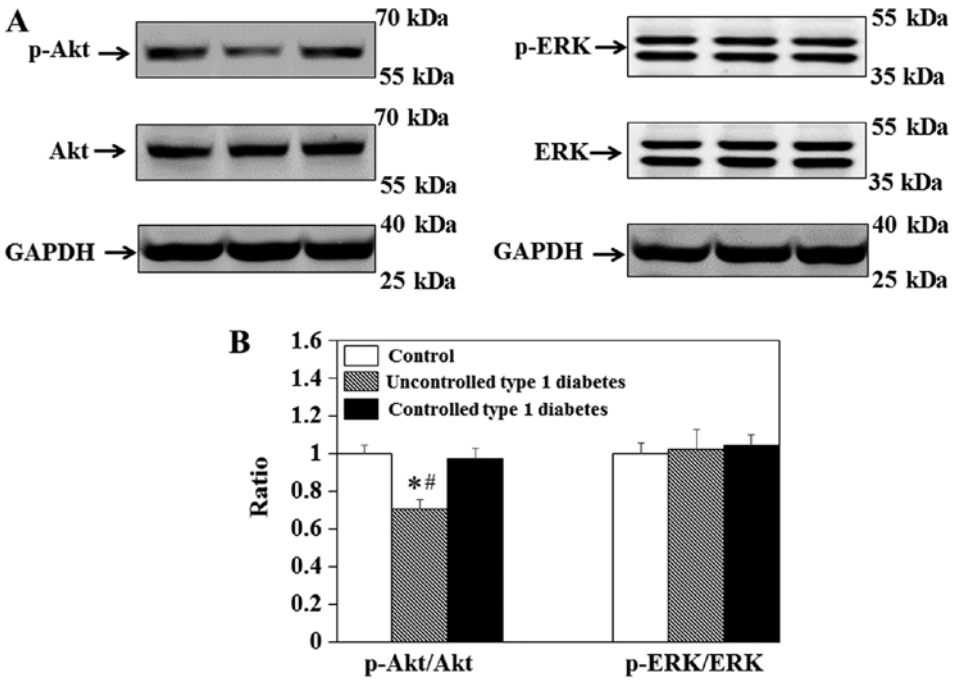

Figure 5. Akt and ERK expression in VSMCs. (A) Immunoblotting of phosphorylated p-Akt (Ser473), total Akt, p-ERK (Thr202/Tyr204), total ERK and GAPDH in VSMCs treated with varying concentrations of glucose (5 or 25 mM) and/or insulin (100 nM). Data are presented as relative to control. (B) Bar graph demonstrated the ratio of p-Akt/Akt and p-ERK/ERK expression in VSMCs. Values are presented as mean ± standard deviation (n=4). *P<0.05 vs. control group; #P<0.05 vs. controlled type 1 diabetes group. VSMCs, vascular smooth muscle cells; p-, phosphorylated; Akt, protein kinase B; ERK, extracellular signal-regulated kinase.

insulin levels, respectively. However, as described previously, in the setting of insulin resistance, the MAPK/ERK signaling pathway may be overactive, promoting proliferation and migration; therefore, a higher ratio of p-ERK/ERK to p-Akt/Akt was associated with increased neointimal hyperplasia following vascular injury (8,11,14). The results of the current study demonstrated that the increased ratio of p-ERK/ERK to p-Akt/Akt did not produce increased neointimal hyperplasia following vascular injury in the setting of uncontrolled type 1 diabetes with low insulin. Nevertheless, the increased ratio of p-ERK/ERK to p-Akt/Akt in controlled type 1 diabetic rats compared with control rats with normal insulin levels may, at least in part, be associated with enhanced neointimal hyperplasia following vascular injury, which is consistent with previously published studies (10,11,14,28).

The current study had limitations. Data were both similar to (8,28) and different from (10,29) published studies, indicating that glucose can stimulate and inhibit VSMC proliferation (8,10,28,29). Regardless, the current study also observed a direct association between *in vitro* and *in vivo* experiments with regard to VSMC proliferation and neointimal hyperplasia in type 1 diabetes with insulin therapy. Notably, the results reported that PI3K/Akt signaling served a further important role in VSMC proliferation in the type 1 diabetic model with different insulin levels. Additionally, the insulin levels in type 1 diabetic rats indicated the damage to the pancreatic tissue induced by STZ, irrespective of insulin administration. However, the pancreatic tissue should be examined to confirm β cell damage in future studies.

The preferential signaling along the PI3K/Akt pathway of insulin action in response to insulin restoration may contribute to neointimal hyperplasia. The present study provided a novel method for further treatment of atherosclerosis in type 1 diabetes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JC conceived the current study, analyzed data and wrote the manuscript. HW and HS acquired and analyzed data. RY, YC, KL and ZQ performed the experiments. MJ, YX, RG and QL analyzed data and revised the manuscript. XL conceived the current study and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were performed according the Guide for the Care and Use of Laboratory Animals published by

the NIH (publication no. 85-23; 1996) and approved by the Institutional Animal Care and Use Committee of Nanjing University, Nanjing, China.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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