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Role of $K_{Ca}3.1$ Channels in Proliferation and Migration of Vascular Smooth Muscle Cells by Diabetic Rat Serum

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Abstract

Proliferation and migration of vascular smooth muscle cells (VSMCs) are important events in the development of diabetic atherosclerosis. Previous studies have suggested that $K_{Ca}3.1$ channels participate in atherosclerosis and coronary artery restenosis. In the present study, we attempted to clarify the roles of $K_{Ca}3.1$ channels in the proliferation and migration of VSMCs using experimental type-2 diabetes rat serum and aortic smooth muscle cells (SMC) prepared from non-diabetic rats. mRNA and protein levels and current density of $K_{Ca}3.1$ channels were greatly enhanced in cultured VSMCs treated with diabetic serum. In addition, diabetic serum promoted cell proliferation and migration in cultured VSMCs, and the effects were fully reversed in the cells treated with the $K_{Ca}3.1$ channels blocker TRAM-34. In conclusion, serum from diabetic rats increases the expression of $K_{Ca}3.1$ channels and promotes proliferation and migration of VSMCs to possibly participate in vascular remodeling in diabetes.

Key Words: intermediate-conductance Ca^{2+} activated K^+ channel, migration, proliferation, smooth muscle cell

Introduction

Diabetes is a major threat to global public health and diabetic macrovascular complication underlies the pathogenesis of diabetic patients' cardiovascular and cerebrovascular accidents (21). As atherosclerotic lesions progress, smooth muscle cells migrate from media and participate in the formation of a fibrous cap. Proliferation and migration of vascular smooth muscle cells (VSMCs) represent an essential event in the diabetic atherosclerosis. In several studies, numerous mitogenic factors, such as insulin, advanced glycosylation end products (AGEs) and hyperglycemia, have been proposed to promote the observed proliferation and migration of VSMCs *in vitro* (1, 3, 33). In recent years, much research has focused on the role of $K_{Ca}3.1$ channels in atherosclerosis (12, 26, 28). The intermediate-conductance calcium-activated potassium channels (IK_{Ca}, $K_{Ca}3.1$) regulates membrane potential and calcium signaling (32). As hyperpolarization and/or depolarization are required for the proliferation in mitogen-stimulated cycling cells, oscillatory intracellular Ca^{2+}-regulated $K_{Ca}3.1$ channels play a crucial role in cell migration and proliferation (4, 6). We and others have previously reported in rats and swine that $K_{Ca}3.1$ expression is significantly increased in VSMCs during vascular remodeling followed by balloon catheter injury, myocardial infarction, hypertension and early atherosclerosis (24, 26). In the present study, we determined whether expression of $K_{Ca}3.1$ channels was regulated by serum of diabetic rats in cultured rat VSMCs.

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Furthermore, we explored the role of $K_{Ca}3.1$ channels in proliferation and migration in VSMCs. We found that the expression of $K_{Ca}3.1$ channels increased in VSMCs treated with diabetic serum. Pharmacological blockade $K_{Ca}3.1$ activities resulted in the suppression of proliferation and migration in VSMCs. We, therefore, propose that diabetic serum promoted migration and proliferation of VSMCs by upregulating $K_{Ca}3.1$ channels, for which it might participate in angiopathy in diabetes.

Materials and Methods

Experimental Animals

Experimental protocols were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA. Male Sprague-Dawley rats (n = 45, 173 ± 18 g), obtained from the Laboratory Animal Centre of Xi'an Jiaotong University, were housed under conditions of 22 ± 2°C, humidity of 55% ± 5%, and a 12 h/12 h light/dark cycle. The animals were randomly divided into non-diabetic and diabetic groups. Type-2 diabetes was induced by a single intraperitoneal injection (i.p.) of 30 mg/kg Streptozotocin (STZ) after 8 weeks on a modified high fat and glucose diet (10% grease, 20% sucrose, 1% bile salt, and 2.5% cholesterol) (31). Blood glucose was measured by using One Touch SureStep Glucose Meter (LifeScan Inc., Milpitas, CA, USA) 1 week after STZ injection. Only animals with > 11.1 mM of blood glucose were included in the diabetic group. Age-matched animals fed with standard rat chow and injected with the drug vehicle citric acid buffer served as the control. One month after STZ injection, rats in the two groups were fed with normal diet for an additional two months. When animals were sacrificed, the blood sera were prepared. Non-diabetic control rats were age-matched with the diabetic rats at the time of sacrifice. The type-2 diabetes was characterized with increase of blood serum insulin (0.035 *vs.* 0.013 μIU/ml of control), fasting blood glucose (22.8 *vs.* 5.2 mM), serum cholesterol (3.6 *vs.* 1.5 mM), glycosylated hemoglobin (19.2% *vs.* 10.5%) and mean artery pressure (143.6 *vs.* 95.8 mmHg) as well as reduced body weight (338 *vs.* 523.8 g) as previously described (31).

Culture of VSMC

VSMCs in primary culture were obtained using explant culture method as described previously (11). Briefly, 6-week-old Sprague Dawley rats (either sex) were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the aortae were quickly excised and placed in cold Dulbecco's modified Eagle's medium (DMEM, with 5 mM glucose) (Gibco, Burlington, ON, Canada). After the layers of adventitia and endothelium were carefully stripped off, the medial VSMC layers were cut into 1 mm^3 scraps, explanted in culture flask with DMEM containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) and incubated at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed twice weekly and the cells were subcultured when grown to 80% confluence. Cultured cells of passages 6-9 were used in experiments. The cells were treated with DMEM containing 5% diabetic rat serum or non-diabetic serum for 24 h after incubation with serum-free DMEM for 24 h.

Electrophysiology

Membrane ionic currents in cultured VSMCs were recorded with the whole-cell patch-clamp technique as previously described (25). VSMCs attached to bottom of the cell chamber mounted on an inverted microscope were perfused with Tyrode solution with the following composition (mM): 136 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 0.33 NaH_2PO_4, 10 glucose, and 10 HEPES with pH adjusted to 7.4 with NaOH. Borosilicate glass electrodes (1.2-mm outer diameter) were pulled with a Brown-Flaming puller (Model P-97; Sutter Instrument Co., Novato, CA, USA), and had tip resistances of 2-4 MΩ when filled with pipette solution containing (mM): 120 KCl, 10 NaCl, 0.15 $CaCl_2$, 1.0 $MgCl_2$, 0.2 EGTA, and 10 HEPES with pH adjusted to 7.2 with KOH. The tip potentials were compensated before the pipette touched the cell. After a gigaohm seal was obtained by negative suction, the cell membrane was ruptured by gentle suction to establish whole-cell configuration. Membrane currents were recorded with an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA), filtered at 5 kHz using a low-pass Bessel filter and stored on the hard disk of an IBM-compatible computer for subsequent analysis with Clampfit 9.0.

All electrical recordings were performed at room temperature (21-22°C).

Reverse Transcriptase-Polymerase Chain Reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with the procedure described previously (13). Briefly, total RNA of VSMCs was isolated by using the TRIzol method (Invitrogen), and then treated with DNase I (Invitrogen). Reverse transcription (RT) was performed with a RT system (Fermentas, St. Leon-Rot, Germany) protocol in a

20-µl reaction mixture using oligo $(dT)_{18}$ primers. The cDNA reaction product was used for polymerase chain reaction (PCR). Specific primers were designed with Primer Premier 5 software (Premier Biosoft International, Pola Alto, CA, USA) and synthesized by Pioneer Biotech (Pioneer Biotech Ltd., Xi'an, Shaanxi, PRC). The primers of K_{Ca}3.1 channels (accession no. NM_023021) were: forward primer, 5'-CACGCTGATGTTGTGGTT-3'; reverse primer, 5'-CGATGCTGCGGTAAGACG-3'. The primers of the GAPDH (accession no. NM_017008) were: forward primer, 5'-GTGCTGAGTATGTCGTGGAG-3'; reverse primer, 5'-GTCTTCTGAGTGGCAGT GAT-3'. PCR was performed with 2 × Taq PCR MasterMix (Pioneer Biotech Ltd., Xi'an, Shaanxi, PRC). The cDNA at 3 µl aliquots was amplified by a DNA thermal cycler in 25 µl of reaction mixture under the following conditions: the mixture was denatured at 95°C (30 s), annealed at 55°C (30 s), and extended at 72°C (1 min) for 35 cycles. This was followed by a final extension at 72°C (10 min) to ensure complete product extension. The PCR products were electrophoresed through a 1% agarose gel, and the amplified cDNA bands were imaged by the Chemi-Genius Bio Imaging System (Syngene, Cambridge, UK). When the cDNA was replaced by the RNA sample, no significant bands were detected.

Western Blot Analysis

VSMCs incubated with diabetic rat serum or non-diabetic rat serum for 24 h were lysed with ice-cold modified RIPA buffer (60 mM Tris-HCl, 0.25% SDS, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 10 µg/ml aprotinin and leupeptin). The lysates were then centrifuged at 12,000 ×*g* for 5 min at 4°C. After transferring the supernatant to a fresh ice-cold tube, the protein concentration was determined with a protein assay kit (Bio-Rad, Hercules, CA, USA). Equal concentrations of proteins were mixed with SDS sample buffer and denatured at 100°C for 5 min. The samples were separated on an SDS-10% polyacrylamide gel, then transferred to a PVDF membrane at 50 mA for 1.5 h in a transfer buffer containing 20 mM Tris, 150 mM glycine and 20% methanol. The membranes were blocked with 5% non-fat dried milk in TBST (0.1% Tween-20) for 1 h. After being blocked, the blots were incubated in a primary antibody for K_{Ca}3.1 (1:200) (Alomone Labs Ltd., Jerusalem, Israel) at 4°C overnight, and then incubated with HRP-conjugated secondary antibodies (1:1000) for 1 h at room temperature. The bound antibodies were detected with an enhanced chemiluminescence detection system (ECL, Amersham, Piscataway, NJ, USA), and quantified by densitometry, using a Chemi-Genius Bio Imaging System (Syngene). To ensure equal sample loading, the ratio of band intensity to GAPDH was obtained to quantify the relative protein expression levels.

Cell Migration Assay

The migration of VSMCs was determined by wound healing assay and modified Boyden Chamber technique. Confluent cultures of VSMCs in 6-well plates were wounded with a sterile 200 µl plastic pipette tip as described previously (10). The starting point was marked with a marker pen at the bottom of the plate. After incubation with a K_{Ca}3.1 channels blocker for 30 min followed by incubation in DMEM containing 2% diabetic or normal rat serum for 24 h, the defined area of the wound was photographed under a phase contrast microscope (Olympus, Tokyo, Japan) and the migrated cells were counted. In Boyden Chamber assay, a 24-well Transwell apparatus with each well containing a 6.5-mm polycarbonate membrane with 8-µm pores was used. Serum-starved VSMCs were trypsin-harvested. Cell suspension in basal medium (250 µl, 1×10^5 cell/well) seeded in the upper chamber; 750 µl of basal medium with 2% diabetic or normal rat serum was added to the lower chamber and incubation was performed for 24 h. The cells were labeled by hematoxylin and the migrated cells were quantified after the plating surface cells were removed. Each treatment was repeated in four independent transwells.

Cell Proliferation Assay

Cell proliferation was determined by the MTT assay and cell counting as previously described (25). Briefly, rat VSMCs were plated in 96-well plates at a density of 1×10^4 cells per well in 200 µl DMEM containing 10% FBS. TRAM-34 was used to pretreat the cells for 30 min before 5% diabetic rat serum was added. Following 24 h culture, 20 µl PBS-buffered MTT (5 mg/ml) was added to each well and the cells were maintained at 37°C for an additional 4 h. The medium was then removed and 100 µl/well DMSO was added to each well to dissolve the purple formazan crystals, and the absorbance at 490 nm was measured. Results were standardized by using the control group values. In cell counting experiment, cells stimulated for 48 h were detached with trypsin-EDTA (0.05%) and counted by using a standard hemocytometer in a blinded manner. Cell viability was established by trypan blue exclusion.

Statistical Analysis

Results are presented as means ± SEM. Paired and/or unpaired Student's *t*-tests were used as appropriate to evaluate the statistical significance of differences

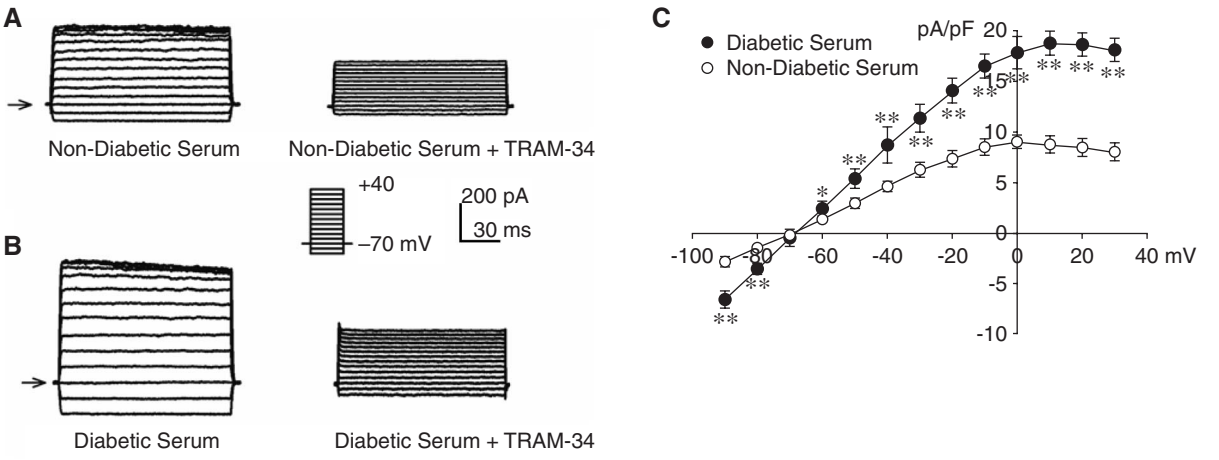

Fig. 1. $K_{Ca}3.1$ channels currents in rat VSMCs. A: Membrane currents recorded in a normal VSMC with the protocol as shown in the inset display the property of weak inward rectification at positive potentials. The current is sensitive to inhibition by the specific $K_{Ca}3.1$ blocker TRAM-34 at 100 nM. The arrow indicates zero current level. B: Membrane currents recorded in a representative cell treated with diabetic rat serum before and after TRAM-34 application. C: I-V relationships of membrane currents in cells with non-diabetic and diabetic serum treatment (n = 5, $*P < 0.05$, $**P < 0.01$ *vs.* non-diabetic serum).

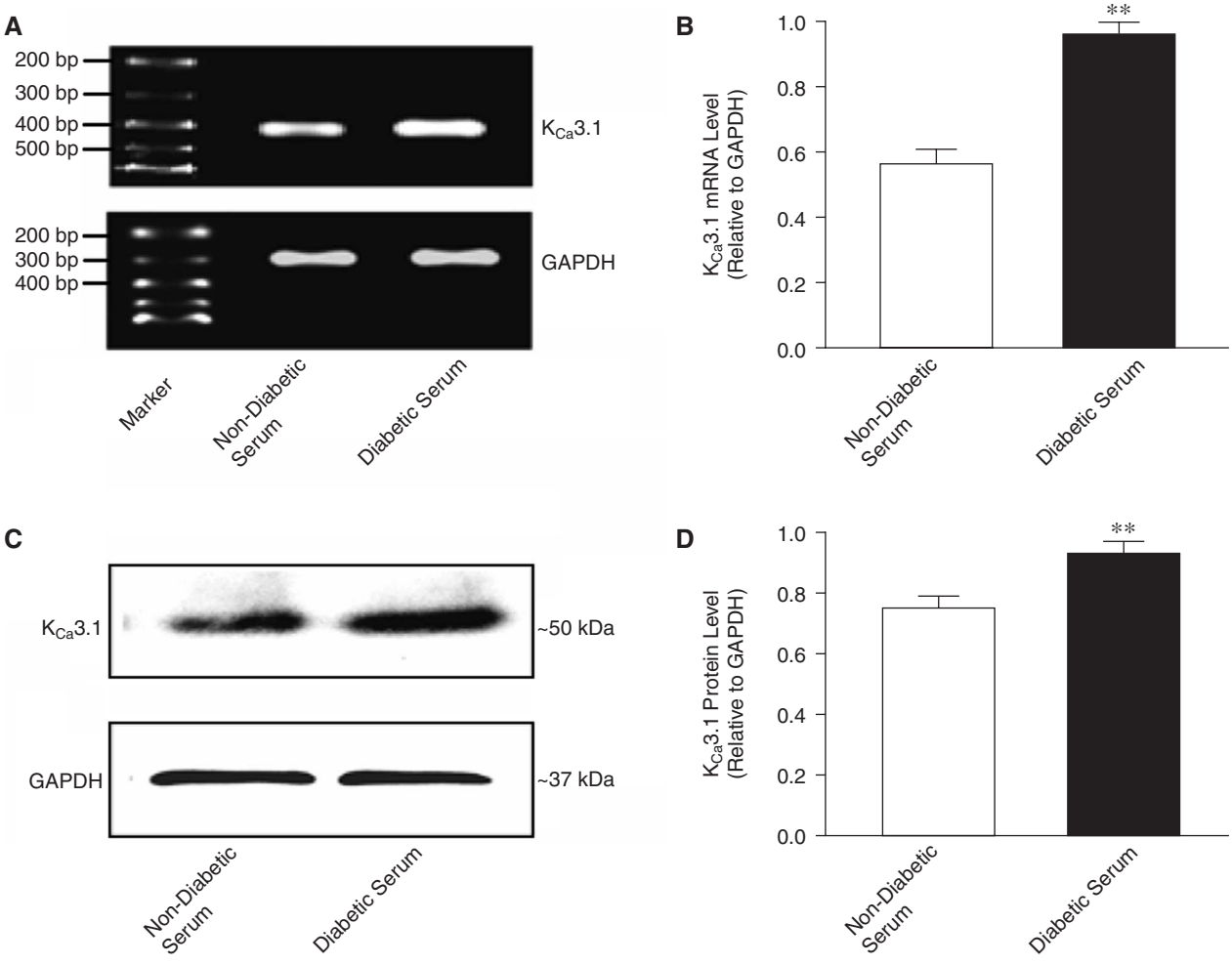

Fig. 2. $K_{Ca}3.1$ channels expression in rat VSMCs. A: Gel image of $K_{Ca}3.1$ channels mRNA in rat VSMCs treated without or with diabetic serum. B: Semi-quantitative analysis of $K_{Ca}3.1$ channels mRNA levels in cultured VSMCs treated without or with diabetic serum (n = 6, $**P < 0.01$ *vs.* non-diabetic serum) by RT-PCR. C: Representative western blot images of $K_{Ca}3.1$ channels and GAPDH protein in rat VSMCs treated without or with diabetic serum. D: Quantitative analysis of $K_{Ca}3.1$ channels protein in rat VSMCs treated without or with diabetic serum (n = 6, $**P < 0.01$ *vs.* non-diabetic serum).

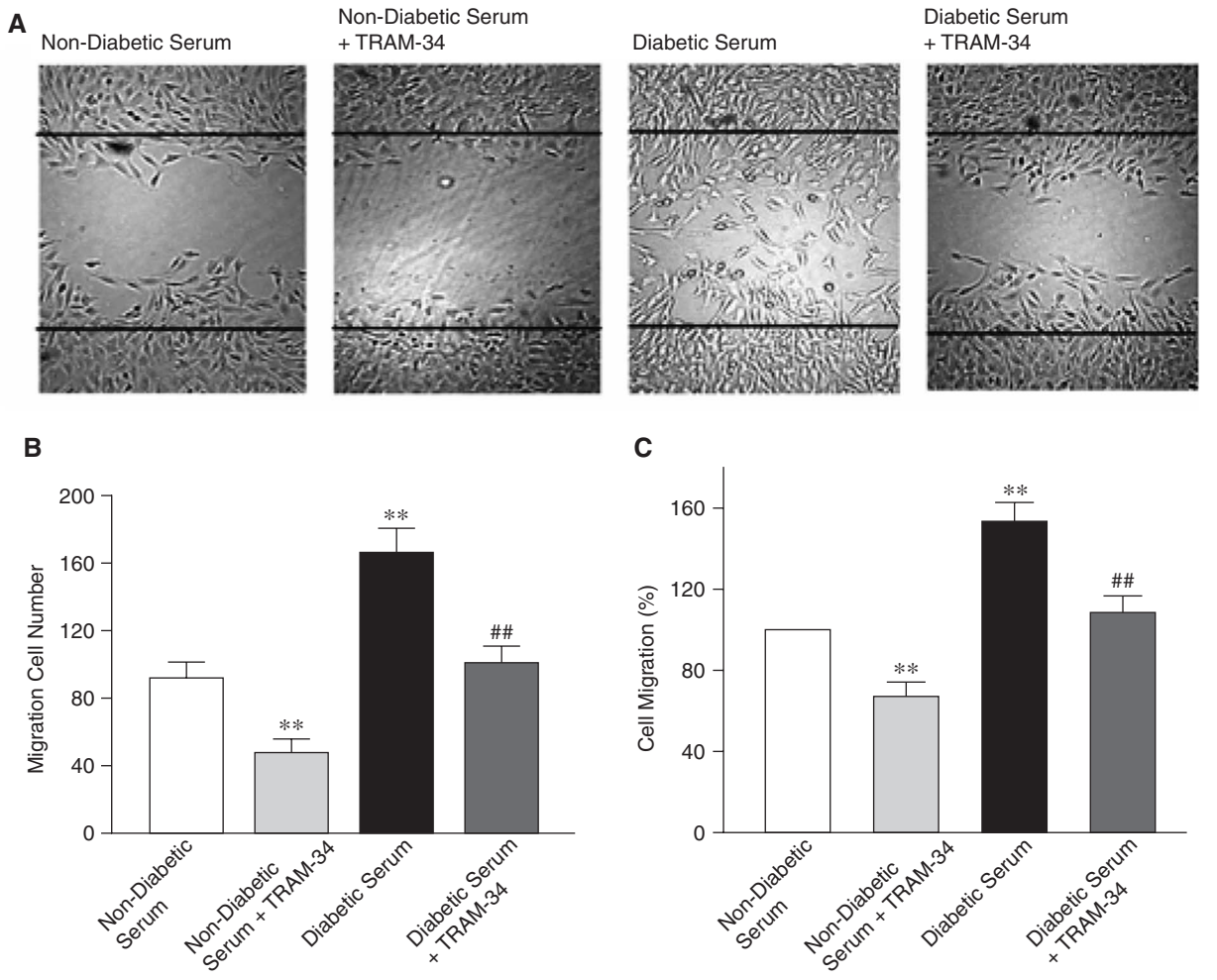

Fig. 3. Effects of diabetic serum and K_{Ca}3.1 channels on VSMCs migration. A: Wound healing migration assay of VSMCs. Confluent VSMCs were scraped of with a pipette tip to induce acellular areas then treated with non-diabetic or diabetic rat serum. Photos were taken after 24 h (upper panel). Black lines indicate the initial acellular wound regions. B: Mean values for the number of migrated VSMCs counted in areas as marked in A (n = 6, $^{**}P < 0.01$ *vs.* non-diabetic serum, $^{\#\#}P < 0.01$ *vs.* diabetic serum alone). C: Migration measured by the Boyden Chamber technique and expressed as percentage of migrated cells in the cells treated with non-diabetic serum and diabetic serum (n = 6, $^{**}P < 0.01$ *vs.* non-diabetic serum, $^{\#\#}P < 0.01$ *vs.* diabetic serum alone).

between two group means and analysis of variance (ANOVA) was performed for multiple groups. A value of $P < 0.05$ was considered statistically significant.

Results

K_{Ca}3.1 Current in Cultured VSMCs

The membrane currents recorded in a representative VSMC with 150-ms voltage steps to between –90 and +40 mV from a holding potential of –70 mV (inset) were displayed in Fig. 1A. The current showed a weak inward rectification at positive potentials and was sensitive to inhibition by the K_{Ca}3.1 channels blocker TRAM-34 (100 nM); this indicates that the current predominantly present in the cultured rat VSMCs treated with non-diabetic serum is carried by K_{Ca}3.1 channels. Interestingly, diabetic rat serum significantly enhanced K_{Ca}3.1 and co-application of diabetic serum and 100 nM TRAM-34 dramatically reversed the K_{Ca}3.1 (Fig. 1B). Current-voltage (I-V) relationships of membrane currents showed a weak inward rectification in control cells at +10 mV to +40 mV. The current density was greater in cells treated with diabetic serum (Fig. 1C). The results indicate that K_{Ca}3.1 channels are upregulated by diabetic serum in cultured rat VSMCs.

Diabetic Serum Regulates K_{Ca}3.1 Channels Expression in VSMCs

The expression levels of K_{Ca}3.1 channels mRNA and protein in cultured rat VSMCs with or without treatment of diabetic serum are shown in Fig. 2. Dia-

betic serum significantly increased $K_{Ca}3.1$ channels mRNA and protein expression levels (Fig. 2, A and C); the mean values of $K_{Ca}3.1$ channels mRNA and protein levels are illustrated in Fig. 2B and D. Diabetic serum significantly increased $K_{Ca}3.1$ channels mRNA and protein levels ($P < 0.01$ *vs.* control). These results indicate that diabetic serum upregulates $K_{Ca}3.1$ gene and protein expression.

Role of Diabetic Serum and $K_{Ca}3.1$ Channels in Migration of Rat VSMCs

To investigate whether upregulation of $K_{Ca}3.1$ channels by diabetic serum in VSMCs mediates cell activity, the effects of diabetic serum on cell migration were determined in cultured rat VSMCs. We first used a wound-healing assay to investigate the role of diabetic serum and $K_{Ca}3.1$ channels blocker in cell migration (Fig. 3, A and B). Cells in culture were scraped off with a pipette tip and a wide acellular area was produced. VSMCs migrated into this area were counted and expressed as the number of migrated cells. Diabetic serum significantly increased cell migration (n = 6, $P < 0.01$) and the effect was antagonized by TRAM-34.

We then confirmed the effects of $K_{Ca}3.1$ channels on VSMC migration with the Boyden chamber apparatus. As shown in Fig. 3C, VSMC migration was 154 ± 9.24% of the non-diabetic serum group (n = 6, $P < 0.01$) in the cells treated with diabetic serum, 108 ± 8.47% in the cells treated with the diabetic serum plus 100 nM TRAM-34 (n = 6, $P < 0.01$ *vs.* diabetic serum alone). These findings indicate that $K_{Ca}3.1$ channels play an important role in diabetic VSMC migration.

Role of Diabetic Serum and $K_{Ca}3.1$ Channels in Proliferation of Rat VSMCs

The effects of diabetic serum and $K_{Ca}3.1$ channels on VSMC proliferation were determined by cell counting and MTT assay. As shown in Fig. 4A, diabetic serum significantly increased cell number and the effect was antagonized by 100 nM TRAM-34. The cell proliferation determined with MTT assay is shown in Fig. 4B. Cell proliferation was 138.25 ± 17.28% of the non-diabetic serum group (n = 6, $P < 0.01$) in the cells treated with diabetic serum, 104.39 ± 18.49% in the cells treated with 100 nM TRAM-34 (n = 6, $P < 0.01$ *vs.* diabetic serum alone). These results suggest that $K_{Ca}3.1$ channels participate in diabetic-mediated rat VSMC proliferation.

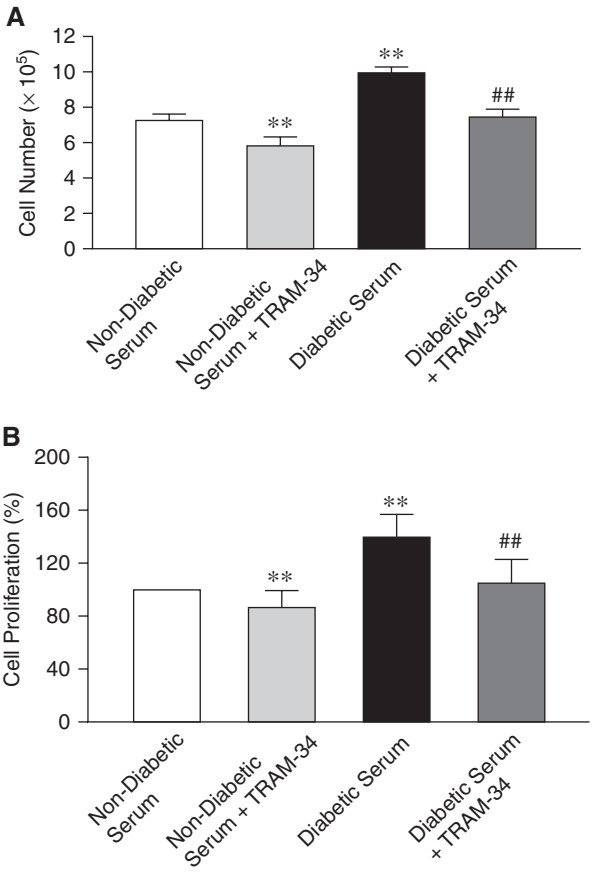

Fig. 4. Effects of diabetic serum and $K_{Ca}3.1$ channels on VSMC proliferation. A: Cell counting shows that diabetic serum increased VSMC number, the effect was countered by 100 nM TRAM-34 (n = 6, $^{**}P < 0.01$ *vs.* non-diabetic serum, $^{\#\#}P < 0.01$ *vs.* diabetic serum alone). B: Cell proliferation determined by MTT (n = 6, $^{**}P < 0.01$ *vs.* non-diabetic serum, $^{\#\#}P < 0.01$ *vs.* diabetic serum alone).

Discussion

Diabetes mellitus is a complex disease comprising a series of metabolic disturbances with hyperglycaemia, hyperinsulinemia and an increase in circulating AGEs, which are associated with vascular complications of diabetes such as nephropathy, retinopathy and the development of atherosclerosis (2, 16, 30). VSMCs may play an influential role in the progression of diabetic macroangiopathy. In atherosclerosis, VSMCs may be activated in response to metabolic factors to migrate from the media to intima of the aorta and proliferate. Hyperglcemia exerts direct effects toward the progression of atherosclerosis (3). The accelerated atherosclerosis in diabetes is most likely to increase the nonenzymatic glycation of protein and lipids, with the formation of reactive AGEs. AGEs can cause mechanical dysfunction in the vessel wall macromoleculars by cross-bridging. AGE accumulation causes circulating blood cells to adhere to vessel wall. AGEs also disturb cellular functions by binding to a variety of receptors that have been identified on various cell

types, including macrophages, endothelial cells, smooth muscle cells and renal and neuronal cells (6, 9, 17, 20, 34). Hyperinsulinemia and insulin resistance are characteristics of type-2 diabetes mellitus. Clinical researches have indicated that hyperinsulinemia is a predictor of cardiovascular disease (18, 21). Recently, studies have shown that insulin exceeding physiological concentrations can accelerate VSMC proliferation and phenotypic transition *in vitro*, which suggests that insulin plays a role in diabetic atherosclerosis (14, 15, 18). The synergism of metabolic factors is an important issue. Until now, few studies have focused on the synergistic effects of these factors on diabetic macrovascular complications. In the present study, the rat model of type-2 diabetes was characterized with hyperinsulinemia, hyperglycemia and increased glycosylated hemoglobin. Thus, our study indicated the synergistic effect of these factors on VSMCs using diabetic serum.

There is growing evidence that several ion channels are of importance in the control of cell proliferation, in particular, K_{Ca}3.1 channels have been shown to regulate cell cycle progression and mitogenesis *in vitro* (7, 22). K_{Ca}3.1 channels are the predominant Ca^{2+}-activated K^+ channels that regulate membrane potential in proliferative smooth muscle cells. It seems to participate in cell proliferation by increasing the ability of cell to hyperpolarization and thereby to promote Ca^{2+} influx, and, thus, sustain a high intracellular Ca^{2+} concentration required for gene transcription, as has been reported in lymphocytes and fibroblasts (8, 19). Upregulation of K_{Ca}3.1 channels has been shown to promote excessive VSMC proliferation and migration induced by either mitogens (PDGF and EGF) or balloon catheter injury (12, 23, 27). K_{Ca}3.1 has been characterized in numerous cell types which contribute to the development of atherosclerosis, such as T-cells, B-cells, endothelial cells, fibroblasts, macrophages and dedifferentiated smooth muscle cells. K_{Ca}3.1 is expressed in all of these cell types, and furthermore, has been demonstrated to play a role in the proliferation of T-cells, B-cells, fibroblasts and VSMCs, as well as in the migration of VSMCs and macrophages and in platelet coagulation (5, 29). Studies have indicated that blockade of K_{Ca}3.1, by specific blockers, such as TRAM-34, could prove to be an effective treatment for vascular disease by inhibiting T-cell activation as well as preventing proliferation and migration of macrophages, endothelial cells and SMCs (26). This vascular protective potential of K_{Ca}3.1 inhibition has been confirmed in both rodent and swine models of restenosis (27, 28). We have recently found that insulin increases the expression of K_{Ca}3.1 channels by enhancing ERK1/2 phosphorylation and, therefore, promoting migration and proliferation of VSMCs, which very likely play at least a partial role in the development of vasculopathy in type-2 diabetes (24). These data demonstrate that K_{Ca}3.1 upregulation is necessary for SMC dedifferentiation, proliferation and migration, contributing to arterial lesion development in diabetes.

In the present study, using the approaches of molecular biology and electrophysiology, we demonstrated that diabetic rat serum augmented K_{Ca}3.1 channels mRNA and protein expression and increased current density. In addiction, we found that diabetic serum enhanced migration and proliferation of rat VSMCs, and the effects were antagonized by the selective K_{Ca}3.1 channels blocker TRAM-34. The new findings provide a direct link between diabetic rat serum and K_{Ca}3.1 channels in the regulation of VSMC migration and proliferation. The limitation of the study was that we did not explore the underlying signal transduction mechanism leading to upregulation of this channels in VSMCs. Upregulation of K_{Ca}3.1 has been demonstrated to be an early and necessary step in smooth muscle cell phenotypic modulation, proliferation and migration all of which exert impact on the onset and development of numerous vascular proliferative diseases, such as atherosclerotic and post-angioplasty restenotic lesions. In diabetic, abnormal metabolic factors are associated with macrovascular complications such as stroke, atherosclerosis, cardiovascular disease and peripheral arterial disease. Therefore, blockade of K_{Ca}3.1 represents a potential treatment for reducing the progression of diabetic atherosclerosis.

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