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K_{Ca}3.1 channels mediate the increase of cell migration and proliferation by advanced glycation endproducts in cultured rat vascular smooth muscle cells

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The mechanisms underlying the involvement of advanced glycation endproducts (AGEs) in diabetic atherosclerosis are not fully understood. The present study was designed to investigate whether intermediate-conductance Ca^{2+}-activated K^+ channels (K_{Ca}3.1 channels) are involved in migration and proliferation induced by AGEs in cultured rat vascular smooth muscle cells (VSMCs) using approaches of whole-cell patch voltage-clamp, cell proliferation and migration assay, and western blot analysis. It was found that the current density and protein level of K_{Ca}3.1 channels were enhanced in cells incubated with AGE-BSA (bovine serum albumin), and the effects were reversed by co-incubation of AGE-BSA with anti-RAGE (anti-receptors of AGEs) antibody. The ERK1/2 inhibitors PD98059 and U0126, the P38-MAPK inhibitors SB203580 and SB202190, or the PI3K inhibitors LY294002 and wortmannin countered the K_{Ca}3.1 channel expression by AGE-BSA. In addition, AGE-BAS increased cell migration and proliferation, and the effects were fully reversed with anti-RAGE antibody, the K_{Ca}3.1 channel blocker TRAM-34, or K_{Ca}3.1 small interfering RNA. These results demonstrate for the first time that AGEs-induced increase of migration and proliferation is related to the upregulation of K_{Ca}3.1 channels in rat VMSCs, and the intracellular signals ERK1/2, P38-MAPK and PI3K are involved in the regulation of K_{Ca}3.1 channel expression.

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It is generally recognized that advanced glycation endproducts (AGEs) are formed by the nonenzymatic glycation reaction of aldose sugars with amino groups of proteins.[1,2] In diabetes mellitus, the high-level glucose reacts with protein and forms the adduct AGEs.[2,3] Atherosclerosis and related microvascular disorders are major vascular complications and constitute the increasing morbidity and mortality in diabetes mellitus. Recent studies have demonstrated that AGEs and the receptors of AGEs (RAGE) are upregulated in atherosclerotic plaques in diabetic subjects, particularly in intimal macrophages and smooth muscle cells.[4–7] Accumulation of AGEs and activation of RAGE are found to mediate proliferation, migration, and inflammatory gene expression in vascular smooth muscle cells (VSMCs), which are believed to accelerate the formation of atherosclerosis in diabetes;[8–10] however, the detailed mechanisms underlying atherosclerosis are not fully understood.

Intermediate-conductance Ca^{2+}-activated K^+ (K_{Ca}3.1 or I_{KCa}) channels are upregulated in the proliferative VSMCs.[11–14] Inhibition of K_{Ca}3.1 channels by the selective blocker TRAM-34 is found to inhibit proliferation mediated by platelet-derived growth factor in VSMCs and decrease the development of restenosis.[15] Local delivery of TRAM-34 via balloon catheter prevents ion channel phenotype switching (K_{Ca}1.1 to K_{Ca}3.1) in VSMCs of coronary artery and reduces subsequent restenosis.[14] Moreover, Toyama *et al*[16] have provided the additional evidence that upregulation of K_{Ca}3.1 channels is related to atherogenesis in mice and humans.

We have recently demonstrated that glycosylated hemoglobin (ie, AGE) level is remarkably increased in a

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diabetic rat model established by feeding high fat and glucose diet and then followed by injecting a small dose streptozotocin,[17] and $K_{Ca}3.1$ channel expression was significantly increased in intimal and medial layers of the aorta in this diabetic rat model.[18] In the present study, we investigated whether the effect of AGEs would be related to the activity of $K_{Ca}3.1$ channels in cultured rat VSMCs using electrophysiology and molecular biological approaches.

MATERIALS AND METHODS

Experimental Regents

The ERK1/2 inhibitor PD98059 and U0126, the P38-MAPK inhibitor SB203580, and the PI3K inhibitor LY294002 were purchased from Promega (Madison, WI, USA). Wortamannin and $K_{Ca}3.1$ antibody were obtained from Alomone Laboratory (Jerusalem, Israel). Anti-RAGE antibody was from R&D system (Minneapolis, MN, USA). Anti-bromodeoxyuridine (BrdU) antibody, SB202190, 1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole (TRAM-34) and other chemicals used in this study were obtained from Sigma-Aldrich (St Louis, MO, USA).

Preparation of AGE-BSA

AGE-bovine serum albumin (AGE-BSA) was prepared as described previously.[19,20] Briefly, cell culture-tested BSA with low endotoxin (2.0 g, Sigma-Aldrich) was dissolved in 10 ml of 0.5 mM phosphate-buffered saline (pH 7.4) and 1.6 mM D-glucose, sterilized by ultrafiltration, and then incubated at 37 °C for 12 weeks, followed by dialysis against phosphate-buffered saline for 24 h to remove unincorporated glucose. Control non-glycated BSA (2.0 g in 10 ml phosphate-buffered saline) was prepared with the same procedure without D-glucose inclusion. AGE-BSA content was estimated by fluorescence spectroscopy (excitation at 390 nm and emission at 460 nm) at a protein concentration of 1 mg/ml, which indicated a 10-fold increase in fluorescence in AGE-BSA compared with control BSA. The prepared AGE-BSA and unmodified BSA were sterilized by ultrafiltration and stored at −20 °C until use. Endotoxin levels of each preparation were measured by Limulus amoebocyte lysate assay (E-Toxate, Sigma, St Louis, MO, USA) and were found to be <0.8 EU/ml.

Cell Culture and Treatments

Rat VSMCs were isolated using explant culture method as described previously.[18] Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells at 70% confluence were starved for 24 h in DMEM containing 0.1% FBS and were treated with anti-RAGE antibody (5 μg/ml), the $K_{Ca}3.1$ channel blocker TRAM-34 (100 nM), the ERK1/2 inhibitors PD98059 (25 μM) and U0126 (10 μM), the P38-MAPK inhibitors SB203580 (10 μM) and SB202190 (10 μM), and the PI3K inhibitors LY294002 (10 μM) and wortamannin (100 nM) for 30 min. Later, AGE-BSA (200 μg/ml) was added into the medium containing 0.1% FBS for continuous culture.

Electrophysiology

Membrane current was recorded in cultured VSMCs with the whole-cell patch-clamp technique as described previously.[21] The detached VSMCs were placed into a 0.2-ml cell chamber mounted on inverted microscope, allowed to settle to the bottom (~20 min), and then superfused with Tyrode solution (pH 7.4) containing (mM): 136 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, and 10 HEPES. Borosilicate glass electrodes (1.2 mm outer diameter) were pulled with a Brown-Flaming puller (Model P-97; Sutter Instrument, Novato, CA, USA) and had a resistance of 2–3 MΩ when filled with pipette solution containing (mM): 120 K-aspartate, 20 KCl, 1.0 $MgCl_2$, 5.0 EGTA, 5 Na-phosphocreatine, 5 Mg-ATP, 0.1 GTP, 10 HEPES, 500 nM free Ca^{2+} with pH adjusted to 7.2 with KOH. The tip potentials were zeroed before the pipette contacted the cell. After a gigaohm seal was obtained by negative suction, the cell membrane was ruptured by a gentle suction to establish whole-cell configuration. Membrane currents were recorded with an EPC-9 amplifier and Pulse software (Heka Elektronik, Lambrecht, Germany). Command pulses were generated by a 12-bit digital-to-analog converter controlled by Pulse software. All current recording experiments were conducted at room temperature (22–23 °C).

Western Blot Analysis

VSMCs incubated with AGE-BSA in the presence or absence of indicated inhibitors for 24 h (for $K_{Ca}3.1$ immunoblotting) or 30 min (for ERK1/2, phospho-specific ERK1/2, P38, phospho-specific P38, Akt, and phospho-specific Akt immunoblotting) were lysed with ice-cold modified RIPA buffer (60 mM Tris-HCl, 0.25% SDS, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 2 μg/ml aprotinin and leupeptin). The lysates were then centrifuged at 12 000 *g* for 15 min at 4 °C. After transferring the supernatant to a fresh ice-cold tube, the protein concentration was determined with BCA protein assay. 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 300 mA for 2 h in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. The membranes were blocked with 1% BSA in TBST (0.1% Tween-20) for 1 h. After blocking, the blots were incubated in primary antibody for $K_{Ca}3.1$ (1:500), anti-ERK1/2 (1:500), phospho-specific anti-ERK1/2 (p-ERK1/2, 1:500), P38 (1:200), phospho-specific anti-P38 (p-P38, 1:200), anti-Akt (1:500), and phospho-specific anti-Akt (1:500) at 4 °C overnight and then incubated with HRP-conjugated secondary antibodies (1:5000) for 1 h at room temperature. The bound antibodies were

detected with an enhanced chemiluminescence detection system (ECL, GE Biotech, USA) and quantified by densitometry using a Chemi-Genius Bio Imaging System (Syngene, Cambridge, UK). To ensure equal sample loading, the ratio of band intensity to GAPDH was obtained to quantify the relative protein expression level.

RNA Interference

Stealth RNAi molecules targeted to $K_{Ca}3.1$ channels were purchased from Invitrogen Life Technology (Invitrogen, USA). Specific small interfering RNA (siRNA) sequences of $K_{Ca}3.1$ were as follows:[22] sense: 5′-GCCACUGGUUCGUGGC CAAACUAUA-3′, antisense: 5′-UAUAGUUUGGCCACGAA CCAGUGGC-3′. In addition, Silencer GAPDH siRNA (Ambion, Austin, TX, USA) was used as the positive control. Stealth RNAi molecules at 100 nM were transfected into the rat VSMCs at 50% confluence for 24 h using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Stealth RNAi of medium GC content (Invitrogen), which had no known target in mammalian genomes, was used as the control. Transfected cells were used for cell migration and proliferation assay or protein extraction after 24 h of transfection. Transfection efficiency was monitored using fluorescent RNA duplex (Invitrogen) according to the manufacturer's instructions.

Cell Migration Assay

The migration of VSMCs was determined by modified Boyden chamber technique.[23] Briefly, a 24-well Transwell apparatus with each well containing a 6.5-mm polycarbonate membrane with 8 μm pores was used. Serum-starved or transfected VSMCs were trypsin-harvested. Cell suspension in basal medium (250 μl, 1×10^5 cells/well or 5×10^4 cells/well in RNAi) was seeded in upper chamber, and 750 μl of basal medium with various reagents was added to the lower chamber. After incubation for 24 h, cells were removed from the top side of the membrane, and the migrated cells from the reverse side were stained with hematoxylin and then quantified. Each treatment was repeated in six independent transwells.

Cell Proliferation Assays

The cell proliferation was determined by cell count and BrdU incorporation assay as previously described.[16] In cell-counting experiment, rat VSMCs were plated in 24-well plates at a density of 1×10^5 cells per well (or 1×10^4 cells per well in RNAi) in 1 ml DMEM containing 10% FBS. After starving for 24 h or 6 h (in RNAi) in DMEM containing 0.1% FBS, the cells were pretreated by $K_{Ca}3.1$ channel blocker or anti-RAGE antibody for 30 min, or $K_{Ca}3.1$ channel siRNA for 24 h before AGE-BSA was added. Following 24 h culture, cells were detached with the solution containing trypsin (0.25%) and EDTA (0.02%) and counted using a standard hemocytometer in a blinded manner. Cell viability was established by trypan blue exclusion. In BrdU-labeling detection, cells

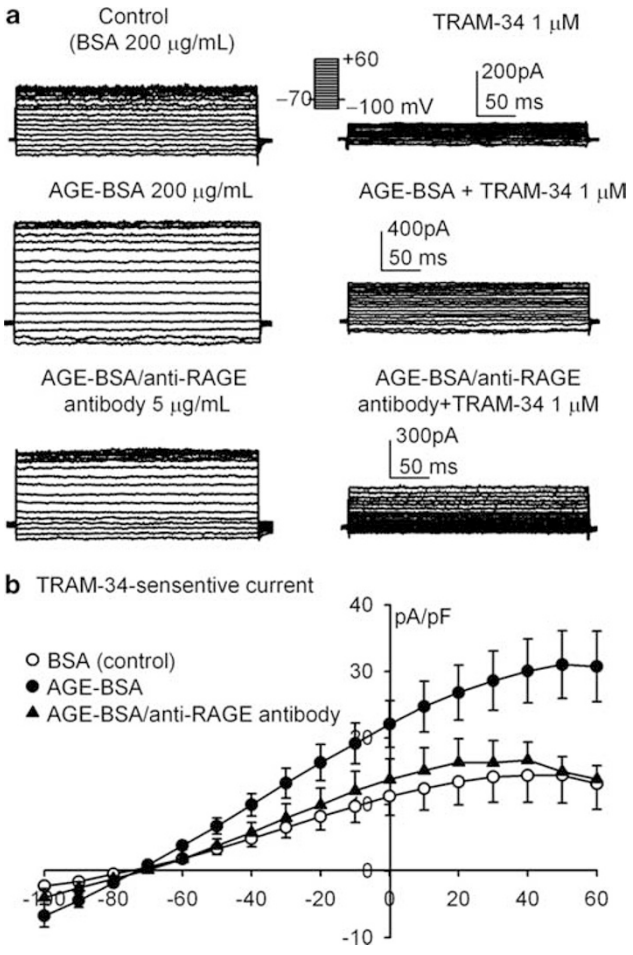

Figure 1 Effect of AGE-BSA on $K_{Ca}3.1$ currents in cultured rat VSMCs. (**a**) Membrane currents recorded with the protocol as shown in the *inset* in cells treated with BSA (200 μg/ml), AGE-BSA (200 μg/ml), and AGE-BSA plus anti-RAGE antibody (5 μg/ml) for 24 h before and after application of TRAM-34 (1 μM). (**b**) *I–V* relationships of the mean values of TRAM-34-sensitive current obtained by digital subtraction of the current before TRAM-34 by the current after TRAM-34 application ($n = 6$ for each groups).

grown on coverslips were treated as indicated in the figures and labeled with 10 μM BrdU for 24 h at 37 °C. The coverslips were then washed with phosphate-buffered saline, fixed in 4% paraformaldehyde for 20 min at room temperature, and DNA was denatured with 2 N HCl for 30 min in an incubator at 37 °C. After incubation in blocking buffer (1% normal BSA and 0.1% Tween-20) for 1 h, the cells were immunostained with mouse anti-BrdU (1:1000) primary antibody overnight in a moist chamber at 4 °C followed by appropriated Rhodamine-labeled goat anti-mouse secondary antibody. Nuclear DNA was counterstained with 4,6-diamidino-2-phenylindole. Images were then captured with a microscope (Olympus IX71, Tokyo, Japan). The total number of nuclei (blue color) and the number of BrdU-stained nuclei (pink color) were counted manually. Proliferation was quantitated as percentage of BrdU-labeled cells in the total number of cells counted.

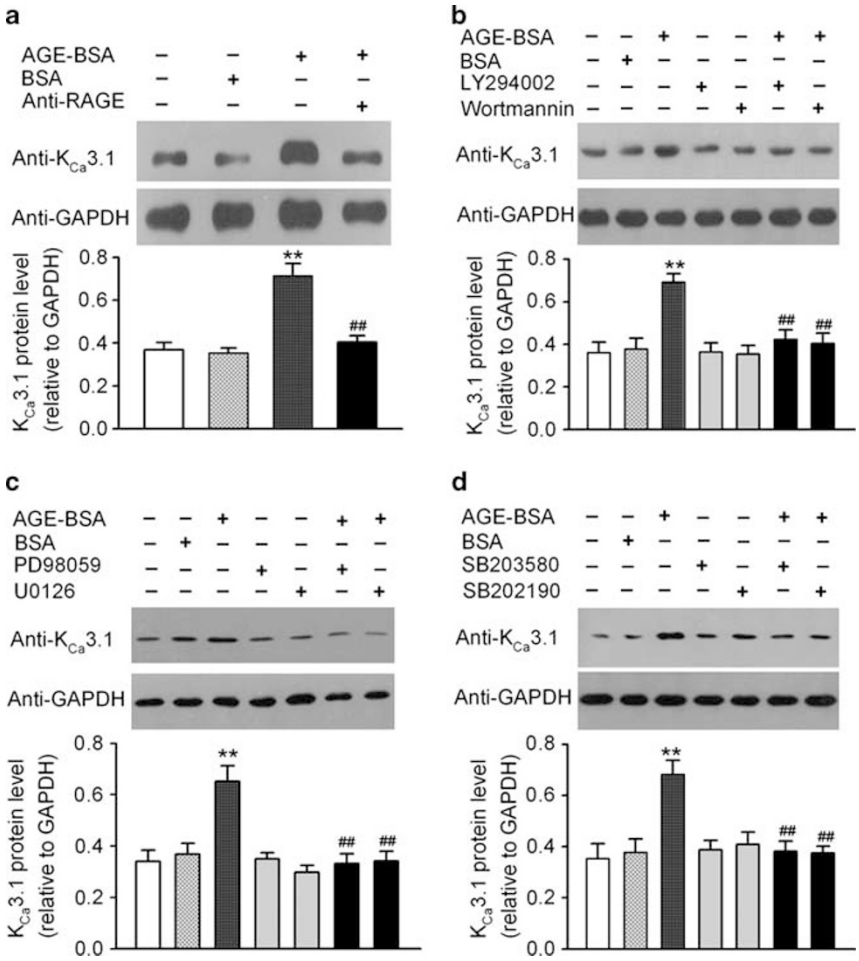

Figure 2 Effect of AGE-BSA on protein expression of K_{Ca}3.1 channels in rat VSMCs. (**a**) Western blots and mean values of K_{Ca}3.1 channel protein in cells treated without (control) or with 200 μg/ml BSA, 200 μg/ml AGE-BSA, and AGE-BSA plus 5 μg/ml anti-RAGE antibody for 24 h ($n=5$, $^{**}P<0.01$ *vs* control or BSA, $^{\#\#}P<0.01$ *vs* AGE-BSA alone). (**b**) Western blots and mean values of K_{Ca}3.1 channel protein in cells treated without (control) or with 200 μg/ml BSA, 200 μg/ml AGE-BSA, AGE-BSA plus 10 μM LY294002 or 100 nM wortmannin, and LY294002 or wortmannin alone for 24 h ($n=5$, $^{**}P<0.01$ *vs* control or BSA, $^{\#\#}P<0.01$ *vs* AGE-BSA alone). (**c**) Western blots and mean values of K_{Ca}3.1 channel protein in cells treated without (control) or with 200 μg/ml BSA, 200 μg/ml AGE-BSA, AGE-BSA plus 25 μM PD98059 or 10 μM U0126, and PD98059 or U0126 alone for 24 h ($n=5$, $^{**}P<0.01$ *vs* control or BSA, $^{\#\#}P<0.01$ *vs* AGE-BSA alone). (**d**) Western blots and mean values of K_{Ca}3.1 channel protein in cells treated without (control) or with 200 μg/ml BSA, 200 μg/ml AGE-BSA, AGE-BSA plus 10 μM SB203580 or 10 μM SB202190, and SB203580 or SB202190 alone for 24 h ($n=5$, $^{**}P<0.01$ *vs* control or BSA, $^{\#\#}P<0.01$ *vs* AGE-BSA alone).

Statistical Analysis

All quantitative measures are presented as mean ± s.e.m. Paired and/or unpaired Student's *t*-tests were used as appropriate to evaluate the statistical significance of differences between two group means, and analysis of variance (ANOVA) was performed for multiple groups. $P<0.05$ was considered statistically significant.

RESULTS

AGEs and K_{Ca}3.1 Current in Cultured Rat VSMCs

Figure 1a shows the membrane currents recorded with 300-ms voltage steps between -100 mV and $+60$ mV from a holding potential of -70 mV (*inset*) in cells treated with BSA (200 μg/ml), AGE-BSA (200 μg/ml), and AGE-BSA plus anti-RAGE antibody (5 μg/ml) before and after the application of the K_{Ca}3.1 channel blocker TRAM-34. The current exhibited a weak inward rectification at positive potentials and was sensitive to the full inhibition by 1 μM TRAM-34, indicating that the K_{Ca}3.1 channels are predominantly expressed in cultured rat VSMCs. K_{Ca}3.1 current was remarkably enhanced in cells treated with AGE-BSA for 24 h. It is interesting to note that the increased K_{Ca}3.1 current was antagonized in cells treated with both AGE-BSA and anti-RAGE antibody. Figure 1b illustrates the mean values of current–voltage (*I–V*) relationships of TRAM-34-sensitive current obtained by digital subtraction of the current before TRAM-34 by the current after TRAM-34 application. The *I–V* curves of TRMA-34-sensitive showed a weak inward rectification. The current density at -100, -90 mV, and -50 to $+60$ mV was greater in cells treated with 200 μg/ml

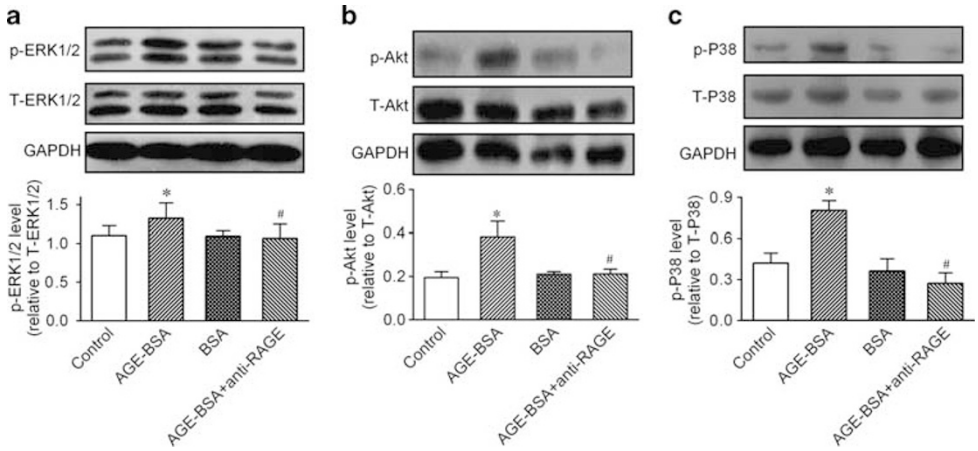

Figure 3 AGE-BSA and phosphorylation of ERK1/2, Akt, and P38 kinases in rat VSMCs. (**a**) Western blots and mean values of p-ERK1/2 in cells treated without (control) or with BSA (200 μg/ml), AGE-BSA (200 μg/ml), or AGE-BSA and anti-RAGE antibody (5 μg/ml) for 30 min ($n=5$, $^{*}P<0.05$ *vs* control or BSA; $^{\#}P<0.05$ *vs* AGE-BSA alone). (**b**) Western blots and mean values of p-Akt in cells treated with the interventions as described in a ($n=4$, $^{*}P<0.05$ *vs* control or BSA; $^{\#}P<0.05$ *vs* AGE-BSA alone). (**c**) Western blots and mean values of p-P38-MAPK in cells treated with the interventions as described in **a**. ($n=4$, $^{*}P<0.05$ *vs* control or BSA; $^{\#}P<0.05$ *vs* AGE-BSA alone).

AGE-BSA ($n=6$, $P<0.05$ or $P<0.01$ *vs* control). The enhanced current by AGE-BSA was almost fully antagonized by anti-RAGE antibody ($n=6$, $P<0.05$ or $P<0.01$ *vs* AGE-BSA alone at -50 to $+60$ mV). These results indicate that the increase of $K_{Ca}3.1$ current by AGE-BSA is mediated by RAGE in cultured rat VSMCs.

AGEs and $K_{Ca}3.1$ Channel Expression in VSMCs

To determine whether the increase of $K_{Ca}3.1$ current by AGE-BSA is related to an enhanced expression of the channel, the protein level of $K_{Ca}3.1$ channels was examined in cultured VSMCs. Figure 2 shows the images of western blot and mean values of relative protein levels of $K_{Ca}3.1$ channels in cells treated with vehicle (control), BSA, AGE-BSA, AGE-BSA with anti-RAGE antibody, or different protein kinase-related inhibitors. AGE-BSA (200 μg/ml), but not BSA (200 μg/ml), increased $K_{Ca}3.1$ channel protein expression, the effect was countered by 5 μg/ml anti-RAGE antibody (Figure 2a) or the PI3K/Akt inhibitors LY294002 (10 μM) and wortmannin (100 nM, Figure 2b), the ERK1/2 inhibitors PD98059 (25 μM) and U0126 (10 μM, Figure 2c), and the P38-MAPK inhibitors SB203580 (10 μM) and SB203580 (10 μM, Figure 2d, $n=5$, $P<0.01$ *vs* control or BSA, $P<0.01$ *vs* AGE-BSA alone). $K_{Ca}3.1$ channel protein was not influenced by application of protein kinase-related inhibitors alone. These results demonstrate that the increase of $K_{Ca}3.1$ current by AGEs is related to the upregulation of the channel protein in cultured rat VSMCs, and ERK1/2, P38-MAPK, as well as PI3K/Akt signals participate in the regulation of $K_{Ca}3.1$ expression.

AGEs and Intracellular Signal Activity

The above results suggest that ERK1/2, P38-MAPK, and PI3K are involved in the upregulation of $K_{Ca}3.1$ channel expression by AGEs. To determine whether the phosphorylation of these signals are affected by AGEs, western blot analysis was used to determine total and phosphorylated levels of ERK1/2, P38, and Akt kinases in VSMCs treated without (control) or with BSA (200 μg/ml), AGE-BSA (200 μg/ml), and AGE-BSA plus anti-RAGE antibody (5 μg/ml). Figure 3 shows the western blots and the mean values of total and phosphorylated ERK1/2, P38, and Akt. It is interesting to note that AGEs significantly increased phosphorylated ERK1/2, P38, and Akt ($n=4$-5, $P<0.05$ *vs* control or BSA), and the effect was fully antagonized by anti-RAGE antibody ($P<0.05$ *vs* AGE-BSA alone). These results indicate that the upregulation of $K_{Ca}3.1$ channels induced by AGEs are related to the RAGE-mediated activation of ERK1/2, P38, or Akt in cultured rat VSMCs.

Role of AGEs in Regulating Migration of Rat VSMCs

To investigate whether the upregulation of $K_{Ca}3.1$ channels by AGE-BSA would influence cell function, we tested the effect on migration in cultured VSMCs. Figure 4 illustrates the cell migration as determined by Boyden chamber assay. AGE-BSA (200 μg/ml), but not BSA (200 μg/ml), increased cell migration by 64% ($n=6$, $P<0.01$ *vs* control or BSA), and the effect was countered by 100 nM TRAM-34 or 5 μg/ml anti-RAGE antibody (Figure 4a, $n=6$, $P<0.01$ *vs* AGE-BSA alone). These results indicate that AGEs-induced cell migration is likely mediated by $K_{Ca}3.1$ channels.

To rule out the possible non-specific effect of the $K_{Ca}3.1$ channel blocker TRAM-34 on cell migration, the test was performed in cells treated with the specific siRNA targeting $K_{Ca}3.1$ channels. The protein level of $K_{Ca}3.1$ channel was reduced in cells transfected with $K_{Ca}3.1$ siRNA (100 nM) but not with control siRNA (Figure 4b). AGE-BSA significantly increased the migration in cells transfected with control siRNA but not with $K_{Ca}3.1$ siRNA (Figure 4c, $n=6$, $P<0.01$ *vs* BSA plus control siRNA; $P<0.01$ *vs* AGE-BSA plus control

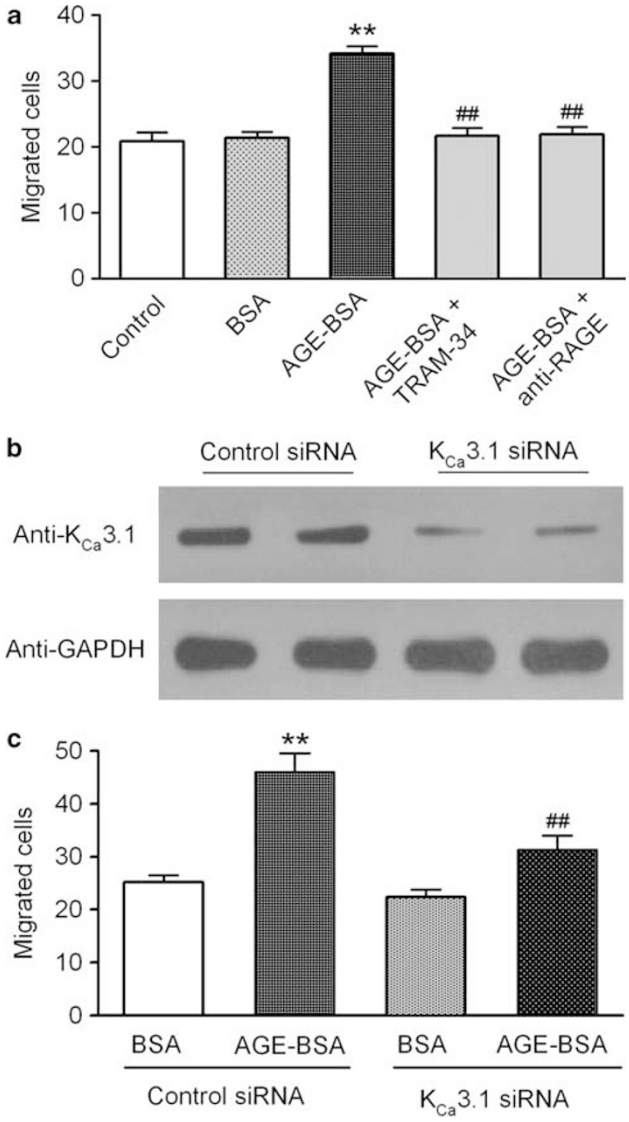

Figure 4 Effects of AGE-BSA and $K_{Ca}3.1$ channels on cell migration in rat VSMCs. (**a**) Mean values of migrated cell number determined with Boyden chamber technique in cells treated with 200 μg/ml BSA, 200 μg/ml AGE-BSA, AGE-BSA plus 100 nM TRAM-34, or 5 μg/ml anti-RAGE antibody for 24 h ($n=6$, $^{**}P<0.01$ *vs* control or BSA, $^{\#\#}P<0.01$ *vs* AGE-BSA alone). (**b**) Western blots of $K_{Ca}3.1$ channels in cells transfected with 100 nM control siRNA or $K_{Ca}3.1$ siRNA for 24 h. (**c**) Mean values of migrated cell number in cells transfected with control siRNA or $K_{Ca}3.1$ siRNA (100 nM) and treated with 200 μg/ml AGE-BSA or 200 μg/ml BSA ($n=6$, $^{**}P<0.01$ *vs* control siRNA with BSA; $^{\#\#}P<0.01$ *vs* control siRNA with AGE-BSA).

siRNA). These results further suggest that AGEs-induced cell migration was mediated by $K_{Ca}3.1$ channels in rat VSMCs.

Role of AGEs in Regulating Proliferation of Rat VSMCs

The effect of AGEs on proliferation was determined by cell counting and BrdU incorporation assay in cultured VSMCs. Figure 5a displays the cell number counted in cells incubated with BSA (200 μg/ml), AGE-BSA (200 μg/ml), AGE-BSA plus TRAM-34 (100 nM), and anti-RAGE antibody (5 μg/ml). The cell number was increased by 41.2% with AGE-BSA ($n=6$, $P<0.01$ *vs* control or BSA), and the effect was antagonized by TRAM-34 or anti-RAGE antibody ($n=6$, $P<0.01$ *vs* AGE-BSA alone). Figure 5b illustrates the images of VSMCs stained with anti-BrdU antibody, showing the newly synthesized DNA. The cells were incubated with AGE-BSA (200 μg/ml) for 24 h in the absence or presence of TRAM-34 or anti-RAGE antibody. AGE-BSA, but not BSA, significantly increased the number of cells with BrdU-stained nuclei, and the effect was countered by TRAM-34 or anti-RAGE antibody (Figure 5c, $n=6$, $P<0.01$ *vs* AGE-BSA alone). These results suggest that AGEs-RAGE interaction promotes cell proliferation through $K_{Ca}3.1$ channels in cultured rat VSMCs.

To further study the involvement of $K_{Ca}3.1$ channels in AGEs-mediated stimulation of proliferation, cell proliferation was determined in cells transfected with control siRNA or $K_{Ca}3.1$ siRNA. Figure 5d illustrates the effects of the specific $K_{Ca}3.1$ siRNA (100 nM) on proliferation. The cell number was increased by 111.5% in AGE-BSA-treated cells with control siRNA transfection ($n=6$, $P<0.01$ *vs* BSA), and the effect was antagonized by the downregulation of $K_{Ca}3.1$ channels ($n=6$, $P<0.01$ *vs* control siRNA with AGEs). Figure 5e shows the BrdU-stained cells treated with BSA (200 μg/ml) or AGE-BSA (200 μg/ml). In cells transfected with control siRNA, AGE-BSA increased cell proliferation by 624%, and the effect was significantly reduced in cells transfected with $K_{Ca}3.1$ siRNA (Figure 5f, $n=6$, $P<0.01$ *vs* control siRNA). These results demonstrate that $K_{Ca}3.1$ channels are involved in enhancement of cell proliferation by AGEs in rat VSMCs.

DISCUSSION

In the present study, we demonstrate for the first time that AGE-BSA upregulates $K_{Ca}3.1$ channels in cultured VSMCs and increases cell migration and proliferation, which is involved in the activation of ERK1/2, P38-MAPK, and PI3K signaling pathways. This study provides the novel information that AGEs are likely involved, at least in part, in the excessive neointimal VSMC proliferation and the increased $K_{Ca}3.1$ channel expression in the aorta of diabetic rats.[18]

AGEs are the products generated from nonenzymatic glycation and oxidation of proteins and lipids. Persistent hyperglycemia and oxidative stress accelerate the formation of AGEs in diabetic subjects, which has an important role in the development of accelerated atherosclerosis. AGEs can induce VSMC proliferation and migration although the mechanisms remain not fully understood. Upregulation of $K_{Ca}3.1$ channels has been found to promote excessive VSMC proliferation and migration induced by either mitogens (platelet-derived growth factor and EGF) or balloon catheter injury.[13–15] However, it is unknown whether AGEs is involved in the $K_{Ca}3.1$ channel regulation. In the present study, we demonstrated that AGE-BSA increased $K_{Ca}3.1$ current density and enhanced $K_{Ca}3.1$ channel protein expression in

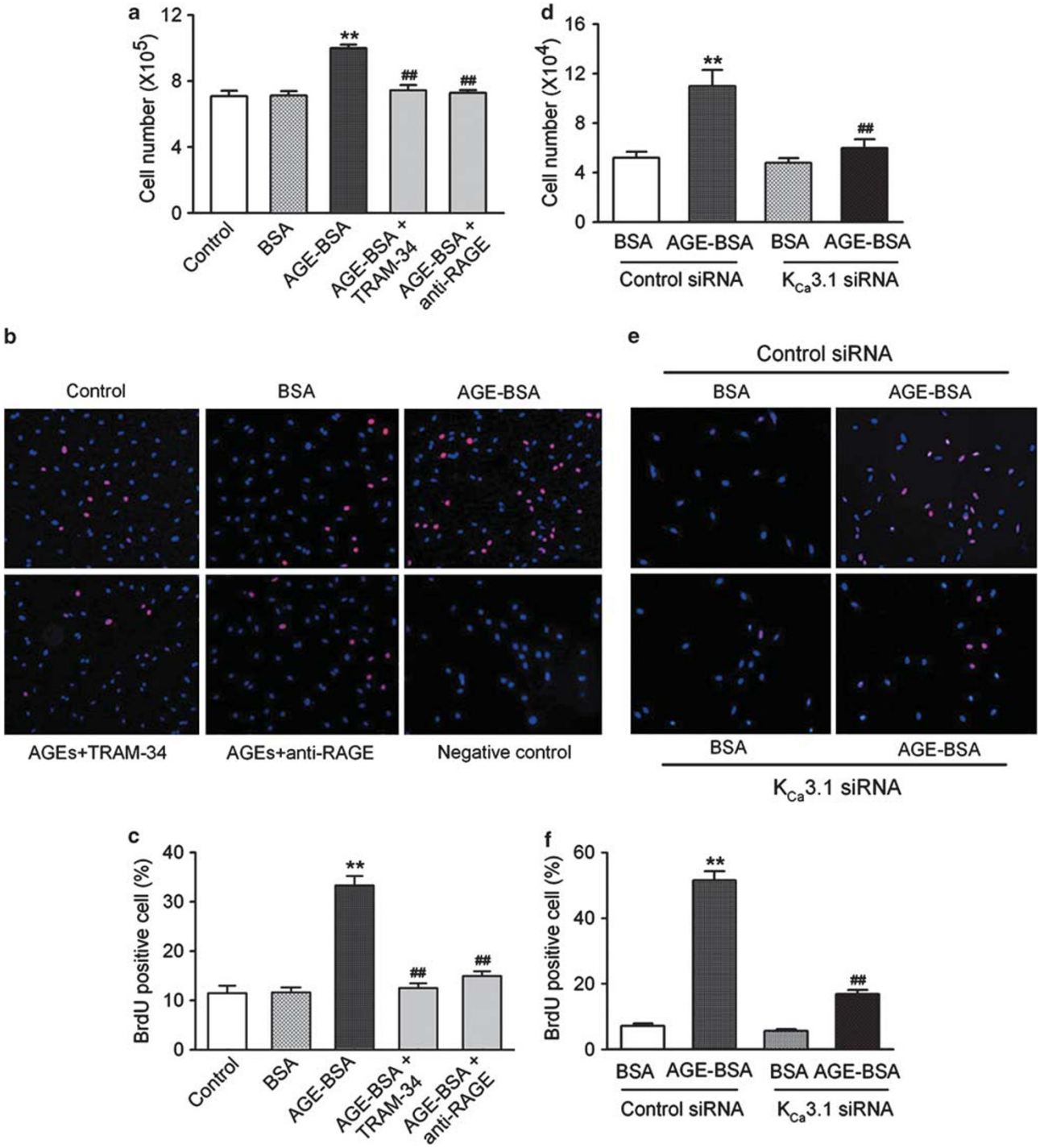

Figure 5 AGE-BSA increases cell proliferation by activating K_{Ca}3.1 channels in rat VSMCs. (**a**) Mean values of cell counting in cells treated without (control) or with BSA (200 μg/ml), AGE-BSA (200 μg/ml), and AGE-BSA plus 100 nM TRAM-34 or 5 μg/ml anti-RAGE antibody 24 h ($n=6$, $^{**}P<0.01$ *vs* control or BSA, $^{\#\#}P<0.01$ *vs* AGE-BSA alone). (**b**) Immunofluorescent BrdU-staining images ($\times 100$) of the nuclei in VSMCs treated with the interventions as described in **a**. Nuclei counterstained in blue (4,6-diamidino-2-phenylindole) and BrdU-positive nuclei shown in pink. No visible pink color (the color of rhodamine-labeled second antibody) was observed in negative control without incubation of the primary antibody. (**c**) Mean percent values of BrdU-incorporated nuclei in VSMCs treated with the interventions as described in **a**. ($n=6$, $^{**}P<0.01$ *vs* control or BSA; $^{\#\#}P<0.01$ *vs* AGE-BSA alone). (**d**) Mean values of cell number counted in cells transfected with control siRNA or K_{Ca}3.1 siRNA (100 nM) and treated with BSA or AGE-BSA (200 μg/ml) for 24 h ($n=6$, $^{**}P<0.01$ *vs* control siRNA with BSA; $^{\#\#}P<0.01$ *vs* control siRNA with AGE-BSA). (**e**) BrdU-incorporation staining ($\times 100$) in cells treated with the interventions as described in **d**. (**f**) Mean percentage of values of proliferation cells with pink color in cells treated with the interventions as described in **d** ($n=6$, $^{**}P<0.01$ *vs* control siRNA with BSA; $^{\#\#}P<0.01$ *vs* control siRNA with AGE-BSA).

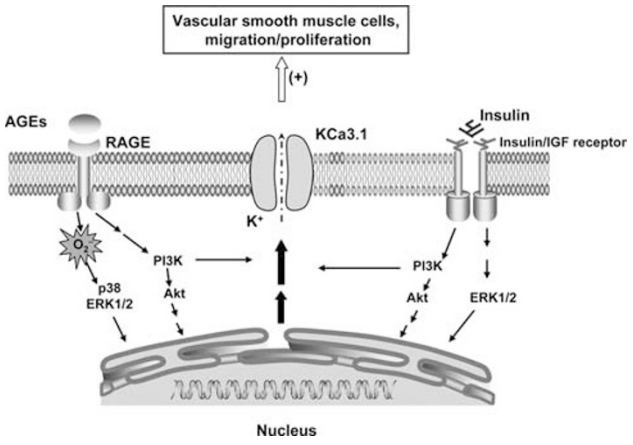

Figure 6 Schematic graph showing the effects of AGEs and insulin on intracellular pathways. AGEs bind to their receptor RAGE and activate P38, ERK1/2, and PI3K/Akt and then promote gene expression of $K_{Ca}3.1$ channels, which has a crucial role in regulating migration and proliferation in VSMCs. Insulin regulates cell proliferation and migration via a similar mechanism.

cultured rat VSMCs. In addition, we found that AGE-BSA increased cell migration and proliferation. These effects were antagonized by anti-RAGE antibody, the $K_{Ca}3.1$ channel blocker TRAM-34, or $K_{Ca}3.1$ siRNA. These results provide a direct link between AGEs and $K_{Ca}3.1$ channels in the regulation of VSMC migration and proliferation.

It is generally recognized that cellular effects of AGEs were mediated by the specific cellular receptor RAGE (Figure 6). Previous studies showed that the interaction of AGEs with RAGE in rat VSMCs leads to an increased phosphorylation of ERK1/2, P38-MAPK,[9,4] Src kinases[10] and promotes cell proliferation and migration. The phosphorylation of PI3K is also essential for RAGE ligand-induced VSMC migration and proliferation.[24] In the present study, we found that specific signal inhibitors, PD98059 and U0126 for ERK1/2, SB203580 and SB202190 for P38-MAPK, and LY294002 and wortmannin for PI3K/Akt, markedly decreased AGEs-induced upregulation of $K_{Ca}3.1$ channel expression in rat VSMCs, indicating that activation of these cellular signaling pathways mediate the AGEs-induced increase of $K_{Ca}3.1$ channels. This is supported by previous reports, in which ERK induces an increase of proliferative gene expression, eg, the AP-1 family member c-fos,[25–27] and the increased production of c-fos leads to induction of $K_{Ca}3.1$ expression through AP-1 promoter elements.[28]

In addition to the upregulation of $K_{Ca}3.1$ current and $K_{Ca}3.1$ expression by AGEs, the recent studies reported that $K_{Ca}3.1$ channels were activated by PI(3)P, which is produced by PI3K from phosphatidylinositol.[29–31] Decreasing intracellular level of PI(3)P can reduce $K_{Ca}3.1$ channel activity.[32] Besides, recombinant human $K_{Ca}3.1$ channels induce HEK293 cell proliferation by a direct interaction with ERK1/2 pathways[33] and downregulating ERK reduces $K_{Ca}3.1$ channel expression.[32] These reports support the notion that $K_{Ca}3.1$ channels are the downstream target of ERK1/2, P38-MAPK and PI3K in regulating cell proliferation and migration of rat VSMCs (Figure 6).

It is believed that atherosclerosis involves a switch to a proliferative phenotype in VSMCs. $K_{Ca}3.1$ channels are the predominant Ca^{2+}-activated K^+ channels in proliferative smooth muscle cells but not in mature contractile cells.[11–14] The expression of $K_{Ca}3.1$ channels is increased in VSMCs in coronary arteries from patients and in aortas of $ApoE^{-/-}$ mice with atherogenesis, and $K_{Ca}3.1$ channel blocker markedly slows down the development of atherosclerosis in $ApoE^{-/-}$ mice.[16] However, reports related to the regulation of $K_{Ca}3.1$ channels are limited in diabetes. Our results suggest that AGEs, as insulin,[18] have a crucial role in upregulating $K_{Ca}3.1$ channel activity and expression in VSMCs (Figure 6). More effort is required in the future to investigate whether blockade of AGEs signal with RAGE antibody or specific $K_{Ca}3.1$ channel blocker would be beneficial in managing diabetic vascular complications *in vivo*.

In summary, the results of the present study provide the novel information that AGEs increase the expression of $K_{Ca}3.1$ channels in a RAGE-dependent manner by activating ERK1/2, P38-MAPK and PI3K signal pathways and therefore promote VSMC migration and proliferation, which may have a role in the development of diabetic vascular complications.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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