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Downregulation of BK channel function and protein expression in coronary arteriolar smooth muscle cells of type 2 diabetic patients

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Aims Type 2 diabetes (T2D) is strongly associated with cardiovascular morbidity and mortality in patients. Vascular large conductance Ca^{2+}-activated potassium (BK) channels, composed of four pore-forming α subunits (BK-α), and four regulatory β1 subunits (BK-β1), are densely expressed in coronary arterial smooth muscle cells (SMCs) and play an important role in regulating vascular tone and myocardial perfusion. However, the role of BK channels in coronary microvascular dysfunction of human subjects with diabetes is unclear. In this study, we examined BK channel function and protein expression, and BK channel-mediated vasodilation in freshly isolated coronary arterioles from T2D patients.

Methods and results Atrial tissues were obtained from 16 patients with T2D and 25 matched non-diabetic subjects during cardiopulmonary bypass procedure. Microvessel videomicroscopy and immunoblot analysis were performed in freshly dissected coronary arterioles and inside-out single BK channel currents was recorded in enzymatically isolated coronary arteriolar SMCs. We found that BK channel sensitivity to physiological Ca^{2+} concentration and voltage was downregulated in the coronary arteriolar SMCs of diabetic patients, compared with non-diabetic controls. BK channel kinetics analysis revealed that there was significant shortening of the mean open time and prolongation of the mean closed time in diabetic patients, resulting in a remarkable reduction of the channel open probability. Functional studies showed that BK channel activation by dehydrosoyasaponin-1 was diminished and that BK channel-mediated vasodilation in response to shear stress was impaired in diabetic coronary arterioles. Immunoblot experiments confirmed that the protein expressions of BK-α and BK-β1 subunits were significantly downregulated, but the ratio of BK-α/BK-β1 was unchanged in the coronary arterioles of T2D patients.

Conclusions Our results demonstrated for the first time that BK channel function and BK channel-mediated vasodilation were abnormal in the coronary microvasculature of diabetic patients, due to decreased protein expression and altered intrinsic properties of BK channels.

Keywords BK channels • Vasodilation • Coronary microvasculature • Smooth muscle cells • Type 2 diabetic patients

Introduction

Diabetes mellitus has become a global epidemic. According to the World Health Organization report (http://www.who.int/features/factfiles/diabetes/en/), about 422 million people worldwide have diabetes. Diabetes is one of the leading causes of death in the world, directly contributing to 1.5 million deaths in 2012 with an additional 2.2 million deaths associated with increased the risks of cardiovascular and other diseases in diabetes. In addition, diabetes imposes an enormous financial burden on individuals and society, estimated that losses in the gross

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domestic product worldwide, including both the direct and indirect costs of diabetes, will total 1.7 trillion US dollars from 2011 to 2030. Type 2 diabetes (T2D), accounting for 90% of the total cases of diabetes, is associated with a two- to four-fold increase in the risks of cardiovascular diseases.[1–3] It has been reported that the poor clinical prognosis of acute myocardial infarction and cardiac sudden death in patients diagnosed with T2D is attributed to compromised coronary blood flow and increased susceptibility to myocardial ischaemia.[4–6] The causes of diabetic coronary dysfunction are multifactorial, involving both vascular endothelial-dependent and -independent mechanisms. The molecular bases of endothelial dysfunction in patients with T2D have been well studied[7]; however, those of vascular smooth muscle pathology are still poorly understood.

Coronary BK channels are composed of four pore-forming α (BK-α) subunits (encoded by the *KCNMA1* gene) and four regulatory β1 (BK-β1) subunits (encoded by the *KCNMB1* gene) and are densely expressed in coronary arterial smooth muscle cells (SMCs), linking intracellular Ca^{2+} homeostasis with cell membrane potentials and playing an important feedback role in the regulation of coronary circulation and myocardial perfusion.[8,9] The BK-α conducts K^+ across cell membrane, and the BK-β1 regulates the BK-α sensitivity to free Ca^{2+} and voltage activation.[10] A large body of work from animal studies has shown that malfunction of vascular BK channels in diabetes contributes to impaired BK channel-mediated coronary vasodilation[11–15] and worsens ischaemia–reperfusion injury in diabetic hearts.[16] Because of the technical difficulties in isolating coronary microvascular SMCs and the limited availability of human heart tissues, our knowledge regarding BK channelopathy in diabetes is mainly obtained from animal models and the results vary depending on the models and vascular beds examined.[17] Whether the BK channel pathophysiology observed in animal studies occurs in human subjects with T2D is unclear. In this study, we have successfully measured BK channel activity in freshly isolated coronary arteriolar SMCs from T2D patients who underwent cardiac surgery. We found that BK channel function and protein expression in the coronary microvessels of surgically removed atrial appendages from diabetic patients were down-regulated. Hence, targeting BK channel activation could be a novel strategy for the treatment of coronary microvascular complications in diabetic patients.

Methods

Patient enrolment and atrial tissue collection

This study recruited 16 patients diagnosed with T2D more than 5 years and 25 patients without any history of diabetes who were scheduled for coronary artery bypass grafting surgery in Mayo Clinic Rochester (MN, USA) during 2011–2017. The exclusion criteria included: dilated or hypertrophic cardiomyopathy, congenital heart disease, uncontrolled hypertension (>160/90 mmHg), untreated obstructive sleep apnoea, hypothyroidism, hyperthyroidism, and other vulnerable patients who had critical condition, such as severe heart failure, liver, and kidney dysfunction which might affect coronary BK channel functions. A written informed consent was obtained from all participants prior to their participation in the study. All protocols were approved by the Mayo Clinic Institutional Review Boards (IRB #10-002575) according to the policy of the National Institute of Health (NIH) of the USA.

During atriotomy for the heart–lung bypass procedure, an atrial biopsy, 3–5 cm^3, was surgically removed and immediately put in ice-cold Krebs' buffer (in mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, and glucose 11.1, equilibrated with 95% O_2 + 5% CO_2, pH 7.4 with NaOH. The sample was immediately delivered to the Pathology Station in the operating room and then transferred to the research laboratory. Usually, only 2 or 3 vessels were found and carefully dissected from the atrial biopsy. There was not different in the diameter of coronary microvessels between non-diabetic controls (114.17 ± 8.19 μm, $n = 25$) and T2D patients (122.19 ± 14.34 μm, $n = 16$, P = N.S.).

Coronary arteriolar SMC isolation

Single coronary microvascular SMCs were enzymatically isolated from atrial coronary arterioles as we have previously described.[11,12,15] Briefly, human coronary arterioles were carefully dissected from the atria in ice-cold dissociation buffer (in mM): NaCl 145.0, KCl 4.0, $CaCl_2$ 0.05, $MgCl_2$ 1.0, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10.0, glucose 10.0, and pH 7.2 with NaOH. The vessels were placed in dissociation buffer containing 0.1% w/v bovine serum albumin (BSA) and incubated in a shaking water bath at 37°C for 30 min. The buffer was replaced by fresh 0.1% w/v BSA dissociation buffer containing 1.5 mg/mL papain and 1.0 mg/mL dithiothreitol and the vessels were incubated in a shaking water bath at 37°C for another 30 min, followed by digestion in fresh 0.1% w/v BSA dissociation buffer containing 1.0 mg/mL collagenase and 1.0 mg/mL of trypsin inhibitor in a shaking water bath at 37°C for 30 min. The vessels were then stored in 2 mL dissociation buffer and gently triturated with a fire-polished glass pipette until the cells were completely dissociated. The specificity of single coronary arteriolar SMCs was confirmed by immunofluorescence staining with anti-α-smooth muscle actin antibody (Supplementary material online).

Immunoblot analysis and confocal immunofluorescence microscopy

Protein expressions of BK-α and BK-β1 in human coronary resistance arteries were determined using western blot analysis[13,18] with rabbit anti-BK-α (1:1000, Alomona Labs Ltd., Israel, #APC-021) and rabbit anti-BK-β1 antibodies (1:1000, Abcam Plc., Cambridge, MA, USA. #AB3587). Blots were also probed with mouse anti-β-actin antibody (1:2000, Santa Cruz Biotechnology Inc., Dallas, TX, USA. #sc47778) as loading controls. Optical densities of the bands were measured using Scion Image (Scion Corp., Frederick, MA, USA). Protein expression was normalized to β-actin and expressed as relative densitometry units.

Single BK channel recording and single channel kinetics analysis

Single BK channel currents were recorded in inside-out configuration using an Axopatch 200B integrating amplifier and Clampex 10.4 software (Molecular Device LLC., Sunnyvale, CA, USA) as previously reported.[11] In brief, the output signals were filtered with an eight-pole Bessel filter (902 LPF, Frequency Devices Inc., Haverhill, MA, USA) at 2 kHz and digitized at 50 kHz. Patch pipettes had a typical tip resistance of 10 MΩ when filled the pipette solution contained (in mM): KCl 140, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 10.0, and ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 1.0, pH 7.4 with KOH. The bath solution contained (in mM): KCl 140, $MgCl_2$ 0.5, EGTA 1.0, and HEPES 10.0, pH 7.35 with KOH. Various amounts of Ca^{2+} were added in the bath solution to obtain the desired concentrations of free Ca^{2+} (from 10^{-9} to 10^{-4} M), calculated using Chelator software as previously described.[11,19] BK channels were identified by their unitary conductance and sensitivity to voltage and Ca^{2+}.

Po-voltage curves were characterized by the Boltzmann equation: $P_o/P_{o,max} = P_o/\{1 + \exp[(V_{1/2} - V_m)/\kappa]\}$, where, $P_{o,max}$ represents the maximal P_o, $V_{1/2}$ is the voltage at half of $P_{o,max}$. V_m is the membrane potential and κ is the slope factor associated with the Boltzmann distribution.

Ca^{2+} concentration-dependent curves were fitted using the Hill equation: $P_o/P_{o,max} = 1/\{1+([Ca^{2+}]/EC_{50})^{nH}\}$, where $P_{o,max}$ represents the maximal P_o, $[Ca^{2+}]$ represents free Ca^{2+} concentration, EC_{50} is the concentration at half-maximal effect, and nH is the Hill coefficient.

BK channel unitary conductance was obtained from the current unitary amplitude (i) and voltage relationship (i–V curve) was fitted with a linear equation.[20] Curve fittings were performed using Igor 6.37 software (WaveMetrics Inc., Lake Oswego, OR, USA).

For single channel kinetics analysis, BK currents were recorded at +60 mV in the presence of 200 nM free Ca^{2+} in the bath solution. Only excised patches containing one single channel was used. Data were further filtered at 2 kHz bandwidth with a digital Gaussian filter to achieve the appropriate signal-to-noise ratio. Dwell-time histograms were fitted with the sums of exponential probability density functions using TAC software (Bruxton Inc., Seattle, Washington, DC, USA) as previously described.[11] The number of exponential components was determined by the likelihood ratio test, and additional exponential component was included only when the probability was >0.95.[11] Data were analysed using TAC software with an imposed dead time of 100 μs. The mean open time and the mean closed time were calculated by the sum of each time constant component (τ) × weight (A): $T=\sum_j \tau j \cdot Aj$, where $\tau j \cdot Aj$ is the total time of j component during the recording.[21]

Shear stress-induced coronary vasodilation

Shear stress-induced vasodilation in freshly isolated human coronary microvessels from atrial tissues was determined by videomicroscopy as previously described.[22] Briefly, 1- to 2-mm long segment of coronary arterioles was mounted in a vessel chamber filled with Krebs' buffer, secured between two borosilicate glass micropipettes with 10-O ophthalmic sutures, and placed on the stage of an inverted Olympus CK40 microscopy (Olympus America Inc.) equipped with a Olympus OLY-105 CCD camera and a video micrometer (VIA-100, Boeckeler Instruments Inc., Tucson, AZ, USA). The intraluminal pressure of the mounted coronary arterioles filled with Krebs' solution was maintained at 60 mmHg using a syringe microinjection pump and a pressure servo controller (Living Systems Ins., St Albans City, VT, USA). After a 1-h equilibration in the physiological Krebs' buffer, the maximal vasodilation was achieved by exposure to Ca^{2+}-free Krebs' solution for additional 30 min. Coronary arteriolar myogenic tone at 60 mmHg was calculated using the formula[23,24]: $(D_{Ca^{2+},free} - D_{Physol})/D_{Ca^{2+},free}$, where, $D_{Ca^{2+}}$ represents the lumen diameter in the presence of the Krebs' buffer, and $D_{Ca^{2+},free}$ is the diameter in the Ca^{2+}-free Krebs' buffer. Shear stress (Q; dyn/cm^2) was calculated according to the following formula as we have described[25]: $Q = \pi D^3/32\eta\tau$, where Q is the flow rate, D is the lumen diameter, τ is the shear stress, and η is the viscosity of fluid. The vessels were subjected to incremental levels of physiological shear stress (1, 5, 10, 15, 20, and 25 dynes cm^{-2}). The contribution of BK channels in shear stress-induced coronary vasodilation was defined as iberiotoxin (IBTX)-sensitive component that was obtained from vessels after a 45-min equilibration with the Krebs' buffer containing a water-soluble IBTX (100 nM). A 45-min equilibration with the Krebs' buffer without IBTX served as a time control.[25]

Chemicals

Dehydrosoyasaponin-1 (DSH-1) was kindly provided by Merck & Co. (Kenilworth, NJ, USA). All other chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MI, USA).

Statistical analysis

Data are presented as mean ± S.E.M. Statistical analysis was performed using SigmaStat 3.5 software (Systat Software Inc., San Jose, CA, USA). Student's t-test was used to compare mean between two groups, and Paired t-test was used to compare data before and after treatment. One-way analysis of variance (ANOVA) followed by the Tukey's test was employed to compare mean from multiple groups. The Fisher's exact test was used to compare categorical variables between groups. Statistical significance was defined as $P < 0.05$.

Results

Patient's information

There were no significant difference in gender, age, body mass index, history of smoking, hypertension, cardiovascular diseases, lung diseases, cardiac function, and kidney function between two groups. Diabetic patients had significant elevated HbA1C levels despite insulin and oral hypoglycaemic drug therapy. Demographic characteristics of controls and diabetic patients are listed in *Table 1*.

Table 1 Clinical information of type 2 diabetic patients and non-diabetic controls

	Non-diabetes	Type 2 diabetes	*P*-value
Gender: male/female	14/11	10/6	N.S
Age (years)	65.9 ± 1.9	69.9 ± 1.8	N.S
Body mass index (kg/m^2)	29.2 ± 1.2	29.3 ± 1.3	N.S
Heart rate (b.p.m.)	71.5 ± 2.4	72.2 ± 2.3	N.S
Systolic pressure/diastolic pressure (mmHg)	129.3 ± 3.9/71.9 ± 2.0	125.9 ± 3.9/72.8 ± 2.8	N.S
Left ventricular ejection fraction (%)	60.0 ± 2.4	56.3 ± 3.6	N.S
Coronary artery disease (%)	16	12	N.S
Valvular disease (%)	15	10	N.S
Arrhythmias, atrial fibrillation (%)	5	4	N.S
Chronic obstructive pulmonary disease (%)	4	3	N.S
History of hypertension (%)	20	14	N.S
History of smoking (%)	16	12	N.S
HbA1C (%)	5.8 ± 0.2	7.6 ± 0.7	<0.05
Total cholesterol (mg/dL)	168.1 ± 6.3	152.6 ± 11.7	N.S
Serum creatinine (mg/dL)	1.0 ± 0.1	0.9 ± 0.1	N.S
Hypoglycaemic therapy (%)	0	14	<0.001

Data were obtained from 25 non-diabetic subjects and 16 T2D patients and are presented as mean ± S.E.M. or the number of patients. The Student's t-test was employed to compare the mean and the Fisher's exact test was used to determine the frequency between two groups.

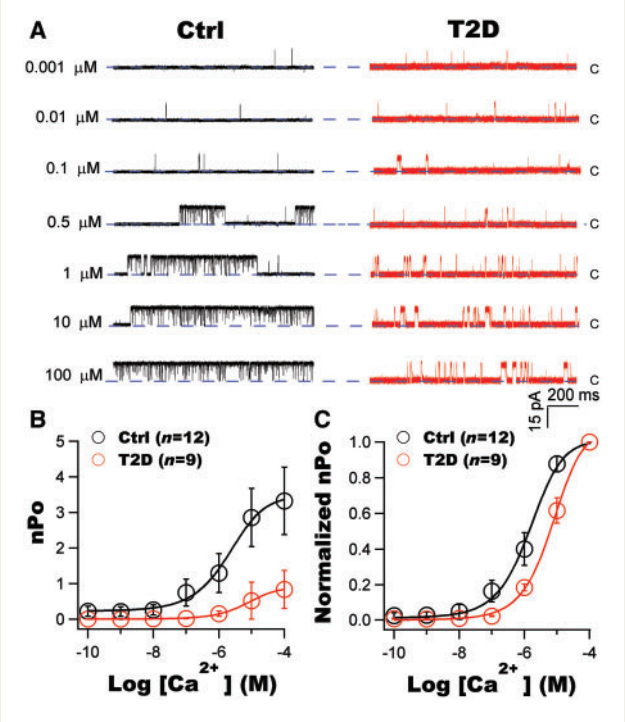

Figure 1 Deminished BK channel Ca^{2+} sensitivity in the coronary arterioles of T2D patients. (*A*) Representative tracings of inside-out single BK channel currents recorded at +60 mV in an excised patch of freshly isolated atrial coronary arteriolar myocytes from non-diabetic (Ctrl) and T2D patients. With increase in free Ca^{2+} concentrations, BK channel open probability (nPo) was robust in controls but not in T2D patients. Dashed lines indicate the closed state (c) of channel. (*B*) The nPo plotted against logarithm Ca^{2+} concentrations (nPo-log[Ca^{2+}] curve) was fitted using the Hill equation. There were significant reductions in Ca^{2+} log[EC_{50}] and BK channel maximal nPo in T2D patients ($n = 9$), compared to those in non-diabetic controls ($n = 12$). (*C*) A rightward shift on the normalized nPo-log[Ca^{2+}] curve of T2D patients. Data are presented as mean ± S.E.M. The BK channel maximal nPo and log[EC_{50}] were significantly reduced in diabetic patients (using Student's *t*-test).

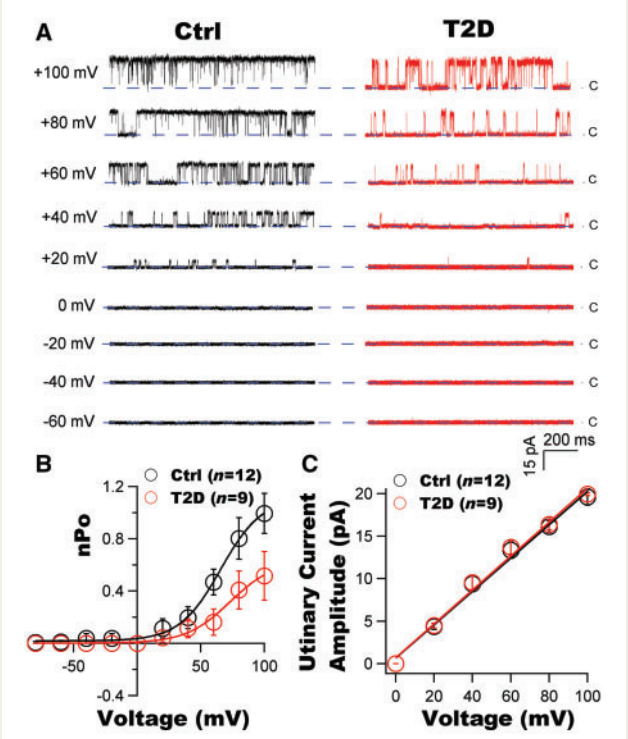

Figure 2 Impaired BK channel voltage sensitivity in the coronary arterioles of T2D patients. (*A*) Representative tracings of inside-out single BK channel currents elicited at different testing voltages in the presence of 200 nM free Ca^{2+} in freshly isolated coronary arteriolar SMCs from non-diabetic controls and T2D patients. BK channel was activated by membrane depolarization with educed effect in diabetes. Dashed line indicates the closed state (c) of channel. (*B*) BK channel open probability and voltage (nPo–V) relationships were fitted using the Boltzmann equation. The maximal nPo and voltage at half of maximal channel activation ($V_{1/2}$) were significantly decreased in T2D patients ($n = 9$), compared with controls ($n = 12$). (*C*) BK channel unitary current amplitude plotted against membrane voltages (*i–V* curves) were fitted using a linear equation. The unitary conductance of BK channel was not different between controls and T2D patients. Data are presented as mean ± S.E.M. There was a significant decrease in BK channel maximal nPo and $V_{1/2}$ in diabetic patients (using Student's *t*-test).

Impaired BK channel Ca^{2+}-sensitivity in the coronary arteriolar SMCs of T2D patients

Single microvascular SMCs were freshly isolated from the atrial coronary arterioles of patients, and the specificity of vascular SMCs was confirmed by immunofluorescence staining (Supplementary material online, *Figure S1*).

In the presence of symmetrical K^+ (140.0 mM), single BK currents were elicited at +60 mV in inside-out configuration and the channel Ca^{2+}-sensitivity was determined by exposure to incremental free Ca^{2+} concentrations from 10^{-9} to 10^{-4} M in the bath solution. As shown in *Figure 1A*, Ca^{2+} concentrations dependently enhanced the total channel open probability (nPo) in non-diabetic controls, but the effect was diminished in diabetic patients. The concentration-dependent relationship of Ca^{2+} on the channel nPo was fitted to a Hill equation (*Figure 2B*). The Po, max was 3.45 ± 0.16 in non-diabetic controls ($n = 12$) and 0.91 ± 0.07 in T2D patients ($n = 9$) ($P < 0.05$ vs. controls). The log[EC_{50}] was -6.39 ± 0.22 M in non-diabetic controls and increased to -5.70 ± 0.10 M in T2D patients ($P < 0.05$ vs. controls) without changing the Hill coefficient (nH = 1.04 ± 0.06 for controls vs. 1.07 ± 0.02 for diabetes, P = N.S.). *Figure 1C* illustrates a right ward shift on the normalized nPo-log[Ca^{2+}] curves in diabetic patients. Hence, these results indicate the downregulation of Ca^{2+} potency and efficacy on coronary arteriolar BK channel activation in T2D patients.

Reduced BK channel voltage sensitivity in the coronary arteriolar SMCs of T2D patients

Inside-out single BK channel currents were elicited from excised patches by different testing potentials in the presence of 200 nM free Ca^{2+} (the physiological intracellular Ca^{2+} concentration is about 100–200 nM). BK channel nPo was robustly increased during

Table 2 BK channel open and closed dwell-time constants and their relative weights in patients

Time constant (weight)	τo_1 (ms) (Ao_1)	τo_2 (ms) (Ao_2)	τo_3 (ms) (Ao_3)	τc_1 (ms) (Ac_1)	τc_2 (ms) (Ac_2)	τc_3 (ms) (Ac_3)	τc_4 (ms) (Ac_4)
Control ($n = 6$)	17.73 ± 2.20	4.51 ± 0.97	0.39 ± 0.08	350.54 ± 68.67	65.61 ± 18.97	1.89 ± 0.30	0.30 ± 0.02
	(0.41 ± 0.07)	(0.46 ± 0.07)	(0.13 ± 0.02)	(0.27 ± 0.06)	(0.14 ± 0.04)	(0.18 ± 0.02)	(0.41 ± 0.06)
T2D ($n = 6$)	11.03 ± 1.00	3.0 ± 0.34	0.54 ± 0.13	*590.34 ± 96.42	70.70 ± 16.38	*3.37 ± 0.80	0.34 ± 0.05
	(0.50 ± 0.07)	(0.29 ± 0.03)	(0.21 ± 0.06)	(*0.44 ± 0.06)	(0.16 ± 0.04)	(0.12 ± 0.03)	(0.28 ± 0.04)

Data were obtained from six non-diabetic subjects and six T2D patients. τo_1, τo_2, and τo_3 represent the slow, intermediate, and fast components of channel open dwell-time durations. τc_1, τc_2, τc_3, and τc_4 represent the very slow, slow, intermediate, and fast components of channel closed dwell-time durations. Ao_{1-3} and Ac_{1-4} are the relative weight of each open and closed dwell-time component, where $Ao_1 + Ao_2 + Ao_3 = 1$, and $Ac_1 + Ac_2 + Ac_3 + Ac_4 = 1$. Data are presented as mean ± S.E.M.
*$P < 0.05$ vs. controls (using one way ANOVA followed by Tukey's test).

membrane depolarization in controls. However, BK channel activation by voltages was impaired in diabetic patients (*Figure 2A*). The Po–voltage (*V*) relationships were fitted using the Boltzmann equation with the Po,max of 1.10 ± 0.06 ($n = 12$) in non-diabetic controls and 0.63 ± 0.10 ($n = 9$) in T2D patients ($P < 0.05$ vs. controls). The $V_{1/2}$ was 65.55 ± 2.46 mV in non-diabetic controls and 81.62 ± 7.01 mV in T2D patients ($P < 0.05$ vs. controls), but no change in the slope factor was noted ($\kappa = 16.22 \pm 1.71$ mV/e-fold in controls vs. 19.43 ± 4.02 mV/e-fold in diabetes, P = N.S.) (*Figure 2B*). BK channel unitary conductance was fitted from the relationship of single channel unitary current amplitude (*i*) and membrane voltage (*i–V* curve) using a linear equation (*Figure 2C*), which was identical between control and diabetic patients. Hence, our results demonstrate that coronary arteriolar BK channels had reduced voltage sensitivity, but the channel K^+ permeability remained unchanged in T2D patients.

Altered BK channel kinetics in the coronary arteriolar SMCs of T2D patients

Single channel kinetics analysis was performed in membrane patches containing only one channel as we have described previously.[11] *Figure 3* shows typical dwell-time histograms of BK channels from non-diabetic subjects and diabetic patients, recorded at +60 mV in the presence of 200 nM free Ca^{2+}. For the best fit, the open dwell-time distribution histograms required at least three time constant components: the slow (τo_1), the intermediate (τo_2), and the fast (τo_3); while the closed dwell slightly but not significantly changed time distribution histograms had at least four time constant components: the very slow (τc_1), the slow (τc_2), the intermediate (τc_3), and the fast (τc_4). Each BK channel open and closed dwell-time constant (τ) and its relative weight (*A*) are listed in *Table 2*. In diabetic patients, the individual τo and Ao were, while the τc_1 and τc_3 were significantly prolonged respectively, accompanied by a remarkable increase in Ac_1, in comparison with those of non-diabetic controls. Interestingly, there was a significant shortening of the mean open time of BK channels (9.28 ± 1.14 ms, $n = 8$ in controls, vs. 6.38 ± 0.93 ms, $n = 7$ in diabetes, $P < 0.05$) and prolongation of the mean closed time of BK channels (101.11 ± 27.40 ms of controls, vs. 295.83 ± 74.41 ms of diabetes, $P < 0.05$) in diabetic patients. These results indicate that the major changes in BK channel kinetics occurred in the closed-states, but an accumulation of small changes in BK channel open-states could be also significant in diabetic patients. The shortening of the mean open time and prolongation of the closed time lead to a decrease of BK channel maximal Po in diabetes.

Diminished BK channel activation by DHS-1 in the coronary arteriolar SMCs of T2D patients

Since BK-β1 is the key determinant of channel Ca^{2+} sensitivity, we examined the effects of 100 nM DHS-1 on BK channel activation in freshly isolated coronary arteriolar SMCs from control and T2D patients. In non-diabetic controls ($n = 9$), the BK channel nPo (expressed as a logarithm value, log nPo) was -1.32 ± 0.26 at baseline and was increased to -0.48 ± 0.18 with DHS-1 ($P < 0.05$ vs. baseline). In T2D patients ($n = 8$), the BK channel log nPo at baseline and in the presence of DHS-1 was -3.08 ± 0.39 and -2.49 ± 0.43, respectively (P = N.S. vs. T2D baseline), and both were significantly lower than those of non-diabetic controls ($P < 0.05$). Moreover, there was a remarkable reduction in BK channel response to DHS-1 in T2D patients (log nPo = -2.88 ± 0.55, $n = 8$), compared to non-diabetic controls (log nPo = -0.68 ± 0.20, $n = 9$, $P < 0.05$) (*Figure 4A*). These results indicate that the effects of DHS-1 on BK channel activation were diminished in the coronary arterioles of diabetic patients.

Reduced BK channel-mediated vasodilation in the coronary arterioles of T2D patients in response to shear stress stimuli

Shear stress is the mechanical force from blood flow on the vascular endothelium and is a potent physiological vasodilation signal.[26] Shear stress-induced vasodilation is associated with the release of endothelium-derived relaxing factors such as nitric oxide and prostacyclin[27] at least partially through activating BK channels in vascular SMCs.[28,29] We examined the physiological role of BK channels in shear stress-mediated coronary arteriolar vasodilation in control and T2D patients. At baseline, there was a significant increase of coronary arteriolar myogenic tone at 60 mmHg (0.35 ± 0.06, $n = 5$), comparted to controls (0.21 ± 0.05, $n = 11$, $P < 0.05$), accompanied by a 2.5-fold decrease of water-soluble IBTX (100 nM)-induced coronary arteriolar vasoconstriction in T2D patients (21.2 ± 2.4%, $n = 5$), compared to that of non-diabetic controls (52.3 ± 12%, $n = 5$). After a 45-min equilibration with the physiological Krebs' buffer, the coronary arteriolar vasodilation to shear stress was examined. *Figure 4B* shows the vasodilation (% maximal vasodilation in the absence of Ca^{2+}) plotted against different shear stress levels (the shear stress response curve) from non-diabetic controls and T2D patients. The area under curve (AUC) was 773.9 ± 47.3 in controls ($n = 16$) and 561.9 ± 64.4 in T2D patients ($n = 9$, $P < 0.05$ vs. controls). Moreover, after a 45-min equilibration with the physiological Krebs' buffer contained 100 nM IBTX, the AUC (non-BK channel-mediated

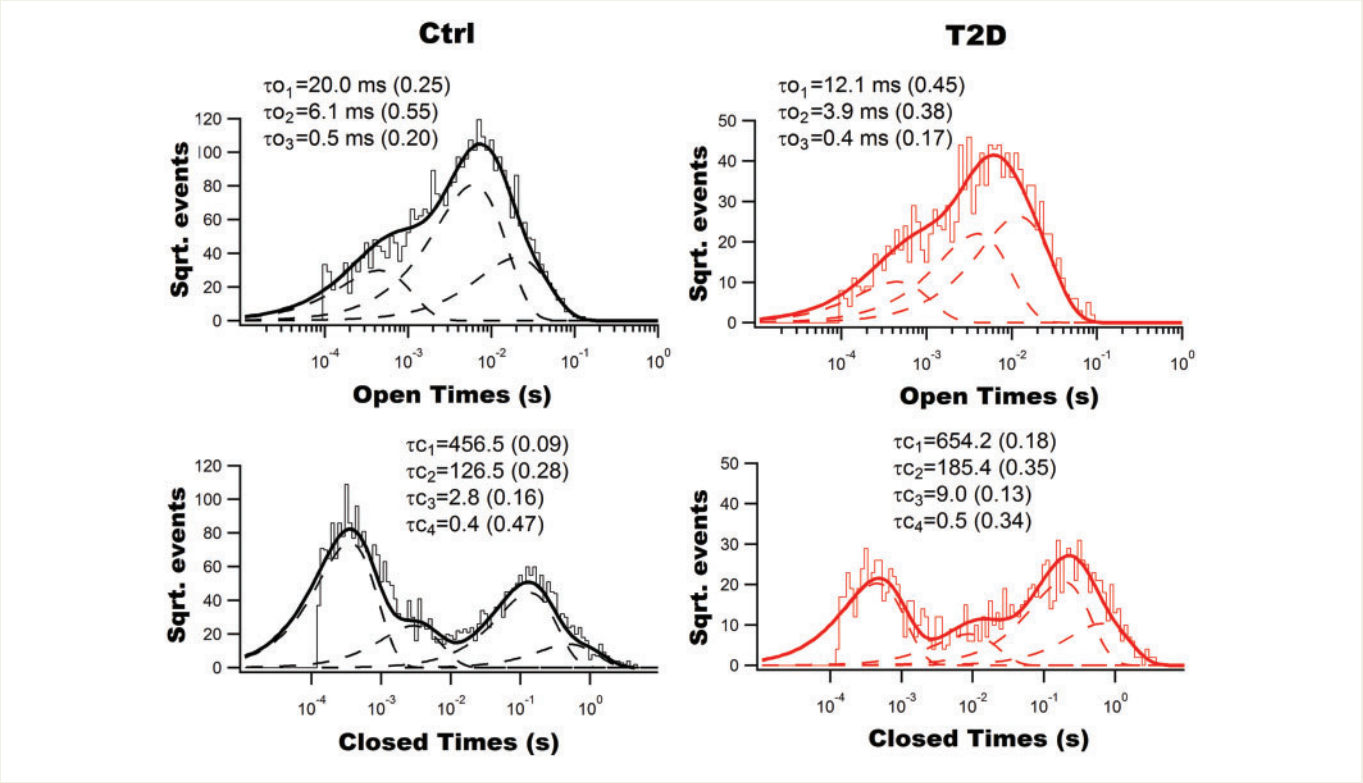

Figure 3 Altered BK channel kinetics in the coronary arterioles of T2D patients. Typical histograms of BK channel open and closed dwell-time durations are illustrated. Data were obtained from inside-out patches at +60 mV in the presence of 200 nM free Ca^{2+} in the both solution. Dwell-time distributions were best fitted by the sum of exponential probability density functions with three open time constant components (the slow τo_1, the intermediate τo_2, and the fast τo_3) and four closed time constant components (the very slow τc_1, the slow τc_2, the intermediate τc_3, and the fast τc_4). Dashed lines represent the distribution of exponential components determined by the logarithm likelihood ratio test. The values of each time constant component and its relative weight (in parentheses) are given above each histogram. Group data of each time constant and associated weight are summated in *Table 2*.

vasodilatation components) was similar between controls (269.1 ± 58.6, $n = 6$) and T2D patients (244.0 ± 64.3, $n = 5$, P = N.S. vs. controls). These results indicate that shear stress-induced vasodilation in human coronary arterioles was mainly dependent on BK channel activation, which was reduced by 37.0% in diabetic patients (*Figure 4C*).

Downregulated protein expressions of BK-α and BK-β1 subunits in the coronary arteriolar SMCs of T2D patients

We examined the protein expressions of BK-α and BK-β1 in freshly isolated coronary arterioles from human atria. *Figure 5* illustrates that the protein expressions of BK-α and BK-β1 were downregulated by 68.1% and 66.5%, respectively in the atrial coronary arterioles of T2D patients ($n = 6$) compared with controls ($n = 4$), while the ratio of two proteins remained unchanged (BK-α/BK-β1, 1.15 ± 0.15 of controls vs. 1.18 ± 0.10 of diabetes, P = N.S.). These results are consistent with our electrophysiological findings.

Discussion

This is the first study to determine human BK channel abnormalities that underlie coronary microvascular BK channelopathy in patients with T2D. We have made several important findings: (1) Coronary arteriolar BK channel response to Ca^2 and voltage activation is reduced in T2D patients, while the channel unitary conductance remains unchanged. (2) BK channels in diabetic vessels have a significantly shortened mean open time but a prolonged mean closed time, resulting in a remarkable reduction in the channel maximal Po. (3) Protein expressions of BK-α and BK-β1 are markedly downregulated in the coronary arterioles of T2D patients, but the ratio of BK-α/BK-β1 is unchanged. (4) BK channel-mediated vasodilation to shear stress stimuli is impaired in the coronary arterioles of T2D patients. Hence, our results have an important clinical ramification, which reveals a novel ionic mechanism underlying coronary microvascular dysfunction in diabetes and help establish a new strategy for the treatment of diabetic patients with cardiovascular diseases.

BK channel activity is allosterically regulated by intracellular free Ca^{2+} concentrations and membrane potentials. Because of their large conductance and high density in coronary SMCs, BK channels are the key determinant of coronary circulation. It has been reported that activation of BK channels contributes to more than 70% of total vasodilation-induced by bradykinin in human coronary resistance vessels.[30] It is well-known that diabetes is an independent risk factor for the morbidity and mortality of cardiovascular diseases in human; however, the role of BK channels in cardiovascular abnormalities in diabetic patients has not been delineated previously. It worth noting that most of our knowledge about coronary BK channelopathy in diabetes was obtained from animal studies, and the results varied depending on vascular size, vascular bed and species.

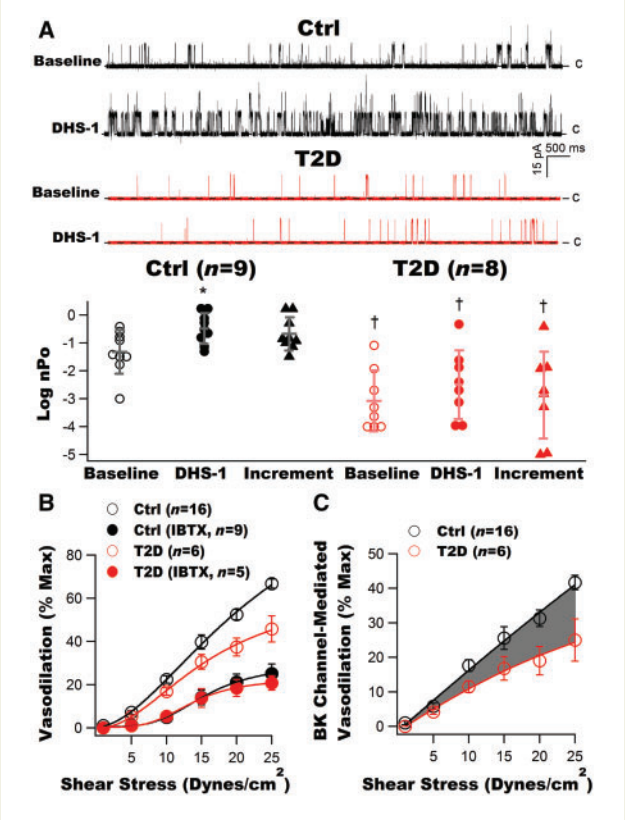

Figure 4 Reduced BK channel activation by DHS-1 and BK channel-mediated vasodilation by shear stress in the coronary arterioles of T2D patients. (*A*) Representative tracings of single BK channel currents of coronary arterial myocytes were recorded at +60 mV in the inside-out configuration and in the presence of 200 nM free Ca^{2+} before and after exposure to 100 nM DHS-1. Dashed line indicates the closed state (c) of channels. Scatter plots with statistical differences are shown in the lower panel. The BK channel logarithm nPo (log nPo) at baseline was significantly lower in diabetic patients than that of non-diabetic controls. Application of DHS-1 robustly augmented BK channel nPo in controls but the effect was diminished in T2D patients. There was a significant reduction in the increment of BK channel nPo between non-diabetic controls and T2D patients in response to DHS-1. Data are presented as mean ± S.E.M. $^{*}P < 0.05$ vs. baseline (using Paired *t*-test); $^{\dagger}P < 0.05$ vs. non-diabetic controls (using Student's *t*-test). (*B*) Shear stress-induced coronary arteriolar vasorelaxation was performed after a 45-min equilibration with Krebs' buffer (a time control). The area under curve (AUC) of shear stress response was significantly reduced in T2D patients, compared to non-diabetic controls. Moreover, after a 45-min equilibration with the physiological Krebs' buffer contained 100 nM IBTX, the AUC of non-BK channel-mediated vasodilatation components was similar between controls and diabetic patients (using Student's *t*-test), indicating that shear stress-induced vasodilation in human coronary arterioles was mainly dependent on BK channel activation. (C) The BK channel-mediated vasodilation to shear stress was obtained by digital subtraction of IBTX insensitive components from the total vasodilation at each shear stress level. The AUC was decreased by 37.0% in T2D patients. Data are presented as mean ± S.E.M.

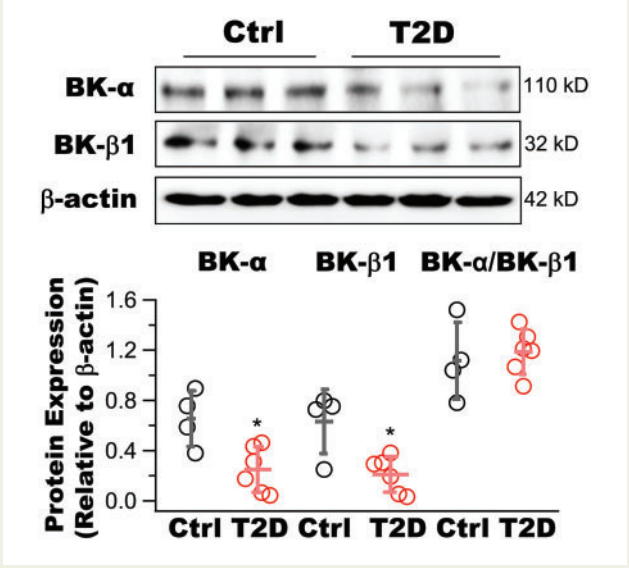

Figure 5 Downregulated BK channel protein expression in the coronary arterioles of T2D patients. Immunoblots show the downregulation of BK-α and BK-β1 protein expressions without changing the ratio of BK-α/BK-β1 expression in freshly isolated atrial coronary arterioles from non-diabetic subjects ($n = 4$) and T2D patients ($n = 6$). Protein expression was normalized to β-actin and expressed as relative densitometry units. Group data are presented as mean±S.E.M. $^{*}P < 0.05$ vs. controls (using Student's *t*-test).

For example, in streptozotocin (STZ)-induced type 1 diabetic mice, type 2 *db*/*db* diabetic mice, high fat diet (HFD)-induced obesity/diabetic mice, and Zucker diabetic fatty (ZDF) rats, impaired BK channel function is found in coronary arteries,[11–14,18,31] cerebral arteries,[32–34] and mesentery arteries.[35] However, in HFD-fed diabetic Yucatan pigs, the downregulation of BK channel activity was only found in coronary microvessels,[36,37] but not in the epicardial coronary arteries.[38] In addition, single BK channel kinetics analysis showed an increase of channel Po with reduced mean closed time and channel conductance in the aortic SMCs of STZ-induced diabetic mice,[39] whereas a decrease of BK channel Po and mean open time, an increase of mean closed time and no change in its conductance were found in the coronary SMCs of ZDF rats.[11] Similar vascular bed specificity of vascular BK channel pathology also occurs in human diabetes. Recently, Nieves-Cintron *et al.* reported that BK channel activity and spontaneous transient outward currents (STOCs) are decreased in the mesentery arteries of diabetic patients without altering the protein expressions of BK-α and BK-β1, and the amplitude and frequency of Ca^{2+} sparks, suggesting that uncoupling of Ca^{2+} signal and BK channel activation may occurs in diabetic vessels.[40] In contrast, we found that coronary arteriolar BK channel activation to physiological Ca^{2+} concentration and voltage was downregulated in human subjects with T2D. Moreover, single channel analysis reveals that the changes of BK channel kinetics in diabetes were mainly in the closed-states with minor changes in the open-states. However, accumulation of these minor changes in the open-states also produced a significant shortening of the mean open time, contributing to a remarkable decrease of BK channel Po in the coronary SMCs of diabetic patients. We have previously reported that in diabetic animals impaired Ca^{2+}-mediated BK channel activation was associated with a reduction of BK-β1 protein expression and physical dissociation between BK-α and BK-β1 coupling in diabetic vessels.[11,16] In addition, it has been shown that the oxidation of cysteine residue at 911 in the C-terminus of BK-α resulted in a functional knockdown of BK-β1 and reduction of Ca^{2+} activation in heterologous expression system.[41]

Since the increased oxidative stress is the hallmark feature of diabetic vasculopathy including human being,[42] we believe that both BK-α and BK-β1 abnormality contribute to BK channelopathy and coronary microvasculopathy in T2D patients.

In addition to our electrophysiological observations, another key finding of BK channel pathology in the coronary arterioles of human diabetes is a 67–68% downregulation of BK-α and BK-β1 protein levels without altering the ratio of BK-α/BK-β1 protein expression. DHS-1 is the most specific BK channel activator that works at BK-β1 on the cytoplasmic surface of membrane. Since the ratio of BK-α/BK-β1 expression remained unchanged in diabetic vessels, we examined DHS-1 effects on BK channel activation to confirm whether reduced BK channel Ca^{2+} sensitivity was associated with BK-β1 dysfunction in diabetic patients. We found that BK channel activation to DHS-1 was lost in T2D patients, suggesting that cooperativity of BK-α and BK-β1 was diminished in the coronary arterioles of T2D patients.

The molecular mechanisms leading the downregulation of coronary BK protein expression in diabetic patients are currently unknown but warrant further investigation. According to animal studies, the underlying mechanisms are multifactorial and remain controversial. For instance, the reduction of BK-β1 expression was due to accelerated protein degradation through the ubiquitin-proteasome system in the aortas of STZ-induced diabetic mice,[18,31] HFD-induced obese/prediabetic mice,[15] and *db/db* diabetic mice,[14] while it was also attributed to the dysregulation of BK-β1 mRNA transcription in HFD-fed mouse vessels.[15,34] In contrast, BK-α protein expression was reportedly increased in the cerebral arteries of *db/db* diabetic mice,[43] decreased in the mesenteric arteries of ZDF rats[35] and unchanged in the coronary arteries and aortas of STZ-induced diabetic rats and mice.[12,18,31,44] Hence, each animal model only partially represents the phenotype of BK channel pathology in coronary arterioles of human diabetes. It has been known that BK channel function is affected by the renin-angiotensin-aldosterone system,[45] the sympathetic and parasympathetic nervous systems,[46] and steroid hormones.[47] We carefully enrolled diabetic patients and matched non-diabetic controls to avoid unexpected results. It has also been reported that hypertension and aging are associated with pathological alterations of vascular BK channels in human.[48] It would not be the case in our study, because age, hypertension, and other risk factors were well-controlled in the diabetic patients and no different from non-diabetic subjects.

The limitation of current study is that coronary BK channel Ca^{2+} and voltage response was examined in a fixed free Ca^{2+} concentration and testing voltage, which may not reflect a shift in the overall Ca^{2+} and voltage sensitivity. However, these experiments were performed in a physiological free Ca^{2+} concentration (200 nM) and at a voltage of +60 mV in inside-out configuration that is equivalent to a normal vascular SMC membrane potential (-60 mV). Nevertheless, we have found that patients with T2D have altered BK channel kinetics, impaired BK channel activation to a physiological Ca^{2+} and voltage, reduced BK channel maximal activation, diminished BK channel-mediated vasodilation to shear stress, and downregulated BK channel protein expressions in the coronary microvessels. Such BK channel pathophysiology may contribute to diabetic patients with cardiovascular complications.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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