Reactive Oxygen Species Signaling Facilitates FOXO-3a/FBXO-Dependent Vascular BK Channel β_1 Subunit Degradation in Diabetic Mice

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Activity of the vascular large conductance Ca^{2+}-activated K^+ (BK) channel is tightly regulated by its accessory β_1 subunit (BK-β_1). Downregulation of BK-β_1 expression in diabetic vessels is associated with upregulation of the forkhead box O subfamily transcription factor-3a (FOXO-3a)–dependent F-box–only protein (FBXO) expression. However, the upstream signaling regulating this process is unclear. Overproduction of reactive oxygen species (ROS) is a common finding in diabetic vasculopathy. We hypothesized that ROS signaling cascade facilitates the FOXO-3a/FBXO-mediated BK-β_1 degradation and leads to diabetic BK channel dysfunction. Using cellular biology, patch clamp, and videomicroscopy techniques, we found that reduced BK-β_1 expression in streptozotocin (STZ)-induced diabetic mouse arteries and in human coronary smooth muscle cells (SMCs) cultured with high glucose was attributable to an increase in protein kinase C (PKC)-β and NADPH oxidase expressions and accompanied by attenuation of Akt phosphorylation and augmentation of atrogin-1 expression. Treatment with ruboxistaurin (a PKCβ inhibitor) or with GW501516 (a peroxisome proliferator–activated receptor δ activator) reduced atrogin-1 expression and restored BK channel-mediated coronary vasodilation in diabetic mice. Our results suggested that oxidative stress inhibited Akt signaling and facilitated the FOXO-3a/FBXO-dependent BK-β_1 degradation in diabetic vessels. Suppression of the FOXO-3a/FBXO pathway prevented vascular BK-β_1 degradation and protected coronary function in diabetes.
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Diabetes is a major cause of morbidity and mortality worldwide and is associated with increased risks of vascular complications such as coronary artery disease, stroke, nephropathy, neuropathy, and retinopathy. The large conductance Ca^{2+}-activated K^+ (BK) channels, abundantly expressed in vascular smooth muscle cells (SMCs), have an established role in mediating vasodilation and regulating tissue perfusion. Functional vascular BK channels are composed of four pore-forming α subunits (BK-α) and four regulatory β_1 subunits (BK-β_1). BK-α activity is tightly regulated by BK-β_1, which significantly enhances the channel voltage- and Ca^{2+}-sensitivity (1–4). Recent studies indicate that vascular BK channel function is impaired in both type 1 and type 2 diabetic animal models, which are associated with reduced BK-β_1 expression in vascular SMCs (5–7).

The ubiquitin-proteasome system (UPS) is the major mechanism of intracellular protein degradation, accounting for 80–90% of the intracellular protein turnover in mammalian cells (8). To be targeted for degradation by UPS, proteins are ubiquitinated, which requires the action of three enzyme complexes: E1, E2, and E3. E1 activates ubiquitin, whereas E2 conjugates ubiquitin and the substrate protein, and E3 facilitates the ubiquitination of the target protein. The polyubiquitinated proteins are then degraded in the 26S proteasomes (9). We have reported that downregulation of vascular BK-β_1 expression in streptozotocin (STZ)-induced diabetic animals and in human coronary SMCs with high glucose (HG) culture conditions was dependent on the activity of F-box only (FBXO) proteins, a key component of the Skp1-Cullin-F-box (SCF)–type E3 ligase complexes (7). Most notably, atrogin-1 (FBXO-32) expression was upregulated in diabetic vessels and in human coronary SMCs cultured with HG (7). Atrogin-1 expression is controlled by the forkhead box O transcription factor-3a (FOXO-3a) (10,11). The transcriptional activity of FOXO-3a is dependent on its subcellular localization. Akt phosphorylates FOXO-3a at T32, which inhibits FOXO-3a transcriptional function by promoting its nuclear export (12–14). However, the signaling pathways responsible for the FOXO-3a/FBXO-dependent downregulation of BK-β_1 expression in diabetes are unknown.

NADP oxidases (NOX) are a major source of superoxide anion ($O_2^{\cdot -}$) generation in the vasculature (15,16), and protein kinase C β (PKCβ) isoform stimulates NOX activity to overproduce $O_2^{\cdot -}$, which inhibits Akt signaling in diabetic vessels (17,18). $O_2^{\cdot -}$ is rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), and H_2O_2 is further reduced to H_2O by catalase (CAT) and glutathione peroxidase (GPX). We have reported that reduced CAT expression played a pivotal role in cellular oxidative stress under HG culture conditions (19). Because reactive oxygen species (ROS) overproduction is a common feature in diabetic pathology, we hypothesized that the ROS signaling cascade participates in the regulation of vascular BK-β_1 expression through the FOXO-3a/FBXO axis in diabetic vessels. In this study, we demonstrated that increased ROS production in STZ-induced diabetic mouse aortas attenuated Akt-mediated FOXO-3a phosphorylation, resulting in an acceleration of BK-β_1 protein degradation and impairment of coronary vasodilation. Overexpression of BK-β_1 and inhibition of FOXO-3a/FBXO axis in coronary arteries increased BK channel activity and preserved coronary function in diabetic mice. Hence, our results suggest that vascular BK-β_1 is a bona fide therapeutic target for diabetic vascular dysfunction.

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RESEARCH DESIGN AND METHODS

Type 1 diabetic animal. Male mice (strain name: C57BL/CJ) were purchased from the Jackson Laboratory at 4 weeks of age. Animals were made diabetic by an injection of STZ (100 mg/kg body weight, intraperitoneally) (16). Animals with blood glucose >300 mg/dL were considered diabetic and were used for experiments 8 weeks after developing hyperglycemia. For drug treatment, diabetic mice at 6 weeks after the development of hyperglycemia were randomly divided into three groups (each group containing12 mice): placebo-treated group (normal drinking water, by gavage), ruboxistaurin-treated group (10 mg/kg/day, by gavage) and GW501516-treated group (2 mg/kg/day, by gavage). After 2 weeks of treatment, diabetic mice and age-matched control mice were killed for experiments. Handling and care of animals were approved by the Institutional Animal Care and Use Committee of Mayo Clinic.

Vascular SMC isolation. Vascular SMCs were isolated as previously described (16). Briefly, mouse coronary arteries were carefully dissected in ice-cold dissociation buffer (in mmol/L): NaCl 145, KCl 4.0, $CaCl_2$ 0.05, $MgCl_2$ 1.0, HEPES 10, glucose 10, pH 7.2. The vessels were placed in dissociation buffer containing 0.1% weight for volume (w/v) BSA and incubated in a shaking water bath at 37°C for 3 min, and then the vessels were incubated with fresh 0.1% w/v BSA dissociation buffer containing 1.5 mg/mL papain and 1.0 mg/mL dithiothreitol in a shaking water bath at 37°C for another 3 min. This was 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 3 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.

BK channel recordings. BK currents were recorded from freshly isolated coronary SMCs (16,19). Whole-cell BK currents were elicited from a holding potential of −60 mV to testing potentials of −40 mV to +160 mV in 10-mV increments with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The pipette solution contained (in mmol/L): KCl 140, $MgCl_2$ 1.0, Na_2ATP 5.0, Na_2GTP 0.5, EGTA 1.0, $CaCl_2$ 0.814 (1 μmol/L free Ca^{2+}), and HEPES 10, pH 7.35 with potassium hydroxide (KOH). The bath solution contained (in mmol/L): NaCl 145, KCl 4.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, HEPES 10 and glucose 10, pH 7.40 with NaOH. The K^+ currents inhibited by 0.1 μmol/L iberiotoxin (IBTX) were referred as the IBTX-sensitive BK currents.

Single BK channel currents were elicited at +60 mV from the inside-out excised membrane patches. The output signals were filtered with an 8-pole Bessel filter (902 LPF; Frequency Devices Inc., Haverhill, MA) at 5 kHz and digitized at 50 kHz. Patch pipettes had a typical tip resistance of 5–10 mol/LΩ when filled with the pipette solution that contained (in mmol/L): KCl 140, $CaCl_2$ 1, $MgCl_2$ 1, EGTA 1, and HEPES 10, pH 7.4 with KOH. The bath solution contained (in mmol/L): KCl 140, $MgCl_2$ 1, EGTA 1, $CaCl_2$ 0.465 (0.2 μmol/L free Ca^{2+}) and HEPES 10, pH 7.35 with KOH. The effects of dehydrosoyasaponin-1 (DHS-1) on BK channel activity were determined in the same membrane patch before and after drug application to the cytoplasmic surface. All patch-clamp experiments were performed at room temperature (22–24°C). Data analysis was performed with Clampfit 10.2 software (Axon Instruments).

Adenoviral expression of BK-β_1 in the isolated coronary arteries of mice. Freshly isolated mouse coronary arteries were incubated with Clonetics SmBM medium (Lonza Group Ltd., Walkersville, MD), and were transduced with adenovirus carrying the EGFP-tagged human *KCNMB1* gene (Ad-BK-β_1-EGFP) or with control adenovirus carrying the *LacZ* gene (Ad-LacZ-EGFP) at 50 multiplicity of infection for 12 h as we have described (19). The expression level of BK-β_1 protein was determined by immunoblotting.

Videomicroscopy. Vasoreactivity was measured as previously described (20). Isolated mouse coronary arteries (1–2 mm in length and 80–130 μm in diameter) were mounted in a vessel chamber filled with Krebs solution containing (in mmol/L): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11.1, pH 7.4, and were secured between two borosilicate glass micropipettes with 10-0 ophthalmic suture. The lumen of each vessel was filled with Krebs solution through the micropipettes and maintained at a constant pressure (no flow) of 60 mmHg, followed by equilibration for 60 min in oxygenated (20% O_2+5% CO_2, balanced with N_2, 37°C) Krebs solution. The vessels were deemed unacceptable for experiments if they demonstrated leaks or failed to produce a <50% constriction to graded doses of endothelin-1. Integrity of endothelial function was determined by its response to acetylcholine. Concentration-response relationships of NS-1619 (10^{-9} to 10^{-5} mol/L) on vasodilatation were measured, and comparisons were made among control and diabetic mice receiving placebo, GW501516, or ruboxistaurin treatment. At the end of each experiment, vessels were maximally dilated with a Ca^{2+}-free solution, and the percentage dilatation in response to NS-1619 was normalized to the maximal diameter.

Immunoblot analysis. Isolated aortas from mice and cultured human coronary SMCs were homogenized, electrophoresed, transferred to nitrocellulose membrane, and blotted against anti-atrogen-1 (1:200; ECM Bioscience, Versailles, KY), anti-Akt (1:1000; Cell Signaling Technology Inc., Danvers, MA), anti-*p*-Akt(S473) (1:1000; Cell Signaling Technology Inc.), anti-BK-α (1:200, customer made), anti-BK-β_1 (1:200, customer made), anti-PKCβ (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-NOX-1 (1:500, Santa Cruz, Biotechnology), anti-NOX-4 (1:1000; Abcam Inc., Cambridge, MA), anti-FOXO-3a (1:1000, Cell Signaling Technology Inc.), anti-*p*-FOXO-3a(T32) (1:1000, Cell Signaling Technology Inc.), anti-MnSOD (1:1000; Enzo Life Science Inc., Farmingdale, NY), anti-Cu/ZnSOD (1:1000, Enzo Life Science Inc.), anti-GPX-1

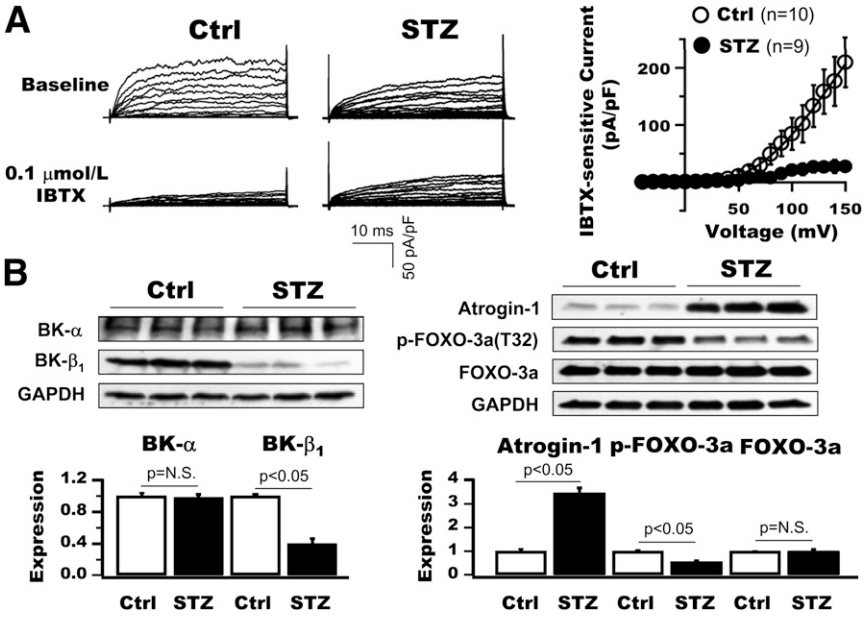

FIG. 1. Reduced BK current density and BK-β_1 protein levels with FOXO-3a-dependent increase in atrogin-1 expression in diabetic mouse vessels. *A*: Whole-cell K^+ current recordings in freshly isolated coronary SMCs from control and diabetic mice, with testing potentials of −40 mV to +160 mV and a holding potential of −60 mV, before and after the application of 0.1 μmol/L IBTX. The BK channel current-voltage (I-V) relationships in coronary SMCs showed a marked reduction of current densities in diabetic vessels. *B*: Immunoblots showing reduced BK-β_1 and *p*-FOXO-3a(T32) expression, whereas that of atrogin-1 was increased in the diabetic mouse aortas. The levels of BK-α and total FOXO-3a expression were unchanged.

(1:500; Laboratory Frontier, Seoul, Korea), and CAT (1:500; Sigma-Aldrich, St. Louis, MO) antibodies. Blots were probed with anti-β-actin antibody (1:2500, Sigma-Aldrich) or with anti-GAPDH antibody (1:2500, Sigma-Aldrich) as loading control. Optical density of the bands was analyzed with Scion Image software (Scion, Frederick, MD). Protein expression was expressed as relative abundance normalized to β-actin or GAPDH.

Intracellular $O_2^{\cdot-}$ measurement. Intracellular $O_2^{\cdot-}$ level was analyzed by HPLC/fluorescence assay that used dihydroethidium as a probe. Aortas were opened longitudinally and incubated in Krebs-HEPES buffer containing 50 μmol/L dihydroethidium (DHE) (Molecular Probes Inc., Eugene, OR) for 15 min at 37°C after which samples were washed and incubated in fresh Krebs-HEPES buffer for 1 h. The aortas were homogenized in cold (4°C) methanol and centrifuged at 12,000 rpm for 10 min. The supernatants were analyzed by HPLC in 37% acetonitrile in 0.1% trifluoroacetic acid aqueous solution. Data were quantified using DHE standard from the reaction between DHE and Fremy's salt as described and normalized against total proteins (21).

Chemicals. GW501516 was purchased from Enzo Life Science International Inc. (Plymouth Meeting, PA). Ruboxistaurin was obtained from Axon Medchem BV (Groningen, The Netherlands). DHS-1 was kindly provided by Merck Research Laboratories (Boston, MA). The other chemicals were purchased from Sigma-Aldrich Co.

Statistical analysis. Data were expressed as mean ± SEM. Student *t* test was used to compare data between two groups. A paired *t* test was used to compare data before and after treatment. One-way ANOVA, followed by the Tukey test analysis was used to compare multiple groups using SigmaStat software (Jandel, San Rafael, CA). A statistically significant difference was defined as $P < 0.05$.

RESULTS

Characterization of STZ-induced diabetic mice. The average body weights and blood glucose levels were 24.7 ± 0.30 g ($n = 35$) and 163.6 ± 18.5 mg/dL ($n = 35$), respectively, in control mice, and 22.1 ± 0.52 g ($n = 35$, $P < 0.05$ vs. control) and 468.5 ± 20.5 mg/dL ($n = 35$, $P < 0.05$ vs. control), respectively, in diabetic mice 8 weeks after STZ injection. Hence, STZ-induced diabetic mice had a 186.4% increase of blood glucose level with a 10.5% decrease in body weight, compared with controls. There was no significant difference in body weight and blood glucose among diabetic mice treated with placebo, ruboxistaurin, or GW501516.

Reduction of BK channel current density and BK-β_1 protein expression in vascular SMCs from diabetic mice. We have recently reported that downregulation of vascular BK-β_1 activity in diabetes was due to enhanced FBXO-dependent BK-β_1 protein degradation in STZ-induced diabetic rat vessels (7). To confirm whether similar results were obtained in STZ-induced diabetic mice, we compared BK channel current density in freshly isolated coronary SMCs and the BK-β_1 expression in the aortas from control and diabetic mice with 8 weeks of hyperglycemia. Figure 1*A* shows representative tracings of total K^+ currents from freshly isolated coronary SMCs of control and diabetic mice at baseline and after exposure to 0.1 μmol/L IBTX (a specific BK channel blocker). The BK channel currents were defined as the IBTX-sensitive K^+ component obtained by subtracting the IBTX-insensitive components from total K^+ currents. The BK current-voltage (I-V) relationships show a significant reduction of BK current density in coronary SMCs of diabetic mice, from 88.44 ± 24.83 picoamperes/picofarad (pA/pF) at a testing potential of +100 mV ($n = 10$) in controls to 20.68 ± 7.67 pA/pF ($n = 9$, $P < 0.05$) in diabetic mice. Figure 1*B* shows that in the aortas of diabetic mice, BK-β_1 protein level was reduced by 2.5-fold, accompanied by a 1.9-fold decrease in *p*-FOXO-3a(T32) and a 3.5-fold increase in atrogin-1 expression, whereas that of BK-α was unchanged.

Impaired Akt signaling downregulated BK-β_1 expression in diabetic mouse aortas and in HG-cultured human coronary SMCs. We have shown that the effects of HG culture on BK-β_1 expression were discernible after 4 days and reached a steady-state level after 10 to 14 days (7). We further determined the role of Akt signaling on the regulation of FOXO-3a phosphorylation and BK-β_1 expression in diabetic vessels and in human coronary SMCs cultured with HG. Hyperglycemia and HG culture produced a 2.0- and a 3.4-fold reduction of *p*-Akt(S473) protein level in the aortas of diabetic mice (Fig. 2*A*) and in human coronary arterial SMCs (Fig. 2*B*), respectively, but did not alter that of total Akt. A 24-h incubation with 7 μmol/L LY294002 (a specific PI3K inhibitor) reduced *p*-Akt(S473) expression by 1.9-fold and *p*-FOXO-3a(T32) expression by 1.8-fold in human coronary SMCs cultured with NG, leading to a 3.3-fold increase in atrogin-1 expression and a 4.3-fold decrease in BK-β_1 protein level (Fig. 2*C*). Thus, the PI3K/Akt signaling pathway regulated vascular BK-β_1 expression in diabetes and in HG culture through a FOX-3a/FBXO-dependent mechanism.

Increased PKCβ and NOX expressions and reduced antioxidant enzyme protein levels in diabetic mouse vessels and in HG-cultured human coronary SMCs. It has been established that PKCβ stimulates ROS generation and contributes to diabetic cardiovascular complications in diabetic rats (22,23). We determined the role of abnormal ROS metabolism in the regulation of vascular BK-β_1 expression. Figure 3*A* shows that the levels of PKCβ, NOX-1, and NOX-4 expression were increased by 1.7-, 1.6-, and 1.8-fold, respectively, in the aortas of diabetic mice compared with those of controls, and by 1.7-, 4.1-, and 3.7-fold, respectively, in human coronary SMCs cultured with HG compared with those cultured with 5 mmol/L glucose (NG). In contrast, the expressions of Cu/ZnSOD, MnSOD, GPX-1, and CAT were decreased by 21.1, 15.8, 31.8, and 76.9%, respectively, in the aortas of diabetic mice (Fig. 3*B*). Notably,

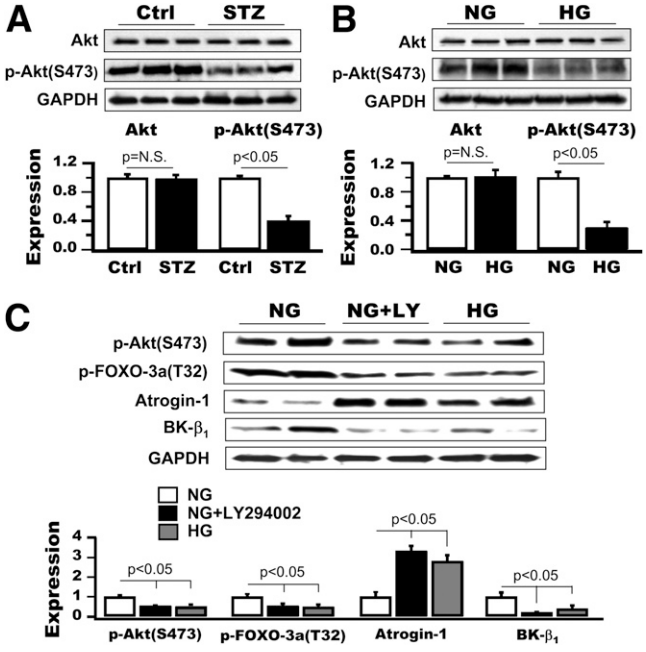

FIG. 2. Role of Akt signaling in the FOXO-3a/atrogin-1-dependent downregulation of BK-β_1 expression. *A* and *B*: Immunoblots against anti-Akt and anti-*p*-Akt(S473) antibodies showed reduced *p*-Akt(S473) level but no change in the total Akt expression in diabetic aortas (*A*) and in human coronary SMCs with HG culture (*B*). *C*: A 24-h incubation with 7 μmol/L LY294002 (LY) mimicked the effects of HG on the levels of *p*-Akt(S473), *p*-FOXO-3a(T32), atrogin-1, and BK-β_1 expression in human coronary SMCs cultured in NG.

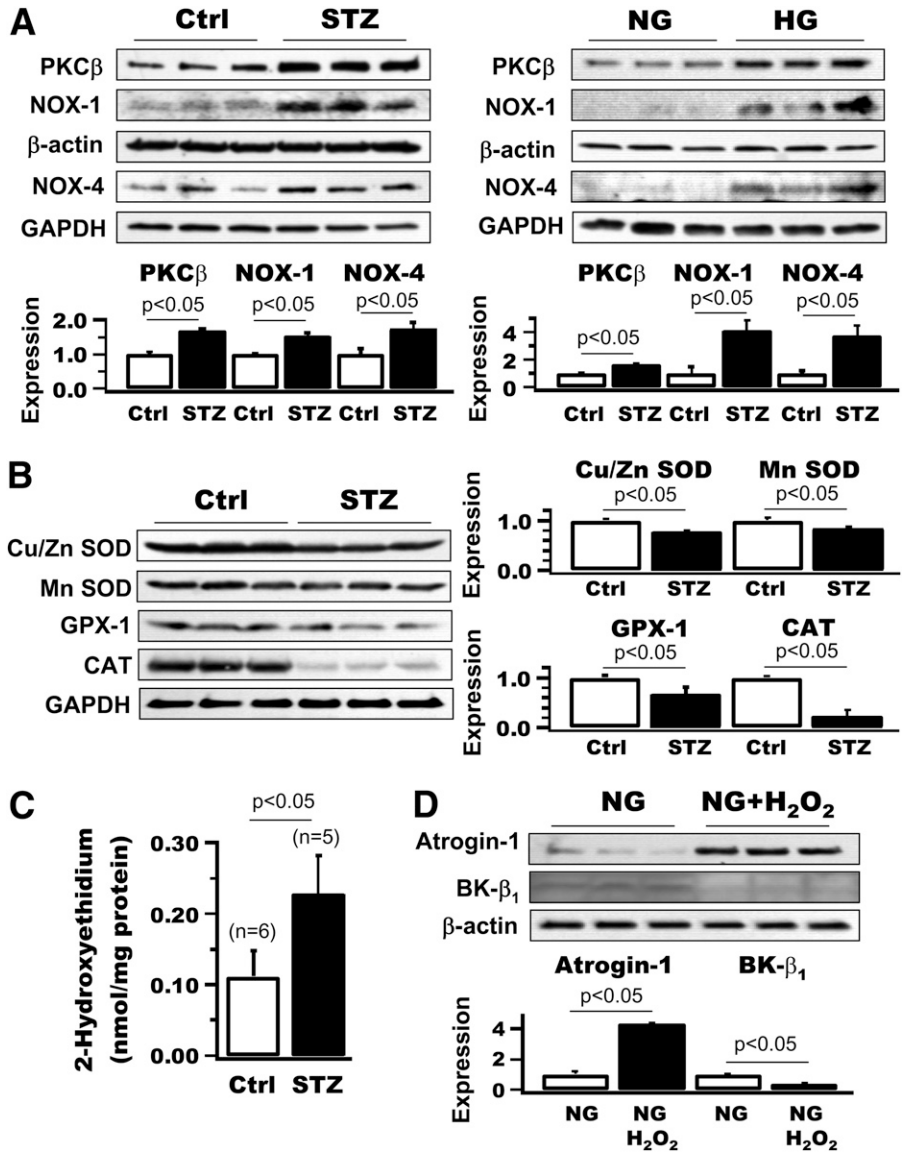

FIG. 3. ROS accumulation stimulated the FOXO-3a/atrogin-1-dependent downregulation of BK-β_1 expression. *A* and *B*: Aortas of diabetic mice and human coronary SMCs cultured in HG showed increased protein levels of PKCβ, NOX-1, and NOX-4 (*A*) and reduced protein expressions of Cu/ZnSOD, MnSOD, GPX-1, and CAT (*B*). *C*: Intracellular $O_2^{\cdot-}$ levels were analyzed by HPLC/fluorescence assay. After incubation with 50 μmol/L dihydroethidium, the aortas from diabetic mice produced a fluorescence signal twice that of control. *D*: A 12-h incubation with 50 μmol/L H_2O_2 markedly increased atrongin-1 expression and decreased BK-β_1 expression in human coronary SMCs cultured in NG.

such changes in NOX and antioxidant enzyme profiles led to a 1.5-fold increase in $O_2^{\cdot-}$ generation in the aortas of diabetic mice, from 0.11 ± 0.04 nmol/mg protein (n = 6) in control mice to 0.16 ± 0.04 nmol/mg protein (n = 5, $P <$ 0.05) (Fig. 3*C*). Most importantly, a 12-h incubation with 50 μmol/L H_2O_2 enhanced atrogin-1 expression by 4.3-fold in human coronary SMCs with NG culture, which was associated with a 2.9-fold reduction in BK-β_1 expression (Fig. 3*D*). Hence, exogenous H_2O_2 mimicked the effects of diabetes and HG on atrogin-1–dependent BK-β_1 protein downregulation.

Adenoviral expression of BK-β_1 restored BK channel-mediated dilation in coronary arteries of diabetic mice. We examined whether overexpression of BK-β_1 in isolated diabetic coronary arteries would restore vessel function. Twelve hours after transduction with Ad-BK-β_1-EGFP, the expression of BK-β_1 in the coronary arteries was confirmed by fluorescence microscopy (Fig. 4*A*) and that in the aortas was determined by Western blot analysis showing a 1.5-fold augmentation in BK-β_1 expression compared with those transduced with Ad-LacZ-EGFP (Fig. 4*B*). The coronary arteries transduced with Ad-BK-β_1-EGFP had a normal dilatory response to NS-1619 (a specific BK channel activator), similar to that in nondiabetic control vessels. In contrast, the diabetic coronary arteries transduced with Ad-LacZ-EGFP had a significantly blunted response to NS-1619 (Fig. 4*C*). These results indicate that reduced BK-β_1 expression was critical for BK channel-mediated coronary artery dysfunction in diabetes.

Regulation of vascular BK-β_1 expression in cultured human coronary SMCs by ROS/Akt signaling. Figure 5*A* shows that incubation with 1 μmol/L ruboxistaurin (a selective PKCβ inhibitor) for 24 h induced a 1.8- and a 6.2-fold increase in *p*-Akt(S473) expression in human coronary

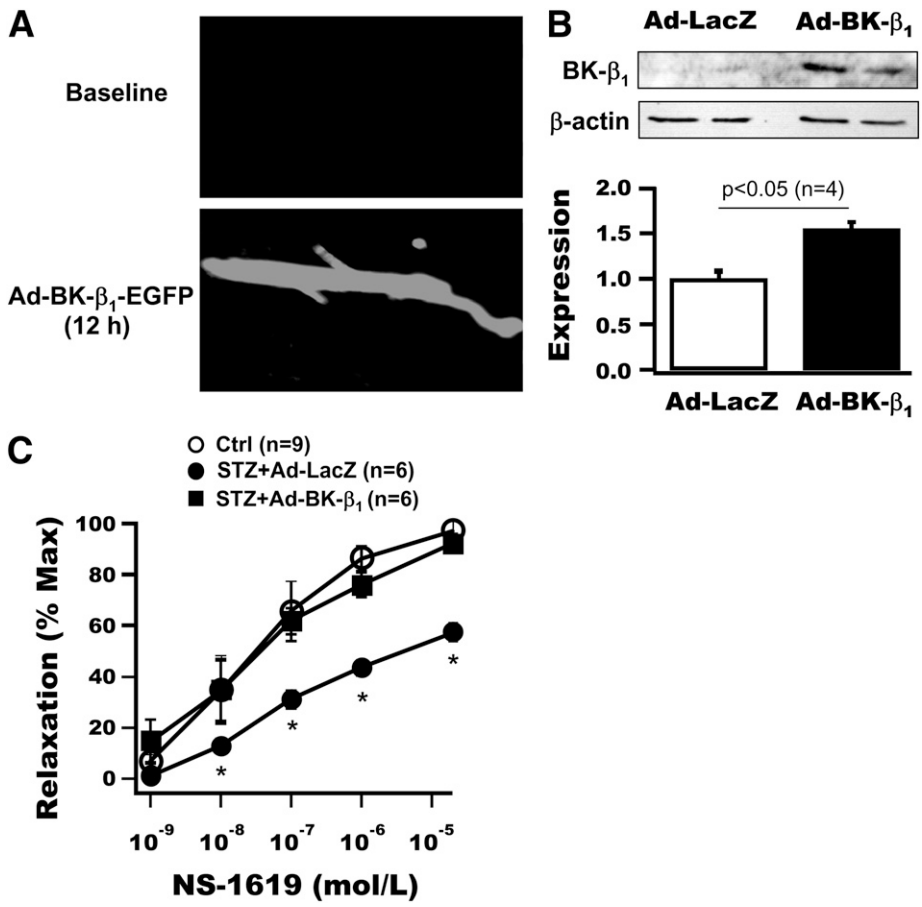

FIG. 4. Adenoviral overexpression of human KCNMB1 gene preserved BK channel-mediated vasodilation in isolated diabetic mouse coronary arteries. *A*: Florescence microscopy images show BK-β_1 expression in diabetic mouse coronary artery after a 12-h transduction with Ad-BK-β_1-EGFP, but not in coronary artery without transduction (baseline) (original magnification: ×4). *B*: Western blot analysis of endothelium-denuded aortas showed a significant increase in BK-β_1 expression in diabetic mouse after a 12-h transduction with Ad-BK-β_1-EGFP, compared with those with Ad-LacZ-EGFP transduction. *C*: Ad-BK-β_1-EGFP transduction produced a normal vasodilation response to NS-1619 in diabetic mouse coronary arteries, similar to that in nondiabetic controls, whereas diabetic coronary arteries with Ad-LacZ-EGFP transduction produced only half the dilatory response to NS-1619 as that in Ad-BK-β_1-EGFP transduction. *$P < 0.05$, Ad-LacZ-EGFP vs. Ad-BK-β_1-EGFP.

arterial SMCs cultured with NG and HG, respectively, stimulating 6.3 and 48.7% increases in FOXO-3a(T32) phosphorylation with NG- and HG-cultured cells respectively, without altering the total Akt and the total FOXO-3a protein levels. Moreover, ruboxistaurin did not change atrogin-1 expression in NG-cultured cells, but produced a 0.77-fold decrease in atrogin-1 expression in HG-cultured cells. Interestingly, ruboxistaurin produced a 4.1-fold increase in BK-β_1 expression in NG-cultured cells, compared with a 1.5-fold increase in HG-cultured cells.

GW501516, a selective agonist of peroxisome proliferator-activated receptor-δ (PPAδ), is known to facilitate Akt phosphorylation in human endothelial cells (24). We examined the effects of GW501516 on Akt phosphorylation and BK-β_1 expression in human coronary arterial SMCs cultured with NG or HG. In HG culture, a 24-h incubation with GW501516 (5 μmol/L) produced a 2.3-fold increase in *p*-Akt(S473) expression and a 8.3-fold increase in *p*-FOXO-3a(T32) expression, whereas that of atrogin-1 was reduced by 2.3-fold and that of BK-β_1 was increased by 11.7-fold (Fig. 5*B*), compared with cells without treatment. In NG culture, however, GW501516 attenuated the Akt(S473) and FOXO-3a(T32) phosphorylation and downregulated BK-β_1 expression in human coronary SMCs. Hence, our results suggest that mechanisms underlying ruboxistaurin- and GW501516-induced regulations of BK-β_1 expression under NG and HG culture conditions are complicated.

Oral administration of GW501516 or ruboxistaurin enhanced BK-β_1 expression and protected BK channel function in coronary arteries of diabetic mice. We determined the therapeutic roles of GW501516 and ruboxistaurin in diabetic coronary dysfunction. Figure 6*A* shows the expressions of *p*-Akt(S473), *p*-FOXO-3a(T32), atrogin-1, and BK-β_1 in the aortas from control and diabetic mice with and without a 2-week treatment with GW501516 or with ruboxistaurin. Compared with the untreated group, the levels of *p*-Akt(S473) and *p*-FOXO-32(T32) were enhanced by 1.1- and 0.8-fold, respectively, in the GW501516-treated diabetic group and increased by 1.2- and 1.3-fold, respectively, in the ruboxistaurin-treated diabetic group. Moreover, the vascular BK-β_1 protein level was upregulated by 3.4-fold in the GW501516-treated group and by 3.9-fold in the ruboxistaurin-treated group, restoring the BK-β_1 expression to 1.3- and 1.4-fold of that in controls. Functional vasoreactivity studies showed there was a 40% reduction of NS-1619–mediated coronary dilation in diabetic coronary arteries, whereas the response to NS-1619 was restored by treatment with GW501516 or with ruboxistaurin (Fig. 6*B*).

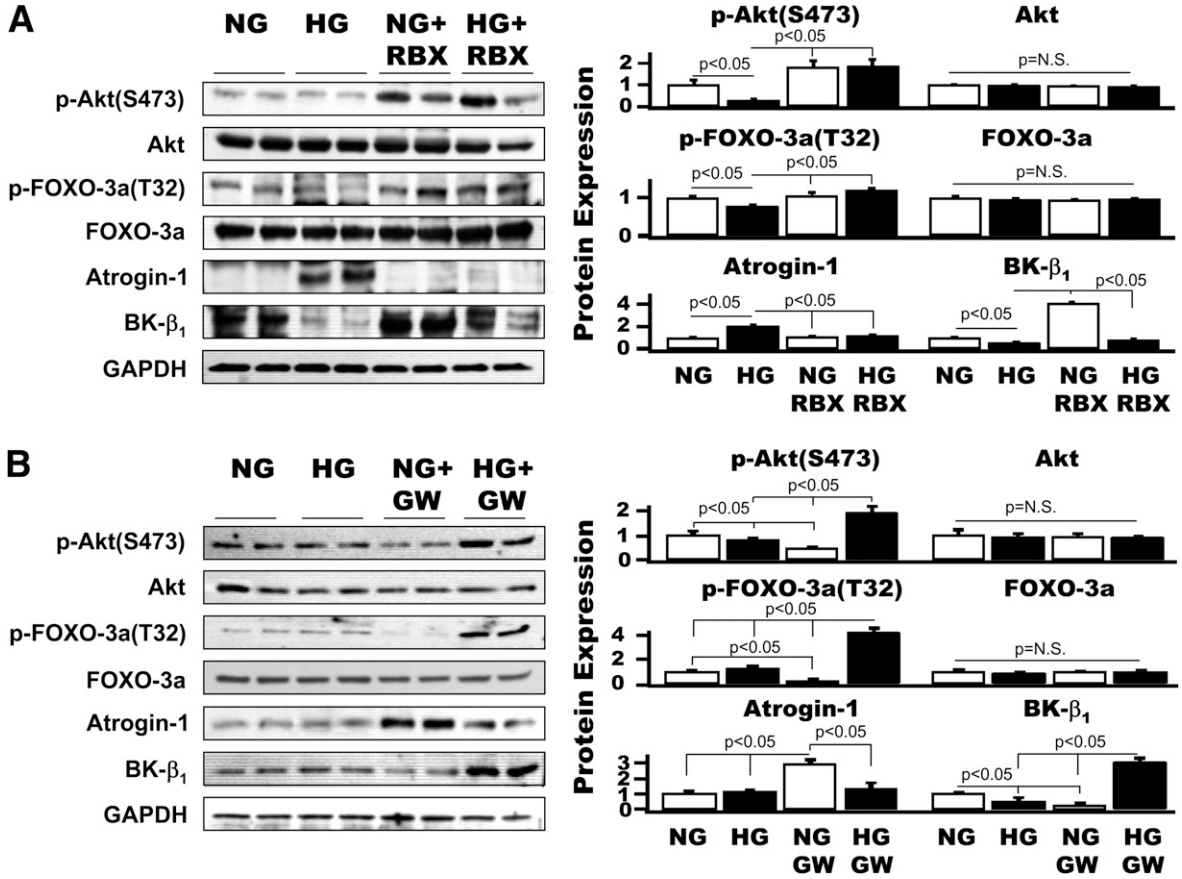

FIG. 5. GW501516 (GW) or ruboxistaurin administration prevented ROS signaling-mediated BK-β_1 protein degradation in cultured human coronary SMCs. *A*: Human coronary SMCs cultured in HG significantly reduced the protein levels of *p*-Akt(S473) and *p*-FOXO-3a(T32) with no change in the expression of total Akt and FOXO-3a. A 24-h incubation with ruboxistaurin (RBX) increased the protein levels of *p*-Akt(S473), *p*-FOXO-3a (T32), and BK-β_1, whereas that of atrogin-1 was reduced in human coronary SMCs cultured in NG and in HG. The effects of RBX on BK-β_1 expression were much more pronounced in the cells cultured with NG. *B*: Cells incubated with GW501516 for 24 h showed marked increase in the levels of *p*-Akt(S473) and *p*-FOXO-3a(T32) protein expression, with a significant decrease in atrogin-1 and an increase in that of BK-β_1 expression. GW had opposite effects on these protein expressions in cells cultured with NG.

Patch clamp experiments confirmed that there were 10- to 12-fold increases in single BK channel activities in response to 0.1 μmol/L DHS-1 (a specific BK-β activator) in freshly isolated coronary SMCs from GW501516- or ruboxistaurin-treated diabetic mice. The BK single channel Po was 0.02 ± 0.004 at baseline and robustly increased to 0.27 ± 0.06 (n = 10, $P < 0.05$) by DHS-1 in SMCs from GW501516-treated diabetic mice. Similarly, cells from ruboxistaurin-treated mice showed BK channel Po was markedly increased from 0.03 ± 0.007 at baseline to 0.25 ± 0.05 (n = 11, $P < 0.05$) by DHS-1. The effects of DHS-1 were comparable with those observed in controls (Fig. 7). In contrast, DHS-1 produced only a very small but significant increase in the Po in cells from untreated diabetic mice (0.018 ± 0.005 at baseline to 0.048 ± 0.014 with DHS-1, n = 11, $P < 0.05$). These results indicate that BK-β_1 is an important therapeutic target for treatment of vascular dysfunction in diabetes.

DISCUSSION

In this study, we reported several novel findings. First, the downregulated BK-β_1 expression in diabetic mouse vessels and in HG-cultured human coronary SMCs was produced by impaired PI3K/Akt signaling, which led to reduced FOXO-3a phosphorylation, upregulated atrogin-1 expression, and accelerated vascular BK-β_1 protein degradation. Second, abnormal ROS metabolism in vascular SMCs promoted the Akt/FOXO-3a/FBXO-dependent downregulation of BK-β_1 expression in diabetes. Third, increased BK-β_1 expression by adenoviral-mediated gene transfer of *KCNMB1* and by pharmacological deterrence of BK-β_1 protein degradation preserved BK channel function and restored normal vasoreactivity in diabetic mouse coronary arteries. Hence, our results have established the fundamental mechanisms that underlie BK channelopathy and BK-channel–mediated vasculopathy in diabetes. These novel observations are scientifically important and clinically relevant, and may help improve our approaches in developing new strategies for the treatment of diabetic vascular complications.

Diabetic vascular diseases result from abnormal endothelial-dependent and -independent mechanisms. Molecular mechanism underlying endothelial-dependent vascular dysfunction in diabetes is well-studied, whereas the endothelial-independent mechanisms are less scrutinized. Vascular smooth muscle is the key determinant of vascular tone and its physiology is profoundly altered in diabetes. BK-α is allosterically regulated by intracellular free Ca^{2+} and membrane potentials, and its activities are significantly potentiated by the presence of BK-β_1, which increases the channel sensitivities to voltage and to Ca^{2+}.

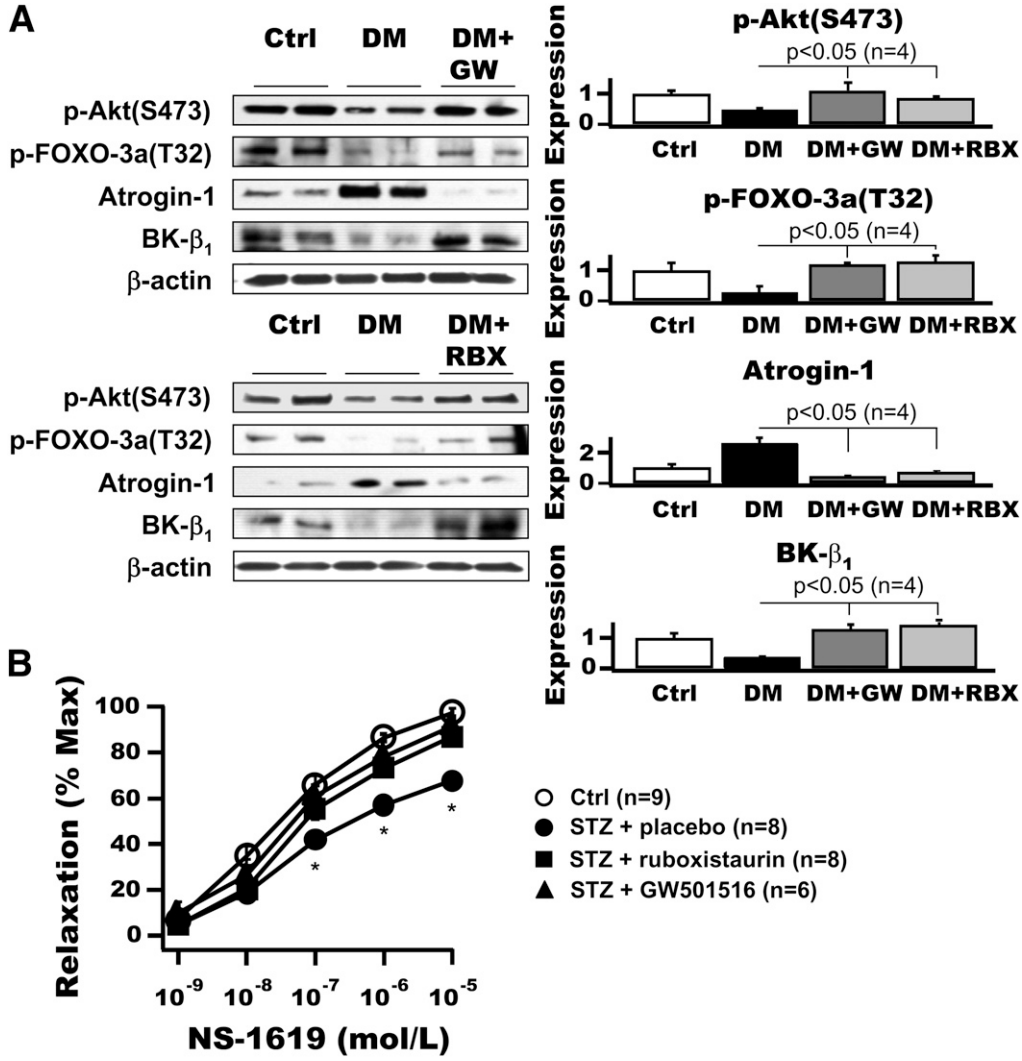

FIG. 6. Treatment with GW501516 (GW) or ruboxistaurin preserved BK-β_1 function and coronary vasodilation in diabetic mice. *A*: Immunoblots show that treatment with GW501516 or ruboxistaurin (RBX) increased the protein levels of *p*-Akt(S473), *p*-FOXO-3a(T32), and BK-β_1 and reduced that of atrogin-1 in the aortas of diabetic mice, compared with nondiabetic controls and to diabetic mice treated with placebo. *B*: Treatment with GW501516 or ruboxistaurin significantly improved the coronary vasodilation to NS-1619 in diabetic mice, compared with nondiabetic control mice and to diabetic mice treated with placebo. *$P < 0.05$.

Downregulation of BK-β_1 is known to be associated with genetic hypertension (25,26) and diabetic vasculopathy (5,27). We have found that reduced BK-β_1 expression is a common feature in vascular pathology for both type 1 and type 2 diabetes, which is attributable to an increase in BK-β_1 protein degradation via the UPS (7). Because BK-β_1 is expressed in vascular SMCs, but not in vascular endothelial cells (28), the BK-β_1 downregulation-associated diabetic vasculopathy must result from endothelial-independent mechanisms. Most notably, adenoviral expression of BK-β_1 in vascular SMCs preserved vascular BK channel function and restored normal vasoreactivity in isolated diabetic mouse coronaries, suggesting that BK-β_1 function plays a critical role in the development of diabetic vasculopathy.

It is well established that ROS-mediated vascular dysfunction in diabetes is linked to vascular endothelial dysfunction; however, the role of ROS in vascular smooth muscle dysfunction in diabetes has not been fully explored. In our STZ-induced diabetic mice, there were significant increases in the aortic expressions of PKCβ and NOX, accompanied by remarkable reductions of antioxidant enzyme expression, particularly that of CAT, resulting in ROS accumulation. We have reported that ROS overproduction in diabetic vessels and in HG culture conditions contributed to the redox-induced protein post-translational modification of BK-α, which suppressed BK-α activity (16,19). In this study, we have provided further evidence that H_2O_2 promoted the upregulation of atrogin-1 expression through inhibition of Akt phosphorylation and stimulation of FOXO-3a transcriptional activity, thereby accelerating vascular BK-β_1 degradation.

Another important observation in our study is that inhibition of the Akt/FOXO-3a/FBXO axis preserved the BK channel activity and coronary artery function in diabetic mice. Ruboxistaurin, a Food and Drug Administration–approved PKCβ inhibitor, is known to improve vascular endothelial function and is being used in clinical trials of diabetic patients with retinopathy (24) and neuropathy (29). However, the mechanism of ruboxistaurin effects remains somewhat controversial. Beckman et al. reported that the beneficial effects of ruboxistaurin on vascular function in type 2 diabetic patients were endothelium-independent

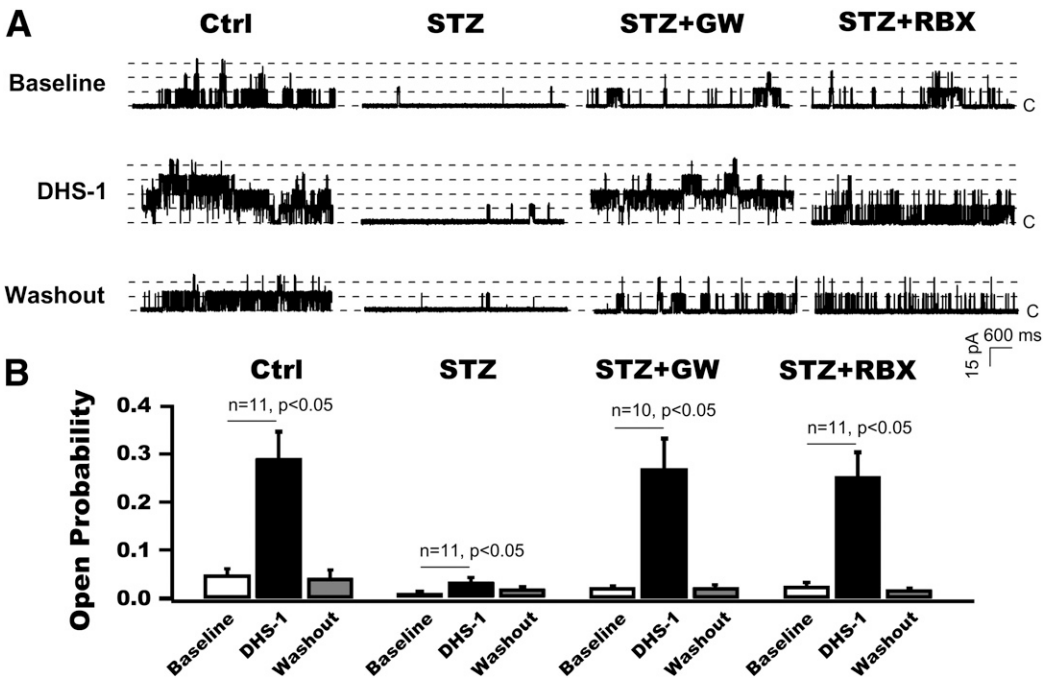

FIG. 7. Treatment with GW501516 or ruboxistaruin restored BK-β_1-mediated BK channel activation in diabetic coronary SMCs. *A*: Representative tracings of inside-out single BK currents were recorded at +60 mV in freshly isolated coronary SMCs obtained at baseline, after exposure to 0.1 μmol/L DHS-1, and upon drug washout. DHS-1 produced robust activation of BK channel activity in coronary SMCs from nondiabetic mice and diabetic mice treated with GW501516 (GW) or with ruboxistaurin (RBX), but not those from diabetic mice treated with placebo. *B*: Bar graphs showing BK channel open probabilities at baseline, after exposure to DHS-1 and after drug washout.

(30). We found that oral administration of ruboxistaurin in diabetic mice significantly increased Akt-mediated FOXO-3a phosphorylation and inhibited atrogin-1 expression, thereby preventing vascular BK-β_1 degradation and preserving the BK channel-mediated coronary vasodilation. Hence, our results are consistent with the clinical observation reported by Beckman et al. It is worthwhile to note that the effects of ruboxistaurin on enhancing BK-β_1 expression in human coronary SMCs were more potent with NG than with HG culture conditions, and we cannot rule out mechanisms other than those discussed. It is obvious that regulation of vascular BK-β_1 expression by the PKCβ/ROS signaling cascades is complex and other downstream pathways of ROS signaling may be involved in regulating BK-β_1 degradation in diabetic vessels.

PPARδ is ubiquitously expressed in all tissues and is involved in the regulation of lipid and glucose metabolism, as well as gene expression (31). Activation of PPARδ is known to have beneficial effects on endothelial function (32), lipid metabolism, and body weight control (33). The selective PPARδ agonist GW501516 is currently in phase I clinical trials as hypolipidemic agents but has not been evaluated for the treatment of diabetic vascular complications (34). GW501516 has been reported to increase Akt phosphorylation in human endothelial cells (32). In this study, we found that GW501516 markedly upregulated Akt phosphorylation, increased FOXO-3a phosphorylation, reduced atrogin-1 expression, and upregulated vascular BK-β_1 levels in diabetes and in HG culture conditions. However, our experiments suggested that GW501516 reduced BK-β_1 expression in NG-cultured cells through Akt inhibition. The different response to GW501516 may ultimately result from changes in intracellular redox homeostasis and signaling transduction. This property could be important for clinical application in the treatment of diabetic vascular complications.

In summary, we found that FOXO-3a/FBXO-dependent downregulation of BK-β_1 expression in diabetic vessels is associated with ROS overproduction. Oral administration of ruboxistaurin and GW501516 significantly improved coronary function by preserving BK-β_1 expression in diabetic vessels. Hence, BK-β_1 is a bona fide molecular target for treating diabetic vascular dysfunction. Validation of the beneficial vascular effects of these drugs in diabetic patients deserves further evaluation in clinical trials.

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T.L. designed and performed the experiments, analyzed the data, and wrote the manuscript. Q.C., L.Y., L.V.d'U., and T.H. performed experiments and analyzed results. Z.K. and H.-C.L. discussed the results and prepared the manuscript. T.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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