Short Communication

Muscle-Specific F-Box Only Proteins Facilitate BK Channel \( \beta_1 \) Subunit Downregulation in Vascular Smooth Muscle Cells of Diabetes Mellitus

Dai-min Zhang,* Tongrong He,* Zvonimir S. Katusic, Hon-Chi Lee, Tong Lu

Rationale: Activity of the large conductance Ca\( ^{2+} \)-activated K\( ^+ \) (BK) channels is profoundly modulated by its \( \beta_1 \) subunit (BK-\( \beta_1 \)). However, BK-\( \beta_1 \) expression is downregulated in diabetic vessels. The ubiquitin–proteasome system (UPS) is a major mechanism of intracellular protein degradation. Whether UPS participates in BK-\( \beta_1 \) downregulation in diabetic vessels is unknown.

Objective: We hypothesize that UPS facilitates vascular BK-\( \beta_1 \) degradation in diabetes.

Methods and Results: Using patch clamp and molecular biological approaches, we found that BK-\( \beta_1 \)-mediated channel activation and BK-\( \beta_1 \) protein expression were reduced in aortas of streptozotocin-induced diabetic rats and in human coronary arterial smooth muscle cells (CASMCs) cultured in high glucose. This was accompanied by upregulation of F-box only protein (FBXO)-9 and FBXO-32 (atrogin-1), the key components of the Skp1-Cullin-F-box (SCF) type ubiquitin ligase complex. BK-\( \beta_1 \) expression was suppressed by the FBXO activator doxorubicin but enhanced by FBXO-9 small interfering RNA or by the proteasome inhibitor MG-132. Cotransfection of atrogin-1 in HEK293 cells significantly reduced Flag-hSlo-\( \beta_1 \) expression by 2.16-fold, compared with expression of Flag-hSlo-\( \beta_1 V146A \) (a mutant without the PDZ-binding motif). After cotransfection with atrogin-1, the ubiquitination of Flag-hSlo-\( \beta_1 \) was increased by 1.91-fold, compared with that of hSlo-\( \beta_1 V146A \), whereas cotransfection with atrogin-1\( \Delta F \) (a nonfunctional mutant without the F-box motif) had no effect. Moreover, inhibition of Akt signaling attenuated the phosphorylation of forkhead box O transcription factor (FOXO)-3a and enhanced atrogin-1 expression, which in turn suppressed BK-\( \beta_1 \) protein levels in human CASMCs.

Conclusions: Downregulation of vascular BK-\( \beta_1 \) expression in diabetes and in high-glucose culture conditions was associated with FOXO-3a/FBXO-dependent decrease in BK-\( \beta_1 \) degradation. (Circ. Res. 2010;107:1454-1459.)

Key Words: ubiquitin–proteasome system • BK channel \( \beta_1 \) subunit • protein degradation • diabetes mellitus

The large conductance Ca\( ^{2+} \)-activated K\( ^+ \) (BK) channels play an important role in the regulation of vascular physiology. Functional BK channels in coronary arterial smooth muscle cells (CASMCs) are composed of the pore-forming \( \alpha \) subunits (BK-\( \alpha \), encoded by the Slo gene) and the regulatory \( \beta \) subunits (BK-\( \beta_1 \)) in 4:4 stoichiometry. However, BK channel function is impaired in diabetes,\(^{1,2} \) which is associated with microvessel complications. Recently, we and other investigators have reported that impaired BK channel activation was attributable to reduced BK-\( \beta_1 \) expression in diabetic vessels.\(^{3,4} \) However, the underlying molecular mechanisms is unknown.

The ubiquitin–proteasome system (UPS) accounts for 80% to 90% of intracellular protein turnover.\(^{5} \) UPS-mediated protein degradation involves 3 enzyme systems: ubiquitin-activation enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3.\(^{6} \) There are 1 E1, >25 E2, and >1000 E3 enzymes. Each E3 recognizes a specific motif on substrate proteins.

F-box only proteins (FBXOs) are key components of the Skp1-Cullin-F-box (SCF) type ubiquitin ligase complex, functioning as sites for enzyme–substrate interaction.\(^{7} \) FBXO expression is controlled by the forkhead box O family transcription factor (FOXO). FOXO activities are negatively regulated by Akt, which phosphorylates FOXO at T32, S253, and S315. Phosphorylated FOXO is extruded from the nucleus with loss of transcriptional function.\(^{8} \) FBOX-9 and FBXO-32 (atrogin-1) are muscle-specific proteins.

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subtypes and are abundantly expressed in myocardium and skeletal muscles.\textsuperscript{9,10} Atrogin-1 may bind to the PDZ-binding motif (T/S-X-V; X is any amino acid) in substrates.\textsuperscript{9} Interestingly, the PDZ-binding motif is present in most BK-\(\beta_1\) isoforms in different species including human. However, the role of FBXOs in the regulation of BK-\(\beta_1\) expression is unknown. Here, we hypothesized that enhanced UPS activity facilitates BK-\(\beta_1\) protein degradation in diabetes. We found that expression of atrogin-1 and FBXO-9 was augmented in human CASMCs under high-glucose (HG) culture and in streptozotocin (STZ)-induced diabetic rat vessels, leading to downregulation of BK-\(\beta_1\) expression. Moreover, expression of FBXOs and BK-\(\beta_1\) was regulated by FOXO-3a phosphorylation. Hence, we have delineated a novel fundamental mechanism that underlies vascular BK-\(\beta_1\) dysfunction in diabetes.

Methods
Male Sprague–Dawley rats were used. Handling and care of animals were approved by the Institutional Animal Care and Use Committee of Mayo Clinic.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results
Reduced BK Current Density and Impaired DHS-1–Mediated Channel Activation in CASMCs of Diabetic Rats
Figure 1A shows whole-cell K\(^+\) currents from freshly isolated CASMCs of control and STZ-induced diabetic rats before and after application of 100 nmol/L iberiotoxin (IBTX) (a specific BK channel inhibitor). The current-voltage curves of IBTX-sensitive K\(^+\) currents (defined as BK currents) were significantly decreased by 4.5-fold in diabetic rats, compared with control. DHS-1 (100 nmol/L, a specific BK-\(\beta_1\) activator) applied to the cytoplasmic membrane surface of CASMCs remarkably increased BK channel open probability from 0.11±0.04 at baseline to 0.33±0.11 with DHS-1 (P<0.05 versus baseline) in control rats but had no effect in diabetic rats (Figure 1B), suggesting that the \(\beta_1\)-mediated BK channel activation is lost.

Downregulated BK-\(\beta_1\) Expression and Enhanced BK-\(\beta_1\) Ubiquitination in Diabetic Vessels and in Human CASMCs With HG Culture
BK-\(\beta_1\) protein levels were decreased by 3.06±0.07-fold (P<0.05 versus control) in STZ-induced diabetic rats (Figure 1C) and by 2.10±0.09-fold in human CASMCs with HG (22 mmol/L), compared with those with normal glucose (NG) (5 mmol/L, P<0.05) (Figure 1D), whereas BK-\(\alpha\) expression remained unchanged. The effects of HG on BK-\(\beta_1\) downregulation were detectable at day 7 and plateaued at days 10 to 14 of HG culture (Online Figure I). However, BK-\(\beta_1\) mRNA level was not reduced in diabetic rats but actually increased in human CASMCs cultured with HG (Online Figure II).

Accordingly, the ubiquitination of BK-\(\beta_1\) in diabetic rat aortas and in human CASMCs cultured with HG was increased by 3.23±0.36-fold (P<0.05 versus controls) and by 2.40±0.33-fold (P<0.05 versus NG), respectively (Figure 1E).

Increased FBXO Expression in Diabetic Rat Vessels and in Human CASMCs With HG Culture
Atrogin-1 and FBXO-9 were expressed in rat aortas and in human CASMCs (Online Figure III). Protein levels of FBXO-9 and atrogin-1 were increased in aortas of STZ-induced diabetic rats by 1.65±0.16-fold and 4.87±1.17-fold, respectively (P<0.05 versus control in both) (Figure 2A), as well as in human CASMCs cultured with HG by 2.43±0.27-fold and 2.33±0.36-fold, respectively (P<0.05 versus NG in both) (Figure 2B). Seventy-two hours after FBXO-9 small interfering (si)RNA (50 nmol/L) was transfected into human CASMCs, FBXO-9 protein level was suppressed by 80%, whereas BK-\(\beta_1\) expression was increased by 1.65±0.16-fold, compared with control siRNA (P<0.05) (Figure 2C).

After 24 hours of incubation with 0.1 \(\mu\)mol/L doxorubicin (DXR) (a FBXO activator),\textsuperscript{11} BK-\(\beta_1\) expression was reduced by 1.92±0.11-fold (P<0.05 versus control) in NG (Figure 2D) and by 1.79±0.05-fold (P<0.05 versus control) in HG (Figure 2E). The DXR effects were abolished by 4-hour treatment with 10 \(\mu\)mol/L MG-132 (a proteasome inhibitor). BK channel openings were less frequent, with no response to DHS-1 in nondiabetic rat CASMCs after treatment with DXR (P=NS versus baseline). In CASMCs incubated with DXR+MG-132, however, BK channel activity was robust in the presence of DHS-1.

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BK</td>
<td>large conductance Ca(^{2+})-activated K(^+) channel</td>
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<tr>
<td>BK-(\alpha)</td>
<td>BK channel (\alpha) subunit</td>
</tr>
<tr>
<td>BK-(\beta_1)</td>
<td>BK channel (\beta_1) subunit</td>
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<tr>
<td>CASMC</td>
<td>coronary arterial smooth muscle cell</td>
</tr>
<tr>
<td>DHS-1</td>
<td>100 nmol/L doxorubicin (a BK-(\beta_1) activator)</td>
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<tr>
<td>DXR</td>
<td>doxorubicin</td>
</tr>
<tr>
<td>FBXO</td>
<td>1-box only protein</td>
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<tr>
<td>FOXO</td>
<td>forerkhead box 0 family transcription factor</td>
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<tr>
<td>HG</td>
<td>high glucose</td>
</tr>
<tr>
<td>IBTX</td>
<td>iberiotoxin</td>
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<tr>
<td>NG</td>
<td>normal glucose</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin–proteasome system</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
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Lack of the PDZ-Binding Motif in hSlo-β₁ Abolished UPS-Mediated Protein Degradation

The PDZ-binding motif is well conserved in BK-β₁ (Figure 3A). We measured protein expression of Flag-hSlo-β₁ wt and Flag-hSlo-β₁V146A (a mutation in the PDZ-binding motif) after cotransfection with ubiquitin and atrogin-1 or atrogin-1/F (a nonfunctional mutant with deletion of the F-box). Seventy-two hours after transfection, expression of hSlo-β₁ wt was 2.16 ± 0.16-fold lower than that of hSlo-β₁V146A (P < 0.05) (Figure 3B). Also, ubiquitination of hSlo-β₁ wt was 1.91 ± 0.08-fold higher than that of hSlo-β₁V146A (P < 0.05) in cells cotransfected with atrogin-1, whereas atrogin-1ΔF transfection had no effect (Figure 3C). Hence, our results suggest that FBXO interacts with BK-β₁ through the PDZ-binding motif and facilitates BK-β₁ ubiquitination and degradation.

Regulation of Atrogin-1 and BK-β₁ Expression by Akt/FOXO-3a Signaling in Human CASMCs

We found that FOXO-3a (T-32) phosphorylation was downregulated by 1.54 ± 0.01-fold (P < 0.05 versus control) in STZ-induced diabetic rat aortas and by 4.23 ± 0.02-fold (P < 0.05 versus NG) in human CASMCs in HG, whereas total FOXO-3a protein remained unchanged (Figure 4A and 4B).

We further determined the role of Akt/FOXO-3a signaling in the regulation of atrogin-1 and BK-β₁ expression in human CASMCs. After 24 hours of incubation with 7 μmol/L LY294002 (a phosphatidylinositol 3-kinase [PI3K] inhibitor), Akt (S473) phosphorylation was attenuated by 2.86 ± 0.03-fold (P < 0.05 versus control) with unchanged total Akt. Phosphorylated FOXO-3a protein was reduced by 1.86 ± 0.04-fold (P < 0.05 versus control), whereas total FOXO-3a was upregulated by 1.70 ± 0.16-fold (P < 0.05 versus control) with LY294002, resulting in 2.44 ± 0.23-fold increase (P < 0.05 versus control) in
atrogin-1 expression and 3.22±0.06-fold reduction (P<0.05 versus control) in BK-β₁ expression in human CASMCs (Figure 4C).

Discussion

The BK-β₁ subunit significantly enhances BK channel voltage sensitivity and Ca²⁺ cooperativity, and its physiological importance is underscored by BK-β₁ knockout mice, which showed increases in myogenic tone and arterial blood pressure, with uncoupling of Ca²⁺ sparks to BK channels in vascular smooth muscle cells (SMCs). We and others have demonstrated that BK channel dysfunction in both type I and type II diabetic vessels is associated with reduced BK-β₁ expression. In this study, we provided the first evidence that downregulation of BK-β₁ expression in vasculature was attributable to increased FBXO expression in diabetes and in HG culture conditions.

Abnormal expression of atrogin-1 is associated with muscle diseases, and its increased expression leads to skeletal muscle atrophy, whereas its reduced expression produces cardiac hypertrophy. We have found that atrogin-1 and FBXO-9 expression are upregulated in vascular SMCs in diabetes and HG. This upregulation of FBXOs plays a pivotal role in UPS-mediated BK-β₁ degradation and BK channel malfunction. Perturbation in atrogin-1 and hSlo-β₁ interaction attenuated hSlo-β₁ ubiquitination and preserved its protein level. Atrogin-1 and FBXO-9 are the target genes of FOXO-3a. We found that in diabetes and in human CASMCs with HG, FOXO-3a phosphorylation level was significantly reduced, accompanied by enhanced FBXO expression. The changes in FOXO-3a, FBXOs, and BK-β₁ expression in human CASMCs with HG were mimicked by PI3K/Akt inhibition. It is well known that the PI3K/Akt pathway is activated by insulin and suppressed by protein kinase C. Insulin receptor signaling is diminished and protein kinase C activity is upregulated in type I and type II diabetes, and these may lead to enhanced FBXO transcription and impaired BK channel function (Figure 4D). Hence, our findings provide novel insights into the pathophysiology of...
Figure 3. Mutation in the PDZ-binding motif of hSlo-β₁ prevented hSlo-β₁ ubiquitination and degradation. A, Sequence alignment of human, rabbit, cattle, rat, and mouse KCNMB1 cDNA shows a conserved PDZ-binding motif. ▼, applied mutation site. B, Seventy-two hours after cotransfection with atrogin-1 and ubiquitin, Flag-hSlo-β₁/V146A expression was significantly higher than that of Flag-hSlo-β₁ wt. C, Immunoprecipitates of anti-Flag antibody against HEK293 cell lysates with following transfection conditions were resolved and blotted against anti-ubiquitin antibody; no transfection, Flag-hSlo-β₁/ubiquitin, Flag-hSlo-β₁/V146A/atrogin-1/ubiquitin, and Flag-hSlo-β₁/atrogin-1/ubiquitin.

Figure 4. Modulation of vascular BK-β₁ expression by Akt/FOXO-3a/FBXO signaling pathway. A and B, Reduced FOXO-3a(T-32) phosphorylation with unchanged total FOXO-3a expression in STZ-induced diabetic rats (A) and in human CASMCs cultured with HG (B). C, Twenty-four-hour treatment with 7 μmol/L LY294002 (LY) attenuated the phosphorylation of Akt and FOXO-3a, enhanced the expression of total FOXO-3a and atrogin-1, and reduced BK-β₁ protein level in human CASMCs cultured with NG. D, Illustration showing the signaling mechanisms that underlie vascular BK-β₁ down-regulation in diabetes. Ins indicates insulin; InR, insulin receptor.
diabetic vasculopathy in both type 1 and type 2 diabetes mellitus and render BK-β1 as a potential therapeutic target in treatment of these conditions.

Acknowledgments

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Vascular BK channels are key determinants of vascular tone. They are composed of the pore-forming α subunits (BK-α) and the regulatory β1 subunits (BK-β1). The expression of the BK-β1 protein is downregulated in vascular smooth muscle cells (SMCs) in diabetes, leading to loss of the β1-mediated channel function, including channel sensitivity to Ca2+ and voltage.
- The ubiquitin-proteasome-system (UPS) is a major mechanism of intracellular protein degradation, accounting for 80% to 90% of intracellular protein turnover.

What New Information Does This Article Contribute?

- Impaired β1-mediated BK channel activation is associated with increase in UPS-dependent BK-β protein degradation in vascular SMCs in diabetes mellitus and in high glucose culture conditions.
- Muscle-specific F-box only proteins (FBXOs), FBXO-9 and FBXO-32 (atrogin-1), which are integral components of E3 ubiquitin ligase complexes, are abundantly expressed in vascular SMCs. The expression of these proteins is upregulated in diabetic rat aortas and in human coronary arterial SMCs cultured with high glucose.
- Expression of FBXOs is controlled by Akt and the forkhead box O family transcription factor 3a (FOXO-3a) signaling. Inhibition of Akt reduces FOXO-3a phosphorylation, increases FOXO-3a transcriptional function and facilitates FBXO expression, which in turn accelerates BK-β1 protein degradation in vascular SMCs.

The BK-β1 subunit plays a pivotal role in BK channel function by modulating channel voltage- and Ca2+ sensitivity. Down-regulation of BK-β1 expression in vascular SMCs is a common finding in diabetic vessels that produces BK channel dysfunction. However, the molecular mechanisms underlying downregulation of BK-β1, protein expression is unknown. In this study, we report that impaired β1-mediated channel activity in diabetes is associated with FOXO-3a/FBXO-dependent increase in BK-β1 protein degradation. These results indicate that BK-β1 subunits and Akt/FOXO-3a/FBXOs signaling cascade are potential therapeutic targets in the treatment of diabetic vascular complications.
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Methods

Development of Type 1 Diabetic Rats Male Sprague-Dawley rats were purchased from Harlan Inc. (Indianapolis, IN). To develop type I diabetic animal model, rats at 6 to 8 weeks of age received an injection of streptozotocin (STZ) (100 mg/kg body weight, ip.), followed by a second injection given on the next day. Control animals received vehicle injections. Animals with blood glucose > 300 mg/dl were considered diabetic. 8 weeks after the development of hyperglycemia, the average body weight and blood glucose level were 223.4±5.7 g and 571.3±6 mg/dl respectively in STZ-induced diabetic rats respectively (n=14) and 316.6±6.7 g and 167.2±7.5 mg/dl in age-matched controls respectively (n=17 p<0.05 between control and diabetic rats for both groups).

Cell culture, Subcloning, Site-Directed Mutagenesis, DNA Transfection and mRNA Knockdown HEK293 cells were cultured in DMEM. Primary human coronary arterial smooth muscle cells (CASMCS) were purchased from Lonza Walkersville Inc. (Walkersville, MD) and were cultured with Clonetics SmBM (Lonza Walkersville Inc.) containing 5 mmol/L or 22 mmol/L glucose. The CASMCs between 5 to 8 passages were used for all experiments. Human KCNMB1 in pGEM (obtained from Dr. Olaf Pongs, University of Hamburg, Hamburg, Germany) was subcloned into pIRE2-EGFP with two Flag-tags in the N-terminal of KCNMB1. The Flag-hSlo-β1 V146A mutant was created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Orientations of constructs and correctness of the mutation were verified by DNA sequencing (DNA facility core of Mayo Clinic).

Flag-hSlo-β1 and flag-hSlo-β1 V146A in pIRE2-EGFP, atrogin-1 and atrogin-1ΔF in pCMC-Tag 3B were transfected into HEK293 cells using FuGENE 6 transfection kit (Roche Diagnostics, IN). FBXO-9 in human CASMCs were knocked down using 50 nmol/L human FBXO-9 siRNA (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as previously described.

Reverse Transcription (RT) PCR and Quantitative Real-Time (qRT) PCR Total RNA was isolated from rat aortas and human CASMCs using RNeasy Plus Mini kit (Qiagen, Valencia, CA) and was reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA). The PCR products were detected by agarose gel electrophoresis.

Quantitative expression of human BK-β1 and human FBXO-9 mRNA were determined by qRT-PCR using the iCycler iQ Real Time Detection System (BIO-RAD, Hercules, CA). Each sample was performed in triplicates and averaged. The reaction underwent a 40-cycle amplification with the following conditions: denaturalization for 15 seconds at 94 °C, annealing for 30 seconds at 55 °C and extension for 30 second at 70 °C. Copy numbers of the target gene were expressed as 2^ΔCt (where ΔCt = Ct of target gene - Ct of internal control gene of GAPDH). Oligonucleotide primers were synthesized by IDATE Co. (Coralville, IA). The forward and reverse primer sequences are listed:

Human:
Atrogin-1 forward: 5’-GCCATGCCCATTCTCCTGGGCA-3’
Atrogin-1 reverse: 5’- TGGGCAGATGCCACCTCAGGGA-3’
BK-β1 forward: 5’-CTTCTCCGCACCTCGGGGA-3’
BK-β1 reverse: 5’-CGGTCAGCAGGAAGGTGGGC-3’
FBXO-9 forward: 5’-AGTGCTGCAATGGAGGTGCTTGGA-3’
FBXO-9 reverse: 5’-CCGTCAGCAGGAAAGGTGGGC-3’
GAPDH forward: 5’-ACCACAGTCCATGCAATGAGTGTGAGG-3’
GAPDH reverse: 5’-ACCAGGAAATGAGCTTGCACAAAAGT-3’
Rat:  
Atrogin-1 forward: 5’- TCCCTGAGTGGCATCGCCCA-3’  
Atrogin-1 reverse: 5’- TGTTGCCCACCAGCACGGAC-3’  
BK-β1 forward: 5’-TGGTCGTGTGTGCCGCCATC-3’  
BK-β1 reverse: 5’-GCATGGCCCATCTGCCCACA-3’  
FBXO-9 forward: 5’-TGCAGCTGTGCTCCAGTGGC-3’  
FBXO-9 reverse: 5’-CTGGCGAAGAACAAGGGGTGT-3’  
GAPDH forward: 5’-AAGGTGGGTGAAGCAGGCGGC-3’  
GAPDH reverse: 5’-GAGCAATGCCAGCCCCAGCA-3’

Co-immunoprecipitation and Western Blot Analysis  
Co-immunoprecipitation was performed as previously described. In brief, isolated rat aortas were cut into small pieces. Transfected HEK293 cells or human CASMCs were washed with PBS three times. The aortas or collected cells were incubated with 200 μl RIPA buffer (in mmol/L): Tris 50, NaCl 150, NaF 2, EDTA 1, EGTA 1, NaVO4 1, and 1% Triton X-100 and 1 μl protease inhibitor on ice for 1 h, homogenized and then centrifuged at 8000 rpm at 4 °C for 10 min. The supernatant (about 200 μg in 200 μl) was incubated with anti-BK-β1 (Alomone Labs, Jerusalem, Israel) or anti-Flag (Cell Signaling, MA) antibodies at a final concentration of 4 μg/ml for each at 4 °C overnight. The samples were then incubated with 20 μl Protein G Plus-Agarose (Santa Cruz, CA) at 4 °C for 2 h with rotation. After centrifugation at 1000 rpm for 7 min and washed twice with RIPA/protease inhibitor buffer, the immunoprecipitates were collected and eluted from Agarose with 30 μl SDS-PAGE loading buffer per tube.

Western blotting was performed as previously reported. Isolated aortas from rats and cultured cells were homogenized, electrophoresed, transferred to nitrocellulose membrane, and immunoblotted against anti-atrogen-1 (1:200, ECM Bioscience, KY), anti-Akt (1:1000, Cell Signaling Technology Inc., Danvers, MA), anti-p-Akt(S473) (1:1000, Cell Signaling Technology Inc., Danvers, MA), anti-BK-β1 (1:200, Alomone Labs, Israel), anti-Flag (1:200 Sigma-Aldrich, MO), anti-FBXO-9 (1:200, Rocklan Inc., Gilbertsville, PA), anti-ubiquitin (1:200, Santa Cruz, CA), anti-FOXO-3a (1:1000, Cell Signaling Technology Inc., Danvers, MA), anti-p-FOXO-3a(T32) (1:1000, Cell Signaling Technology Inc., Danvers, MA) antibodies. Blots were probed with anti-β-actin (1:2500, Sigma-Aldrich, MO) or with anti-GAPDH (1:2500, Sigma-Aldrich, MO) as loading control. Following extensive washing, horseradish peroxidase-conjugated secondary antibodies were added. Signals were developed by Immun-Star HRP Chemiluminescent Kit (Bio-Rad, Hercules, CA). Optical density of the bands was analyzed using Scion Image software (Scion, Frederick, MD). Protein expression was expressed as relative abundance normalized to β-actin or GAPDH.

Rat CASMC Isolation and BK Current Recording  
Rat CASMCs were dissociated enzymatically as previously reported. Briefly, rat hearts were rapidly excised and placed in cold (4°C) physiological saline solution that contained (in mmol/L): NaCl 145, KCl 4, CaCl2 0.05, MgCl2 1, HEPES 10, glucose 10, pH 7.2. The septal, the right, and the left anterior descending coronary arteries were carefully dissected free of surrounding myocardium and connective tissue, and placed in 1 ml physiological saline solution containing bovine serum albumin (0.1%, w/v) for a 10 min incubation at 37°C in a shaking water bath. The vessels were treated with 1.75 mg papain and 1.25 mg dithiothreitol in 1 ml saline solution for 10 min and further digested with 1.25 mg collagenase and 1.25 mg trypsin inhibitor in 1 ml saline solution at 37°C for 10 min. The vessels were then washed three times with 1 ml aliquots of saline solution, and gently triturated with a fire-polished glass pipette until the cells were completely dissociated. Isolated CASMCs were kept in 4°C and were used within 48 h.
Single BK channel currents were elicited from freshly isolated rat CASMCs at +60 mV in the inside-out configuration using an Axopatch 200B integrating amplifier and Clampex 10.2 software (Axon Instruments, Foster City, CA). 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 to 10 MΩ when filled with the pipette solution which contained (in mmol/L): KCl 140, CaCl₂ 1, MgCl₂ 1, HEPES 10 and EGTA 1, pH 7.4 with KOH. The bath solution contained (in mmol/L): KCl 140, MgCl₂ 1, EGTA 1, HEPES 10, CaCl₂ 0.465 (0.2 μmol/L free Ca²⁺), pH 7.35 with KOH.

Whole-cell K⁺ currents were recorded at a voltage range from -40 mV to +160 mV in 10-mV increments from a holding potential of -60 mV. BK currents were obtained as the IBTX (100 nmol/L)-sensitive components by digital subtraction. Pipette resistance was 0.5~1.0 MΩ when filled with the pipette solution which contained (in mmol/L): KCl 140.0, MgCl₂ 0.5, Na₂ATP 5.0, Na₂GTP 0.5, HEPES 1.0, EGTA 1.0, CaCl₂ 0.465 (200 nmol/M free Ca²⁺), pH 7.35 with KOH. The bath solution for whole-cell recordings contained (in mmol/L): NaCl 145.0, KCl 5.6, MgCl₂ 1.0, CaCl₂ 0.5, HEPES 10.0, and glucose 10.0, pH 7.4 with NaOH. Data were analyzed using Clampfit 10.2 software.

All patch-clamp experiments were performed at room temperature (22-24 °C).

**Chemicals** Unless otherwise mentioned, all chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO).

**Statistical Analysis** Data were expressed as mean±SEM. Student t test was employed to compare data between 2 groups. A paired t-test was used to compare data before and after treatment. One-way ANOVA, followed by Tukey’s 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**

**Time-Course of BK-β₁ Down-Regulation in Human CASMCs Cultured with HG** We determined the time frame of HG-induced BK-β₁ down-regulation in human CASMCs. BK-β₁ protein levels in human CASMCs were measured after 0, 4, 7, 10 and 14 days in HG culture (Online Figure I). BK-β₁ expression remained unchanged at day 4 of HG culture, but it was reduced by 50% at day 7 of HG culture and reached a steady-state suppression of 70% at days 10 to 14 of HG culture (p<0.05 versus baseline, n=3).

**BK-β₁ mRNA expression in Human CASMCs with HG Culture and in Diabetic Vessels** The down-regulation of BK-β₁ protein expression was not accompanied by a corresponding reduction in mRNA expression. In contrast, BK-β₁ mRNA expression was increased in human CASMCs after 2 week culture in HG, compared with cells cultured in NG (3.6±1.5 x10⁻⁵ of HG versus 8.2±1.2 x10⁻⁵ of NG, n=6, p<0.05) (online Figure IIA). We also measured the expression of BK-β₁ mRNA in aortas of control and STZ-induced diabetic rats. There was no statistical difference between them (3.8±1.2 x10⁻² of diabetes versus 5.2±1.3 x10⁻² of control, n=6, p=N.S.) (Online Figure IIB). These results suggest that accelerated protein degradation rather than reduced protein biosynthesis may account for the down-regulation of vascular BK-β₁ protein levels in diabetes and in HG culture conditions.

**Expression of Atrogin-1 and FBXO-9 in Rat Aortas and in Cultured Human CASMCs** To determine whether atrogin-1 and FBXO-9 are expressed in vascular smooth muscle cells, we measured the expressions of FBXO-9 and atrogin-1 mRNA by RT-PCR. Online Figure III shows the agarose gel electrophoresis of PCR products of the total RNA isolated from rat aortas and
human CASMCs, demonstrating that atrogin-1 and FBXO-9 were abundantly expressed in rat aortas and in cultured human CASMCs.

References
Online Figure I. Time Course of HG Effect on BK-β₁ Down-Regulation in Human CASMCs

Immunoblot shows BK-β₁ protein expression in human CASMCs at 0, 4, 7, 10 and 14 days of culture in HG. Reduced BK-β₁ expression was detectable on day 7 of HG culture and the down-regulation reached steady-state level at days 10 to 14 of HG culture. Group results with statistic significance are illustrated in the bar graph.

Online Figure II. Comparison of BK-β₁ mRNA Expression in Aortas between Control and Diabetic Rat Aortas, and between NG and HG Culture of Human CASMCs

Bar graphs show the expression of BK-β₁ mRNA in human CASMCs cultured with NG and HG (A) and in aortas of control and STZ-induced diabetic rats (B). BK-β₁ mRNA expression was significantly increased in human CASMCs with HG culture, but was not changed in STZ-induced diabetic rat aortas.
Online Figure III. Expression of atrogin-1 and FBXO-9 mRNA in rat aortas and in human CASMCs. RT-PCR was performed in rat aortas and in cultured human CASMCs cultured with NG. The PCR products of atrogin-1 and FBXO-9 mRNA in rat aortas (A) and in cultured human CASMCs (B) were detected by agarose gel electrophoresis. Distilled water was served as a sample control. The predicted size of each product is illustrated in parentheses.