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Muscle-Specific F-Box Only Proteins Facilitate BK Channel β_1 Subunit Down-Regulation in Vascular Smooth Muscle Cells of Diabetes Mellitus

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Abstract

Rationale—Activity of the large conductance Ca^{2+}-activated K^+ (BK) channels is profoundly modulated by its β_1 subunit (BK-β_1). However, BK-β_1 expression is down-regulated in diabetic vessels. The ubiquitin-proteasome-system (UPS) is a major mechanism of intracellular protein degradation. Whether UPS participates in BK-β_1 down-regulation in diabetic vessels is unknown.

Objective—We hypothesize that UPS facilitates vascular BK-β_1 degradation in diabetes.

Methods and Results—Using patch clamp and molecular biological approaches, we found that BK-β_1-mediated channel activation and BK-β_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 up-regulation of F-box only (FBXO) protein-9 (FBXO-9) and FBXO-32 (atrogin-1), the key components of the Skp1-Cullin-F-box (SCF) type ubiquitin ligase complex. BK-β_1 expression was suppressed by the FBXO activator doxorubicin, but enhanced by FBXO-9 siRNA or by the proteasome inhibitor MG132. Co-transfection of atrogin-1 in HEK293 cells significantly reduced Flag-hSlo-β_1 expression by 2.16-fold, compared to expression of Flag-hSlo-β_1V146A (a mutant without the PDZ-binding motif). After co-transfection with atrogin-1, the ubiquitination of Flag-hSlo-β_1 was increased by 1.91-fold, compared with that of hSlo-β_1V146A, while co-transfection with atrogin-1ΔF (a non-functional mutant without the F-box motif) had no effect. Moreover, inhibition of Akt signaling attenuated the phosphorylation of forkhead box O transcription factor-3a (FOXO-3a) and enhanced atrogin-1 expression, which in turn suppressed BK-β_1 protein levels in human CASMCs.

Conclusions—Down-regulation of vascular BK-$\beta_1$expression in diabetes and in high glucose culture conditions was associated with FOXO-3a/FBXO-dependent increase in BK-β_1 degradation.

Keywords

ubiquitin-proteasome system; BK channel β_1 subunit; protein degradation; diabetes mellitus

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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 α subunits (BK-α, encoded by the Slo gene) and the regulatory β_1 subunits (BK-β_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 investigator have reported that impaired BK channel activation was due to reduced BK-β_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 three enzyme systems: ubiquitin-activation enzyme E1, ubiquitin conjugating enzyme E2, and ubiquitin ligase E3.[6] There are one E1, more than 25 E2 and more than 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 T-32, 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 subtypes and are abundantly expressed in myocardium and skeletal muscles.[9, 10] Atrogin-1 may bind to the PDZ-binding motif (T/S-X-V, X is any amino acid) in substrates.[9] Interestingly, the PDZ-binding motif is present in most BK-β_1 isoforms in different species including human. However, the role of FBXOs in the regulation of BK-β_1 expression is unknown. Here, we hypothesized that enhanced UPS activity facilitates BK-β_1 protein degradation in diabetes. We found that expression of atrogin-1and 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-β_1 expression. Moreover, expression of FBXOs and BK-β_1 was regulated by FOXO-3a phosphorylation. Hence, we have delineated a novel fundamental mechanism that underlies vascular BK-β_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 I-V 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-β_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 β_1-mediated BK channel activation is lost.

Down-regulated BK-β_1 Expression and Enhanced BK-β_1 Ubiquitination in Diabetic Vessels and in Human CASMCs with HG culture

BK-β_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 to those with normal glucose (NG, 5 mmol/L, $p<0.05$) (Figure 1D), while BK-α expression remained unchanged. The effects of HG on BK-β_1 down-regulation were detectable at day 7 and plateaued at days 10 to 14 of HG culture (Online Figure I). However, BK-β_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-β_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). 72-h after, FBXO-9 siRNA (50 nmol/L) was transfected into human CASMCs, FBXO-9 protein level was suppressed by 80%, while BK-β_1 expression was increased by 1.65±0.16 fold, compared with control siRNA ($p<0.05$) (Figure 2C).

After 24-h incubation with 0.1 μmol/L doxorubicin (DXR, a FBXO activator),[11] BK-β_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-h treatment with 10 μmol/L MG132 (a proteasome inhibitor). BK channel openings were less frequent with no response to DHS-1 in non-diabetic rat CASMCs after treatment with DXR (p=N.S. versus baseline). In CASMCs incubated with DXR + MG-132, however, BK channel activity was robust in the presence of DHS-1 ($p<0.05$ versus baseline) (Figure 2F), consistent with the immunoblotting results.

Lack of the PDZ-Binding Motif in hSlo-β_1 Abolished UPS-Mediated Protein Degradation

The PDZ-binding motif is well-conserved in BK-β_1 (Figure 3A). We measured protein expression of Flag-hSlo-β_1 wt and Flag-hSlo-β_1V146A (a mutation in the PDZ-binding motif) after co-transfection with ubiquitin and atrogin-1 or atrogin-1ΔF (a non-functional mutant with deletion of the F-box). 72-h after transfection, expression of hSlo-β_1 wt was 2.16±0.16 fold lower than that of hSlo-β_1V146A ($p<0.05$) (Figure 3B). Also, ubiquitination of hSlo-β_1 wt was 1.91±0.08 fold higher than that of hSlo-β_1V146A ($p<0.05$) in cells co-transfected with atrogin-1, while atrogin-1ΔF transfection had no effect (Figure 3C). Hence, our results suggest that FBXO interacts with BK-β_1 through the PDZ-binding motif and facilitates BK-β_1 ubiquitination and degradation.

Regulation of Atrogin-1 and BK-β_1 Expression by Akt/FOXO-3a Signaling in Human CASMCs

We found that FOXO-3a (T-32) phosphorylation was down-regulated 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, while 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-β_1 expression in human CASMCs. After 24 h incubation with 7 μmol/L LY294002 (a 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) while 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-β_1 expression in human CASMCs (Figure 4C).

Discussion

The BK-β_1 subunit significantly enhances BK channel voltage sensitivity and Ca^{2+} cooperativity [4] and its physiological importance is underscored by BK-β_1 knockout mice, which showed increases in myogenic tone and arterial blood pressure, with uncoupling of Ca^{2+} sparks to BK channels in vascular SMCs.[12] We and others have demonstrated that BK channel dysfunction in both type I and type II diabetic vessels is associated with reduced BK-β_1 expression.[3, 4] In this study, we provided the first evidence that down-regulation of BK-β_1 expression in vasculature was due 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;[9] whereas its reduced expression produces cardiac hypertrophy.[10] 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-β_1 degradation and BK channel malfunction. Perturbation in atrogin-1 and hSlo-β_1 interaction attenuated hSlo-β_1 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-β_1 expression in human CASMs with HG were mimicked by PI3K/Akt inhibition. It is well known that PI3K/Akt pathway is activated by insulin (Ins) and suppressed by protein kinase C. Insulin receptor (InR) signaling is diminished and PKC 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 diabetic vasculopathy in both type 1 and type 2 diabetes mellitus, and render BK-β_1 as a potential therapeutic target in treatment of these conditions.

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 β_1 subunits (BK-β_1). The expression of the BK-β_1 protein is down-regulated in vascular smooth muscle cells (SMCs) in diabetes, leading to loss of the β_1-med$_i$ated channel fu$_n$ction, including channel sensitivity to Ca^{2+} 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 β_1-mediated BK channel activation is associated with increase in UPS-dependent BK-β_1 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-β_1 protein degradation in vascular SMCs.

Summary

The BK-β_1 subunit plays a pivotal role in BK channel function by modulating channel voltage- and Ca^{2+}-sensitivity. Down-regulation of BK-β_1 expression in vascular SMCs is a common finding in diabetic vessels that produces BK channel dysfunction. However, the molecular mechanisms underlying downregulation of BK-β_1 protein expression is unknown. In this study, we report that impaired β_1-mediated channel activity in diabetes is associated with FOXO-3a/FBXO-dependent increase in BK-β_1 protein degradation. These results indicate that BK-β_1 subunits and Akt/FOXO-3a/FBXOs signaling cascade are potential therapeutic targets in the treatment of diabetic vascular complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard Abbreviations and Acronyms

BK	large conductance Ca^{2+}-activated K^+ channel
BK-α	BK channel α subunit
BK-β_1	BK channel β_1 subunit
CASMC	coronary arterial smooth muscle cell
DXR	doxorubicin
FBXO	f-box only protein
FOXO	forkhead box O family transcription factor
HG	high glucose
IBTX	iberiotoxin
InR	insulin receptor
Ins	insulin
NG	normal glucose
siRNA	small interfering RNA
STZ	streptozotocin
UPS	ubiquitin-proteasome system

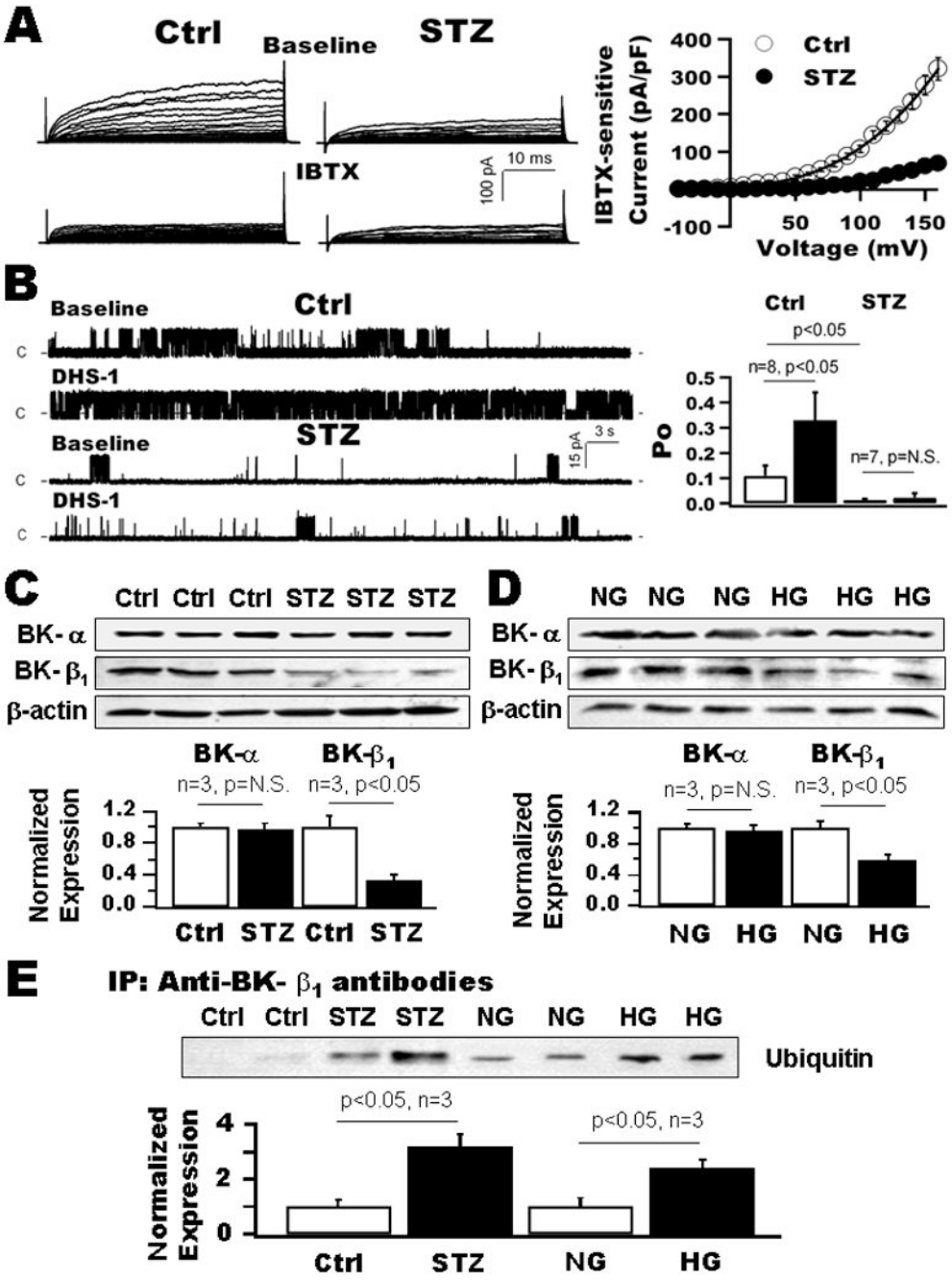

Figure 1. Impaired BK-β_1 function and increased BK-β_1 ubiquitination in diabetes and in HG culture conditions

Whole-cell K^+ currents before and after exposure to 100 nmol/L IBTX and the I-V relationship of IBTX-sensitive currents from freshly isolated CASMCs of control and STZ-induced diabetic rats (**A**). Inside-out single BK channel currents in CASMCs from control and diabetic rats at baseline and after application of DHS-1 (**B**). "c" represents the channel closed state. Western blots of BK-α and BK-β_1 in control and diabetic aorta (**C**) and in human CASMCs in NG and HG (**D**). Increased BK-β_1 ubiquitination in diabetic aorta and CASMC in HG (**E**).

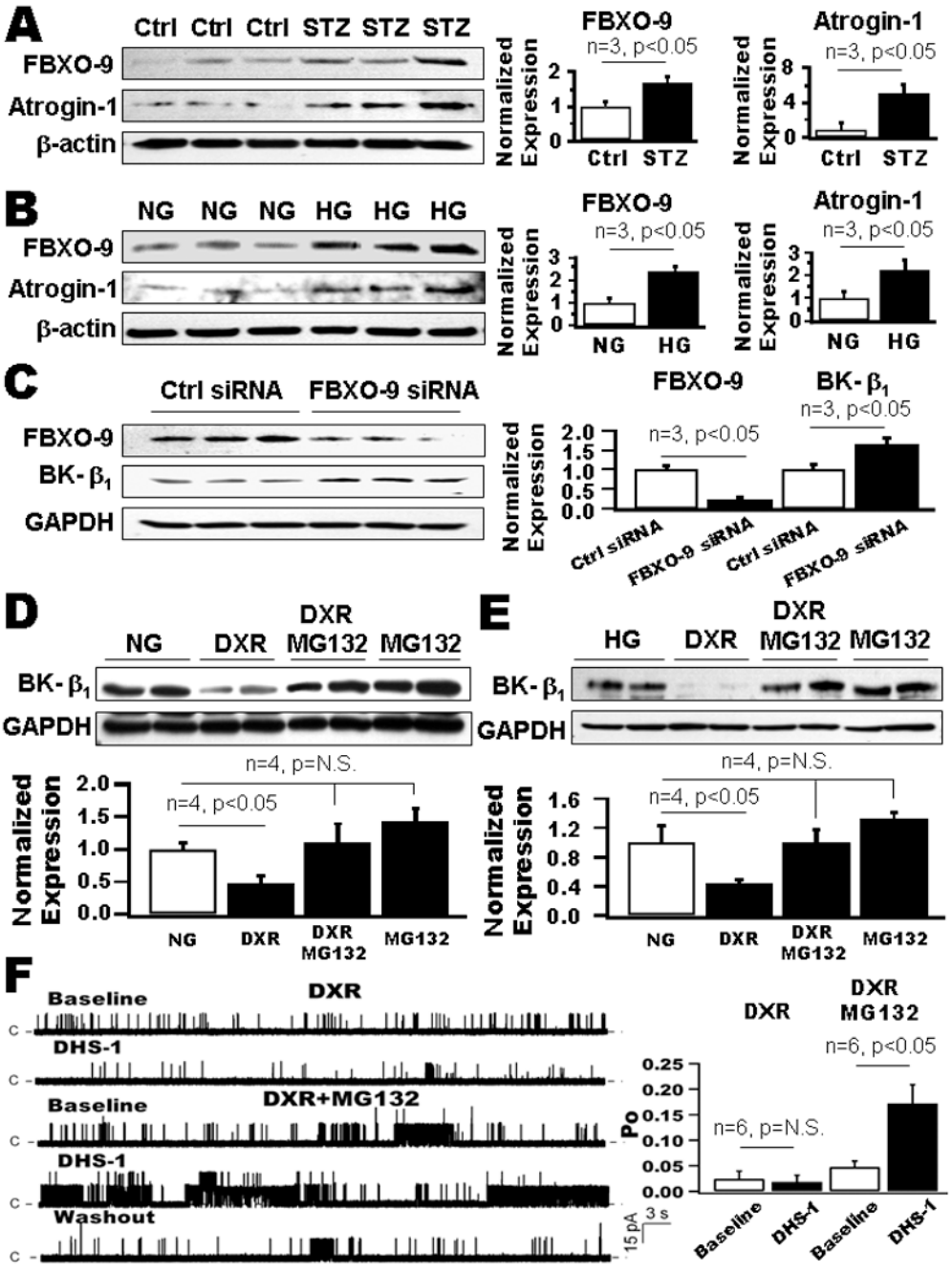

Figure 2. Enhanced FBXO expression and reduced BK-β_1 expression in diabetic vessels and in HG culture conditions

Increased expression of FBXO-9 and atrogin-1 in STZ-induced diabetic rat aortas (**A**) and in human CASMCs cultured with HG (**B**). FBXO-9 siRNA suppressed FBXO-9 but enhanced BK-β_1 expression in human CASMCs **(C)**. BK-β_1 expression in human CASMCs cultured with NG **(D)** and HG **(E)** after treatment with 0.1 μmol/L DXR (24 h), 10 μmol/L MG132 (4 h), or DXR (24 h) + MG132 (4 h). DHS-1 had no effect in rat CASMCs pretreated with DXR, but BK channel activation was preserved in cells treated with DXR + MG-132 (**F**).

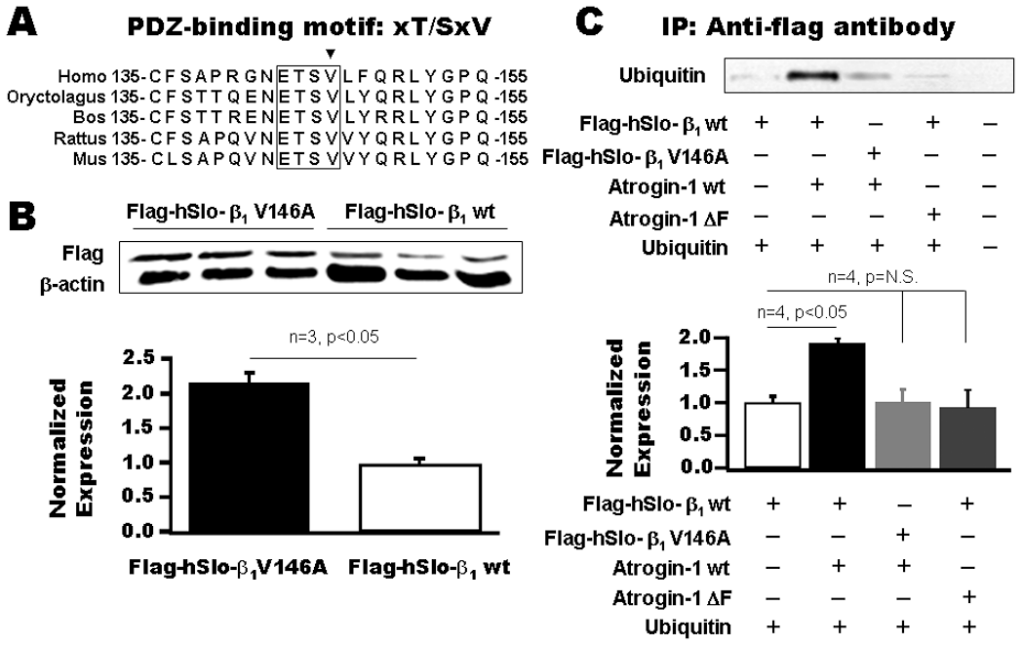

Figure 3. Mutation in the PDZ-binding motif of hSlo-β_1 prevented hSlo-β_1 ubiquitination and degradation

Sequence alignment of human, rabbit, cattle, rat, and mouse KCNMB1 cDNA shows a conserved PDZ-binding motif (**A**). ▼: applied mutation site. 72-h after co-transfection with atrogin-1 and ubiquitin, Flag-hSlo-β_1V146A expression was significantly higher than that of Flag-hSlo-β_1 wt (**B**). Immunoprecipitates of anti-Flag antibody against HEK293 cell lysates with following transfection conditions: no transfection, Flag-hSlo-β_1/ubiquitin, Flag-hSlo-β_1/atrogin-1/ubiquitin, Flag-hSlo-β_1V146A/atrogin-1/ubiquitin and Flag-hSlo-β_1/atrogin-1ΔF/ubiquitin, were resolved and blotted against anti-ubiquitin antibody (**C**).

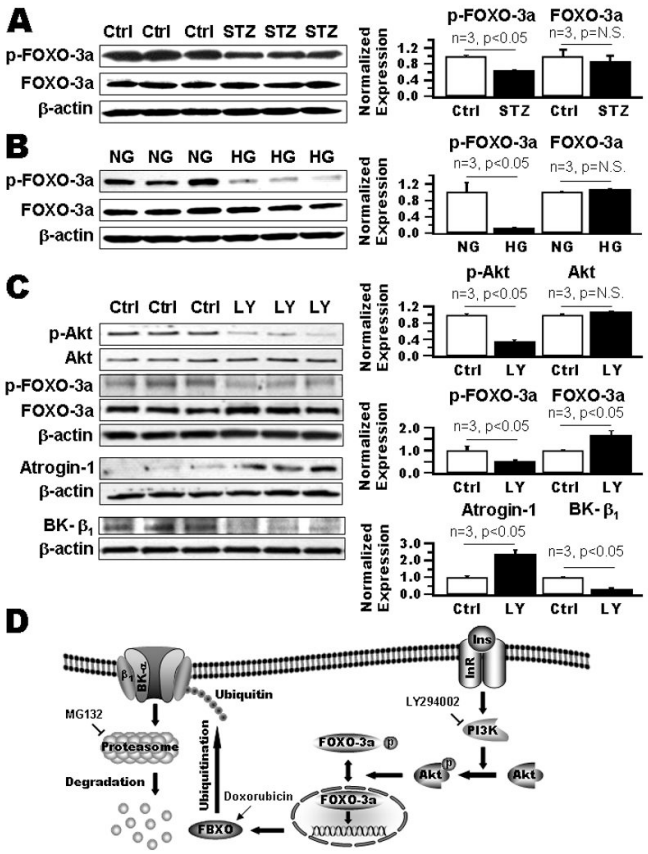

Figure 4. Modulation of vascular BK-β_1 expression by Akt/FOXO-3a/FBXO signaling pathway

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**). 24-h 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-β_1 protein level in human CASMCs cultured with NG (**C**). Illustration showing the signaling mechanisms that underlie vascular BK-β_1 down-regulation in diabetes (**D**).