Regulation of Coronary Arterial BK Channels by Caveolae-Mediated Angiotensin II Signaling in Diabetes Mellitus

Tong Lu, Dai-Min Zhang, Xiao-Li Wang, Tongrong He, Ru-Xing Wang, Qiang Chai, Zvonimir S. Katusic, Hon-Chi Lee

Rationale: The large conductance Ca²⁺-activated K⁺ (BK) channel, a key determinant of vascular tone, is regulated by angiotensin II (Ang II) type 1 receptor signaling. Upregulation of Ang II functions and downregulation of BK channel activities have been reported in diabetic vessels. However, the molecular mechanisms underlying Ang II–mediated BK channel modulation, especially in diabetes mellitus, have not been thoroughly examined.

Objectives: The aim in this study was to determine whether caveolae-targeting facilitates BK channel dysfunction in diabetic vessels.

Methods and Results: Using patch clamp techniques and molecular biological approaches, we found that BK channels, Ang II type 1 receptor, G_αq/11 (G protein q/11 α subunit), nonphagocytic NAD(P)H oxidases (NOX-1), and c-Src kinases (c-Src) were colocalized in the caveolae of rat arterial smooth muscle cells and the integrity of caveolae in smooth muscle cells was critical for Ang II–mediated BK channel regulation. Most importantly, membrane microdomain targeting of these proteins was upregulated in the caveolae of streptozotocin-induced rat diabetic vessels, leading to enhanced Ang II–induced redox-mediated BK channel modification and causing BK channel and coronary dysfunction. The absence of caveolae abolished the effects of Ang II on vascular BK channel activity and preserved BK channel function in diabetes.

Conclusions: These results identified a molecular scheme of receptor/enzyme/channel/caveolae microdomain complex that facilitates the development of vascular BK channel dysfunction in diabetes. (Circ Res. 2010; 106:1164-1173.)

Key Words: BK channel • caveolin-1 • angiotensin II • reactive oxygen species • coronary smooth muscle cells

Diabetic vascular complications account for a 2- to 4-fold increase in the risk of heart attack, heart failure and stroke, causing more than 200 000 deaths per year in the United States. Diabetic patients with acute coronary syndrome have a significant increase in mortality resulting from poor microcirculation and vascular dysfunction.¹ The large conductance Ca²⁺-activated K⁺ (BK) channel is an important determinant of vascular tone. Activation of vascular BK channel hyperpolarizes the membrane potential of smooth muscle cells (SMCs), closes the voltage-gated Ca²⁺ channels, and produces vasorelaxation. However, BK channel function is impaired in diabetes mellitus because of oxidative stress in the vascular wall with enhanced production of reactive oxygen species (ROS), such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and peroxynitrite (OONO⁻),²,³ accompanied by a decrease in the production and bioavailability of vasodilators including nitric oxide and prostaglandin.⁴,⁵ NAD(P)H oxidase activity is thought to be the major source of O₂⁻ generation in vascular SMCs.⁶,⁷ Vascular NAD(P)H oxidases are structurally different from those in phagocytic cells, and the nonphagocytic NAD(P)H oxidases (NOXs) include NOX-1, NOX-4, p22phox, NOXO1 (or p47phox), NOXA1 (or p67phox), and Rac-1 subunits.⁸ In vessels from patients with diabetes, expression and activity of NOXs are significantly increased, whereas those of antioxidant enzymes are reduced.⁹,¹⁰ Hence, a misbalance between ROS generation and scavenging represents a fundamental mechanism underlying the development of intracellular oxidative stress in diabetes.
Angiotensin II (Ang II) plays a key role in the regulation of cardiovascular homeostasis through binding to the type 1 (AT$_1$,R) and type 2 (AT$_2$,R) receptors. AT$_1$,R is a G protein–coupled receptor, activating G$_{q/11}$ and G$_{q/11}$. The G$_{q/11}$-mediated phospholipase C/phosphatidylinositol-4,5-bisphosphate/Ca$^{2+}$ signaling activates protein kinase (PK)C and is a primary mechanism through which Ang II exerts its physiological and pathological effects.$^{11,12}$ In addition, G$_{q/11}$ activates c-Src kinase (c-Src), which in turn activates c-AbI tyrosine kinase, causes tyrosine 14 phosphorylation of caveolin (cav)-1, and facilitates the AT$_1$,R translocation into caveolae.$^{13,14}$ Because c-Src is also activated by ROS, these steps result in a self-perpetuated activation loop promoting sustained ROS generation in response to Ang II stimulation. Interestingly, NOX-1 is localized in the caveolae of SMCs$^{15}$ and activation of AT$_1$,R by Ang II is accompanied by receptor translocation into the caveolae of vascular SMCs.$^{16}$ The physiological importance of NOX-1 is underscored by studies using NOX-1 knockout (KO) mice, which lack Ang II–induced ROS generation and have reduced blood pressure.$^{14,17}$

Caveolae are unique flask-shaped, non–clathrin-coated plasma membrane microdomains, 50 to 100 nm in diameter, and are characterized by their signature structural protein cav.$^{18,19}$ Cav-1 is the primary isoform in vascular SMCs. The N terminus of cav-1 (residues 1 to 101) contains an important functional structure: the caveolin scaffolding domain (residues 82 to 101), which is essential for membrane binding and for interaction with signaling proteins that contain the caveolin binding motifs (ΦXXXΦXXXΦ and ΦXΦXXXΦ, where Φ represents an aromatic amino acid and X is any amino acid), including those of AT$_1$,R signaling proteins$^{13}$ and BK channels.$^{20,21}$ However, the functional role of caveolae targeting for BK channel regulation is unknown, especially in diabetic vessels.

In this study, we hypothesized that caveolae/Ang II signaling complexes may play an important role in the ROS-associated BK channel modulation, including cysteine oxidation, tyrosine nitration, and tyrosine phosphorylation, leading to vascular BK channel dysfunction in diabetes. We found that BK channels, AT$_1$,R, G$_{q/11}$ (G protein q/11 α subunit), c-Src, and NOX-1 were physically associated in caveolae and the integrity of caveolae in SMCs was critical for mediating the regulation of BK channel function by Ang II. In addition, cav-1 expression was upregulated in the vasculature of streptozotocin (STZ)-induced diabetic rats, accompanied by increased physical association between BK channels and AT$_1$,R signaling complex, resulting in enhanced AT$_1$,R–mediated oxidative modification and dysfunction of BK channels. These results highlight the critical role of caveolae in the inhibition of BK channel function by Ang II, which leads to abnormal vascular function in diabetes.

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

### Type 1 Diabetic Animal Development and Vascular SMC Isolation
Diabetic rats and mice were produced by STZ injection. Handling and care of animals, as well as animal procedures, were approved by the Institutional Animal Care the Use Committee (Mayo Clinic). SMCs from rat coronary arteries and mouse aortas were enzymatically isolated as previously reported.$^{22}$

### Electrophysiology
Whole-cell K$^+$ currents were recorded using standard patch-clamp techniques,$^{22}$ and BK currents were defined as the 0.1 μmol/L iberiotoxin (IBTX)-sensitive component.

### Measurement of Coronary Ring Tension
Rat coronary left anterior descending artery was used for contraction and relaxation experiments.

### Sucrose Gradient Density Centrifugation
The cellular distribution of cav-1 in rat aortas was determined by sucrose density gradient fractionation as previously described.$^{21}$ Ten fractions of 1.2 mL each were collected and analyzed by Western blotting.

### Cav-1 Knockdown by Small Interfering RNA
Cav-1 in SMCs was knocked down using human cav-1 small interfering (si)RNA as previously described.$^{20}$

### Coimmunoprecipitation and Immunoblotting
Immunoprecipitation and Western blotting were performed as described previously.$^{2,23}$

### Results

**Type 1 Diabetic Animals**
Eight weeks after the development of hyperglycemia, blood glucose was significantly increased and body weight was significantly reduced in diabetic animals (see expanded Results section in the Online Data Supplement).
Regulation of Vascular BK Channels by Ang II and Caveolae Targeting

We first examined the effects of Ang II on BK channel activities in coronary arterial SMCs from control rats. K⁺ currents were continuously recorded at baseline and after superfusion with Ang II (2 μmol/L) and IBTX (0.1 μmol/L). Ang II produced significant inhibition of K⁺ currents, and this effect was reversible on washout (Figure 1A). BK currents were obtained by subtracting the IBTX-insensitive K⁺ currents from total K⁺ currents, and the BK current–voltage (I-V) relationships (holding potential, −70 mV; testing potentials [TP], from −100 mV to +160 mV) before and after exposure to Ang II are shown in Figure 1B. Ang II suppressed BK currents by 50.3% in control rats, from 272 ± 26.6 pA/pF at baseline to 135.2 ± 14.2 pA/pF (TP = +150 mV, n = 7, P < 0.05 versus baseline).

The effect of Ang II on BK channel function was confirmed by outside-out single BK channel recordings (Online Figure I). Extracellular application of 2 μmol/L Ang II resulted in 49.4% reduction in channel open probability, from 0.45 ± 0.06 at baseline to 0.23 ± 0.03 with Ang II (P < 0.05, n = 4).

To determine the role of caveolae in Ang II signal transduction, we examined the effects of cav-1 knockdown in rat coronary arterial SMCs using siRNA on BK channel inhibition by Ang II. Figure 1C shows the protein expression of cav-1 in coronary arterial SMCs 48 hours after transfection with cav-1 siRNA at 0, 20, 40, 60, and 100 nmol/mL, and control siRNA at 100 nmol/mL. Cav-1 siRNA at 40 nmol/L or higher significantly suppressed cav-1 expression by 80% to 90%, compared with control siRNA. Figure 1D shows the time course of whole-cell K⁺ currents in rat coronary arterial SMCs 48 hours after transfection with 100 nmol/mL cav-1 siRNA or 100 nmol/mL control siRNA, in response to 2 μmol/L Ang II and 0.1 μmol/L IBTX. Ang II suppressed BK currents in cells with control siRNA transfection but failed to inhibit BK currents in cells with cav-1 siRNA transfection. Instead, a small increase in BK currents was noted. Group data (n = 6) are shown in Figure 1E.

Impaired Vascular BK Channels and Coronary Vasoreactivity in STZ-Induced Diabetic Rats

To determine the role of AT₁R-mediated BK channel regulation in diabetic vessels, we examined the effects of Ang II on BK currents in coronary arterial SMCs from STZ-induced diabetic rats. Whole-cell K⁺ currents were 61.5 ± 10.6 pA/pF at baseline (TP = +150 mV, n = 8, P < 0.05, versus control baseline) and 61.2 ± 15.7 pA/pF with 2 μmol/L Ang II (n = 8, P = NS versus diabetic baseline), indicating the loss of Ang II effect on vascular BK currents in diabetes. Representative tracings and the BK channel I-V curves before and after exposure to Ang II are shown in Figure 2A. Very little BK currents were present in diabetic coronary arterial SMCs, and Ang II effects were absent, indicating marked vascular BK channel dysfunction in diabetes.

To determine the physiological relevance of these findings, the effects of Ang II and NS-1619 (BK channel specific activator) on the contraction/relaxation of coronary rings from control and STZ-induced diabetic rats were measured. We found that in diabetic rats, coronary constriction by Ang II (2 μmol/L) and by IBTX (0.1 μmol/L) was reduced by 76.6% and 46.2%, respectively, whereas NS-1619-mediated coronary relaxation was reduced by 59.0% (Figure 2B). These results suggest that BK channel–mediated coronary vasoreactivity was abnormal in diabetes.

Colocalization of BK Channels, AT₁R, Gᵦq/₁₁, NOX-1, and c-Src in the Caveolae of Vascular SMCs

To better understand the molecular mechanism whereby caveolae-targeting modulates BK channel activity, we determined the cellular distribution of BK channels, AT₁R, Gᵦq/₁₁, NOX-1, and c-Src in vessels from control and STZ-induced diabetic rats by sucrose density gradient fractionation. Be-
cause the BK channels in aortic SMCs and coronary arterial SMCs showed similar abnormalities in STZ-induced diabetic rats (Online Figure II), we used the aorta for further biochemical characterization. Figure 3A and 3B shows immunoblots of the cell lysates and the fractions (1 to 10, with 1 being the lightest and 10 the heaviest) of control and diabetic rat aortas, respectively, blotted against anti–cav-1, anti–BK channel, anti–c-Src, anti–NOX-1, anti-G$_\alpha$q/11, and anti-AT$_1$R antibodies. BK channels, NOX-1, G$_\alpha$q/11, and c-Src were detected in the low buoyant density, caveolae-rich fractions of control and diabetic rat aortas. In contrast, very little AT$_1$R was detected in the caveolae-rich fractions of both control and diabetic rats under baseline condition. However, aortas obtained from animals after treatment with Ang II showed that AT$_1$R was readily detected in the low buoyant density fractions, suggesting receptor translocation into caveolae on agonist activation, similar to previous reports. Analysis of the distribution of AT$_1$R in the membrane fractions showed that it was very different between control and STZ-induced diabetic rats after Ang II treatment. In control rats, only 28.5% of the total AT$_1$R was in the low buoyant density fractions (fractions 1 to 6), whereas 83.4% of the total AT$_1$R was found in the low buoyant density fractions of STZ-induced diabetic rats (Figure 3C). These results suggest that caveolae targeting of AT$_1$R is enhanced in diabetic vessels.

**Inhibition of BK Channel Activity by Ang II–Induced Posttranslational Modulation**

We proceeded to determine the roles of PKC, NOX-1, and c-Src on the Ang II–mediated development of BK channel fractions, suggesting receptor translocation into caveolae on agonist activation, similar to previous reports. Analysis of the distribution of AT$_1$R in the membrane fractions showed that it was very different between control and STZ-induced diabetic rats after Ang II treatment. In control rats, only 28.5% of the total AT$_1$R was in the low buoyant density fractions (fractions 1 to 6), whereas 83.4% of the total AT$_1$R was found in the low buoyant density fractions of STZ-induced diabetic rats (Figure 3C). These results suggest that caveolae targeting of AT$_1$R is enhanced in diabetic vessels.

**Inhibition of BK Channel Activity by Ang II–Induced Posttranslational Modulation**

We proceeded to determine the roles of PKC, NOX-1, and c-Src on the Ang II–mediated development of BK channel
dysfunction. We found that after a 1-hour incubation with the membrane permeable PKC peptide inhibitor (50 μmol/L), BK currents in coronary arterial SMCs were no longer suppressed by Ang II. BK current densities were 229.9±67.5 pA/pF at baseline and 274.8±64.5 pA/pF (TP=+150 mV, n=10, P=NS versus baseline) after exposure to 2 μmol/L Ang II (Figure 4). Similarly, a 1-hour pretreatment with the c-Src inhibitor lavendustin A (LavA) (10 μmol/L) or with the NOX-1 inhibitor diphenylene iodonium (DPI) abolished the Ang II effects on BK channel activity. After pretreatment with LavA, BK current densities were 210.1±58.8 pA/pF at baseline versus 249.0±67.2 pA/pF (n=11, P=NS versus baseline) with Ang II (Figure 4B). After pretreatment with diphenylene iodonium, BK currents were 180.7±31.3 pA/pF at baseline versus 182.0±42.3 pA/pF (TP=+150 mV, n=8, P=NS) with Ang II (Figure 4C). In contrast, pretreatment with 10 μmol/L lavendustin B (the negative control of LavA) did not inhibit the Ang II effects (data not shown). Hence, these results suggest that Ang II inhibits BK channels through PKC-, c-Src–, and NOX-mediated mechanisms.

Comparison of BK Channel AT1R and Cav-1 Expression in the Aortas Between Normal and STZ-Induced Diabetic Rats

We determined the expression of BK channels, AT1R, and Cav-1 in diabetic vessels. Figure 5A shows the immunoblot of aortic homogenates from control and diabetic rats against

Figure 4. Effects of inhibitors of PKC, c-Src, and NOX-1 on the inhibition of BK channels by Ang II. BK currents in coronary arterial SMCs after a 1-hour incubation with membrane permeable PKC peptide inhibitor (A), LavA (B), and diphenylene iodonium (DPI) (C) at baseline and after exposure to 2 μmol/L Ang II. The Ang II effects were abrogated by these inhibitors. I-V curves from group results (n=8 to 10, P=NS vs baseline) are shown.

Figure 5. Upregulation of Cav-1 expression and caveolae-targeting in the aortas of STZ-induced diabetic rats. A, Immunoblotting against BK channels, AT1R, and Cav-1 from 3 pairs of aortas from control and diabetic rats. Cav-1 expression was significantly increased but not BK channel or AT1R. B, Immunoprecipitates (IPs) of anti-Cav-1 antibody on aorta homogenate from control and diabetic rats were analyzed using anti-AT1R, anti-NOX-1, and anti-c-Src antibodies. C, IPs of anti-3-nitrotyrosine antibody were blotted against anti-BK channel antibody. D, IPs of anti-phosphotyrosine antibody were blotted against anti-BK channel antibody.
anti–BK channel, anti–AT_1R, and anti–cav-1 antibodies, as well as anti–β-actin antibodies as loading control. There was no significant difference in BK channel and AT_1R expression between control and diabetic rats, but cav-1 expression was increased by 3.1±0.2-fold (n=3, P<0.05 versus control) in diabetic rats. These results suggest that reduction of BK channel activity in diabetic vessels was not attributable to downregulation of channel expression but was associated with altered channel function in the presence of increased caveolae abundance.

Enhanced Caveolae Targeting of BK Channels, AT_1R, NOX-1, and c-Src and Oxidative Modification of BK Channels in Diabetic Vessels

Figure 5B shows results from 2 representative pairs of control and STZ-induced diabetic rat aorta homogenates, in which immunoprecipitates with anti–cav-1 antibody were analyzed for the presence of BK channels, c-Src, and NOX-1. Cav-1–associated BK channels, AT_1R, c-Src, and NOX-1 were significantly increased in diabetic rats, by 1.9±0.2 (n=4), 2.6±0.4 (n=3), 2.1±0.4 (n=3), and 1.6±0.1 fold (n=3), respectively, compared to control rats.

Because ROS are prominent products of AT_1R signaling and the machinery for producing oxidative modulation are in the vicinity of BK channels in caveolae of SMCs, we examined the potential consequence of the augmented caveolae-mediated association between BK channels and the AT_1R signaling cascade in diabetes by determining BK channel oxidative modification. We found that in diabetic vessels, there was a 3.3±0.4-fold (n=3, P<0.05 versus control) increase in the BK channel tyrosine nitration (Figure 5C) and a 2.1±0.3-fold (n=4, P<0.05 versus control) increase in BK channel tyrosine phosphorylation (Figure 5D). These results suggest that the enhanced caveolae-targeting of BK channels and AT_1R signaling molecules in diabetic vessels may underlie the enhanced BK channel protein posttranslational oxidative modification, accounting for the molecular mechanisms of BK channelopathy in diabetes.

Preserved BK Channel Activity in Cav-1 KO Diabetic Mice

To determine the role of caveolae on BK channel function in control and diabetes, we used cav-1 KO mice for further studies. Figure 6A and 6B show the time course and representative tracings of K^+ currents in aortic SMCs from nondiabetic wild-type (WT) and KO mice at baseline, after exposure to Ang II and to IBTX, and on washout of chemicals. The I-V curves of BK currents before and after the application of 2 μmol/L Ang II are illustrated in Figure 6C. Ang II produced 50% inhibition of BK currents in WT mice, whereas there was no Ang II effect in cav-1 KO mice. The results are similar to those from rat coronary arterial SMCs after cav-1 siRNA treatment. In diabetic WT mice, vascular BK channels showed very little response to Ang II (Figure 7). The current density was 27.2±6.9 pA/pF at baseline and was 26.8±12.8 pA/pF after exposure to 2 μmol/L Ang II (TP=+150 mV, n=9, P=NS versus baseline). In diabetic cav-1 KO mice, BK channels were also insensitive to Ang II; BK current density was 155.5±28.3 pA/pF at baseline (TP=+150 mV, n=9, P<0.05 versus WT) and 133.1±30.7 pA/pF after Ang II treatment (TP=+150 mV, n=9, P=NS versus baseline). These results indicate that in diabetes, there was profound loss of vascular BK channel activity with no further suppression by Ang II. However, in KO mice, vascular BK channel function is preserved in diabetes because the absence of caveolae spared the channels from Ang II–mediated inactivation.

Discussion

In this study, we have provided compelling evidence on the critical role of caveolae in mediating the inhibition of vascular BK channels by Ang II. First, we have shown that BK channels and the AT_1R signaling cascade, including...
Gq/11, c-Src, and NOX-1, are colocalized in the caveolae of vascular SMCs. Ang II inhibits BK channel function through activation of its downstream pathways. Second, Ang II loses its effects on BK channel function with lack of caveolae by cav-1 knockdown using siRNA in vascular SMCs and by cav-1 gene ablation in cav-1 KO mice. Third, coronary arterial BK channel activity and coronary vaso-reactivity are impaired in STZ-induced diabetic rats. With the development of diabetes, cav-1 expression is upregulated, and this is accompanied by increased oxidative modification of BK channels by tyrosine phosphorylation and tyrosine nitration. Fourth, the absence of caveolae preserves BK channel function in diabetes. These results indicate that vascular BK channel functions are importantly modulated by Ang II/AT1R signaling and caveolae targeting is critical in facilitating the Ang II/AT1R-mediated effects (Figure 8). BK channel function is significantly compromised by the heightened Ang II/AT1R/caveolae oxidative stress signaling in diabetes.

Caveolae have emerged as important membrane microdomains where signal transduction mechanisms are facilitated because a wide variety of signaling molecules are found to reside in caveolae of vascular SMCs, including BK channels and AT1R signaling proteins. However, the functional consequence of caveolae targeting and the molecular mechanisms of caveolae-mediated modulation of vascular BK channel function are unclear. In cultured bovine aortic endothelial cells, BK channels are quiescent but can be activated on isoproterenol stimulation or by dissolution of caveolae using β-cyclodextrin.20 Based on BK current measurements in inside-out macropatches with similar pipette resistance (assuming that each one has a similar surface area of pipette tip), Alioua et al21 reported that cav-1 affects Slo surface expression. Specifically, coexpression with cav-1 in HEK293 cells reduced Slo currents (in nA*M2) by 70%, whereas the channel sensitivity to voltage- and Ca2+ was unaltered. Further deletion of the YNMLCFGIIY caveolin binding motif in Slo abolished channel surface expression. Using whole-cell

Figure 7. Effects of Ang II on BK channels of aortic SMCs from STZ-induced diabetic WT and cav-1 KO mice. A, Whole-cell K+ currents in aortic SMCs from diabetic WT (left) and KO mice (right) at baseline and after the application of Ang II, IBTX, and washout of chemicals. B, I-V curves of BK currents in diabetic WT and KO mice before and after exposure to Ang II. Dashed line represents the BK current density in WT control.
recorded in IBTX-sensitive K⁺ currents (in pA/pF) in SMCs between WT and cav-1 KO mice. These results suggest that the mechanism of BK channel modulation by cav-1 in native vascular SMCs may be different from those in heterologous expression systems.

A key finding in this study is that caveolae integrity is crucial for AT₃R-mediated BK channel regulation. Inhibition of BK channels by Ang II was abolished in rat coronary SMCs with cav-1 knockdown and in aortic SMCs of cav-1 KO mice. Our finding that targeting of AT₃R to vascular caveolae requires agonist stimulation suggests the presence of an elegant balance and control mechanism that precludes nonspecific incidental activation of AT₃R signaling cascade, consistent with previous observations.16 Furthermore, our finding that BK channels and AT₃R signaling proteins are colocalized in the caveolae of SMCs, as demonstrated by sucrose density gradient fractionation, confocal imaging analysis (Online Figure III), and coimmunoprecipitation with cav-1, carries physiological significance; as such, an organization brings BK channels to the vicinity and in close proximity to c-Src, PKC, and NOX-1, which are downstream effectors of AT₃R signaling. Stimulation of AT₃R by Ang II activates 2 major downstream pathways, with activation of c-Src and PKC, which would lead to activation of NOXs. NOX-1 and NOX-4 are the major NOX isoforms in human coronary SMCs, and they have distinct subcellular distributions with NOX-1 localized in caveolae and NOX-4 in the cytoplasm.15 Most importantly, we found a 3.1-fold increase in cav-1 expression in diabetic rat aortas, similar to previous report.24 Furthermore, on Ang II stimulation, 83.4% of the total AT₃R moved to the low buoyant density fractions in diabetic rats, compared to 28.5% in control rats. Such cellular remodeling has contributed a 1.6- to 2.6-fold increase in the abundance of BK channels, AT₃R, NOX-1, and c-Src in caveolae.

Ang II–mediated effects are dependent on ROS generation and the renin–angiotensin system is activated in diabetes.25 Yoshimoto et al26 reported that a 2-hour incubation with 10 μmol/L Ang II produced a significant increase in ROS generation and NOX-1 expression in SMCs of rat aortas. We have confirmed that production of intracellular ROS in cultured human coronary SMCs is significantly reduced after treatment with a NOX inhibitor, but not with a mitochondrial electron transport complexes II inhibitor (Online Figure IV). Moreover, incubation with 2 μmol/L Ang II significantly increases ROS generation in freshly isolated aortic SMCs of WT mice, but not cav-1 KO mice (Online Figure V). These results suggest that in arterial SMCs, caveolae-associated NOX-1 constitutes the major source of intracellular O₂⁻⁻ generation in response to AT₃R stimulation. However, the elevated activities of the AT₃R signaling cascade in diabetes and the proximity to ROS generating enzymes has rendered BK channels particularly vulnerable to redox modulation. We have previously reported hSlo expressed in HEK293 cells in the presence of high glucose is susceptible to the inhibitory modulation by H₂O₂ and OONO⁻. In addition, O₂⁻⁻ could further enhance c-Src activity,27 leading to channel tyrosine phosphorylation, and O₂⁻⁻ could also react with NO to generate ONOO⁻, resulting in channel tyrosine nitration.28 Tyrosine phosphorylation and nitration are 2 important mechanisms of protein posttranslational modification. Indeed, there is 2.1- and 3.3-fold increase in BK channel tyrosine phosphorylation and tyrosine nitration, respectively, in STZ-induced diabetic rat arteries. ONOO⁻ is known to directly inhibit BK channel function,28 and we found that BK channel activity in coronary arterial SMCs was lost after incubation with 0.1 μmol/L ONOO⁻ for 2
hours (data not shown), a time when protein nitration has reached a maximal effect.\textsuperscript{28} Incubation with LavA abolished the Ang II effects on BK currents, which is in agreement with the report by Alioua et al.\textsuperscript{30} With the upregulation of cav-1 expression and increased localization of BK channels and AT\textsubscript{1}R signaling proteins in the caveolea of diabetic vessels, we believe that Ang II–mediated ROS generation via caveolea-associated NOX-1 activation plays a central role in BK channel regulation. In contrast, the absence of caveolea prevents the deleterious effects of Ang II on BK channel activities in diabetes.

Our study has potential limitations. First, density gradient fractionation and immunoprecipitation experiments require a lot of proteins and these experiments were done using aortas instead of coronary arteries. Second, BK channel activity in WT and cav-1 KO mice was characterized in aortic SMCs. However, we have shown that Ang II/BK channel regulation is the same in SMCs from rat coronaries, rat aortas, and mouse aortas. Hence, we believe the conclusions derived from these experiments are valid as they all bear the same results.

In summary, we have shown that BK channels and AT\textsubscript{1}R signaling proteins are colocalized in vascular caveolea microdomains and caveolea targeting is critical for mediating the regulation of BK channel function by Ang II. In Figure 8, we present a working model to illustrate that caveolea of vascular SMCs facilitate the assembly of BK channels and AT\textsubscript{1}R signaling proteins into a molecular complex, leading to increase of BK channel posttranslational modulation. Hence, our results delineated a molecular mechanism through which caveolea microdomain organization facilitates the inhibition of BK channel function in diabetic vessels, which in turn regulates coronary blood flow and may affect the clinical outcome of diabetic patients with acute coronary syndrome.

Sources of Funding

This work is supported by American Diabetes Association grant ADA-JFA-07-39 and NIH grants HL74180 and HL080118.

Disclosures

None.

References


2. Lu T, He T, Katusic ZS, Lee HC. Molecular mechanisms mediating inhibition of human large conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels by high glucose. Circ Res. 2006;99:607–616.


---

**Novelty and Significance**

**What Is Known?**

- Caveolae provide a central platform for signaling transduction, including that of angiotensin II (Ang II) type 1 receptor signaling (AT1-R).
- The effects of Ang II are mediated through reactive oxygen species generation.
- Vascular large conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channel activity is impaired in diabetes, contributing to diabetic vascular dysfunction.

**What New Information Does This Article Contribute?**

- BK channels are colocalized with AT1-R and its signaling proteins such as protein kinase C, nonphagocytic NAD(P)H oxidase (NOX)-1, and c-Src in the caveolae of vascular smooth muscle cells, forming a channel/receptor/enzyme/caveolae microdomain complex.
- Cav-1 expression is upregulated in diabetic vessels with enhanced BK channel/AT1-R signaling caveolae targeting, resulting in increased redox-mediated BK channel modification.
- Cav-1 gene ablation protects vascular BK channel function in diabetes.
- This is the first report that caveolae/Ang II signaling participates in vascular BK channel regulation and facilitates BK channel and coronary dysfunction in diabetes. Our results delineate a fundamental mechanism underlying diabetic vascular dysfunction and may help to establish the BK channels as a therapeutic target in the treatment of diabetic vascular complications.
Regulation of Coronary Arterial BK Channels by Caveolae-Mediated Angiotensin II Signaling in Diabetes Mellitus
Tong Lu, Dai-Min Zhang, Xiao-Li Wang, Tongrong He, Ru-Xing Wang, Qiang Chai, Zvonimir S. Katusic and Hon-Chi Lee

Circ Res. 2010;106:1164-1173; originally published online February 18, 2010; doi: 10.1161/CIRCRESAHA.109.209767

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/106/6/1164

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/02/18/CIRCRESAHA.109.209767.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Data Supplement

Regulation of Coronary Arterial BK Channels by Caveolae-Mediated Angiotensin II Signaling in Diabetes Mellitus

Running title: BK channel regulation by Ang II in diabetic vessels

Tong Lu¹
Dai-Min Zhang¹
Xiao-Li Wang¹
Tongrong He²
Ru-Xing Wang³
Qiang Chai¹
Zvonimir S. Katusic²
Hon-Chi Lee¹

From the Departments of Internal Medicine¹ and Anesthesiology², Mayo Clinic, Rochester, Minnesota 55905, USA, and from the Department of Cardiology³, Affiliated Hospital of Nanjing Medical University in Wuxi, Wuxi People’ Hospital, Wuxi 214023, P. R. China

Corresponding Author: Tong Lu, M.D., Ph.D., Division of Cardiovascular Diseases, Department of Internal Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN55905. Tel: 507-255-9653; Fax: 507-538-6418; E-mail: lu.tong@mayo.edu.
Methods

Type I diabetic animal development and vascular SMC isolation
Sprague-Dawley rats were purchased from Harlan Inc. (Indianapolis, IN). Cav-1(-/-) knockout (KO) mice and cav-1(+/-) (WT) mice were obtained from Jackson Laboratories (Bar Harbor, ME). To develop type I diabetic animals, male Sprague-Dawley rats, cav-1 WT and KO mice at 6 to 8 weeks of age received an injection of STZ (100 mg/kg body weight, ip.) and a second dose was given on the next day. Control animals received vehicle injections. Animals with blood glucose > 300 mg/dl were considered diabetic and were used for experiments in 8 weeks.

Isolated vascular SMC were prepared as we have described 1. Briefly, rat coronary arteries or mouse aortas were carefully dissected in ice-cold dissociation buffer (in mmol/L): NaCl 145, KCl 4.0, CaCl2 0.05, MgCl2 1.0, HEPES 10, glucose 10, pH 7.2. 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 10 min. The vessels were replaced with fresh 0.1% w/v BSA dissociation buffer containing 1.5 mg/ml papain and 1.0 mg/ml dithiothreitol and incubated in a shaking water bath at 37 °C for another 10 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 10 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. Dissociated cells were used for patch clamp experiments within 8 h.

Whole-cell patch clamp recording
Whole-cell BK currents were recorded from freshly isolated SMC with a holding potential (HP) of -60 mV and a testing potential (TP) of -40 mV to +160 mV in increments of 10 mV. The pipette solution contained (in mmol/L): KCl 140, HEPES 10, MgCl2 1.0, Na2ATP 5.0, Na2GTP 0.5, EGTA 1.0, and CaCl2 0.814 (1 μmol/L free Ca2+), pH 7.35. The bath solution contained (in mmol/L): NaCl 145, KCl 4.0, MgCl2 1.0, CaCl2 1.0, HEPES 10, and glucose 10, pH 7.40. The effects of chemicals on BK channel activity were determined in the same cell before and after drug superfusion. The K+ currents inhibited by 0.1 μmol/L iberiotoxin (IBTX) were referred as the IBTX-sensitive BK currents.

Single channel recording
Outside-out single channel recordings were made at +60 mV in freshly isolated SMC of rat coronary arteries. The BK current was identified by IBTX-sensitivity and its unitary conductance. The output signals were filtered with an 8-pole Bessel filter (902 LPF, Frequency Devices, Inc., Haverhill, MA) at 10 kHz and digitized at 50 kHz. Single channel analysis was performed using TAC software (Bruxton, Inc., Seattle, WA) as previously described 1. The pipette solution and bath solution were the same as those used for whole-cell recording.

Measurement of coronary artery ring tension
Tension in rat coronary artery rings was measured using a multi-wire myograph system (610M, DMT-USA, Atlanta GA) as previously reported 2. The left anterior descending coronary artery was carefully dissected from the isolated rat heart in ice-cold Krebs' solution that contained (in mmol/L) NaCl 118, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO 35, CaCl2 2.5 and glucose 5.6 (pH=7.4). The artery was cut into rings of 1.5 to 2 mm in length, and each ring was suspended by two stainless-steel wires and mounted in an 8 ml organ chamber filled with Krebs' solution. Basal tension of the artery ring was maintained at 1.25 fold of the basal diameter of the vessel and equilibrated for 60 min with 96-4% O2-CO2. Changes in artery ring tension were recorded using a PowerLab 4/25 Data Acquisition Systems (AD Instruments, Medford, MA) and analyzed by LabChart 5.0 software (AD Instruments, Medford, MA). Changes in coronary artery ring tension
in response to chemicals were expressed as the percentage of maximal contraction produced by 100 mmol/L KCl. NS-1619-mediated relaxation was expressed as the percentage of the changes in the tension produced by 5 nmol/L endothelin-1 (ET-1).

**Sucrose gradient density centrifugation**
Cellular distribution of cav-1 and caveolae targeting of the AT$_1$R-enzyme-channel complex in rat aortas was determined by sucrose density gradient fractionation as previously described. Aortas were homogenized in 500 mmol/L Na$_2$CO$_3$ with 2 % protease inhibitors (v/v), and then centrifuged at 5,000 rpm at 4 °C for 10 min. The supernatant was adjusted to 40% sucrose, placed to the bottom of a 10-ml ultracentrifuge tube, layered with 4-ml discontinuous sucrose gradients (40%, 30% and 5%), and centrifuged at 32,000 rpm at 4 °C for 20 h. Ten fractions of 1.2 ml each were collected and analyzed by Western blotting.

To determine the effects of Ang II on caveolae targeting of AT$_1$R, some rats were infused with Ang II (100 μg/kg, iv.) 1 h prior to the isolation of aortas, which were homogenized and fractionated by ultracentrifugation.

**Co-immunoprecipitation and immunoblotting**
Immunoprecipitation was performed as described previously. Isolated rat aortas were cut into small pieces and incubated with 200 μl RIPA buffer (in mmol/L): Tris 50, NaCl 150, NaF 2, EDTA 1, EGTA 1, NaVO$_4$ 1, and 1% Triton X-100) and 1 μl protease inhibitor on ice for 1 h, homogenized and then centrifuged at 500 rpm at 4 °C for 10 min. The supernatant (about 200 μg in 200 μl) was incubated with anti-cav-1 (Santa Cruz, CA), anti-phosphotyrosine (Cell Signaling, MA) or anti-3-nitrotyrosine (Upstate, NY) 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 mice were homogenized, electrophoresed, transferred to nitrocellulose membrane, and immunoblotted against rabbit anti-human BK channel antibodies (1:200, custom made), anti-cav-1 (1:200 Santa Cruz, CA), anti-AT$_1$R (1:200, Santa Cruz, CA), anti-c-Src (1:200, Santa Cruz, CA), anti-NOX-1 (1:200, Santa Cruz, CA) and anti-G$_{αq/11}$ (1:200, Santa Cruz, CA) antibodies. Blots were probed with mouse anti-β actin antibodies (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.

**Cav-1 knockdown by small interfering (si)RNA**
Cav-1 in SMC was knocked down using human cav-1 siRNA as previously described. Cav-1 siRNA and control scrambled siRNA were obtained from Dharmacon (Lafayette, Co). 48 h after transfection with 100 nM cav-1 siRNA or control siRNA, cells were analyzed for cav-1 abundance.

**Confocal immunofluorescence microscopy**
Freshly isolated aortic smooth muscle cells from control and diabetic rats, as well as from cav-1 WT and KO mice, were seeded on glass slides, fixed with 4% formaldehyde for 30 min, and then permeated with 0.1% Triton X-100 in PBS for 2 min. After incubation with 10% normal goat serum in PBS for 30 min, cells were incubated with a monoclonal anti-caveolin-1 antibody (1:200 dilution) plus either a polyclonal anti-BK α-subunit antibody (1:100 dilution) or a polyclonal anti-
NOX-1 antibody (1:100). The primary antibodies were detected with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:1000 dilution) or with Texas Red-conjugated goat-anti-rabbit secondary antibody (1:500 dilution) and mounted in Prolong Anti-fade Reagent (Molecular Probes, Eugene, OR). Samples were washed with PBS after both the primary and the secondary antibody incubations. Cells were visualized using a confocal laser microscope (LSM 510, Zeiss, Germany) with a 63X water immersion lens.

**Chemicals**

OONO$^-$ was purchased from Upstate Cell Signaling and Solutions (Lake Placid, NY). Lavendustin A (LavA), Lavendustin B (LavB), membrane permeable PKC peptide inhibitor, diphenylene iodonium (DPI) and myxothiazole (MXTZ) were obtained from BIOMOL International (Plymouth Meeting, PA). The other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO).

**Statistical analysis**

Data are presented as mean±S.E.M. One way ANOVA followed by Tukey’s test was employed to compare data from multiple groups. Student’s t test was used to compare data between two groups and paired t test was used to compare data before and after treatment.

**Results**

**Characterization of STZ-induced type I diabetic animals**

8 weeks after the development of hyperglycemia, the average weight and blood glucose level was 223.4±5.7 g and 571.3±6 mg/dl respectively in STZ-induced diabetic rats (n=14) and was significantly different in those of age-matched controls, 316.6±6.7 g and 167.2±7.5 mg/dl respectively (n=17, p<0.05 between control and diabetic rats for both). The average weight and blood glucose level of age-matched non-diabetic WT mice was 24.8±0.6 g and 175.8±28.4 mg/dl (n=18) respectively and 18.8±0.7 g and 490.2±19.1 mg/dl (n=17, p<0.05 vs. non-diabetic WT for both) respectively for diabetic WT mice. The average weight and blood glucose level of age-matched non-diabetic KO mice was 24.6±0.8 g and 148.2±10.8 mg/dl (n=10) respectively, and 20.8±0.6 g and 522.4±29.0 mg/dl (n=17, p<0.05 vs. non-diabetic KO for both) respectively for diabetic KO mice.

**Vascular SMC capacitance**

The cell capacitance of SMC isolated from control and STZ-induced diabetic rats was 11.8±0.8 pF (n=41) and 11.1±1.0 pF (n=35) respectively (p=N.S. vs. control). There was also no significant difference in SMC capacitance among cells isolated from control and diabetic cav-1 WT and KO mice (7.1±0.5 pF for non-diabetic WT, n=30; 6.8±0.5 pF for diabetic WT, n=28; 10.6±1.1 pF for non-diabetic KO, n=10 and 8.1±1.2 pF for diabetic KO SMC, n=11, respectively).

**Effects of Ang II on single BK channel activity**

Single BK currents were recorded at +60 mV from freshly isolated coronary arterial SMC in outside-out configuration. 2 μmol/L Ang II applied extracellularly reduced the channel open probability (Po) by 49.4%, from 0.45±0.06 at baseline to 0.23±0.03 of (p<0.05, n=4). For the best fit, the open dwell-time distribution histograms required at least three components: slow (τ-o1), intermediate (τ-o2) and fast (τ-o3) time constants. The closed-dwell time distribution of histograms had four components: very slow (τ-c1), slow (τ-c2), intermediate (τ-c3) and fast (τ-c4) time constants. The representative tracings and kinetic analysis of BK channel was illustrated in Figure I.
Effects of Ang II on BK channel current density of aortic arterial SMC from control and STZ-induced diabetic rats

Figure II shows that 2 μmol/L Ang II reduced aortic SMC BK current density from 206.5±36.2 pA/pF at baseline to 82.6±19.1 pA/pF (TP=+150 mV, n=6, p<0.05) in control rats and from 57.4±7.7 pA/pF (TP=+150 mV, n=6, p<0.05 vs. baseline of controls) at baseline to 54.2±11.6 pA/pF in STZ-induced diabetic rats (TP=+150 mV, n=6, p=N.S. vs. baseline of diabetic rats). I-V curves of BK channels from control and diabetic rat aortic SMC before and after exposure to Ang II are shown in online Figure II. Hence, the response of BK currents to treatment with Ang II was similar between coronary and aortic SMC from control and diabetic rats.

Co-localization of BK channel and NOX-1 in caveolae of SMC

To corroborate the results of immunoprecipitation, we performed confocal fluorescence microscopy analysis to demonstrate the targeting of BK channel and NOX-1 to the caveolae of SMC. The results showed that the BK channel and NOX-1 are colocalized in the caveolae of SMC, BK channel and NOX-1 targeting to caveolae appeared to be upregulated in diabetes (Figure III).

Role of NAD(P)H and mitochondrial respiratory chain complexes in high glucose-induced ROS generation in coronary arterial SMC

It is well known that culture with high glucose increases ROS generation in SMC. To further determine the source of high glucose-induced ROS generation in coronary arterial SMC, we measured the effects of DPI (a NOX inhibitor) or MXTZ (the mitochondrial respiratory chain complex II inhibitor) on the peroxide level in human coronary arterial SMC after a 2-week culture in 22 mmol/L glucose. Cellular peroxide level was significantly reduced after 1 h incubation with DPI, but not with MXTZ (Figure IV), suggesting that the major source of high glucose-mediated peroxide generation in the cells is from NAD(P)H oxidases, consistent with previous reports.

Ang II treatment increased ROS generation only in the SMC of cav-1 WT, but not in those of cav-1 KO mice (Figure V).
Figure I. Effects of Ang II on single BK channel activity of rat coronary arterial SMC

A: Representative tracings of single BK current were recorded at +60 mV from freshly isolated coronary arterial SMC in outside-out configuration at baseline and after extracellular application of 2 μmol/L Ang II. Selected segments with expanded details (lower tracings) show higher frequency of channel openings in baseline with reduction of channel activity by Ang II. “c” with solid lines indicate the channel closed state.

B: The histograms of BK channel open- and closed-dwell durations have at least three open-dwell time constant components: $\tau_{o1}$, $\tau_{o2}$ and $\tau_{o3}$; and four closed-dwell time constant components: $\tau_{c1}$, $\tau_{c2}$, $\tau_{c3}$ and $\tau_{c4}$. Dashed lines represent the distribution of each exponential component of dwell times. The value of each time constant and its relative weight (in parentheses) are given above each histogram. Ang II reduced the $\tau_{o1}$ and prolonged $\tau_{c1}$ and $\tau_{c2}$, compared with those at baseline. C: BK channel open probability (Po) was reduced by 49.4% after application of Ang II.
Figure II. Effect of Ang II on aortic arterial SMC BK channel activity of control and STZ-induced diabetic rats

I-V curves of BK current densities in freshly isolated SMC from the aortas of control and STZ-induced diabetic rats. Ang II (2 μmol/L) inhibited BK current density by 50% in control rats. 8 weeks after the development of diabetes, BK current density was significantly reduced at baseline Ang II did not produce any further inhibition, suggesting that the vascular BK channel function was profoundly impaired in diabetes.
Figure III. Colocalization of BK channel and NOX-1 in the caveolae of coronary arterial SMC from STZ-induced diabetic rats

A: After incubation with anti-BK and anti-cav-1 antibodies, fluorescence images were acquired separately for Texas Red for BK channel (red in left column), fluorescein for cav-1 (green in middle column). BK channel and cav-1 are colocalized on the SMC membrane (yellow in right column) when images are merged. B: Similarly, NOX-1 colocalization in the caveolae of diabetic SMC is increased.
Figure IV. Inhibition of high glucose-mediated ROS generation by NOX and mitochondrial electron transport complex inhibitors in human coronary arterial SMC

Human coronary arterial SMC were cultured in 22 mmol/L glucose for two weeks. After pretreatment with DPI (20 μmol/L) for 1 h or with MXTZ (10 μmol/L) for 10 min, cells were incubated with 2 μmol/L dihydroethidium (DHE) for 30 min and then examined by confocal laser scanning microscopy. DHE is oxidized by O$_2$•⁻ and intercalates DNA, producing a bright red fluorescence. DHE was excited at 488 nm and fluorescence emission was detected with a 585- to 615-nm band-pass filter. Laser settings were identical for acquisition of all images. Bar graphs showing a significant reduction of fluorescence in cells after pretreated with DPI, but not with MXTZ.

Figure V. Increased ROS production in aortic arterial SMC of WT but not cav-1 KO mice by Ang II

Freshly isolated aortic SMC from WT and cav-1 KO mice were incubated with 0 (control) or 2 μmol/L Ang II for 1 h, followed by incubation with DHE for 30 min. Ang II enhanced the intensity of bright red fluorescence in SMC from WT mice, but not in those from KO mice.
References