## Coronary Arterial BK Channel Dysfunction Exacerbates Ischemia Reperfusion-Induced Myocardial Injury in Diabetic Mice

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<th>Journal:</th>
<th><em>Applied Physiology, Nutrition, and Metabolism</em></th>
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<td>Manuscript ID</td>
<td>apnm-2016-0048.R2</td>
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<td>Manuscript Type:</td>
<td>Article</td>
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<td>Date Submitted by the Author:</td>
<td>10-May-2016</td>
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| Keyword: | BK channel, angiotensin II type 1 receptor, vascular smooth muscle cell, myocardial ischemia-reperfusion injury, diabetes |
Coronary Arterial BK Channel Dysfunction Exacerbates Ischemia Reperfusion-Induced Myocardial Injury in Diabetic Mice

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Abstract

The large conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels, abundantly expressed in coronary artery smooth muscle cells (SMCs), play a pivotal role in regulating coronary circulation. A large body of evidence indicates that coronary arterial BK channel function is diminished in both type 1 and type 2 diabetes. However, the consequence of coronary BK channel dysfunction in diabetes is not clear. We hypothesized that impaired coronary BK channel function exacerbates myocardial ischemia reperfusion (I/R) injury in streptozotocin (STZ)-induced diabetic mice. Combining patch-clamp techniques and cellular biological approaches, we found that diabetes facilitated the colocalization of Angiotensin II (Ang II) type 1 receptors (AT1R) and BK channel α-subunits (BK-α), but not BK channel β1-subunits (BK-β1), in the caveolae of coronary SMCs. Such caveolae compartmentalization in vascular SMCs not only enhanced Ang II-mediated inhibition in BK-α, but also produced a physical disassociation between BK-α and BK-β1, leading to increased infarct size in diabetic hearts. Most importantly, genetic ablation of caveolae integrity or pharmacological activation of coronary BK channels protected the cardiac function of diabetic mice from experimental I/R injury in both in vivo and ex vivo preparations. Our results demonstrate a vascular ionic mechanism underlying the poor outcome of myocardial injury in diabetes. Hence, activation of coronary BK channels may serve as a therapeutic target for diabetic cardiovascular complications.

Key words: BK channel, angiotensin II type 1 receptor, vascular smooth muscle cell, myocardial ischemia-reperfusion injury, diabetes.
Introduction

Diabetes mellitus has become an epidemic worldwide. The global prevalence of diabetes is estimated to be 9% among adults according to the World Health Organization global status report. Diabetes is associated with a 2- to 4-fold increase in the risks of cardiovascular diseases which remain the leading cause of death in both men and women with diabetes (Snell-Bergeon & Wadwa, 2012). A large body of clinical evidence has shown that the poor prognosis of acute myocardial infarction and cardiac sudden death in patients diagnosed with diabetes is attributed to compromised coronary blood flow and increased susceptibility of myocardial ischemia (Kurisu et al., 2003; Cubbon et al., 2007; Yeung et al., 2012). Despite advances in the management of diabetes, the cardiovascular morbidity and mortality in diabetic patients remain twice that of non-diabetic patients (Wackers, 2005; Norhammar et al., 2007). However, the molecular mechanisms that underlie the poor cardiovascular outcome in diabetic patients are not fully understood.

Because of high unitary conductance and high density in coronary smooth muscle cells (SMCs), the large conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels play a negative feedback role in the regulation of vascular tone and cardiac perfusion. Activation of vascular BK channels by elevated intracellular free Ca\(^{2+}\) concentrations gives rise to the generation of spontaneous transient outward currents that hyperpolarize the membrane potentials, shut off the voltage-dependent Ca\(^{2+}\) channels in vascular SMCs and lead to vascular relaxation (Nelson et al., 1995; Jaggar et al., 1998). Functional vascular BK channels are octameric complexes with four pore-forming \(\alpha\) subunits (BK-\(\alpha\), encoded by Slo1 gene) and four regulatory \(\beta\)1 or \(\gamma\)1 subunits (BK-\(\beta\)1 or BK-\(\gamma\)1) (Tanaka et al., 1997; Evanson et al., 2014). In diabetes, however, the BK channel
function is diminished in many vascular beds including those of coronary arteries (Lu et al., 2010; Zhang et al., 2010; Wang et al., 2012; Yi et al., 2014). Whether coronary BK channel dysfunction contributes to worsened outcome of cardiovascular diseases with diabetes has not been studied.

Caveolae are flask-shaped nonclathrin-coated plasma membrane structures that contain the signature protein, caveolin (Cav). Three Cav isoforms are known: Cav-1, Cav-2 and Cav-3. Cav-1 and Cav-2 are present in vascular endothelial cells and SMCs, while Cav-3 is primarily expressed in cardiac and skeletal muscles (Krajewska & Maslowska, 2004). The N-terminus of Cav-1 contains an important functional structure: the Cav scaffolding domain, which is essential for interaction with other proteins containing the Cav binding motifs (ΦXΦXXXXΦ or ΦXXXXΦXXΦ, where Φ represents an aromatic amino acid and X is any amino acid) (Krajewska & Maslowska, 2004). Such Cav binding motifs are present in human BK-α (1102-YNMLCΦGIY-1110) and human AT1R (304-FLGKKΦKRY-312), but not in human BK-β1 or BK-γ1. Caveolae are membrane microdomains that assemble signaling molecules including those of Ang II (Ushio-Fukai & Alexander, 2006). Two types of Ang II receptors are expressed in the heart: type 1 (AT1R) and type 2 (AT2R) with AT1R being the dominant isoform that mediates the major cardiovascular effects of Ang II (Dasgupta & Zhang, 2011). We have demonstrated that Ang II promoted in AT1R trafficking into the caveolae of vascular SMCs, forming the BK channel-AT1R-caveolae microdomain complexes. Importantly, such caveolae compartmentation was enriched in diabetic vessels, potentiating the inhibitory effect of Ang II on BK channels in the vicinity and promoting coronary vasoconstriction (Lu et al., 2010). In addition, it has been reported that caveolae facilitated direct coupling between BK channels and
L-type Ca$^{2+}$ channels in vascular SMCs to regulate vascular tone (Suzuki et al., 2013). However, the role of BK channel-AT1R-caveolae microdomain complexes in cardiovascular diseases, particularly with diabetes, has not been established. In this study, we found that dysregulation of coronary smooth muscle BK channel activity contributes to exacerbated ischemia/reperfusion (I/R) injury in diabetic mouse hearts, due to an increase of BK channel-AT1R-caveolae microdomain compartmentation in coronary SMCs. Activation of coronary BK channels and suppression of AT1R-caveolae signaling minimized myocardial I/R injury in diabetic mice. Hence, protection of coronary BK channel function may serve as a novel molecular target for the treatment of cardiovascular complications in diabetes.

Materials and methods

Diabetic mouse model — Male Cav-1 null mice (Cav-1$^{-/-}$, Cav1tm1M1s/J) and wild type (wt) control mice (C57BL/6J) were purchased from the Jackson Laboratory at 4 weeks of age. Mice received a STZ injection (100 mg/kg body weight, ip.) and those with blood glucose higher than 300 mg/dl were considered diabetic. Eight weeks after developing hyperglycemia, mice and age-matched controls were sacrificed and used for experiments. All animal protocols were approved by the Mayo Clinic Institute Animal Care and Use Committee (Rochester, MN).

Coronary SMC isolation and BK channel current recording — Mouse coronary SMCs were enzymatically isolated for whole-cell K$^{+}$ current recordings as we have previously described (Lu et al., 2010; Lu et al., 2012). The pipette solution contained (in mM): KCl 140, MgCl$_2$ 0.5, Na$_2$ATP 5.0, Na$_2$GTP 0.5, HEPES 10.0, EGTA 1.0, CaCl$_2$ 0.465 (~0.2 µM free Ca$^{2+}$) and pH at 7.38. The bath solution contained (in mM): NaCl 145, KCl 5.6, MgCl$_2$ 1.0, CaCl$_2$ 1.0, HEPES 10.0, glucose 5.0 and pH at 7.40. BK currents were defined by its sensitivity to 0.1 µM
iberiotoxin (IBTX, a membrane impermeable BK channel-specific blocker) and were obtained by subtraction of the IBTX-insensitive component from the total K⁺ currents. All patch clamp experiments were conducted at room temperature (22 °C).

Sucrose gradient density centrifugation — The cellular distributions of caveolae-targeted BK channels and AT1R in mouse aortic SMCs were determined by sucrose density gradient fractionation as previously described (Wang et al., 2005; Lu et al., 2010). Cells were homogenized in 500 mM Na₂CO₃ with 2% protease inhibitors (v/v), and then centrifuged at 5,000 rpm at 4 °C for 10 min. One ml of the supernatant were adjusted to 40% sucrose-MBS (2-[N-morpholino]-ethanesulfonic acid) by adding 1 ml of 80% sucrose-MBS, placed to the bottom of a 6-ml ultracentrifuge tube, layered with a 2-ml discontinuous sucrose gradient (40%, 30% and 5%), and centrifuged at 32,000 rpm at 4 °C for 20 h. Eight fractions of 1.25 ml each were collected and analyzed by immunoblotting.

Co-immunoprecipitation (Co-IP) and immunoblotting — Co-IP and immunoblotting were performed as we have previously reported (Lu et al., 2010; Lu et al., 2012). Mouse aortas were incubated with 200 µl RIPA buffer (in mM): Tris 50, NaCl 150, NaF₂ 1, EDTA 1, EGTA 1, NaVO₄ 1, and 1% Triton X-100 (pH 7.5) and 1 µl protease inhibitor on ice for 30 min, homogenized and then centrifuged at 2000 rpm at 4 °C for 10 min. The supernatant (200 µg protein in 200 µl) was incubated with 4 µg anti-Cav-1 antibody (Santa Cruz Biotechnology, Inc., catalog number: sc-7875) at 4 °C overnight. Incubation with same amount of IgG severed as a negative control. The samples were then incubated with 20 µl Protein G Plus-Agarose (Santa Cruz Biotechnology, Inc.) at 4 °C for 2 h. 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, resolved by polyacrylamide gel electrophoresis and blotted against rabbit anti-BK-α antibodies (1:200, custom made) and rabbit anti-AT1R antibody (1:200, Santa Cruz Biotechnology, Inc., catalog number sc-1173). Custom-made rabbit polyclonal anti-BK-α and anti-BK-β1 antibodies against rat BK α-subunit peptide (KTKEAQQINNGSSQADGTLKPVDE) and rat BK-β1 peptide (HTEDTRDQNQCSYPRNL) were made in Mayo Clinic Antibody Core Facility as we have previously published (Wang et al., 2005; Lu et al., 2010; Zhang et al., 2010; Yi et al., 2014). Optical density of the bands was analyzed using Scion Image software (Scion Corp.).

Confocal immunofluorescence microscopy — Freshly isolated mouse coronary SMCs were fixed with 4% formaldehyde on glass slides for 30 min and permeated with 0.1% Triton X-100 in PBS for 2 min. After incubation with 10% normal goat serum in PBS for 1 h, cells were incubated with monoclonal anti-Cav-1 antibody (1:200) plus either polyclonal anti-BK-α (1:100) or anti-AT1R (1:100) antibodies. Cells were washed with PBS and the fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:1000) or Texas Red-conjugated goat-antirabbit secondary antibody (1:500) was applied for 1 h. After washing with PBS, a coverslip was applied to the glass slide with a drop of ProLong Gold antifade reagent (Molecular Probes-Invitrogen Co., catalog number 36934). Cells were visualized using a confocal laser microscope (LSM 510, Zeiss) with a 60X water immersion lens (Wang et al., 2005; Lu et al., 2010). Quantitative correlation of the fluorescence intensities of two images was analyzed using ImageJ JaCop (Bolte & Cordelieres, 2006; Schneider et al., 2012).

HEK293 cell line stably expressing hSlo gene and site-directed mutagenesis — 1 μg hSlo cDNA/pTracer-CMV2 (accession no. NM_001014797) was transfected into HEK293 cells using
Effectene Transfection Reagent kit (Qiagen Co.). After 48-h transfection, the cells expressing hSlo gene at high levels were selected by growth in 10% FBS culture medium (Gibco-Invitrogen Ltd.) containing 1.0 mg/ml Geneticin (Gibco-Thermo Fisher Scientific Inc.) for four weeks. Only the transfected cells (hSlo-HEK293 cell line) with resistance to Geneticin survived in long-term cultures. Human EGFP-tagged Cav-1 cDNA/pDNA3.1 (accession no. NM_001753) was transiently co-transfected with human AT1R cDNA/pDNA3.1 (accession no. NM_000685) in the hSlo-HEK293 cells. The transfected cells were detected by the presence of strong green fluorescent protein under ultraviolet microscopy (IX70, Olympus) 48 h after transfection. The AT1R F309A mutation was generated by PCR (forward: 5’-GGCTTTCTGGGGAAAAAAGCTAAAAGATATTTTCTCCAGC-3’; reward: 5’-GCTGGAGAAATATCTTTTTAGCTTTTTTTCCCCAGAAAGCC-3’) according to the manufacture’s protocol of the QuickChange site-directed mutagenesis kit (Stratagene, Inc.). The construct orientation and correctness of the mutation were verified by DNA sequencing (DNA Facility Core of Mayo Clinic, Rochester, MN).

Ex vivo myocardial I/R studies — Isolated mouse hearts were rapidly mounted on the cannulus of a modified Langendorff apparatus and perfused with Tyrode’s solution (in mM): NaCl 119, KCl 4.8, KH_{2}PO_{4} 1.2, MgSO_{4} 1.2, CaCl_{2} 1.0, NaHCO_{3} 24.9, glucose 10.0, pyruvate 5.0, heparin 1200 U/L, pH=7.4), equilibrated with 95% O_{2}-5% CO_{2}, using a peristaltic pump (P720, Instech Laboratories Inc.) at 3.0 mL/min at 37 °C. The left atrium was cut open, and a balloon catheter was inserted through the mitral valve into the left ventricular chamber. The balloon catheter was connected to a pressure transducer (Gould Inc.) for monitoring left ventricular pressure. A bipolar electrode was placed on the ventricular epicardium for pacing (350 beats/min) and for
monitoring heart rate. Hearts were paced at 350 beats/min throughout all experiments. The left ventricular developed pressure (LVDP) was calculated as the left ventricular systolic pressure minus the left ventricular diastolic pressure before the onset of experimental ischemia and at the end of reperfusion as previously described (Gelpi et al., 2002). The ischemia-reperfusion protocols (Gelpi et al., 2002) for isolated hearts were conducted as follows: (i) Control group: 30 min of normal perfusion with Tyrode’s solution, then 40 min of global ischemia (no flow), followed by 60 min of reperfusion with Tyrode’s solution; (ii) Ang II group: 30 min of perfusion with 2 µM Ang II, then 40 min of global ischemia, followed by 60 min of perfusion with 2 µM Ang II; (iii) Ang II+Losartan (an AT1R specific blocker) group: 30 min of perfusion with 2 µM Ang II and 10 µM Losartan, then 40 min of global ischemia, followed by 60 min of perfusion with 2 µM Ang II and 10 µM Losartan; (iv) Ang II+NS1619 (a BK channel activator) group: 30 min of perfusion with 2 µM Ang II and 10 µM NS1619, then 40 min of global ischemia, followed by 60 min of perfusion with 2 µM Ang II and 10 µM NS1619; (v) IBTX (a membrane impermeable BK channel-specific blocker) group: 30 min of perfusion with 0.2 µM IBTX, then 40 min of global ischemia, followed by 60 min of perfusion with 0.2 µM IBTX; (vi) IBTX+NS1619: 30 min of perfusion with 0.2 µM IBTX and 10 µM NS1619, then 40 min of global ischemia, followed by 60 min of perfusion with 0.2 µM IBTX and 10 µM NS1619.

At the end of the I/R procedure, the heart was perfused with 1% 2,3,5-triphenyltetrazolium chloride (TTC) in 0.1 M phosphate buffer (pH 7.4) at 37 °C for 10 min (Gao et al., 2002). Viable myocardium containing dehydrogenase enzymes was stained brick red by reacting with TTC, whereas infarct tissue remained unstained (white color) because of the lack of the enzymes. After
incubation overnight with 10% formaldehyde, the heart was cut into 6 to 8 slices (each 0.1 mm thick) and the infarct weight was calculated as:

\[ A_1W_1 + A_2W_2 + \cdots + A_7W_7 + A_8W_8, \]

where A is the area of infarct as a percentage of the total area of the slice and W is the weight of the respective section. TTC-stained area and TTC negative area were measured digitally using ImageJ software (Schneider et al., 2012). The cardiac infarct size (\( \sum A \cdot W \)) was presented as a percentage of the total weight of the heart.

*In vivo myocardial I/R studies* — The surgical *in vivo* ischemia-reperfusion procedure was performed as previously described (Suzuki et al., 2002). Mice were anesthetized with pentobarbital (60 mg/kg, ip.) and anesthesia was maintained by 1.5% isoflurane with O\(_2\) through a vaporizer (Midmark Co.). Anesthetized mice were placed on a heating plate (at 37 °C) to keep the body temperature stable. Mice were tracheotomized, intubated, and ventilated with a small animal ventilator (Kent Scientific Co.). The tidal volume and ventilation rate were 0.5 to 0.6 ml and 100 to 120 strokes per min, respectively. Thoracotomy was performed through the third left intercostal space, and the pericardium was opened. A 7-0 silk suture was passed around the left anterior descending coronary artery (LAD) using a tapered needle. Electrocardiogram (ECG) limb leads were placed on the fore- and hind-limbs to record cardiac ECG throughout the experiment. A balloon catheter was inserted into the femoral artery to monitor systemic blood pressure. Acute myocardial ischemia was induced by ligation of the LAD for 40 min, followed by a 60-min of reperfusion achieved by releasing the ligation. After reperfusion, the heart was rapidly excised and mounted on the cannulus connected to a syringe. 1% TTC (10 ml, at 37 °C) was injected into the coronaries retrogradely through the aorta. Then, the heart was cut into 6 to 8 transverse slices for infarct size measurements.
Statistical analysis — Data were presented as mean±S.E.M. Statistical analysis was performed using SigmaStat 3.5 software (Systat Software, Inc.). 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. Paired *t* test was used to compare data from same subjects before and after treatment. Statistical significance was defined as *p*<0.05.

Results

Myocardial I/R injury studies in vivo — *In vivo* myocardial I/R injury was accomplished by a 40-min period of LAD ligation followed by a 60-min period of reperfusion (Fig. 1A). Myocardial infarct size was 20.2±2.7% (n=5) in control wt mice but 42.2±5.6% in diabetic wt mice (n=5, *p*=0.007 vs controls). However, the infarct size was 24.9±4.6% in non-diabetic Cav-1−/− mice (n=5, *p*=N.S. vs non-diabetic wt mice) and was 27.0±1.5% in diabetic Cav-1−/− mice (n=5, *p*=0.69 vs non-diabetic Cav-1−/− mice) (Fig. 1B). There was 55.3% reduction in diabetic effects on I/R-induced cardiac infarct size in Cav-1−/− mice. Hence, the absence of caveolae integrity reduced the myocardial injury and infarct size produced by I/R in diabetic mice to the level of non-diabetic mice.

Myocardial I/R injury studies ex vivo — To determine the role of coronary BK channel function on I/R-induced myocardial infarction, we performed *ex vivo* myocardial I/R injury studies in Langendorff-perfused mouse hearts subjected to 30 min of pretreatment with saline (control) or chemicals (Ang II, IBTX or NS1619), followed by 40 min of global ischemia and 60 min of reperfusion with saline or chemicals. In non-diabetic wt mouse hearts, infarct size was 44.1±2.3% (n=8) under control conditions but increased to 80.6±4.3% (n=7) after pretreatment with 2 µM Ang II (p=5.1x10^{-6} vs non-diabetic controls). The effect of Ang II on myocardial
infarct size was antagonized by 10 μM Losartan (49.0±4.6%, n=8, p=2.4x10^{-4} vs Ang II and p=0.35 vs non-diabetic controls) and by 10 μM NS1619 (49.0±7.1%, n=7, p=0.002 vs Ang II; p=0.47 vs non-diabetic controls). Pretreatment with 0.2 μM IBTX also increased the I/R-induced infarct size (72.5±7.6%, n=7, p=0.003 vs non-diabetic controls), but the effect of IBTX was not salvaged by the presence of 10 μM NS1619 (69.4±1.8%, n=7, p=0.72 vs IBTX treatment) (Fig. 2). These results indicate that sarcolemmal BK channels of coronary SMCs are critically involved in I/R-induced myocardial injury since the protective effect of NS1619 was abolished by the membrane-impermeable IBTX. In addition, diabetic wt mice had a myocardial infarct size (90.7±4.0%, n=9, p=1.3x10^{-3} vs non-diabetic controls) as large as those of non-diabetic wt mice with Ang II or IBTX pretreatment (p=0.28 vs Ang II treatment; p=0.81 vs IBTX treatment). Strikingly, pretreatment with 10 μM NS1619 dramatically reduced I/R-induced infarct size (27.7±2.3%, n=8, p=3.1x10^{-6} vs diabetes) in wt diabetic mouse hearts, to a level even smaller than those of non-diabetic control mice (p=1.4x10^{-4}) (Fig. 2), suggesting that the NS1619 effects on cardiac infarct size is more complicated than our original thoughts.

In non-diabetic Cav-1^{-/-} mouse hearts, the infarct size (28.8±2.4%, n=7) induced by I/R procedure was about half that of non-diabetic wt controls (p=5.6x10^{-4}). Pretreatment and reperfusion with 2 μM Ang II produced significantly larger infarcts (46.7±3.6%, n=7, p=0.003 vs non-diabetic Cav-1^{-/-} controls), similar to those of non-diabetic wt mice without treatment. IBTX pretreatment also greatly augmented the infarct size (84.9±3.9%, n=7, p=2.2x10^{-7} vs non-diabetic Cav-1^{-/-} controls), which was twice that of Ang II-treated Cav-1^{-/-} mice (p=1.0x10^{-4}) and comparable to that of non-diabetic wt with IBTX treatment (p=0.23) (Fig. 3). It is worth noting that the effect of IBTX on I/R-induced myocardial infarction was independent of Cav-1
expression in coronary SMCs, suggesting that AT1R-caveolae signaling is the upstream of coronary BK channel modulation. Furthermore, the presence of diabetes significantly enlarged I/R-induced myocardial infarct size in Cav-1\(^{-/-}\) mice (44.7±6.0%, n=9, p=0.040 vs non-diabetic Cav-1\(^{-/-}\) controls), but it was smaller than that of diabetic wt mice (p=5.8x10\(^{-4}\)) and IBTX-treated non-diabetic Cav-1\(^{-/-}\) mice (p=2.9x10\(^{-4}\)) (Fig. 3). In addition, pretreatment with 10 \(\mu\)M NS1619 significantly minimized myocardial infarct size in diabetic Cav-1\(^{-/-}\) mice to 23.6±5.4% (n=7, p=0.023 vs diabetic Cav-1\(^{-/-}\) mice), similar to that of non-diabetic Cav-1\(^{-/-}\) mice without treatment (Fig. 3). The changes in LVDP of wt and Cav-1\(^{-/-}\) mice before and after I/R procedure are summarized in Table 1.

**Colocalization of BK-\(\alpha\) and AT1R in the caveolae of coronary SMCs of mice** — We determined the cellular distribution of BK-\(\alpha\), BK-\(\beta\)1 and AT1R in cultured mouse coronary SMCs by sucrose density gradient and immunoblot analysis. As shown in Fig. 4A, BK-\(\alpha\) and AT1R proteins were abundantly found in the low buoyant density, caveolae-rich membrane fractions (No. 2 to 4) and in whole cell lysates, while BK-\(\beta\)1 protein expressions in those caveolae-rich membrane fractions were very little, compared to those in the cell lysate and the heavy density fractions (No. 7 to 8). Confocal immunofluorescence imaging of freshly isolated mouse coronary SMCs also showed that the signals of BK-\(\alpha\) with Cav-1, and BK-\(\alpha\) with AT1R were overlapped (Fig. 4B). Pixel-wise correlation between the staining intensities of two images was estimated using the ImageJ JaCop software, with the slope (Pearson correlation coefficient, \(\gamma\)) of 0.77 between BK-\(\alpha\) with Cav-1 signals, and 0.82 between BK-\(\alpha\) with AT1R signals respectively. Note: \(\gamma\) is from 1 to -1, with 1 standing for complete positive correlation and -1 for negative
correlation, with 0 standing for no correlation). In addition, co-IP studies detected both BK-α and AT1R in the immunoprecipitates pulled down by anti-Cav-1 antibody from the cell lysates of mouse aortas, suggesting there was physical interaction of Cav-1, BK-α and AT1R. Moreover, the colocalization of BK-α and AT1R in caveolae microdomain complexes was enriched in diabetic mouse vessels, compared to that of non-diabetic controls (Fig. 4C).

* Cav binding motif of AT1R is essential for Ang II-mediated BK channel inhibition — We have reported that pretreatment of Ang II facilitated AT1R trafficking into the caveolae of aortic SMCs of rats and enhance its effect on BK channel inhibition, and ablation of Cav-1 preserved BK channel activity in diabetic vessels (Lu et al., 2010). To further delineate the molecular mechanism underlying BK channel dysregulation by caveolae-targeting, we generated an AT1R mutation in the Cav binding motif by substituting the phenylalanine residue at 309 with an alanine (AT1R F309A) and examined the effects of Ang II on hSlo-HEK293 cell line 48 h after co-transfection of Cav-1 wt cDNAs with AT1R wt or the AT1R F309A mutant. Fig. 5A shows the time course of hSlo currents recorded at +100 mV in hSlo-HEK293 cell line transiently co-transfected with Cav-1 wt and AT1R wt cDNAs after exposure to Ang II (2 µM), Losartan (10 µM) and IBTX (0.1 µM). Ang II suppressed about 40% of hSlo currents and the Ang II effect was reversible upon washing out and blocked by Losartan (Fig. 5B). Ang II had no effect on hSlo currents stably expressed in HEK293 cells after co-transfection with AT1R F309A and Cav-1 wt cDNAs, while BK channel expression remained intact as shown by sensitivity to IBTX (Fig. 6A). Fig. 6B shows whole-cell hSlo currents tracings and the current-voltage relationships (I-V curve) at baseline and after exposure to 2 µM Ang II. A critical substitution in the Cav binding motif in AT1R completely abolished the inhibitory effect of Ang II on hSlo, revealing
the functional consequences of physical interaction between AT1R and Cav-1. Our results demonstrate that caveolae in vascular SMCs is a central platform for Ang II biological effects on BK channel regulation.

Discussion

The clinical outcome of patients with acute coronary events is dependent on the maintenance of effective myocardial perfusion (Wackers, 2005; Esposito et al., 2014). Studies over the last couple of decades improved our understanding on the pathological mechanisms of myocardial I/R injury which involve myocardial factors and vascular factors (Yellon & Hausenloy, 2007). The vascular factors are attributed to microvessel obstruction and blood flow deficiency in previously ischemic region of the heart after coronary angioplasty, contributing to the “no-reflow” phenomenon (Hoffmann et al., 2003). It is well known that pathological and functional changes in diabetic coronary endothelium contribute to exacerbated I/R-induced myocardial injury, including increased platelet activation and clot coagulation, as well as aggravated inflammatory response (Hausenloy & Yellon, 2013). In this study, we have further demonstrated that coronary smooth muscle BK channel activity is also important for the I/R-induced myocardial infarction, particularly in diabetic hearts. Activation of AT1R by Ang II and suppression of BK channels by IBTX enlarged myocardial infarct size in non-diabetic mice, similar to the effects of diabetes. Blockade of AT1R by Losartan and activation of BK channel by NS1619 reduced Ang II-induced myocardial infarct size and improved cardiac contractility. More importantly, the infarct size in diabetic hearts was reduced by NS1619 to a level even smaller than that in diabetic controls and in Ang II-treated mice, suggesting coronary BK channel dysfunction may play a fundamental role in the outcome of myocardial I/R injury, particularly in diabetes. It has been reported that NS1619 protects the heart by activating
myocardial mitochondrial BK channels (Singh et al., 2013), cardiac neuron BK channels (Wojtovich et al., 2013) and other non-selective ion channels (Cancherini et al., 2007). Indeed, activation of mitochondrial BK channels has protective effects on cardiac I/R injury (Sakamoto et al., 2008; Borchert et al., 2011; Singh et al., 2013; Testai et al., 2013). We believe that activation of sarcolemma BK channels in coronary SMCs is also critical in reducing I/R-induced myocardial infarct size in diabetic mice, based on following reasons: First, the effects of NS1619 on cardiac protection were abolished by IBTX which is membrane impermeable, indicating the involvement of sarcolemmal BK channels rather than mitochondrial BK channels. It is worth noting that BK channels are not found in the sarcolemma of cardiac myocytes (Singh et al., 2012) and are poorly expressed in the most freshly isolated vascular endothelial cells (Feletou, 2009). Second, significant reduction in myocardial infarct size was associated with ablation of the Cav-1 gene that is expressed in the vasculature, but not in myocardium which expresses Cav-3. A recent study also showed that forskolin-induced myocardial infarct size reduction was blocked by IBTX, demonstrating the protective role of vascular BK channels in whole heart I/R injury (Heinen et al., 2014).

The molecular mechanisms underlying BK channel regulation by caveolae-targeting could be complicated. It has been reported that inhibition of BK channel activity by Ang II is mediated through posttranslational modification of BK-α protein (Lu et al., 2010) or a direct interaction between AT1R and BK-α proteins (Zhang et al., 2014). In addition, binding of BK-α to Cav scaffolding domain results in a decrease of BK channel current density in cultured endothelial cells (Wang et al., 2005; Riddle et al., 2011). Interestingly, we found that there is an increase of colocalization of BK-α and AT1R, but not BK-β1, in the caveolae of diabetic vascular SMCs.
Such cellular distribution of protein profiles will greatly suppress BK-α activity not only through proximity with AT1R and its downstream signaling, but also through a physical dissociation with BK-β1. This contention is supported by the finding that with the loss of caveolae integrity, coronary BK channel activity is preserved and I/R-induced myocardial infarct size in diabetic mice is attenuated.

Ang II plays an essential role in the regulation of cardiovascular homeostasis. AT1R and its associated signaling proteins, such as G-proteins, non-phagocytic NAD(P)H oxidases (NOXs) and c-Src kinase, are colocalized in the caveolae of vascular SMCs (Ushio-Fukai & Alexander, 2006; Lu et al., 2010). Upon stimulation by Ang II, AT1R translocates into the caveolae, activates protein kinase C and NOXs, promotes reactive oxygen species (ROS) generation (Touyz & Schiffrin, 2000) and leads to the redox-mediated protein modification of BK channels (Lu et al., 2010). It is known that development of diabetic vascular dysfunction is associated with augmented Ang II signaling and increased ROS production (Van Linthout et al., 2008; Giacco & Brownlee, 2010). However, the roles of the renin-angiotensin system in cardiac I/R injury remain controversial. Autoradiography and immunohistochemistry experiments found that AT1R expression in the infarct area of rats was upregulated after myocardial I/R (Higuchi et al., 2010). AT1R antagonists preserved cardiac function against I/R injury (Flynn & Akers, 2003; Han et al., 2013). In contrast, some studies suggested that Ang II had protective effects on cardiac I/R injury through Ang II initiated myocardial preconditioning as a result of increase in mitochondrial ROS generation with activation of JNK and p38 MAP kinase pathways (Kimura et al., 2005; Das et al., 2006). Reasons for the discrepancy among these studies are unclear, but they may reflect the differences in the animal species used and experimental conditions employed. In particular, the range of Ang II concentration for vasoconstriction studies was
varied from $10^{-12}$ to $10^{-4}$ M and the efficiency of Ang II was vascular bed dependent (Pallone, 1994; Batenburg et al., 2004; Patzak et al., 2005; Park et al., 2012). Since there was a 10-fold increase of Ang II production in cardiomyocytes of STZ-induced diabetic rats (Singh et al., 2008), we applied Ang II in $10^{-6}$ M range to Langendorff-perfused hearts to mimic a gain of activity of renin-angiotensin in diabetic hearts as we have published (Lu et al., 2010). We found that the worsening of I/R-induced myocardial injury by Ang II was caveolae-dependent and mediated through the inhibition of coronary BK channel function. In Cav-1<sup>−/−</sup> hearts, the I/R-induced myocardial infarct size produced by Ang II was about 40% smaller than that in wt mice, suggesting the role of caveolae in mediating the detrimental effects of Ang II. However, the effect of IBTX on I/R-induced myocardial infarction was similar between Cav-1<sup>−/−</sup> and wt mice, indicating that coronary BK channels are the downstream of AT1R signaling.

Our study has potential limitations. First, cardiovascular complications are commonly associated with type 2 diabetes mellitus. However, STZ injection usually induces type 1 diabetes rather than type 2 diabetes. Use of high fat-diet or genetic induced type 2 diabetic mouse models will have a better clinical relevant. Second, the maximal efficacy of Ang II is about in $10^{-7}$~$10^{-8}$ M range. We applied a relative high Ang II concentration to isolated perfusion hearts to mimic an increase of Ang II production in diabetes, although we did not measure serum Ang II level in STZ diabetic mice. However, we have shown deleterious roles of caveolae and diabetes in myocardial I/R study in vivo, similar to the results observed from ex vivo study. Hence, we believe that conclusions derived from these experiments are valid.

In summary, our study demonstrated a novel mechanism by which the receptor-channel-caveolae microdomain complexes in coronary SMCs suppressed BK channel function and exacerbated myocardial infarction in diabetic mice. Protection and activation of coronary BK
channel function may provide a new strategy in the prevention and the treatment of cardiovascular complications in diabetes.

**Conflict of interest:** The authors declare that they have no conflict of interests in the contents of this article.

**Acknowledgement**

This work was supported by grants from the American Diabetes Association (1-12-BS-119 and 1-16-IBS-195), and from the National Institutes of Health (HL-74180 and HL-080118).

**Author contributions:** T. L. designed research, performed research, analyzed data and wrote paper; B. J. and X-L.W. performed experiments and analyzed results. H-C.L. discussed and prepared the manuscript.

**Footnotes:** Ang II, Angiotensin II; AT\(_1\)R, Ang II type 1 receptor; BK channel, the large conductance Ca\(^{2+}\)-activated K\(^+\) channel; BK-\(\alpha\), BK channel \(\alpha\) subunit; Cav, Caveolin; Co-IP, co-immunoprecipitation; ECG, Electrocardiogram; IBTX, iberiotoxin; IPs, immunoprecipitates; I/R, ischemia/reperfusion; SMC, smooth muscle cell; STZ, streptozotocin; TTC, 2,3,5-triphenyltetrazolium chloride; wt, wild type.
References


Gelpi, R.J., Morales, C., Cohen, M.V., and Downey, J.M. 2002. Xanthine oxidase contributes to preconditioning's preservation of left ventricular developed pressure in isolated rat heart: developed pressure may not be an appropriate end-point for studies of preconditioning. Basic Res. Cardiol. 97: 40-46. PMID:11998976


Singh, H., Stefani, E., and Toro, L. 2012. Intracellular BK$_{Ca}$ (iBK$_{Ca}$) channels. J. Physiol. 590: 5937-5947. PMID:22930268


Suzuki, Y., Yamamura, H., Ohya, S., and Imaizumi, Y. 2013. Caveolin-1 Facilitates the Direct Coupling between large conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) and Cav1.2 Ca$^{2+}$ channels and their clustering to regulate membrane excitability in vascular myocytes. J. Biol. Chem. 288: 36750-36761. PMID:24202214


Zhang, Z., Li, M., Lu, R., Alioua, A., Stefani, E., and Toro, L. 2014. The angiotensin II type 1 receptor (AT1R) closely interacts with large conductance voltage- and Ca\(^{2+}\)-activated K\(^+\) (BK) channels and inhibits their activity independent of G-protein activation. J. Biol. Chem. 289: 25678-25689. PMID:25070892
Table 1. Left ventricular developed pressure (LVDP, mm Hg) of Langendorff-perfused mouse hearts before after ischemia/reperfusion procedure

<table>
<thead>
<tr>
<th>Mice</th>
<th>WT, Ctrl</th>
<th>WT, Ang</th>
<th>WT, Ang</th>
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<th>WT, STZ</th>
<th>WT, STZ</th>
<th>Cav-1&lt;sup&gt;−/−&lt;/sup&gt;,</th>
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<td>Before I/R</td>
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<td></td>
<td>86.2±7.3</td>
<td>64.2±5.3</td>
<td>81.9±7.5</td>
<td>83.6±5.6</td>
<td>90.5±4.2</td>
<td>83.2±4.9</td>
<td>72.2±8.7</td>
<td>76.4±5.0</td>
<td>78.8±3.4</td>
<td>86.6±4.0</td>
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<tr>
<td>After I/R</td>
<td>53.4±4.1*</td>
<td>34.1±2.6*</td>
<td>34.2±3.9*</td>
<td>48.1±4.7*</td>
<td>52.9±3.0*</td>
<td>30.1±5.3*</td>
<td>52.9±3.1*</td>
<td>49.2±5.3*</td>
<td>38.7±5.9*‡</td>
<td>48.1±4.2*</td>
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LVDP was significantly decreased in each group after ischemia reperfusion (I/R) procedure. NS1619, losartan and caveolin-1 knockout (Cav-1<sup>−/−</sup>) counteract the exacerbated effects of diabetes, Ang II and IBTX on LVDP after I/R. Data are presented as mean±S.E.M. *: p<0.05 vs before I/R; †: p<0.05 vs wt controls after I/R; ‡: p<0.05 vs Cav-1<sup>−/−</sup> control mice after I/R.
Figure Captions

Figure 1. In vivo myocardial I/R injury studies in wt and Cav-1<sup>−/−</sup> mice. (A) Myocardial infarction of mice was induced by a 40-min occlusion of the left anterior descending (LAD) coronary artery followed by a 60-min period of blood reflow. Body surface ECG and femoral artery blood pressure were recorded in diabetic wt mice throughout I/R procedure. Selected ECG recordings before and after 40 min of LAD occlusion are expanded to show S-T segment elevations (arrows) and pathological Q waves (asterisks) during blood reflow. (B) Representative photographs showing the healthy tissue and the infarct areas in myocardial slices. Diabetic wt mice (STZ) had a significant increase in I/R-induced myocardial infarct size, compared to that of non-diabetic wt controls. However, diabetes has no effect on infarct size in Cav-1<sup>−/−</sup> mice. Group data with statistical analyses are shown in the bar graphs. ∗: p<0.05 vs wt controls; †: p<0.05 vs wt STZ.

Figure 2. Ex vivo myocardial I/R injury studies in wt mice. Representative photographs of myocardial slices from wt control and diabetic mice under different experimental conditions as indicated. Isolated hearts were pretreated with chemicals for 30 min, followed by 40 min of global ischemia and then 60 min of reperfusion with the same chemicals. Treatment with Ang II (2 µM) and IBTX (0.2 µM) produced a significantly larger infarct size, with values similar to that in diabetes (STZ). The effects of Ang II on I/R-induced infarct size were reduced by NS1619 (10 µM) and Losartan (10 µM). However, the effect of IBTX was not altered by NS1619. Group data with statistical analysis are shown in the bar graphs. ∗: p<0.05 vs controls; †: p<0.05 vs STZ; ‡: p<0.05 vs Ang II+NS1619.

Figure 3. Ex vivo myocardial I/R injury studies in Cav-1<sup>−/−</sup> mice. Representative photographs of myocardial slices from Cav-1<sup>−/−</sup> mice under different experimental conditions as indicated.
Myocardial infarct size induced by I/R injury was increased by Ang II (2 µM) and with diabetes, but the lesions were about 40% smaller than those in wt mice. Infarct size in Cav-1−/− mice doubled after pretreatment with IBTX, comparable to those with Ang II and with diabetes (STZ), suggesting the BK channel activity is important in determining infarct size and the absence of Cav-1 is protective against I/R-induced injury. Group data with statistical analysis are shown in the bar graphs. ∗: p<0.05 vs controls; †: p<0.05 vs Ang II and STZ; ‡: p<0.05 vs STZ.

**Figure 4. Colocalization of BK-α and AT1R, but not BK-β1, in the caveolae of vascular SMCs.** (A) Western blots showing BK-α and AT1R proteins were present in the low-buoyant density, caveolae-rich membrane fractions (fraction No. 2 to 4) and in cell lysates of mouse aortas. There were very little protein expression of BK-β1 in the caveolae-rich membrane fractions, compared to that of cell lysate and the heavy density fractions (No. 7 to 8). (B) Freshly isolated coronary SMCs of mice were incubated with anti-Cav-1, anti-BK-α and anti-AT1R antibodies, and the primary antibodies were detected by fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody or with Texas Red-conjugated goat-anti-rabbit secondary antibody. Fluorescence images show the overlap of the signals between BK-α (red) and Cav-1 (green) (upper panel), as well as between AT1R (red) and BK-α (green) (lower panel). Pixel-by-pixel colocalization was estimated using a linear equation with the slope (Pearson correlation coefficient, γ) of 0.77 and 0.82 respectively. (C) Western blotting analysis shows the pulldowns of anti-Cav-1 antibody from two pairs of control and STZ-induced diabetic mouse aorta homogenates blotted against anti-BK-α and anti-AT1R antibodies. BK-α and AT1R proteins were markedly enriched in the pulldowns of diabetic mouse aortas. Group data with statistical significance are illustrated in the bar graphs.
Figure 5. Effects of Ang II on HEK293 cells stably expressing hSlo. (A) hSlo currents were continuously elicited at +100 mV from a holding potential of -60 mV at 10-s intervals in HEK293 cells stably expression hSlo gene (hSlo-HEK293 cell line), 48 h after transient transfection with Cav-1 and AT1R wt cDNAs. The time course (left panel) and representative tracings (right panel) of hSlo currents in response to Ang II (2 µM), Losartan (10 µM) and IBTX (0.1 µM) are shown. Ang II inhibited the hSlo currents by 50% and the effect of Ang II was blocked by Losartan. (B) The I-V curves of hSlo currents recorded from hSlo-HEK293 cell line 48 h after co-expression with AT1R wt cDNA at baseline and exposure to 2 µM Ang II (left) or 2 µM Ang II + 10 µM Losartan (right) show that the inhibitory effects of Ang II on hSlo currents were blocked by Losartan. *: <0.05 (n=5 for both groups).

Figure 6. Mutation in the Cav-1 binding motif in AT1R abolished the Ang II effect on hSlo currents. (A) Time course of the effects of Ang II (2 µM), IBTX (0.1 µM) and wash out of chemicals on hSlo currents stably expressed in HEK293 cells, 48 h after co-transfection with Cav-1 wt and AT1R F309A mutant cDNAs. The Ang II did not inhibit hSlo channels which were inhibited by IBTX, indicating that hSlo channels were present and active but the AT1R mutant was unable to mediate the inhibitory effects of Ang II on BK channels. (B) Whole-cell hSlo currents recorded from hSlo-HEK293 cells co-expressing Cav-1 and AT1R F309A (left column) and their I-V relationships (right column) elicited from -40 mV to +150 mV at a holding potential of -60 mV before and after exposure to Ang II. Ang II failed to inhibit the hSlo channels.