Selective Downregulation of Kv2.1 Function Contributes to Enhanced Arterial Tone During Diabetes

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Running title: AKAP150 suppresses vascular Kv2.1 expression in diabetes

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Background: Kv channels in VSMCs regulate arterial tone.

Results: Kv2.1 in VSMCs is downregulated via AKAP150-CaN-dependent NFATc3 signaling during diabetes.

Conclusions: Transcriptional suppression of Kv2.1 contributes to enhanced arterial tone in diabetes.

Significance: AKAP150-CaN-dependent activation of NFATc3 may be a general mechanism for transcriptional regulation of K+ channels, and a valuable target to prevent and treat diabetic vascular complications.

Abstract
Enhanced arterial tone is a leading cause of vascular complications during diabetes. Voltage-gated K+ (Kv) channels are key regulators of vascular smooth muscle cells (VSMCs) contractility and arterial tone. Whether impaired Kv channel function contributes to enhanced arterial tone during diabetes is unclear. Here, we demonstrate a reduction in Kv-mediated currents (I_Kv) in VSMCs from a high fat diet (HFD) mouse model of type 2 diabetes. In particular, I_Kv sensitive to stromatoxin (ScTx), a potent Kv2 blocker, were selectively reduced in diabetic VSM. This was associated with decreased Kv2-mediated regulation of arterial tone and suppression of Kv2.1 subunit mRNA and protein in VSM/arteries isolated from HFD mice. We identified A-kinase anchoring protein 150 (AKAP150), via targeting of the phosphatase calcineurin (CaN), and the transcription factor nuclear factor of activated T-cells c3 (NFATc3) as required determinants of Kv2.1 suppression during diabetes. Interestingly, substantial reduction in transcript levels for Kv2.1 preceded downregulation of large-conductance Ca2+-activated K+ (BKCa) channel β1 subunits, which are ultimately suppressed in chronic hyperglycemia to a similar extent. Together, our study supports the concept that transcriptional suppression of Kv2.1 by activation of the AKAP150-CaN/NFATc3 signaling axis contributes to enhanced arterial tone during diabetes.

Introduction
Non-insulin dependent type 2 diabetes is a devastating disease affecting millions worldwide due to an aging population, sedentary lifestyle, and overnutrition. Vascular dysfunction, including enhanced arterial tone, is a leading cause of cardiovascular complications contributing to morbidity and mortality in the diabetic population (1). While endothelial dysfunction has long been recognized as a major factor contributing to vascular dysfunction and enhanced arterial tone
during diabetes (2-5), abnormal VSMC function may also play a critical role, although the mechanisms for this remain poorly understood. Thus, advances in this area could prove valuable for the development of rational therapies to treat and prevent diabetic vascular complications.

The level of arterial tone is largely determined by VSMC membrane potential ($V_M$) and Ca$^{2+}$ entry via voltage-gated, L-type Ca$^{2+}$ channels (LTCCs) (6). A major regulator of $V_M$ is the activity of $K_V$ and $BK_{Ca}$ channels (7-9). Physiological activation of these channels hyperpolarizes VSMCs, thereby decreasing LTCC open probability and Ca$^{2+}$ influx leading to vasodilation, whereas their inhibition promotes vasoconstriction (10). Previous reports have shown that in vitro, short-term exposure of coronary, cerebral and mesenteric arteries to extracellular glucose concentrations that resemble diabetic hyperglycemia (e.g. 20 mM) inhibit $K_V$ and $BK_{Ca}$ channel activity in VSMCs (11-15). Thus, inhibition of these channels may contribute to enhanced arterial tone and vascular complications during diabetes. Consistent with this, our group and others have found that the activity of $BK_{Ca}$ channels is suppressed in VSMCs of several mouse models of diabetes (14-17). However, whether impaired $K_V$ channel activity in VSMCs contributes to enhanced arterial tone during diabetes is currently unclear.

We recently demonstrated that suppression of $BK_{Ca}$ channel activity in a high fat diet (HFD) mouse model of type 2 diabetes proceeds through activation of the prohypertensive CaN/NFATc3 signaling pathway (14). Activation of NFATc3 required anchoring of CaN by the scaffolding protein AKAP150 (murine ortholog of human AKAP79) in diabetic cells (14). Considering that this transcription factor is also known to regulate the expression of $K_V$ channels in VSMCs (18), we postulated that activation of this pathway may also modulate $K_V$ function during diabetes.

In the present study, we tested the hypothesis that $K_V$ channel function in VSMCs is impaired and contributes to increased arterial tone in a HFD mouse model of type 2 diabetes, and that the mechanism involves activation of the AKAP150-CaN/NFATc3 signaling pathway. Consistent with this hypothesis, we show that $K_V$ channel function is decreased in VSMCs from wild type (WT) HFD mice due to a reduction in the expression of ScTx-sensitive $K_V2.1$, but not psora-4-sensitive $K_V1.2$ or $K_V1.5$ subunits. Genetic ablation of NFATc3, AKAP150 or disruption of the AKAP150-CaN interaction prevented downregulation of the $K_V2.1$ subunit, suppression of ScTx -sensitive $I_{K_V}$, and enhanced arterial tone in HFD mice. Furthermore, our data indicate that downregulation of $K_V2.1$ occurs at an earlier time point (i.e. 1 hour) compared with $BK_{Ca}$ $\beta_1$ subunits under hyperglycemic conditions. These findings illuminate a critical role for AKAP150-anchored CaN, NFATc3 and $K_V2.1$ function in the regulation of arterial tone during diabetes.

**Materials and Methods**

**Animals:** WT (C57Bl/6J), AKAP150$^{-/-}$, NFATc3$^{-/-}$, and knock-in mice expressing AKAP150 lacking its CaN binding site (ΔPIX) (19) were euthanized with a lethal dose of sodium pentobarbital (250 mg/kg; intraperitoneally), as approved by the University of California, Davis Institutional Animal Care and Use Committee. Mice were sustained on either a low fat (10% kcal; ct) or high fat (60% kcal) diet (Research Diets, New Brunswick, NJ) starting at 5 weeks of age for 24-26 weeks. The composition of these diets and the propensity of mice maintained on this HFD to develop type 2 diabetes and induce vascular dysfunction of small resistance arteries have been well documented in previous studies (14,20,21).

Cerebral arteries were used for functional experiments (i.e. electrophysiology, arterial diameter, and immunofluorescence) while 3rd and 4th order mesenteric arteries were used for electrophysiology experiments in VSMCs from WT LFD and HFD mice and for molecular biology experiments requiring larger tissue samples (i.e. Western blots).

**Isolation of VSMCs:** VSMCs were dissociated from arteries using enzymatic digestion techniques as described previously (18,22,23). Middle and posterior cerebral arteries, as well as third and fourth order mesenteric arteries were dissected in ice-cold dissection buffer composed of (in mM): 140 NaCl, 5 KCl, 2 MgCl$_2$, 10 D-glucose, 10 HEPES, pH 7.4 with NaOH. Cerebral arteries were digested in a dissection solution supplemented with papain (1 mg/mL) and
dithiothreitol (1 mg/mL) at 37°C for 7 minutes, then incubated in dissection buffer supplemented with collagenase type H (0.3 mg/mL) and collagenase type F (0.7 mg/mL) at 37°C for 7 minutes. Cells were then washed in ice-cold dissection buffer. Glass pipettes of decreasing diameters were used to gently triturate arteries and obtain single VSMCs. Isolated cells were maintained in ice-cold dissection buffer until use.

**Electrophysiology:** $I_{K_v}$ from freshly dissociated VSMCs were measured using the conventional whole-cell patch-clamp technique with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Currents were evoked by 0.6 s depolarizing pulses from a holding potential of -70 mV to +80 (for psora-4-sensitive currents) or to +40 mV (for ScTx-sensitive currents) in increments of +10 mV. A voltage error of 15 mV attributable to liquid junction potential of the recording solutions used was corrected offline. $I_{K_v}$ were recorded in the continuous presence of the BK$_{Ca}$ channel blocker iberiotoxin (IbTx; 100 nM) to eliminate BK$_{Ca}$ channel activity. Bath solution consisted of the following components (in mM): 120 NaCl, 3 NaHCO$_3$, 4.2 KCl, 1.2 KH$_2$PO$_4$, 2 MgCl$_2$, 0.1 CaCl$_2$, 10 D-glucose and 10 HEPES, (pH 7.4). Patch pipette solution was composed of (in mM): 110 K-Gluconate, 30 KCl, 0.5 MgCl$_2$, 10 EGTA, 5 HEPES, 5 NaATP, and 1 GTP (pH 7.2 with KOH). Experiments were carried out at room temperature (22-25°C). Currents were sampled at 20 kHz and low-pass filtered at 5 kHz. Electrophysiology recordings were analyzed using pCLAMP 10.

**Quantitative polymerase chain reaction:** $K_v$ transcript expression was analyzed in single isolated cells using the Power SYBR Green Cells-to-CT kit (Life Technologies). For these experiments, single VSMCs were collected after enzymatic isolation using a glass micropipette. RT-product was used for quantitative PCR. Specific primers to detect $K_v$2.1 (NM_008420; reference number: QT00285971), $K_v$1.2 (NM_008417; reference number: QT00128100), and $K_v$1.5 (NM_145983; reference number: QT00268387) were acquired from Qiagen (Valencia, CA). β-actin was used as an internal control (GenBank accession number V01217; sense nt 2384-2404 and antisense nt 3071-3091). Amplification was performed using a Power SYBR Green PCR mix (Life Technologies, Carlsbad, CA) and an Applied Biosystems real-time PCR instrument. Expression for each gene was normalized to β-actin and expressed as a percentage of LFD.

**Western blot analysis:** Cerebral and mesenteric arteries were homogenized in a triton lysis buffer solution containing (in mM) 150 NaCl, 10 Na$_2$HPO$_4$, 1 EDTA with 1% deoxycholic acid, 0.1% sodium dodecyl sulfate and protease inhibitors (Complete Mini protease inhibitor cocktail, Roche, San Francisco, CA), followed by sonication (20 minutes at 4°C). Tissue debris and nuclear fragments were removed by centrifugation at 10,000 rpm (10 min, 4°C) and whole cell lysates were obtained as the supernatant. An equal amount of protein was loaded for each tissue lysate. Proteins were separated under reducing conditions on a 10% polyacrylamide gel (Bio-Rad, Hercules, CA) by electrophoresis at 100 V for 1.5 hr. Proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane at 20 V (overnight, 4°C). Membranes were washed in tris-buffered saline with 0.1% tween 20 (TBS-t) and blocked with 10% nonfat milk in TBS-t (1 hr, room temperature). Membranes were then incubated for 2 hours at room temperature with subunit specific antibodies from NeuroMab (University of California, Davis; Davis, CA). Antibodies and dilutions were as follows: mouse anti-$K_v$2.1 (73-014 clone K89/34; 1:2), anti-$K_v$1.2 (clone 14/16; 1:2) and $K_v$1.5 (clone K7/45; 1:2). Monoclonal antibody against β-actin (MA5-15739; 1:5,000) was from Pierce. Antibodies were prepared in TBS-t with 1% bovine serum albumin and 0.01% sodium azide. Membranes were then incubated (1 hr, room temperature) with horseradish peroxidase-labeled goat anti-mouse (sc-2005; 1:5,000; Santa Cruz) in TBS-t containing 5% nonfat dried milk. Bands were identified by enhanced chemiluminescence and exposure to X-ray film. Densitometry for immunoreactive bands was performed with ImageJ software (National Institutes of Health). β-actin was used as input control for normalization. Density was expressed as a percentage of control (LFD).
**Immunofluorescence**: Immunofluorescent labeling of freshly isolated VSMCs was performed as described previously (24) using a monoclonal antibody specific for Kv2.1 subunits (NeuroMab; 75-159; clone K39/25, University of California, Davis, CA). The secondary antibody was an Alexa Fluor 488-conjugated donkey anti-mouse (5 mg/mL) from Molecular Probes. Cells were imaged (512 X 512 pixel images) using an Olympus FV1000 confocal microscope coupled with an Olympus 60X oil immersion lens (NA = 1.4) and a zoom of 3.5 (pixel size = 0.1 µm). Images were collected at multiple optical planes (z-axis step size = 0.5 µm). The specificity of the primary antibody was tested in negative control experiments in which the primary antibody was substituted with PBS. Kv2.1-associated fluorescence was undetected under this experimental condition. Cells for each group were imaged with the same laser power, gain settings, and pinhole for all the treatments.

**Arterial diameter measurements**: Freshly isolated posterior cerebral arteries were cannulated on glass micropipettes mounted in a 5 mL myograph chamber (Living Systems Instrumentation, St. Albans, VT) as described previously (14). Arteries were pressurized to 20 mmHg and allowed to equilibrate while continuously superfused (37°C, 30 min, 3-5 mL/min) with physiological saline solution consisting of (in mM): 119 NaCl, 4.7 KCl, 2 CaCl₂, 24 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, 0.023 ethylenediaminetetraacetic acid (EDTA), and 10 D-glucose aerated with 5% CO₂/95% O₂. Bath pH was closely monitored and maintained at 7.35-7.40. Following equilibration period, intravascular pressure was increased to 60 mmHg and arteries were allowed to develop myogenic tone. Arteries not exhibiting stable myogenic tone after ~1 hour were discarded. To assess the contribution of Kv1.X and Kv2.1 channel function to regulation of arterial tone, the Kv1.X inhibitor psora-4 (500 nM) and Kv2.1 inhibitor ScTx (50 nM), respectively, were added to the superfusate. Arterial tone data is presented as a percent decrease in diameter relative to the maximum passive diameter at 60 mmHg obtained at the end of each experiment using Ca²⁺-free saline solution containing nifedipine (1 µM).

**Chemicals and Statistical analyses**: All chemical reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Iberiotoxin was from Peptides International and ScTx-1 was from Alomone Labs. Data are expressed as mean ± SEM. Data obtained using multiple vessels from the same animal were pooled for statistical analyses. Data were analyzed using GraphPad Prism software. Statistical significance was determined by Student’s t-test or one-way analysis of variance followed by Tukey multiple comparison test for comparison of multiple groups. \( P < 0.05 \) was considered statistically significant (denoted by * in figures).

**Results**

Freshly isolated, small diameter (~75-150 µm) cerebral and mesenteric arteries and VSMCs from age-matched mice fed ad libitum with either a low fat (LFD, 10% kcal) or high fat (HFD, 60% kcal) diet were used for this study (14,20). This HFD model of type 2 diabetes was employed because it does not depend on genetic manipulations or chemical destruction of pancreatic β-cells. In addition, it recapitulates clinical features observed in patients with type 2 diabetes (20,25), including enhanced arterial tone and increased blood pressure (14). WT mice on a HFD had significantly higher average body weight (42 ± 1.0 g) than WT LFD (30 ± 0.6 g; Table 1). Furthermore, non-fasting blood glucose levels were significantly increased in WT HFD (301 ± 12 mg/dL) compared to WT LFD mice (145 ± 4 mg/dL; Table 1).

**\( I_{Kv} \) function is suppressed in VSM from WT diabetic mice**

Figure 1 shows \( I_{Kv} \) evoked in cerebral VSMCs freshly dissociated from WT LFD and HFD mice in the presence of 100 nM iberiotoxin (IbTx) to inhibit BKCa channels. The average capacitance of cerebral WT LFD and HFD cells was 19.4 ± 1.2 pF and 19.0 ± 1.2 pF, respectively (\( P > 0.05 \)). \( I_{Kv} \) were initially elicited by 600 ms depolarizing pulses from a holding potential of -85 mV to voltages ranging from -85 mV to +65 mV in 10 mV increments. We found that the amplitude of control \( I_{Kv} \) from WT HFD were significantly lower than that from WT LFD (Figure 1Ai). Indeed, the current-voltage relationship of control \( I_{Kv} \), revealed that current densities were smaller in WT HFD
than in WT LFD at most voltages examined (Figure 1Bi; $P < 0.05$). At $+65$ mV, control $I_{K_v}$ were $\sim 42\%$ smaller in WT HFD ($21.0 \pm 1.8$ pA/pF; $P < 0.05$) when compared to WT LFD ($36.4 \pm 3.5$ pA/pF; $P < 0.05$; Figure 1Bi). Similar results were observed in mesenteric VSMCs under same experimental conditions (Figure 1Bi and 1Di, insets). These results indicate impaired $I_{K_v}$ in cerebral and mesenteric VSMCs during diabetes.

**Impaired stromatoxin sensitivity in VSM and arteries from WT diabetic mice**

Previous studies indicate that $K_{V1.2}$, $K_{V1.5}$ and $K_{V2.1}$ subunits make predominant contributions to $I_{K_v}$ in VSMCs from cerebral and mesenteric arteries (8,9,26-28). Thus, we examined whether impaired function of $K_{V1.2}$- and/or $K_{V2.1}$-containing channels contributes to suppressed $I_{K_v}$ in VSMCs during diabetes. This was achieved by selectively inhibiting either $K_{V1}$ channels with psora-4 (500 nM) (29) or $K_{V2}$ channels with ScTx (100 nM) (8,9). These agents have been used to evaluate the relative contribution of $K_{V1.2}$ and $K_{V2.1}$ channels in the same VSMCs employed in the present study (8,9,30,31).

$I_{K_v}$ were recorded from cerebral VSMCs as described above. The addition of psora-4 decreased $I_{K_v}$ at $+65$ mV by $\sim 30\%$ in both LFD and HFD VSMCs (Figure 1A ii). However, the amplitude and current-voltage relationship of the psora-4-sensitive $I_{K_v}$ component was similar between these cells (Figure 1Ai iii and 1Bi ii). Inhibition of $K_{V2}$ channels with ScTx becomes partial at positive potentials greater than $+30$ mV (32). Thus, $K_{V}$ currents were evoked in the presence of ScTx by voltage steps from $-85$ to $+25$ mV. Application of ScTx reduced $I_{K_v}$ at $+25$ mV by $\sim 70\%$ in LFD and $\sim 50\%$ in HFD cells (Figure 1Ci i and 1Di ii). Unlike psora-4-sensitive $I_{K_v}$, the amplitude of ScTx-sensitive $I_{K_v}$ was significantly smaller across a range of voltages in WT HFD compared to those in WT LFD ($5.8 \pm 0.9$ pA/pF vs. $14.2 \pm 2.0$ pA/pF at $+25$ mV, respectively; Figure 1Ci i and 1Di iii; $P < 0.05$). Similar results were observed in mesenteric VSMCs (Figure 1B i and 1D i, insets). These data suggest that a reduction in $I_{K_v}$ observed in cerebral and mesenteric VSMCs from HFD mice is due to reduced ScTx-sensitive $I_{K_v}$.

The functional significance of HFD-associated alterations in $I_{K_v}$ function was assessed by measuring psora-4 and ScTx sensitivity of arterial tone in cerebral arteries from WT LFD and HFD mice. At $60$ mmHg intravascular pressure, WT HFD arteries were significantly more constricted than WT LFD (Figure 2A and 2B). Whereas application of psora-4 (500 nM) caused a significant constriction in both WT LFD ($13 \pm 2\%$) and HFD ($17 \pm 4\%$) arteries (Figure 2A and 2C), ScTx (50 nM) induced constriction only in WT LFD arteries (Figure 2B and 2D). Indeed, ScTx had little effect on arteries from WT HFD ($3 \pm 1\%$) as compared to LFD ($11 \pm 2\%$; $P < 0.05$). Arteries from all groups responded with robust constriction to $60$ mM extracellular K$^+$ concentration (Figure 2E) or phenylephrine (14), suggesting that altered ScTx response was not due to differences in the magnitude of tone development. These results are consistent with impaired ScTx-sensitive $I_{K_v}$ contributing to enhanced arterial tone during diabetes.

**Downregulation of $K_{V2.1}$ subunits in VSM during diabetes**

Impaired sensitivity to ScTx, but not psora-4, in diabetic cells and arteries could reflect selective downregulation of $K_{V2.1}$ subunit expression. To test this, we evaluated $K_{V1.2}$, $K_{V1.5}$ and $K_{V2.1}$ transcript levels using qPCR on single isolated cerebral and mesenteric VSMCs from WT LFD and HFD mice. We found that $K_{V2.1}$ transcript expression was $\sim 65\%$ lower in WT HFD than in WT LFD cells, with no detectable change in the expression of $K_{V1.2}$, and $K_{V1.5}$ transcripts (Figure 3A). Consistent with this, $K_{V2.1}$, but not $K_{V1.2}$ or $K_{V1.5}$, protein levels were significantly reduced ($\sim 75\%$) in WT HFD arterial lysates (Figure 3B).

Immunofluorescence was also used to determine changes in $K_{V2.1}$ subunit expression in isolated cerebral VSMCs from WT LFD and HFD with a $K_{V2.1}$-specific antibody. As illustrated in Figure 3C, we observed a strong intensity of the $K_{V2.1}$-associated fluorescence along the sarcolemma of this WT LFD cell. No $K_{V2.1}$-associated fluorescence was observed when the primary or secondary antibodies were excluded from the preparation. Conversely, the intensity of the $K_{V2.1}$-associated fluorescence along the sarcolemma, under the same experimental conditions, was markedly lower in cells from WT HFD mice (Figure 3C). Altogether, these data
indicate that suppression of ScTx-sensitive \( I_{Kv} \) in VSMCs and impaired ScTx-induced vasoconstriction during diabetes results from downregulation of \( \text{Kv2.1} \) subunit expression.

**AKAP150-anchored CaN and NFATc3 activity are necessary for impaired ScTx-sensitive \( I_{Kv} \) function and downregulation of \( \text{Kv2.1} \) subunit expression during diabetes**

CaN and NFATc3 are known to regulate transcription of \( \text{Kv2.1} \) in VSMCs (18) and their activity is enhanced in diabetic arteries (14). Activation of CaN and CaN-mediated activation of NFATc3 in VSMCs of diabetic animals is dependent upon sarcolemmal phosphatase targeting by AKAP150 (18,33). Thus, to test the involvement of this pathway in the suppression of \( \text{Kv2.1} \) in HFD cells, we first examined the role of AKAP150. To do so, we fed AKAP150-null (AKAP150-/-) mice with either a LFD or HFD. Non-fasting blood glucose levels and body weight in AKAP150-/- LFD and HFD mice were comparable with corresponding aged-match WT mice, and were elevated in HFD (Table 1).

We measured \( I_{Kv} \) in VSMCs from AKAP150-/- LFD and HFD mice before and after ScTx using the voltage protocol described above. VSMCs from AKAP150-/- LFD mice produced robust \( I_{Kv} \) (Figure 4A) that were comparable to those observed in WT LFD cells, suggesting that AKAP150 does not influence basal \( I_{Kv} \) in VSMCs of control mice. The average capacitance of AKAP150-/- LFD and HFD cells was 17.7 ± 1.2 pF and 16.4 ± 0.6 pF, respectively, which is similar to WT cells (P > 0.05). In contrast to diabetic WT cells, the amplitude of control and ScTx-sensitive \( I_{Kv} \) at +25 mV in AKAP150-/- HFD cells (13.0 ± 2.0 pA/pF) was similar to corresponding AKAP150-/- LFD cells (14.0 ± 2.0 pA/pF; Figure 4A and 4B). Likewise, no differences in ScTx-insensitive components were observed. Consistent with the electrophysiological data, \( \text{Kv2.1} \) transcript and protein levels (Figure 4D and 4E) were similar in cells and lysates, respectively, from AKAP150-/- LFD and HFD mice. Ablation of AKAP150 did not alter basal expression of \( \text{Kv2.1} \) subunits (Figure 4E). These data indicate that AKAP150 is required for suppression of \( \text{Kv2.1} \) subunit expression and function in diabetic VSMCs.

Next, we directly tested the role of AKAP150-CaN interaction in \( \text{Kv2.1} \) suppression during diabetes. To do this, we used VSMCs from LFD and HFD knock-in mice expressing mutant AKAP150 that is unable to bind CaN (\( \Delta \text{PIX} \)) (14,19). Non-fasting glucose levels and body weight were significantly elevated in \( \Delta \text{PIX} \) HFD compared to \( \Delta \text{PIX} \) LFD mice (Table 1). Selective disruption of AKAP150-CaN interaction abolished suppression of ScTx-sensitive \( I_{Kv} \) function and \( \text{Kv2.1} \) subunit expression during diabetes (Figure 4C-4E). These results indicate that subcellular targeting of CaN by AKAP150 is necessary for suppression of \( \text{Kv2.1} \) expression and function in VSMCs during diabetes.

The above findings are consistent with the concept that AKAP150-targeted CaN signals \( \text{Kv2.1} \) transcriptional suppression via downstream activation of NFATc3. Therefore, we investigated the role of NFATc3 in suppression of \( \text{Kv2.1} \) expression and function during diabetes by using NFATc3 null (NFATc3-/-) mice in LFD or HFD (Table 1). Figure 5A demonstrated that NFATc3-/- LFD and HFD myocytes produced robust \( I_{Kv} \). Consistent with a role for NFATc3, ScTx-sensitive \( I_{Kv} \) function and \( \text{Kv2.1} \) expression (Figure 5A-D) were similar in VSM and arteries from NFATc3-/- LFD and HFD mice. Together, our data indicate that anchoring of CaN by AKAP150 mediates impairment of \( \text{Kv2.1} \) expression and function during diabetes via downstream activation of NFATc3.

**Loss of AKAP150, AKAP150-targeted CaN or NFATc3 prevents enhanced arterial tone and restores ScTx-sensitive constriction in diabetic mice**

We examined the functional relevance of the AKAP150-anchored CaN and NFATc3 signaling module on arterial tone and ScTx sensitivity. Diameter measurements at 60 mmHg intravascular pressure were performed in cerebral arteries from AKAP150-/-, \( \Delta \text{PIX} \) and NFATc3-/- mice in LFD and HFD. Unlike ScTx observations in arteries from WT mice (see Figure 2B and 2D), arterial tone development and ScTx-induced constriction were similar in LFD and HFD vessels from AKAP150-/-, \( \Delta \text{PIX} \) and NFATc3-/- mice (Figure 6A-C). Altogether, these data indicate that
disruption of AKAP150-CaN interaction or ablation of NFATc3 restores normal Kv2.1 regulation of myogenic tone during diabetes.

**Differential suppression of Kv2.1 and BKCaβ1 expression at earlier stages of diabetic hyperglycemia**

We further examined the CaN/NFATc3 signaling axis in the suppression of Kv2.1 during diabetic hyperglycemia by determining Kv2.1 transcript expression in VSMCs from WT arteries incubated for 48 hours in media containing 10 mM D-glucose, 20 mM D-glucose, 20 mM D-glucose + 10 µM CaN inhibitory peptide (CiP; specific CaN inhibitor), and 20 mM D-glucose + 5 µM VIVIT (a selective NFAT inhibitor). These D-glucose concentrations are within the range of observed non-fasting blood glucose levels reported for LFD (5-10 mM) and HFD (20 mM) mice. As expected, 20 mM D-glucose caused > 60% reduction in Kv2.1 transcript that was not observed when the non-metabolized L-glucose was substituted for D-glucose or when CiP or VIVIT were present in the cultured media (Figure 7A). These results suggest that activation of the CaN/NFATc3 axis in VSMCs is necessary for transcriptional suppression of Kv2.1 during diabetic hyperglycemia.

In a previous study, we found that the regulatory BKCaβ1 subunit in VSMCs is downregulated via activation of AKAP150-CaN/NFATc3 signaling in hyperglycemic animals on HFD leading to BKCa channel impairment (14). Considering a potential common mechanism of AKAP150-anchored CaN in Kv2.1 and BKCaβ1 suppression during diabetic hyperglycemia, 20 mM D-glucose had no effect on transcript levels for either gene in VSMCs from ΔPIX mice (P > 0.05; Figure 7C). Together, these data suggest a distinct temporal profile of early Kv2.1 suppression during diabetic hyperglycemia, with subsequent concomitant downregulation of both genes to a similar extent that is mediated by direct activation of the AKAP150-anchored CaN/NFAT signaling pathway.

**Discussion**

In this study, we provide evidence implicating suppression of Kv channel function as a significant contributor of enhanced arterial tone during diabetes (Figure 7D). We report the following novel findings: First, I_Kv in cerebral and mesenteric VSMCs are significantly suppressed during diabetes. Second, impaired I_Kv in these cells leading to enhanced arterial tone during diabetes occurs due to selective downregulation of Kv2.1 expression. Third, AKAP150-anchored CaN and NFATc3 are obligatory components in the signaling pathway underlying suppression of I_Kv and Kv2.1 expression during diabetes. Fourth, although Kv2.1 and BKCaβ1 transcripts are reduced to a similar extent during established diabetic hyperglycemia, our data suggest that Kv2.1 suppression may occur at an earlier stage. The implications of these findings are discussed below.

Considerable data attribute impairment of endothelium-dependent vasodilatory mechanisms as a significant contributor to vascular dysfunction and enhanced arterial tone during diabetes (2-5). However, abnormal VSM function may also critically contribute to enhancement of arterial tone during this pathological condition. For instance, enhanced arterial tone could result from a reduction in outward K+ conductance in VSMCs (34). Consistent with this idea, our initial experiments demonstrate that Kv channel function is suppressed in diabetic VSMCs (Figure 1). This discovery is significant as a reduction in Kv channel function will result in membrane depolarization, and increased activity of LTCCs, ultimately leading to elevated global [Ca2+]i, and VSM contraction (6,7). Although an increase in
global cytosolic $[Ca^{2+}]_i$ could promote activation of BKCa channels to compensate for the loss of KV channel function via negative feedback control of $V_M$ depolarization, BKCa channel activity is also suppressed in WT HFD mice (14). Thus, synergistic impairment of KV and BKCa activity in diabetic animals may substantially reduce feedback membrane potential hyperpolarization leading to VSM contraction and enhanced arterial tone during diabetes (Figure 7D). Ultimately, this could significantly contribute to raise mean arterial blood pressure or limit blood flow in diabetic patients.

In this study, we found that psora-4, a selective KV1.X inhibitor (29-31,35), induced robust constriction in pressurized arteries from LFD and HFD mice. Yet, ScTx, which is a selective KV2 inhibitor in VSM (8,9), had a reduced effect on WT HFD vessels indicating impaired KV2 channel function during diabetes. Interestingly, we observed a trend toward a greater psora-4-induced constriction in pressurized arteries from WT HFD as compared to WT LFD mice, perhaps reflecting compensatory activation of additional KV1.X at more depolarized membrane potentials in pressurized arteries from diabetic mice. This difference however was not statistically significant. Note that vasoconstriction of cerebral vessels in response to KV2 or KV1 channel blockers is independent of functional endothelium (8,13). Considering this, it follows that reduced endothelial vasodilatory function, as observed in several experimental models of diabetes, would not significantly impact the contribution of KV2 channels to the regulation of arterial tone. Thus, altered response to ScTx during diabetes observed in this study likely reflects reduced expression of functional KV2 channels. Consistent with this, ScTx-sensitive and psora-4-insensitive (presumably produced by KV2.1 channels) current densities were significantly suppressed at most membrane potentials in HFD as compared to LFD cells. Conversely, the amplitude of the ScTx-insensitive currents, which presumably are predominantly mediated by KV1.X, and psora-sensitive currents were not different in VSM isolated from LFD and HFD mice. These data indicate that KV2.1, but not KV1.2 or KV1.5, transcript and protein in HFD cells/arteries when compared to LFD cells/arteries. Altogether, these results are the first indication that selective KV2.1 downregulation underlies reduced $I_{K_V}$ in VSM contributing to enhanced arterial tone during diabetes, and point to impairment of KV function as a mediator of vascular dysfunction.

An interesting observation in this study is that KV2.1 and BKCa β1 genes are differentially suppressed during diabetic hyperglycemia (Figure 7B). Our data indicate that within the first hour of incubation in a hyperglycemic solution that resembles the levels of plasma glucose in HFD mice, KV2.1, but not BKCa β1 expression, is significantly downregulated. Subsequently, concomitant suppression of both genes occurs to a similar extent within 48 hours of hyperglycemic exposure. These results suggest that KV2.1 and BKCa β1 genes have different thresholds for hyperglycemia-induced transcriptional suppression. Mechanisms of early temporal differences in KV2.1 and BKCa β1 expression during hyperglycemic stimuli are unclear, but may involve dissimilar thresholds for NFATc3-dependent suppression or differential mRNA degradation independent of the rate or magnitude of transcriptional suppression (36). While future studies should further investigate this issue, it is intriguing to speculate that early initiation of KV2.1 suppression could represent a key feature of pre-diabetic hyperglycemia, which drives pathophysiological engagement of the AKAP150-CaN/NFATc3 signaling pathway via membrane depolarization, LTCC activity and $Ca^{2+}$ influx (37,38). Subsequently, further NFATc3 nuclear accumulation reaches levels sufficient for BKCa β1 downregulation. Data demonstrating that disruption of AKAP150-CaN interaction or specific inhibition of CaN and NFAT in VSMCs prevents the downregulation of KV2.1 and BKCa β1 expression (see also (14)) and impaired arterial tone during diabetic hyperglycemia and in HFD gives credence to this hypothesis. However, future experiments should investigate the relationship between NFATc3 activity and KV2.1 and BKCa β1 expression in further detail.
Our data support a mechanistic model whereby targeting of CaN by AKAP150 drives NFATc3 activation during diabetes. Once activated, NFATc3 translocates into the nucleus of VSMCs where it reduces K\textsubscript{V}2.1 and subsequently BK\textsubscript{Ca}\beta1 expression (Figure 7D). Consistent with a critical role for AKAP150-anchored CaN, disruption of the interaction between these two proteins prevented NFATc3 dephosphorylation and nuclear accumulation during diabetes (14). This is correlated with restoration of K\textsubscript{V}2.1 (Figures 4, 6 and 7) and BK\textsubscript{Ca}\beta1 subunit expression and function, and attenuation of blood pressure in diabetic AKAP150\textsuperscript{+/-} and ΔPIX mice (Figure 7B-C, and (14)).

While our data argue against a role for AKAP150 in basal regulation of K\textsubscript{V} or BK\textsubscript{Ca} channel function (see Figure 1 and 4 and (14)), this anchoring protein is known to interact with LTCCs (39). Hence, AKAP150 may also function to position CaN near LTCCs (23) to efficiently activate Ca\textsuperscript{2+}-dependent CaN/NFATc3 signaling during diabetes. Indeed, we have previously found that NFATc3 is preferentially activated in VSMCs by LTCC-dependent Ca\textsuperscript{2+} microdomains (i.e. Ca\textsuperscript{2+} sparklets (38,40,41)), which are significantly elevated during diabetes (42), rather than by elevations in global [Ca\textsuperscript{2+}]i (40). Similar increases in LTCC-dependent Ca\textsuperscript{2+} microdomains have been observed in angiotensin II-induced hypertensive VSMCs (24,40) and after activation of reactive oxygen species (43). Thus, increases in local LTCC activity leading to activation of the AKAP-CaN/NFATc3 signaling pathway may represent a wide-ranging mechanism for development of vascular dysfunction in many pathological conditions. In the present model, NFATc3 nuclear accumulation and subsequent K\textsubscript{V}2.1 and BK\textsubscript{Ca}\beta1 downregulation is a dynamic process highly dependent on NFAT nuclear import and export rate (44), as well as non-fasting plasma glucose levels in diabetic animals that could be sufficient for NFAT activation.

In addition to transcriptional suppression of K\textsubscript{V}2.1 subunit expression, post-translational modification of this (and other K\textsubscript{V}) subunit may also contribute to a reduction in I\textsubscript{Kv} during diabetes (11-13,45-47). Hence, multiple mechanisms may potentially synergize and contribute to impaired K\textsubscript{V} function, and vascular complications during diabetes. Note however that activation of divergent mechanisms may vary between vessels and animal models of diabetes. Thus, the relative contribution of transcription-dependent and independent pathways to altered K\textsubscript{V} expression and function during diabetes warrants further investigation.

To summarize, our data demonstrate that suppression of K\textsubscript{V} channel function via selective downregulation of K\textsubscript{V}2.1 contributes to enhanced arterial tone during diabetes. AKAP150-CaN/NFATc3 signaling is central to impaired K\textsubscript{V}2.1 expression and function. Our findings also support the view that activation of this signaling pathway may be a general mechanism for transcriptional regulation of K\textsuperscript{+} channels and that with K\textsubscript{V}2.1, may be novel therapeutic targets to prevent and/or treat vascular complications during diabetes, and perhaps other pathological conditions.
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**Footnotes**
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The abbreviations used are: VSMCs, vascular smooth muscle cells; Kᵥ, voltage-gated K+ channels; Iᵥ, Kᵥ currents; Vₛ, membrane potential; BKᵥ, large conductance Ca²⁺-activated potassium channel; BKᵥ β1, BKᵥ beta 1 subunit; LTCC, L-type Ca²⁺ channels; [Ca²⁺], intracellular Ca²⁺ concentration; AKAP150, A-kinase anchoring protein 150; WT, wild type; LFD, low fat diet; HFD, high fat diet; ct, control; CaN, calcineurin; NFATc3, nuclear factor of activated T cells, c3 isoform; ΔPIX, AKAP150 lacking binding site for calcineurin; ScTx, stromatoxin; IbTx, iberiotoxin.
Figure Legends

Figure 1. Stromatoxin-sensitive $I_{Kv}$ are suppressed in VSM from WT HFD mice. (A-B) Exemplar whole-cell $I_{Kv}$ evoked by depolarizations from -85 to +65 mV and corresponding current-voltage relationships from cerebral and mesenteric (insets) LFD (n = 7 cerebral cells and 8 mesenteric cells from 5 mice) and HFD (n = 7 cerebral and 7 mesenteric cells from 5 mice) VSMCs before (i) and after (ii) psora-4 (500 nM) application and resultant psora-4-sensitive component (iii). Average capacitance of mesenteric WT LFD and HFD cells is 18.8 ± 1.9 pF and 19.5 ± 1.5 pF, respectively. Average peak $I_{Kv}$ at +65 mV in LFD and HFD mesenteric cells, respectively, were as follow; control: 37.4 ± 6.0 and 21.8 ± 3.2 pA/pF; +psora-4: 22.7 ± 3.0 and 11.9 ± 3.5 pA/pF; psora-4-sensitive: 17.2 ± 4.0 and 13.0 ± 2.4 pA/pF. (C-D) Representative $I_{Kv}$ evoked by depolarization from -85 to +25 mV and current-voltage relationship from cerebral and mesenteric (insets) LFD (n = 12 cerebral and 7 mesenteric cells from 7 mice) and HFD (n = 14 cerebral and 7 mesenteric cells from 5 mice) cells before (i) and after (ii) application of ScTx-1 (100 nM) and resultant ScTx-sensitive current (iii). Average capacitance of mesenteric WT LFD and HFD cells is 18.8 ± 1.9 pF and 19.5 ± 1.5 pF, respectively. Average peak $I_{Kv}$ at +25 mV in LFD and HFD mesenteric cells, respectively, were as follow; control: 21.4 ± 3.3 and 11.5 ± 2.8 pA/pF; +ScTx: 5.0 ± 1.5 and 5.9 ± 2.0 pA/pF; ScTx-sensitive: 16.5 ± 2.5 and 5.9 ± 1.3 pA/pF. Psora-4- and ScTx-sensitive components were obtained by digital subtraction of currents in the presence of the specific inhibitor from the currents before application of the inhibitor (control). *$P < 0.05$.

Figure 2. Impaired ScTx-induced constriction in arteries from WT HFD mice. Representative diameter recordings from pressurized (60 mmHg) WT cerebral arteries from LFD and HFD mice before and after (A) psora-4 (500 nM) or (B) ScTx (50 nM). (C) Bar plot summarizing arterial tone in WT LFD (n = 5 arteries from 5 mice) and HFD (n = 5 arteries from 5 mice) arteries in the absence (-) and presence (+) of (C) psora-4. (D) Arterial tone in the absence (-) or presence (+) of ScTx in WT LFD (n = 7 arteries from 5 mice) and HFD (n = 7 arteries from 6 mice) arteries. (E) Amalgamated data of arterial tone from WT LFD (n = 8 arteries from 5 mice) and HFD (n = 10 arteries from 5 mice) at 60 mmHg in response to 60 mM extracellular K+. *$P < 0.05$.

Figure 3. $K_{V2.1}$ subunit expression is downregulated in VSM and arteries from WT HFD mice. (A) Bar plot summarizing quantitative real-time PCR data for $K_{V1.2}$, $K_{V1.5}$, $K_{V2.1}$ and $K_{V9.3}$ transcript levels in WT VSMCs from LFD and HFD mice relative to control (normalized to $\beta$-actin; n ≈ 40-50 VSMCs from 7 mice per condition). (B) Representative blots of immunoreactive bands of expected molecular weight for $K_{V1.2}$ (~64 kDa), $K_{V1.5}$ (~66 kDa), $K_{V2.1}$ (~110 kDa) and $\beta$-actin (~43 kDa) (left), and corresponding (right) densitometry summary data (n = 4 lysates per condition). (C) Confocal images of $K_{V2.1}$-associated fluorescence in WT LFD and HFD VSMCs. Lower panels show DIC images for representative cells in the top panel (n = 14 cells from 3 mice per condition). *$P < 0.05$.

Figure 4. AKAP150-anchored CaN is necessary for impaired $K_{V2.1}$ expression and function in HFD cells. (A) Representative whole-cell $K_{V}$ currents from VSMCs isolated from LFD and HFD AKAP150−/− mice recorded before and after application of ScTx (100 nM), and the corresponding ScTx-sensitive component (n=11 cells from 5 LFD mice and 9 cells from 5 HFD mice). Current-voltage relationships of $I_{Kv}$ before and after ScTx and the corresponding ScTx-sensitive component in LFD and HFD (B) AKAP150−/− and (C) ΔPIX cells. (D) Bar plot of $K_{V2.1}$ transcript levels in AKAP150−/− and ΔPIX HFD cells relative to LFD normalized to $\beta$-actin (n ≈ 40-50 VSMCs from 3 mice per condition). (E) Western blot of immunoreactive bands of expected molecular weight for $K_{V2.1}$ (~110 kDa) and $\beta$-actin (~43 kDa) in control, LFD and HFD arteries from AKAP150−/− and ΔPIX mice (left) and corresponding (right) densitometry summary data (n = 4 lysates from 4 mice per condition). *$P < 0.05$.

Figure 5. NFATc3 is necessary for downregulation of $K_{V2.1}$ expression and function in HFD cells. (A) Representative whole-cell $K_{V}$ currents from VSMCs isolated from LFD and HFD NFATc3−/− mice
recorded before and after application of ScTx (100 nM) and the corresponding ScTx-sensitive component (n = 6 cells from 3 LFD mice and 8 cells from 3 HFD mice). 

**B** Current-voltage relationships of $I_{K_v}$ before and after ScTx and the corresponding ScTx-sensitive component in LFD and HFD NFATc3−/− cells. 

**C** Bar plot of $K_{v}2.1$ transcript levels in NFATc3−/− HFD cells relative to LFD normalized to β-actin (n ≈ 40-50 cells from 3 mice per condition). 

**D** Western blot of immunoreactive bands of expected molecular weight for $K_{v}2.1$ (~110 kDa) and β-actin (~43 kDa) in LFD and HFD arteries from NFATc3−/− mice (left) and corresponding (right) densitometry summary data (n = 3 lysates from 3 mice per condition). *P < 0.05.

**Figure 6. AKAP150-anchored CaN and NFATc3 are necessary for impaired arterial tone in HFD arteries.** Representative diameter recordings (left panel) and summary arterial tone data (right panel) from pressurized (60 mmHg) (A) AKAP150−/− LFD (n = 7 arteries from 5 mice) and HFD (n = 6 arteries from 4 mice), (B) ΔPIX LFD (n = 7 arteries from 3 mice) and HFD (n = 8 arteries from 3 mice) and (C) NFATc3−/− LFD (n = 6 arteries from 3 mice) and HFD (n = 6 arteries from 3 mice) cerebral arteries in the absence (−) and presence (+) of ScTx (50 nM). Maximum passive diameters for representative recordings are as follows: AKAP150−/− LFD: 150 µm, AKAP150−/− HFD: 145 µm; ΔPIX LFD: 161 µm, ΔPIX HFD: 154 µm; NFATc3−/− LFD: 153 µm, NFATc3−/− HFD: 155 µm. *P < 0.05 as compared to (−).

**Figure 7. Time course of $K_{v}2.1$ and $BK_{ca}$ β1 subunit mRNA expression during diabetic hyperglycemia, involvement of the AKAP150-CaN/NFAT axis, and model for AKAP150-anchored CaN-dependent suppression of $K_{v}$ and $BK_{ca}$ subunits during diabetes.** (A) Bar plot of $K_{v}2.1$ transcript levels in VSMCs from WT arteries incubated in medium supplemented with 10 mM D-glucose, 20 mM D-glucose, 5 mM D-glucose + 15 mM L-glucose, 20 mM D-glucose + 10 µM CiP and 20 mM D-glucose + 5 µM VIVIT (n ≈ 40-50 cells from 3 mice per condition). Time course of $K_{v}2.1$ and $BK_{ca}$ β1 transcripts in (B) WT and (C) ΔPIX VSMCs following incubation of arteries in 20 mM D-glucose (n ≈ 40-50 cells from 3 mice per condition). (D) Proposed model for AKAP150-CaN/NFATc3-dependent suppression of $K_{v}$ and $BK_{ca}$ channel function in VSMCs during diabetes. *P < 0.05.
Table 1: Body weight and non-fasting blood glucose in wild type, AKAP150\(^{-/-}\), ΔPIX and NFATc3\(^{-/-}\) mice fed low and high fat diet.

|                | Body Weight (g) | Blood glucose (mg/dL) |
|----------------|-----------------|-----------------------|
| wild-type      |                 |                       |
| LFD            | 30 ± 0.6        | 145 ± 4               |
| HFD            | 42 ± 1.0*       | 301 ± 12*             |
| AKAP150\(^{-/-}\) |                 |                       |
| LFD            | 25 ± 1.1        | 148 ± 4               |
| HFD            | 36 ± 3.0*       | 264 ± 16*             |
| ΔPIX           |                 |                       |
| LFD            | 36 ± 2.0        | 169 ± 11              |
| HFD            | 45 ± 1.0*       | 264 ± 24*             |
| NFATc3\(^{-/-}\) |                 |                       |
| LFD            | 32 ± 4.0        | 147 ± 8               |
| HFD            | 44 ± 2.0*       | 241 ± 41*             |

Values are mean ± SEM obtained at 24-26 weeks after start of diet at 5 weeks of age. *P<0.05.
Figure 1

A

i. control

ii. psora-4

iii. psora-4-sensitive

B

i.

\[ I_{kV} (\text{pA/pF}) \]

ii.

\[ I_{kV} (\text{pA/pF}) \]

iii.

\[ I_{kV} (\text{pA/pF}) \]

C

i.

control

ii. ScTx

iii. ScTx-sensitive

D

i.

\[ I_{kV} (\text{pA/pF}) \]

ii.

\[ I_{kV} (\text{pA/pF}) \]

iii.

\[ I_{kV} (\text{pA/pF}) \]
Figure 3

A

$K_v$ transcript / $\beta$-actin (fold difference)

LFD | $K_v1.2$ | $K_v1.5$ | $K_v2.1$
--- | --- | --- | ---
HFD | [Bars] | [Bars] | [Bars] with *

B

$K_v1.2$, $\beta$-actin, $K_v1.5$, $\beta$-actin, $K_v2.1$, $\beta$-actin

LFD | HFD
--- | ---

C

LFD | HFD

DIC

5 $\mu$m

$K_v2.1$

HFD
Selective down-regulation of Kv2.1 function contributes to enhanced arterial tone during diabetes.

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