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Heteromeric complexes of aldo-keto reductase auxiliary $K_v\beta$ subunits (AKR6A) regulate sarcolemmal localization of $K_v1.5$ in coronary arterial myocytes

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Abstract

Redox-sensitive potassium channels consisting of the voltage-gated K^+ (K_v) channel pore subunit $K_v1.5$ regulate resting membrane potential and thereby contractility of vascular smooth muscle cells. Members of the K_v1 family associate with cytosolic auxiliary β subunits, which are members of the aldo-keto reductase (AKR) superfamily (AKR6A subfamily). The $K_v\beta$ subunits have been proposed to regulate K_v1 gating via pyridine nucleotide cofactor binding. However, the molecular identity of $K_v\beta$ subunits that associate with native $K_v1.5$ channels in the vasculature is unknown. Here, we examined mRNA and protein expression of $K_v\beta$ subunits and tested whether $K_v\beta$ isoforms interact with $K_v1.5$ channels in murine coronary arteries. We detected $K_v\beta1$ (AKR6A3), $K_v\beta2$ (AKR6A5) and $K_v\beta3$ (AKR6A9) transcripts and $K_v\beta1$ and $K_v\beta2$ protein in left anterior descending coronary arteries by real time quantitative PCR and Western blot, respectively. *In situ* proximity ligation assays indicated abundant protein-protein interactions between $K_v1.5/K_v\beta1$, $K_v1.5/K_v\beta2$ and $K_v\beta1/\beta2$ in coronary arterial myocytes. Confocal microscopy and membrane fractionation analyses suggest that arterial myocytes from $K_v\beta2$ -null mice have reduced abundance of sarcolemmal $K_v1.5$. Together, data suggest that in coronary arterial myocytes, $K_v1.5$ channels predominantly associate with $K_v\beta1$ and $K_v\beta2$ proteins and that $K_v\beta2$ performs a chaperone function for $K_v1.5$ channels in arterial myocytes, thereby facilitating $K_v1\alpha$ trafficking and membrane localization.

Keywords

vascular smooth muscle; potassium channels; oxidoreductase; nicotinamide adenine dinucleotide; myocardial blood flow

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Conflicts of Interest:

The authors declare that there are no conflicts of interest.

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INTRODUCTION

Voltage-gated potassium (K_V) channels, via regulation of membrane potential, control numerous physiological processes such as muscle contraction, neurotransmitter release and hormone secretion. These channels are octameric complexes consisting of membrane-bound α pore-subunits and cytoplasmic auxiliary β subunits [1]. The β subunits can promiscuously assemble as either homo- or hetero-tetramers, with each member ($\beta 1-3$) capable of interacting with the N-terminal T1 docking motif (in K_V1 channels) or the C-terminus (in K_V4 channels) of the adjacent α subunit early in channel biosynthesis [2–5]. The $K_V\beta$ proteins belong to the aldo-keto reductase superfamily (AKR6A subfamily) and possess a core catalytic site and NADPH binding site common to those found in other AKR proteins. Whereas $K_V\beta$ subunits demonstrate weak enzymatic activity with aldehydes and ketones [6], the physiological relevance of $K_V\beta$ -mediated catalysis remains unclear. However, it has been shown previously that $K_V\beta$ subunits participate in the regulation of membrane excitability via distinct modulation of K_V channel gating [7, 8] and membrane trafficking [9, 10].

Shaker-type K_V1 potassium channels play a critical role in the regulation of vascular smooth muscle membrane potential and contractility, and thereby control resistance artery diameter and tissue perfusion. In pulmonary resistance arteries, reduced current through oxygen-sensitive K_V channels contributes to hypoxia-elicited vasoconstriction via a mechanism proposed to involve associated $K_V\beta$ subunits [11, 12]. Conversely, small diameter arteries and arterioles of the coronary circulation exhibit vasodilation in response to low oxygen to maintain myocardial oxygen availability [13, 14]. Channels that are sensitive to inhibition by 4-aminopyridine and consisting of $K_V1.5$ α subunits have been implicated as essential components of coronary vasodilation in response to myocardial oxygen demand [15, 16]. While association with oxygen-sensing auxiliary subunits may impact $K_V1.5$ function under altered metabolic demand, the molecular identity of $K_V\beta$ subunits interacting with $K_V1.5$ channels in coronary arterial myocytes is not known.

In this study, we examined basal mRNA expression and relative protein abundance of $K_V\beta$ subunit isoforms in murine left anterior descending coronary arteries by qRT-PCR and Western blot, respectively. In situ proximity ligation assays performed in isolated coronary arterial myocytes revealed that $K_V1.5$ channels in coronary arterial myocytes are primarily associated with $K_V\beta 1$ and $K_V\beta 2$ subunits in coronary arterial myocytes. Consistent with the composition of $K_V\beta$ complexes found in other excitable cell types, our data suggest that these subunits may be arranged as heteromultimers in native K_V channels within this vascular bed. Furthermore, cellular imaging and subcellular fractionation analyses of arterial myocytes from wild type and $K_V\beta 2$ -null mice suggest that expression of $K_V\beta 2$ subunits promotes surface localization of $K_V1.5$ channels at the sarcolemma of arterial myocytes. Based on these findings, we propose a potential functional role for $K_V\beta$ proteins in K_V1 -mediated regulation of coronary vascular tone.

MATERIALS AND METHODS

Animals and tissue/cell isolation

This study was performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and in strict accordance with protocols approved by the Animal Care and Use Committee of the University of Louisville. Male mice, 16–20 weeks of age, were used. Wild type (C57Bl/6J, 129SvEv), $K_V\beta 2^{-/-}$ (129SvEv background) [17] and $K_V\beta 1.1^{-/-}$ (C57Bl/6NJ background) [18] were euthanized by intraperitoneal injection of sodium pentobarbital (200 mg/kg) and thoracotomy. First- and second-order left anterior descending coronary arteries were immediately dissected in ice-cold buffer containing (in mM): 134 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 7 glucose, pH 7.4. Coronary arterial myocytes were isolated using enzymatic digestion procedures similar to those described previously [19]. Briefly, vessels were incubated in digestion buffer containing (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4 for 1 min at 37°C, before incubation in digestion buffer containing 1 mg/mL papain (Worthington) and 1 mg/mL dithiothreitol (37°C, 5 min with gentle agitation) and then in digestion buffer containing 1.25 mg/mL collagenase type H (Sigma) and 1 mg/mL trypsin inhibitor (Sigma) (37°C, 5 min with gentle agitation). Following incubation in enzyme solutions, the cells were washed three times with ice-cold digestion buffer and triturated with a flame-polished Pasteur pipette. Cells were kept on ice until use (1 h).

Quantitative real time polymerase chain reaction

Total RNA was isolated from approximately 8 pooled coronary arteries per sample ($n = 3$) with Trizol (Invitrogen) and RNeasy MiniKit (QIAGEN) according to manufacturers instructions. RNase-free DNase (QIAGEN) was used to remove any contaminating DNA. RNA was eluted from RNeasy minicolumns into 30 μ L of RNase-free water and the concentration was measured using NANODROP 2000C (Thermo Scientific). Total cDNA was synthesized using iScript cDNA synthesis Kit (BIO-RAD) according to manufacturer's instructions. Briefly, 15 μ L denatured RNA (1 μ g), 4 μ L 5X iScript reaction mix and 1 μ L of iScript reverse transcriptase were mixed (total volume, 20 μ L) and cDNA synthesis was carried out in a Bio-Rad MyCycler thermocycler using the following conditions: 42 °C for 60 min and 94 °C for 5 min. Total cDNA was diluted 5-fold using RNase-free water before use. Real-time quantitative PCR was performed using iTaq Universal SYBR green supermix (BIO-RAD) according to manufacturer's instructions. The following were added to each well of a 384 well plate sequentially: 5.0 μ L iTaq 2X Universal SYBR green supermix, 2 μ L diluted cDNA and 3 μ L of mouse K_V channel primers (see Table 1 for primer sequences; mouse *Kcna3* primer set was purchased from Qiagen- cat. no. PPM0407A) or primers for mouse GAPDH (for a total of 10 μ L final volume) and subjected to qRT-PCR using standard protocols on an Applied Biosystems 7900 HT Real-Time PCR system. For each RNA sample, the cDNAs were run in triplicate for each primer set in the same plate with GAPDH as an internal control.

Western blot analysis

Whole tissue lysates were obtained from coronary arteries and cerebral cortex by sonication (10 min in ice bath) in lysis buffer containing (in mM): 150 NaCl, 10 Na_2HPO_4 , 1 EDTA

with 1% deoxycholic acid, 0.1% sodium dodecyl sulfate and protease inhibitors (Complete Mini protease inhibitor cocktail, Roche). For a set of experiments, crude membrane fractions were obtained as described previously [20]. Briefly, mesenteric arteries were pooled from two mice and were homogenized in a glass micro tissue grinder (Wheaton) with 10 mM Tris-HCl (pH 7.4) with 1.4 mM 2-mercaptoethanol containing protease inhibitors and centrifuged at $8000 \times g$ for 45 minutes. The pellets were discarded and the supernatants were centrifuged at $105,000 \times g$ for 1 hr to obtain the membrane pellets, which were then solubilized in 75 μ l of lysis buffer containing protease inhibitors. The solubilized membrane fractions were then sonicated in an ice bath for 30 minutes, incubated for 4 hrs at 4°C with occasional shaking, and centrifuged at $48,000 \times g$ for 1 hr. Supernatants were boiled in Laemmli sample buffer for 10 min and run on a 10% acrylamide gel (Bio-Rad) and subjected to SDS-PAGE. Following transfer to a polyvinylidene fluoride (PVDF) membrane, non-specific binding was blocked with 5% dry milk in Tris-buffered saline (TBS). Membranes were incubated overnight at 4°C in primary antibodies against K_V1.5 (Alomone), K_Vβ1, K_Vβ2, K_Vβ3 or cadherin (Abcam) in TBS containing 0.1% Tween-20 (TBS-t). After five washes (10 min each, room temp) in TBS-t, membranes were incubated in TBS-t containing 1% BSA and horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling; 1:3000). Detection of HRP was performed with enhanced chemiluminescent reagents (Amersham) and a myECL imaging system (Thermo). Densitometry for immunoreactive bands was performed with FIJI software (National Institutes of Health). Density of immunoreactive bands for K_V1.5 was expressed as a percentage of WT controls.

Immunofluorescent staining and confocal imaging

Enzymatically-isolated coronary arterial myocytes were plated on glass microscope slides and allowed to adhere for 20 min at room temperature before fixation with 4% paraformaldehyde in phosphate buffered saline (PBS). Following fixation, cells were incubated in PBS containing Alexa 555-conjugated wheat germ agglutinin (WGA; 5 μ M) for 10 min at room temperature and washed twice with PBS for 5 min. Non-permeabilized cells were then blocked with 1.5% BSA in PBS for 45 min. Immunofluorescent labeling of freshly isolated arterial myocytes was performed as described previously [19] using a rabbit polyclonal antibody specific for an extracellular epitope of K_V1.5 (Alomone; APC-150). The secondary antibody was an Alexa Fluor 488-conjugated donkey anti-rabbit (Abcam; 5 mg/ml). Cells were imaged (512 $\times$ 512 pixel images) using a Nikon A1 confocal microscopy system coupled with a Nikon 60X oil immersion lens (NA = 1.4). Images were collected at multiple optical planes (z-axis step size = 0.5 μ m). Specificity of secondary antibodies for anti-K_V1.5 primary antibodies was tested in negative control experiments in which the primary antibody was substituted with PBS. K_V1.5-associated fluorescence was not observed under these experimental conditions. Images were taken under identical laser power, PMT gain, and pinhole size for all cells analyzed.

Proximity Ligation Assay

Fixation of isolated coronary arterial myocytes was performed as described above. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. A Duolink in situ proximity ligation assay (PLA) kit (Sigma-Aldrich) was used per

manufacturer's instructions for detection of macromolecular complexes consisting of $K_V1.5$ and $K_V\beta$ subunits. Cells were blocked with Duolink blocking solution and incubated overnight in primary antibodies: mouse anti- $K_V1.5$ (NeuroMab, 1:40), rabbit anti- $K_V1.5$ (Alomone, 1:200), Mouse anti- $K_V\beta1$ (Abcam, 1:200), Rabbit anti- $K_V\beta2$ (Abcam, 1:200) or rabbit anti- $K_V\beta3$ (Abcam, 1:200). In control experiments, the cells were incubated in antibody diluent solution without primary antibodies. Primary antibodies against K_V subunits were detected with oligonucleotide-conjugated secondary antibodies as PLA probes (anti-rabbit PLUS and anti-mouse MINUS) followed by a ligation solution including probe-specific oligonucleotides and ligase to form circular DNA. Cells were then incubated (100 min at 37°C) in a solution consisting of polymerase for rolling circle amplification and generation of a concatemeric product, which was subsequently labeled with fluorophore-tagged oligonucleotides. Slides were mounted with Duolink mounting media and sealed with nail polish. Cellular fluorescent signals were detected using a Nikon Eclipse-ti epifluorescence microscopy system by capturing multiple images in the z axis (1 μ m step). Images from an entire z-series were flattened using the z project function and PLA-associated punctate particles for each cell were counted using the particle analyzer function in ImageJ software (NIH). The number of particles per cell was normalized to the area of the cell footprint, obtained from transmitted light images.

Statistical Analysis

Values are mean $\pm$ SE. Student's *t*-test was used to compare two groups. One-way ANOVA followed by Newman-Keuls multiple comparisons test was used for comparison between >2 groups. In all cases, $P < 0.05$ (*) was considered statistically significant. NS denotes $P > 0.05$.

RESULTS

$K_V\beta$ transcripts and proteins in murine coronary arteries

Expression of K_V1 , K_V2 and $K_V\beta$ subunits has been reported in vascular smooth muscle of portal vein, mesenteric and pulmonary arteries and adipose arterioles [12, 21–23]. However, the expression of auxiliary $K_V\beta$ subunits in the coronary vasculature has not been examined and it remains unclear which, if any, $K_V\beta$ proteins are expressed in coronary vascular smooth muscle cells. Considering that the coronary arteries operate in a unique environment, exhibit several phenotypic differences and are derived from distinct embryonic lineages, the composition of $K_V\alpha/\beta$ complexes in these cells may differ from those observed in vascular smooth muscle cells from other vascular beds. Therefore, we examined the expression profile for K_V1 and K_V2 α subunits and $K_V\beta$ subunits in conduit coronary arteries in mice. We first examined transcript expression in isolated left anterior descending coronary arteries using real-time polymerase chain reaction. Figure 1A shows the detection of PCR amplicons corresponding to $K_V1.2$, $K_V1.5$, $K_V2.1$, $K_V\beta1$, $K_V\beta2$ and $K_V\beta3$ subunits. A summary of relative transcript expression, expressed as a percentage of GAPDH, as well as threshold cycle values for each subunit transcript are shown in Figure 1B. We also examined protein expression for $K_V\beta1$, $K_V\beta2$, and $K_V\beta3$ subunits in isolated coronary arteries relative to brain using Western blot analysis. Robust immunoreactive bands corresponding to $K_V\beta1$ and $K_V\beta2$, but not $K_V\beta3$ subunits were observed in coronary artery lysates. As a positive control, primary antibodies raised against $K_V\beta3$ subunits detected a band at the expected

molecular weight (~39 kDa) in brain lysates, consistent with previous reports of high expression of this subunit in brain tissues [24]. These data demonstrate that coronary conduit arteries express $K_V\beta 1$, $K_V\beta 2$ and $K_V\beta 3$ transcripts, of which $K_V\beta 1$ and $K_V\beta 2$ proteins predominate.

$K_V1.5$ channels in arterial myocytes consist of heteromeric complexes of $K_V\beta 1$ and $K_V\beta 2$ subunits

Auxiliary $K_V\beta$ subunits associate with intracellular domains of K_V1 and K_V4 channels. Given the documented influence of K_V1 channels in the regulation of resistance arterial tone and the importance of $K_V1.5$ channels in redox-dependent coronary vasodilation [15, 16, 25, 26], we determined the identity of $K_V\beta$ subunits that are associated with $K_V1.5$ in coronary arterial myocytes. For this, we used an in situ proximity ligation assay (PLA) to identify sites of physical spatial proximity between epitopes on $K_V1.5$ and $K_V\beta 1$, $K_V\beta 2$, or $K_V\beta 3$ in isolated coronary arterial myocytes. The PLA method relies on dual binding of primary antibodies at epitopes that are located within close proximity (~ 40 nm) to one another to generate circular DNA as a template for multi-fluorophore labeling [27]. Therefore, PLA has been used as an approach to identify in situ protein-protein interactions in complexes at individual molecule resolution.

When PBS was substituted for primary antibodies in the PLA procedure (-1° ab), no fluorescent signals were observed in arterial myocytes (Fig. 2A). However, we observed abundant PLA-associated fluorescent signals in cells that were co-labeled for $K_V1.5$ and $K_V\beta 1$ (Fig. 2A). A similar observation was made in cells co-labeled for $K_V1.5$ and $K_V\beta 2$. Conversely, and in agreement with results from Western blot experiments shown in Figure 1C, the PLA signal density was not significantly different for cells co-labeled for $K_V1.5$ and $K_V\beta 3$ compared to -1° ab controls. To verify specificity of the PLA assay for $K_V\beta 1$ and $K_V\beta 2$ subunits, we performed experiments in which cells from either $K_V\beta 1.1^{-/-}$ or $K_V\beta 2^{-/-}$ animals were co-labeled for $K_V1.5$ and either $K_V\beta 1$ or $K_V\beta 2$. In cells from $K_V\beta 1.1^{-/-}$ animals, PLA signal density in cells co-labeled for $K_V1.5$ and $K_V\beta 1$ was nearly abolished, while cells co-labeled for $K_V1.5$ and $K_V\beta 2$ showed abundant PLA puncta (Fig. 2B). In contrast, cells from $K_V\beta 2^{-/-}$ animals exhibited PLA signal density in cells co-labeled for $K_V1.5$ and $K_V\beta 1$ similar to that of wild type cells, while signal density in cells co-labeled for $K_V1.5$ and $K_V\beta 2$ was abolished (Fig. 2C). These data indicate that the PLA procedure used is specific for these K_V subunits and also suggest that $K_V1.5$ associates with both $K_V\beta 1$ and $K_V\beta 2$ in native channel complexes in coronary arterial myocytes.

We next postulated that K_V1 hetero-oligomeric complexes could consist of $K_V\beta 1$ and $K_V\beta 2$ subunits assembled within the same channel structure. To test this hypothesis, we performed an additional set of PLA experiments in which isolated coronary arterial myocytes from wild type, $K_V\beta 1^{-/-}$, and $K_V\beta 2^{-/-}$ animals were co-labeled with primary antibodies against $K_V\beta 1$ and $K_V\beta 2$. Similar to results obtained in cells co-labeled for $K_V1.5$ and $K_V\beta 1$, and $K_V1.5$ and $K_V\beta 2$, we detected a large number of potential $K_V\beta 1/K_V\beta 2$ -associated PLA fluorescent puncta in cells isolated from wild type animals (Fig. 3A), indicating close proximity between these subunits within native channel complexes. PLA-associated fluorescence in myocytes isolated from $K_V\beta 1.1^{-/-}$ and $K_V\beta 2^{-/-}$ animals was nearly

abolished (Fig. 3A, B). Together, these data suggest that $K_V1.5$ channels in coronary arterial myocytes associate with both $K_V\beta1$ and $K_V\beta2$ subunits and that these auxiliary subunits may co-assemble into heteromeric complexes in native Kv channels in coronary vascular smooth muscle cells.

Genetic ablation of $K_V\beta2$ impacts surface localization of arterial $K_V1.5$

$K_V\beta2$ subunits assemble with K_V1 channels during biosynthesis at the cytoplasmic face of the endoplasmic reticulum upon co-expression in mammalian cells [10]. Previous studies suggest that $K_V\beta2$ acts as a $K_V\alpha$ protein chaperone protein, stabilizing the K_V1 complex to facilitate ER export and promote the intracellular trafficking of pore-forming channel subunits to the plasmalemma [9, 28]. Therefore, we tested the hypothesis that $K_V\beta2$ expression contributes to the localization of $K_V1.5$ channels in the membrane of arterial myocytes. Consistent with our hypothesis, Western blots performed on crude mesenteric artery membrane preparations revealed reduced membrane $K_V1.5$ abundance in vessels isolated from $K_V\beta2^{-/-}$ animals compared with those isolated from wt animals (Fig. 4A–B).

We also performed immunofluorescent staining of isolated myocytes from wild type and $K_V\beta2^{-/-}$ animals followed by confocal imaging and fluorescence intensity analysis. Non-permeabilized myocytes were labeled with a primary antibody against an extracellular epitope of $K_V1.5$ [29] and Alexa Fluor 555-conjugated wheat germ agglutinin (sarcolemmal marker). Fluorescent image analysis revealed that the mean intensity of sarcolemmal $K_V1.5$ -associated fluorescence in myocytes from $K_V\beta2^{-/-}$ animals was reduced by approximately 34% when compared with myocytes from wild type animals (Fig. 4C–D). Together, these data suggest that 2 $K_V\beta$ subunits promote surface expression of $K_V1.5$ channels in coronary arterial myocytes.

DISCUSSION

In this study, we report the following novel findings: 1) Coronary arteries from healthy wild type mice express transcripts for $K_V\beta1$, $K_V\beta2$ and $K_V\beta3$, and protein for $K_V\beta1$ and $K_V\beta2$; 2) In situ proximity ligation assays performed in isolated coronary arterial myocytes suggest protein-protein interactions between $K_V1.5$ α subunits with $K_V\beta1$ and $K_V\beta2$. Low abundance of interaction was demonstrated for $K_V1.5$ and $\beta3$; 3) Close proximity was observed for $K_V\beta1$ and $K_V\beta2$ subunits, suggesting that these subunits may assemble together in heteromeric β subunit complexes of native channels in coronary arterial myocytes; and 4) Genetic ablation of $K_V\beta2$ subunits reduces sarcolemmal expression of vascular $K_V1.5$ channels.

Multiple K_V channel α protein subtypes are widely expressed in smooth muscle cells throughout the vascular network. Yet, little is known regarding the identity of auxiliary $K_V\beta$ proteins that associate with these subunits and how their expression varies between vascular beds. While the functional importance of oxygen-sensitive potassium channels has been reported extensively for arteries and arterioles of the cerebral, coronary, mesenteric, and pulmonary circulation [25, 30–33], recent work has highlighted significant distinctions in channel regulation by oxygen sensing mechanisms. For example, hypoxic inhibition of $K_V1.5$ channels in arterial myocytes of small resistance pulmonary vessels is a purported

contributor to hypoxic pulmonary vasoconstriction [30]. Outside of the pulmonary circulation, increases in tissue oxygen demand generally cause vasodilation to promote enhanced tissue perfusion [34]. While tissue-derived diffusible factors may to some extent contribute to this response in vivo, hypoxia-induced dilation is observable in isolated vessel/myocyte preparations and involves increases in outward K_V current, hyperpolarization, and reduction in $[Ca^{2+}]_i$, suggesting that low oxygen conditions may modulate potassium channel activity in a cell type- and vascular bed-specific manner. Thus, considering the complexity of overlapping oxygen-sensitive membrane potential regulatory mechanisms, expression patterns of oxygen-responsive auxiliary subunits in native channels of the vasculature should be carefully considered.

Our finding that $K_V1.5$ associates with $K_V\beta1$ and $K_V\beta2$ in coronary arterial myocytes is consistent with a previous report demonstrating the presence of mRNAs encoding $K_V1.2$, $K_V1.4$ and $K_V1.5$ and $K_V\beta1.1$, $K_V\beta1.2$, $K_V\beta1.3$, $K_V\beta2.1$ and $K_V\beta2.2$, and protein complexes consisting of $K_V1.2/K_V1.5$ with $K_V\beta1.2$ in co-immunoprecipitation studies of rabbit portal vein [21]. However, it should be noted that K_V subunit expression changes dramatically with progression along the vascular tree from conduit vessels (e.g. aorta) towards resistance vessels (e.g., mesenteric bed) that exhibit a more pronounced myogenic tone response [22]. Likewise, protein abundance of $K_V\beta1.1$, $K_V\beta1.2$ and $K_V\beta1.3$ subunits progressively increase along the vascular network from conduit to fourth order bovine pulmonary arteries [12]. In the current study, $K_V\beta1$ and $K_V\beta2$ expression and interaction with $K_V1.5$ was examined in first and second order left anterior descending coronary vessels of mice. Unlike the $K_V\beta1$ and $K_V\beta3$ subunits, $K_V\beta2$ subunits lack an N-terminal “ball-and-chain” domain and do not significantly affect the inactivation of K_V1 currents, but could alter the voltage-dependence of channel activation [5]. Hence, we propose that the relative stoichiometry of functionally unique $K_V\beta$ s in heteromeric complexes could have important implications regarding the responsiveness of coronary smooth muscle membrane potential, and consequently vasomotor tone, to local metabolic stimuli. Accordingly, further analysis is warranted to examine potential regional differential expression and molecular interactions between K_V channel subunits of large versus small caliber vessels of the coronary microcirculation.

The relationship between the molecular identity of the $K_V\beta$ complex and basal surface expression of K_V1 channels has not yet been examined in the vasculature. While conventional biochemical techniques (e.g., cell surface biotinylation) were not sufficiently sensitive to reliably quantify surface abundance of $K_V1.5$ in acutely isolated coronary arteries of mice due to limited tissue size, we used an immunofluorescent labelling approach to evaluate surface expression of $K_V1.5$ subunits in intact (i.e., non-permeabilized) isolated coronary arterial myocytes. Although similar extracellular antibody labeling strategies have been used to examine subcellular K_V1 localization in past studies (for example, see references [35] and [9]), we acknowledge that the use of such fluorescence-based methods to accurately determine surface protein levels is not without technical limitations. For example, conclusions drawn from these experiments are based on assumptions of equal rate and extent of fluorophore photobleaching and similar intrinsic auto-fluorescence levels of fixed myocytes from wild type and knockout animals. While we cannot completely rule these out as potentially confounding factors, additional data demonstrating reduced $K_V1.5$ abundance

in crude membrane preparations of mesenteric arteries from $K_V\beta 2^{-/-}$ animals compared with those from wild type mice are in agreement with a modest, yet significant reduction in surface $K_V1.5$ expression upon loss of $K_V\beta 2$.

Altered surface expression of native $K_V1.5$ channels in arterial myocytes from $K_V\beta 2^{-/-}$ animals are consistent with previous studies indicating a clear role for this subunit in the intracellular trafficking of $K_V1.2$ channels to subcellular compartments in neurons and in heterologous expression systems [10, 36]. These studies show that $K_V1\alpha/\beta 2$ interactions occur early in channel biosynthesis and support proper folding of nascent $K_V1\alpha$ subunits, which is essential for efficient ER export and plasmalemmal localization of potassium channels [10]. Moreover, interactions between $K_V\alpha$ and $K_V\beta 2$ are quite stable and likely persist for the lifetime of the channel. The enhanced stabilization of the K_V1 channel complex conferred by $K_V\beta 2$ during the biosynthetic process is likely a significant contributor to higher surface expression of K_V channels. Although $K_V\beta$ isoforms exhibit subtype-specific functional effects on channel gating, members of each $K_V\beta$ gene product have been shown to promote surface expression of $K_V1.2$ in COS-1 cells, suggesting a role of the highly conserved ~330 amino acid C-terminal region that contains that AKR core region [9]. Indeed, these previous results suggest that a structurally intact NAD^+ binding domain, but not the catalytic site, was necessary for intracellular trafficking and axonal channel targeting in hippocampal neurons. Additional functional and biochemical analyses are warranted to address whether modulation of channel function could occur as a result of differential trafficking via binding of oxidized versus reduced cofactor in excitable cells. Perhaps more importantly, whether the binding of pyridine nucleotides to the $K_V\beta$ AKR domain imparts metabolic sensitivity to K_V channels in coronary arterial myocytes, such that $NAD(P)^+/NAD(P)H$ ratio modulates channel gating and vascular tone, remains to be tested. It is plausible that this functional property of the $K_V\beta$ proteins could generate substantial diversity in K_V channel regulation in the coronary vasculature, for instance, as a region-specific adaptation mechanism to adjust blood flow in the heart to physiological (e.g., exercise) or pathological (e.g., heart failure) conditions.

In summary, we report that murine coronary arteries express $K_V\beta 1$ and $K_V\beta 2$ subunits at the protein level, both of which interact with $K_V1.5$ in arterial myocytes of these vessels. Our data suggest that alterations in the composition of $K_V\beta$ complexes, for instance through dynamic modulation of $K_V\beta 2$, may impact the surface expression of $K_V1.5$ in coronary arterial myocytes. We propose that diversity among $K_V\alpha/\beta$ interactions could play a role in vascular oxygen sensitivity to control blood flow to the heart via regulating cell excitability in response to altered myocardial metabolic activity.

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HIGHLIGHTS

- Coronary arteries express $K_V\beta 1$, $K_V\beta 2$ and $K_V\beta 3$ mRNAs and $K_V\beta 1$ and $K_V\beta 2$ proteins.
- $K_V1.5\alpha$ interacts with $K_V\beta 1$ and $K_V\beta 2$ subunits in coronary arterial myocytes.
- $K_V\beta 2$ promotes surface expression of $K_V1.5\alpha$ in arterial myocytes.

Figure 1. mRNA expression and protein abundance of K_V1, K_V2 and K_Vβ subunits in murine coronary arteries

A. RT-PCR gel image demonstrating amplification products for K_V channel subunits as indicated in freshly isolated coronary arteries. **B.** Summary of transcript expression, expressed as a percent of GAPDH, for K_V1.2, K_V1.5, K_V2.1, K_Vβ1, K_Vβ2, K_Vβ3 in coronary arteries. Mean ± SEM threshold cycle values are shown for each in table inset. (n = 3) **C.** Images of Western blots for K_Vβ1, K_Vβ2 and K_Vβ3 in murine coronary arteries (CA) and brain (B) lysates. Blots are representative of three independent experiments.

Figure 2. Proximity ligation detection of $K_v1.5$ and $K_v\beta$ proteins in native coronary arterial myocytes

A. Representative transmitted light (i.), dapi (nuclear stain; ii.) and fluorescent PLA signal (iii.) images (*left*) and summary bar plot of PLA signal normalized to cell footprint (μm^2 ; *right*) for isolated coronary arterial myocytes from wild type mice co-labelled with primary antibodies against $K_v1.5$ and $K_v\beta1$ ($n = 7$), $K_v\beta2$ ($n = 9$) or $K_v\beta3$ ($n = 5$). $*P < 0.05$ compared to -1° ab group. Data is also shown from negative control experiments in which primary antibodies were omitted from the PLA procedure (-1° ab). Scale bars in transmitted light images represent $10 \mu\text{m}$. **B–C.** Representative images and summary plots showing PLA puncta/ μm^2 , as in A, for isolated coronary arterial myocytes from $K_v\beta1.1^{-/-}$ (**B**; $n = 6-7$) and $K_v\beta2^{-/-}$ (**C**; $n = 5-6$) animals co-labelled with primary antibodies against $K_v1.5$ and $K_v\beta1$ or $K_v\beta2$. $*P < 0.05$

Figure 3. Proximity ligation detection of $K_v\beta 1$ and $K_v\beta 2$ proteins in native coronary arterial myocytes

A. Transmitted light (i.), dapi (ii.) and fluorescent PLA (iii.) images of freshly isolated coronary arterial myocytes labeled with antibodies against $K_v\beta 1$ and $K_v\beta 2$ in cells from wild type (wt), $K_v\beta 1.1^{-/-}$, and $K_v\beta 2^{-/-}$ animals. Scale bars represent 10 μm . **B.** Summary bar plot of PLA fluorescent signals, normalized to cell footprint (μm^2) for wt (n = 12), $K_v\beta 1.1^{-/-}$ (n = 6), and $K_v\beta 2^{-/-}$ (n = 10) coronary arterial myocytes labeled for $K_v\beta 1$ + $K_v\beta 2$. *P < 0.05

Figure 4. Surface expression of $K_V1.5$ channels in wild type and $K_V\beta 2^{-/-}$ coronary arterial myocytes

A. Representative blot images (*top*) showing immunoreactive bands for $K_V1.5$ and cadherin (pan-cdh; membrane loading control) and corresponding densitometric summary data (*bottom*) for $K_V1.5$ subunits in membrane fractions of mesenteric arteries isolated from wild type and $K_V\beta 2^{-/-}$ animals ($n = 3$ each). **B.** Differential interference contrast (DIC) and confocal fluorescence images showing wt and $K_V\beta 2^{-/-}$ coronary arterial myocytes stained with alexa-555-conjugated wheat germ agglutinin (WGA) membrane stain and an antibody against an extracellular epitope of $K_V1.5$ in non-permeabilized myocytes. **C.** Summary bar plot showing mean intensity (arbitrary units; A.U.) of $K_V1.5$ -associated fluorescence in coronary arterial myocytes from wild type and $K_V\beta 2^{-/-}$ animals ($n = 5$ cells each). * $P < 0.05$.

Table 1qRT-PCR primer sequences used for K_V transcript detection in mouse coronary artery samples

Gene	Primer sequences
K_V1.1	Forward: 5' - AGATCGTGGGCTCCTTGTGT - 3' Reverse: 5' - ACGGGCAGGGCAATTGT - 3'
K_V1.2	Forward: 5' - AGCTGATGAGAGAGATTCCCAGTT - 3' Reverse: 5' - GACTGCCCACCAGAAAGCA - 3'
K_V1.4	Forward: 5' - TTTTGCAGAGGCGGATGAA - 3' Reverse: 5' - CAAAACGCATCTGGAATG CTT - 3'
K_V1.5	Forward: 5' - TCAGGATCACTCCATCACCA - 3' Reverse: 5' - CTCAAGGGATCGAAGTAGCG - 3'
K_V1.6	Forward: 5' - GAGTCCGTTTCTTTGACCCCT - 3' Reverse: 5' - GGCGACCTCCAGATTGATAGTA - 3'
K_V2.1	Forward: 5' - CGTCATCGCCATCTCTCATG - 3' Reverse: 5' - CAGCCCACTCTCTCACTAGCAA - 3'
K_V2.2	Forward: 5' - CACCTGGCTTGAACAGAAAG - 3' Reverse: 5' - TTGCTTCGGATAATGTCCAC - 3'
K_Vβ1	Forward: 5' - GGAAGCCTACTCTGTGCGCAC - 3' Reverse: 5' - CATGTCATTGCACCAACTCC - 3'
K_Vβ2	Forward: 5' - GGGCAATAAACCTACAGCA - 3' Reverse: 5' - ACTTTGGAGGTCGATTCCCT - 3'
K_Vβ3	Forward: 5' - GGAAGGCAAGAAGCAACAAG - 3' Reverse: 5' - TGTAGGGAGCCCAGATGTTC - 3'
GAPDH	Forward: 5' - AGGTCATCCCAGAGCTGAACG - 3' Reverse: 5' - GGAGTTGCTGTTGAAGTCGCA - 3'