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DEDICATION

This thesis is dedicated to my parents Stephen M. Raph and Elizabeth J. Sylvester who have always supported and encouraged me to forge my own path.

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ABSTRACT

ROLE OF Kvβ2 SUBUNITS IN REGULATION OF RESISTANCE ARTERIAL TONE

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Voltage-gated potassium (Kv) channels control vascular resistance and facilitate the augmentation of myocardial blood flow in response to increases in cardiac workload (i.e., metabolic hyperemia). Native sarcolemmal Kv1 channels in arterial myocytes associate with a heteromeric auxiliary complex consisting of intracellular Kvβ1 and Kvβ2 proteins. The Kvβ proteins differentially regulate the function of heterologously expressed Kv channels, as well as native Kv1 channels in excitable cells of the cardiovascular and nervous systems. The physiological importance of vascular Kvβ proteins in mediating vasodilation remains unknown. In this study, I tested the hypothesis that Kvβ proteins functionally regulate vascular tone in response to an altered pyridine nucleotide redox state. I evaluated *ex vivo* vasoreactivity of small diameter mesenteric arteries isolated from wild type (WT), mutant mice in which either Kv channel subfamily a member regulatory beta subunits 1 or 2 (*Kcnab1* or *Kcnab2*, respectively) was deleted (i.e., Kvβ1.1$^{-/-}$ and Kvβ2$^{-/-}$, respectively), transgenic mice in which kcnab1.1 overexpression was induced by addition of doxycycline to the water (SM22α-rtTA: TRE β1) and point mutant mice in which the Kvβ2 subunit was catalytically inactive (Kvβ2^{Y90F}). Loss of Kvβ1.1 or Kvβ2 did not significantly impact vasoconstriction in response to elevated extracellular K^+ (60 mM),

U46619 (thromboxane A_2 analogue), or increases in intravascular pressure. Whereas vasodilation in response to application of adenosine (10^{-6} – 10^{-4} M) was similar between Kvβ-null and WT groups, vasodilation in response to external L-lactate (5-20 mM), which modifies cellular pyridine nucleotide redox state , was sensitive to inhibition by the Kv1-selective inhibitor psora-4 (500 nM) and was abolished in arteries from $Kv\beta2^{-/-}$ mice but not in arteries from $Kv\beta1^{-/-}$ mice. Interestingly, arteries from mice in which overexpression of the Kvβ1 subunit (SM22α-rtTA: TRE β1) and point-mutated inactivation of Kvβ2 ($Kv\beta2^{Y90F}$) enzymatic function, similarly abolished vasodilation in response to external L-lactate (5-20 mM). Our results indicate that vascular tone is differentially regulated by Kv1-associated Kvβ proteins, whereby Kvβ2 promotes and Kvβ1.1 opposes lactate induced vasodilation. Additionally, our data signify the importance of the Kvβ-subunit complex composition and enzymatic function in promoting vasodilation in response to an altered pyridine nucleotide redox state.

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LITERATURE REVIEW

a. Introduction

Adequate blood flow requires an adaptive, dynamic system, capable of matching oxygen and nutrient supply to organ metabolic demand.[1] Small resistance arteries (~100-300 μm) [2] contractile state is modified to alter blood flow and prevent tissue ischemia during increased metabolic activity. Disruptions in blood flow can result in ischemic injury and organ failure that increases the risk of morbidity and death.[3,4] The intricate mechanisms underlying metabolic regulation of blood flow are poorly understood.

The concentration of vascular smooth muscle global cytosolic free Ca^{2+} ($[Ca^{2+}]_i$ regulates arterial tone. In the vascular smooth muscle cell, $[Ca^{2+}]_i$ is predominantly determined by sarcolemmal membrane potential and Ca^{2+} influx via L-type voltage-dependent Ca^{2+} channels (VDCC).[5] This literature review will discuss the major known mechanisms of arterial tone regulation. Part one of this review will discuss vascular smooth muscle mediated arterial tone regulation, focusing on mechanisms of ion channel mediated vascular tone regulation. Part two will discuss the biochemical and physiological properties of K^+ channel associated AKR6A ($K_V\beta$) proteins. This literature review will provide the base evidence for investigating the role of $K_V\beta$ proteins in regulating arterial tone.[a]

[a] Parts of the following thesis have been previously published with Sean Raph listed as co-first author or first author, respectively; 6 Ohanyan, V. *et al.* Myocardial Blood Flow Control by Oxygen Sensing Vascular Kvbeta Proteins. *Circ Res*, doi:10.1161/CIRCRESAHA.120.317715 (2021); 7 Raph, S. M., Bhatnagar, A. & Nystoriak, M. A. Biochemical and physiological properties of K(+) channel-associated AKR6A (Kvbeta) proteins. *Chem Biol Interact* **305**, 21-27, doi:10.1016/j.cbi.2019.03.023 (2019).

b. Part 1: Resistance artery physiology

Resistance Artery Myogenic Tone Regulation

Resistance arteries are those arteries with a diameter in the range of ~100-300 μm.[8] These small resistance arteries exhibit a large dynamic range of arterial distention and significantly influence blood pressure and flow. Adequate supply of blood flow to organs requires arteries that can adequately dilate in response to the metabolic demand of the supplied organs and tissues. To allow for adequate O_2 and nutrient supply, the resistance arteries must allow for greater blood flow. This is achieved in one of two ways; 1) the heart can pump harder, forcing a greater volume of blood through the same diameter 'tube' in turn increasing the pressure in the circulatory system, however this will further exacerbate the O_2 deficit of the heart and therefore is insufficient; or 2) the artery can dilate, increasing the diameter of the vessel through which a greater of volume of blood can travel. In those events where the heart does pump more blood but small resistance arterial dilation is impaired, the tissue becomes starved of oxygen and nutrients.[9] To match organ blood flow demands, the vascular smooth muscle mediate vasoconstriction through a response known as myogenic tone. This is achieved through tight regulation of intracellular $[Ca^{2+}]_i$. For example, influx of Ca^{2+} induces vasoconstriction (decreased diameter), while inhibition of Ca^{2+} influx induces vasodilation (increased diameter).[10] Calcium enters the vascular smooth muscle cells via L-type voltage-dependent Ca^{2+} channels (VDCC), which will be discussed in greater detail later.

Control of blood flow

The movement of blood through the circulatory system is dependent on regions of variable pressure. Blood moves from regions of high pressure (i.e., aorta) to regions of

low pressure (i.e., capillaries). The increase in blood flow parallels this increase in pressure (ΔP); however, resistance opposes blood flow. Blood flow is proportional to ΔP and inversely proportional to vascular resistance (R) such that: Flow≈ ΔP/R. Furthermore, resistance is a function of length (L) and radius (r) of the vessel and viscosity (η) of the fluid moving through the vessel and are best expressed by Poiseuille's Law: $R = 8L\eta/\pi r^4$. Because the circulatory system and blood viscosity are generally constant, the equation can be refined to the following: $R \approx 1/r^4$. Therefore, this equation declares that vascular resistance is inversely proportional to the vessel radius to the fourth power. For example, a change of arterial diameter from 100 μm to 80 μm will result in ~59% reduction in blood flow. For example during hyperemia, a regulatory process by which blood flow is modified to match changes in metabolic and oxygen demand and prevent ischemia, significant changes in arterial diameter are necessary to prevent end organ damage.[11] There are several situational hyperemia responses; 1) reactive, the response to diminished tissue oxygen and increased metabolic waste (ischemia), 2) active, a response to increased exertion (i.e., exercise), and 3) infectious or traumatic, as during infection of a cut or edema.[11] To match these increased demands of the tissues (i.e., brain, skeletal muscle, heart) small resistance arteries must dilate. The K_V channels play an important role in regulating vascular tone during hyperemia.[12] This reaffirms that arterial diameter is a significant driving force of vascular resistance and even small changes can have significant effects on blood flow and end organ perfusion.

Autoregulation and Ca^{2+} dependence of vascular tone

Maintaining constant blood flow during changes in blood pressure requires resistance vasculature to constrict in response to increased intravascular pressure and dilate in response to reduced pressure. This phenomenon of arterial constriction in

response to increased intravascular pressure, or “myogenic tone” was first described by Bayliss in the early 1900’s.[13] These constrictions resulting from increases in intravascular pressure were later identified to be an intrinsic characteristic of arterial wall smooth muscle cells independent of other influences (e.g., endothelial).[14-16] Additionally, increasing intravascular pressure to within the physiological range (60-100 mmHg) results in membrane depolarization of vascular smooth muscle cells and activation of voltage dependent Ca^{2+} channels (VDCC), which results in an increase in $[Ca^{2+}]_i$, vascular smooth muscle contraction, and vessel constriction.[17-19] The effects of pressure and other endogenous mediators of arterial tone are significant for regulating a partially constricted state in order to match blood flow to metabolic demand in small diameter arteries.[20,21] These various factors (e.g., pressure) contribute to the autoregulation of vascular tone.

Regulation of smooth muscle membrane potential

The activity of VDCCs and smooth muscle $[Ca^{2+}]_i$ is dependent on the membrane potential. The change in membrane potential of vascular smooth muscle cells and $[Ca^{2+}]_i$ mediated constriction is tightly regulated and is modified by efflux of potassium ions (K^+). The intracellular and extracellular K^+ concentrations, determined by the high permeability of the cell membrane and chemical gradient for K^+ ions, drive the resting membrane potential. This is due to the differing intracellular (~140 mM) and extracellular (~4-6 mM) K^+ concentrations. However, when the cell membrane becomes depolarized, voltage gated potassium channels open to drive hyperpolarization and return to the E_K Nernst potential. This efflux of K^+ diminishes positive charge increasing the electrical driving force inward toward the cytoplasm. A major family of K^+-selective channels regulates vascular smooth muscle cell membrane potential. This section will first describe how regulation of ion flow affects membrane potential and then will describe the properties of K^+ channels

that are expressed in small resistance arteries: voltage dependent (K_V), large conductance Ca^{2+} activated (BK), ATP-sensitive (K_{ATP}), and inwardly rectifying (K_{IR}) K^+ channels.

Membrane polarization of excitable cells: The polarization state of excitable cells (e.g. neurons) depends on the flow of positively and negatively charged ions, predominantly this includes K^+, Na^+, and Cl^-.[22] The movement of ions across the membrane is determined by their respective electrical and chemical gradients. When the electrical forces driving ions into or out of the cell matches the chemical forces driving ions into or out of the cell, this determines the membrane potential at which there is no net flow of that ion in either direction across the membrane, this is known as the equilibrium potential. The equilibrium potential for each respective ion (X) can be calculated using the Nernst Equation,

$$E_X = (RT/zF)*\ln([X]_O/[X]_i)$$

where R is the gas constant, T is temperature, z is the charge of the ion, F is the Faraday constant, and $[X]_O$, $[X]_i$ are the external and internal ion concentrations (mM). Further simplified, this can be written as

$$E_X = 58\ mV/1*\log([X]_O/[X]_i)$$

Using this we can calculate the equilibrium potential (E_K) for K^+, to be approximately -90 mV, when $[K^+]_O$ = ~4.7 and $[K^+]_I$ = 150.[19] These equations are used to determine the contributions of the different ions to the resting membrane potential. By using the Goldman equation,[23] Hodgkin and Huxley analyzed the respective contributions of ions to membrane potential of the squid giant axon.[24-26] They observed that K^+ had the greatest contribution to the resting membrane potential. Furthermore, the resting membrane potential for many excitable cells is driven by E_K (~90 mV) meaning the membrane is most permeable to potassium. Since Hodgkin, Huxley, and Katz established that membrane

excitability is largely maintained by K^+ channels[27-31], numerous investigations have found numerous K^+ specific channels[32], which regulate a multitude of different physiological processes.[33-35]

Inwardly rectifying channels: Inwardly rectifying (K_{IR}) channels have evolved voltage independent mechanisms of activation such as gating by G-proteins, pH, and ATP. Unlike other K^+ channels (e.g. K_V) the K_{IR} are comprised of two membrane spanning helices.[36] Additionally, these channels more readily pass K^+ ions inward than outward when membrane potential is negative to E_K; however, arterial myocyte membrane potential is positive to E_K and currents through these K_{IR} channels is low. Hyperpolarization occurs when external K^+ is elevated and K^+ efflux through K_{IR} occurs.[37] Additionally, K_{IR} may by activated by cellular release of K^+ during hypoxia and ischemia.[38] As a result, K^+-induced dilation during reduced oxygen and nutrient delivery in metabolically active cells is a result of K_{IR} and may be an important mechanism to increase blood flow and prevent ischemia.

ATP inactivates the ATP-sensitive potassium channels: The inward rectifying ATP-sensitive potassium channels (K_{ATP}) are inhibited when the intracellular ATP:ADP ratio increases. The K_{ATP} channels are comprised of pore-forming subunits and sulfonylurea receptor subunits. This gives rise to the sensitivity of K_{ATP} channels to sulfonylurea inhibitors like glibenclamide.[39] Activation of K_{ATP} channels mediates vasodilation via the sulfonylurea receptor subunits and adenosine A2 receptor.[40] ATP hydrolysis produces adenosine, this binds to adenosine type 2A receptors, a G-coupled protein which stimulates adenylate cyclase and increases cyclic-AMP.[41] Through this mechanism, K_{ATP} channels are activated, hyperpolarizing the cell membrane, inhibiting VDCC mediated Ca^{2+} influx and inducing vasodilation.[41] As adenosine is a product of increased metabolic

activity, K_{ATP} channels are included in the growing list of modifiers of blood flow; however, recent reviews provide collected evidence that K_{ATP} channels may not be key mediators of coronary vasodilation in response to exercise.[2,42-45]

Large conductance Ca^{2+} activated potassium channels: The large conductance Ca^{2+} activated potassium channels (BK) are comprised of four α-subunits, which form an ion conducting pore and associated β subunits. Genetic ablation (i.e. $BK^{-/-}$) and or pharmacological inhibition of BK channels induces constriction and greater myogenic tone, thus reducing blood flow in $BK^{-/-}$ mice as compared with wildtype.[46-48] The BK channels mediate K^+ efflux in response to local release of sarcoplasmic reticulum Ca^{2+} by ryanodine receptor mediated calcium sparks.[49-52] The BK-mediated efflux of K^+ drives membrane hyperpolarization, which inhibits VDCC facilitated Ca^{2+} influx and induces vasodilation.[49] The release of sarcoplasmic intracellular Ca^{2+} mediated by ryanodine receptors, which in turn activate BK channels signify yet another important mechanism that regulates blood flow.

Voltage dependent K+ channels: The voltage dependent potassium (K_V) channels are comprised of ~35 genes that can be further categorized into 12 families expressed in excitable cells. There are several K_V channels expressed in small resistance arteries including members of the K_V1, K_V2 and K_V7 families.[53] The K_V channels are comprised of a heteromeric pore formed by K_Vα-subunits, which consist of six transmembrane helices (S1-S6) with S5-S6 forming the ion conducting pore and S4 containing the voltage sensor region.[53] Additionally, the K_Vα complex associates with intracellular β-subunit complex, which binds the α-subunit at the intracellular T1 region. The β-subunits bind endogenous cofactors and substrates, which modify K_Vα channel activity. K_V channels play a significant

role in mediating vasodilatory control in response to altered metabolic demand.[54] There is significant evidence that functional K_V channels are crucial in regulating arterial tone.[19,55] Additionally, K_V channels are crucial to modifying coronary blood flow in mice[56], and play a role in adaptive and innate immune cell development[57], neuronal diseases such as epilepsy[58], and sleep[59], and hyperpolarization of excitable cells.[60]

Endothelial influences

Since the discovery of the endothelium in the late 1800's, it has been perceived as an entity separate and inert from other segments of the vasculature. However, the mechanisms of endothelial regulation of vascular function have been intently investigated. This section will provide an overview of several major endothelial originating factors that influence vasodilation, including nitric oxide (NO), endothelium derived relaxing factor (EDRF), and endothelin-1 (ET-1).

Nitric oxide: Nitric oxide (NO) plays an important role in regulating vascular tone.[61,62] Acetylcholine, first isolated and synthesized in the 19th century by Adolf von Baeyer and later by Dudley and others[63,64], stimulates vasodilation via increased nitric oxide synthase (NOS).[65] The effect of acetylcholine induced vasorelaxation was first recognized by Mott and Halliburton in the early 1900's, when small concentrations decreased blood pressure in laboratory animals.[66] Decades later, a functional role for the endothelium was observed when Furchgott and Zawadski directly noted acetylcholine induced smooth muscle relaxation.[67] It was not until 1987 when, separately, Ignarro[68,69] and Palmer[70], independently identified nitric oxide (NO) as the mediating factor. After these influential discoveries, several ligands were identified (e.g., bradykinin, acetylcholine) which bind to endothelial receptors and mediate a cascade that initiates activation of enzymes in the

nitric oxide synthase (NOS) pathway. Both agonists and sheer stress induce increased production of endothelial NO.[71,72] Due to either endothelial agonist or sheer stress stimulation of the NOS enzymatic machinery converts L-arginine to L-citrulline and NO is produced as a byproduct.[73] NO then diffuses from endothelial cells to vascular smooth muscle cells where NO activates soluble guanylate cyclase (sGC), which increases cyclic guanosine monophosphate (cGMP) production.[74] Elevated cGMP activates PKG, which inhibits IP3R mediated sarcoplasmic Ca^{2+} release, this decreases intracellular Ca^{2+}, and results in vasorelaxation.[74,75] However, there are other mechanisms of vasodilation discussed in great detail in these reviews.[76-78] Additionally, NO plays a role in numerous other mechanisms such as, angiogenesis[79], apoptosis[73], neurotransmission, inflammation response, and thrombosis.[80] Although NO has been proposed as a key mediator of coronary vasodilation several studies have shown that blockade of NO does not significantly influence coronary dilation in response to increased cardiac work (i.e. exercise).[72,81] It has also been shown that blockade of NOS has a negligible effect on metabolic coronary vasodilation during exercise.[8,81-83] It has been proposed that endothelial mediated control acts to preserve a reserve dilatory capacity in order to prevent excessive shear stress.[8,84]

Endothelial derived relaxing factors: There have been numerous endothelial derived relaxing factors (EDRF) identified, including ADP, CGRP, thrombin, P450 metabolites of arachidonic acid, H_2S, and H_2O_2.[85-87] EDRF molecules activate soluble guanylate cyclase, this increases cyclic-GMP (c-GMP) in the vascular smooth muscle. NO binds to soluble c-GMP and starts a signaling cascade that drives vasorelaxation (described in further detail above in "*Nitric oxide*"). These EDRF molecules exact local or direct change to vascular

tone in response to changes in metabolic demand.[88] This is further evidence of a multifaceted compensatory mechanism to ensure proper blood flow regulation.[89]

Endothelin-1: The vasoconstrictor endothelin (ET) is expressed as three isoforms, though endothelial cells express only ET-1. The precursor to ET-1, Big ET-1, is converted to ET-1 by endothelin converting enzyme.[90] Receptors for ET-1 have been identified in both vascular smooth muscle and endothelial cells and distribution of different receptors is dependent on the type of vascular bed. Extracellular Ca^{2+} enters the cells when ET-1 binds ET_A or ET_{B2} receptors; however, the ET_{B1} receptor is also linked to NO- and PGI_2-mediated vasodilation.[75] ET-1 increases the sensitivity of myosin light chain (MLC) to Ca^{2+}, inducing vasoconstriction.[91] Additionally, ET-1 induced Ca^{2+} increase is inhibited by nicardipine, and vasoconstriction is inhibited by the inhibitor of the ET_A receptor BQ-123, but not the ET_B agonist IRL 1620.[91] The ET-1-induced constriction is impaired by either BQ-123 or $GDP_{\beta S}$ in the presence of increased Ca^{2+}.[91] Additionally, there is evidence supporting a role for ET-1 in the development of vascular diseases in which blood flow is impaired.[92] Despite being an essential regulator of vascular tone, high levels of ET-1 contribute to enhancement of hypoxia induced hypertension via inhibition of K_{Ca} channel conductance.[93] Importantly, ET-1 at low concentrations increases K_{Ca} activity, but in excess of 5 nM, ET-1 inhibits K_{Ca} conductance.[93] Importantly, high levels of ET-1 mediated vasomotor control can detrimentally contribute to dysregulated blood flow.

Taken together, these different pathways act as complements to one another in regulation of arterial tone and therefore blood flow. It is crucial to remember that no single regulator is sufficient to modify blood flow or arterial tone in response to all stimuli. Therefore, the vasculature has evolved numerous complementary mechanisms that

regulate the vasodilatory response to exogenous and endogenous signals. However, understanding the various modifiers of arterial tone (e.g., K_V channels) requires a deeper understanding. We will describe the link between the role of K_V channels and their associated auxiliary subunits as crucial regulators of arterial tone in further detail in this review and with the entirety of this work.

c. *Part 2: Biochemical and physiological properties of K^+ channel associated AKR6A ($K_V\beta$) proteins*

Voltage-gated potassium (K_V) channels play an essential role in the regulation of membrane excitability and thereby control physiological processes such as cardiac excitability, neural communication, muscle contraction, and hormone secretion. Members of the Kv1 and Kv4 families associate with auxiliary intracellular Kvβ subunits, which belong to the aldo-keto reductase superfamily. Electrophysiological studies have shown that these proteins regulate the gating properties of Kv channels. Although the three gene products encoding Kvβ proteins are functional enzymes in that they catalyze the nicotinamide adenine dinucleotide phosphate (NAD[P]H)-dependent reduction of a wide range of aldehyde and ketone substrates, the physiological role for these proteins and how each subtype may perform unique roles in coupling membrane excitability with cellular metabolic processes remains unclear. Here, we discuss current knowledge of the enzymatic properties of Kvβ proteins from biochemical studies with their described and purported physiological and pathophysiological influences.

The aldo-keto reductases (AKRs) comprise a group of oxidoreductase enzymes that catalyze the reduction of endogenous and xenobiotic carbonyl compounds. These enzymes are ubiquitous among eukaryotic and prokaryotic organisms and share significant structural identity in that they all possess a C-terminal active site region within

a triose-phosphate isomerase (TIM) barrel (α_8/β_8) motif with three loops at the base of the barrel that govern substrate binding.[94,95] The utility of this structural arrangement among the AKRs allows for flexibility in binding and metabolizing a wide range of chemical substrates that includes aliphatic and aromatic aldehydes and ketones, monosaccharides, steroids, and polycyclic aromatic hydrocarbons.[96-98] All AKRs require nicotinamide adenine dinucleotides (i.e., NAD(P)H) as a cofactor for hydride transfer [99] and their function can thus be modulated by the cellular redox state of electron carriers used in many intermediary metabolic reactions.

Most human AKRs are soluble monomeric proteins that are found in the cytosolic compartment. An exception to this are members of the AKR6 subfamily, which form tetrameric complexes that are associated with the pore-domains of voltage-gated potassium (Kv) channels (i.e., the Kvβ proteins).[100,101] The Kv channels are a large family of transmembrane K^+-permeable ion channels that, via regulation of membrane potential in excitable cell types, control numerous physiological processes, including neuronal excitability, hormonal secretion, and muscle contraction.[35,102,103] While this assembly between a catalytically active AKR and ion channel has stimulated several intriguing hypotheses regarding its evolutionary conservation and potential physiological role(s) [104], there is limited information about the potential *in vivo* role for the Kvβ proteins in the cardiovascular, endocrine, and nervous systems, and it is unclear how these proteins may regulate diverse cellular physiological processes and pathophysiological development. While the enzymatic properties and cellular functions of the AKR family have been reviewed (readers are referred to [95,105]), we will discuss the enzymatic properties of the Kvβ proteins, including how these properties may relay metabolic information to the Kv channel gating apparatus. Additionally, these subunits may serve as molecular transducers that couple metabolism and membrane electrical signaling in excitable cell

types. While underscoring key remaining questions that require further investigation, we discuss the potential efficacy of small molecules or peptides that selectively modulate Kvβ expression or functionality as a novel class of therapeutics that could prevent or reverse pathological changes, and therefore may be useful interventions for controlling excitability under a variety of different physiological and pathological conditions.

Molecular and structural biology

In the human genome, there are ~35 genes encoding Kv channel pore proteins belonging to 12 subfamilies (i.e., Kv1.x – Kv12.x).[106] The basic Kv channel tertiary structure consists of a multi-subunit complex of pore-forming proteins with a diverse repertoire of associated auxiliary and regulatory proteins. The pore domain is formed by the tetrameric assembly of four distinct transmembrane subunits (α) that are arranged around a central axis to form a membrane-traversing ion conduction pore that is highly selective (~10,000 fold more selective for K^+ than for Na^+) and efficient for K^+ transport (~10^7 K^+ ions $channel^{-1}$ sec^{-1}).[107-109] Kvα subunits are 70-100 kDa in mass and consist of six membrane-spanning α helices (S1-S6) with S1-S4 forming the voltage-sensor domain and the S5-S6 segments of each contributing to the pore lining with selectivity filter. A highly conserved series of positively charged arginine residues within the S4 region form the voltage sensor of the channel that responds to changes in membrane voltage to constrict or dilate the central pore.[110] In native channel complexes, members of a particular Kv family (e.g., Kv1) are known to interact with other functional members of the same family, giving rise to heteromeric alpha pore complexes with variable gating properties, which could ultimately increase diversity among functional channels.[111,112] This is thought to occur through highly conserved regions within the intracellular T1 domain, which also

serves as a docking site for intracellular subunits. Association with conserved regions among accessory Kv proteins also allows for the formation of heterotetrameric auxiliary subunit complexes, which, as described below, may further add to the functional diversity of native channels.

Although the expression and assembly of four Kvα subunits is the minimum requirement to form a functional channel, association of the pore-domain with a diverse set of accessory subunits, such as Kvβ, KChAP, KChIP, and MinK, imparts multimodal regulatory features to Kv channels *in vivo.*[113] Members of the *Shaker* (Kv1) and *Shal* (Kv4) families are known to associate with Kvβ subunits.[114,115] The human genome contains three genes that encode Kvβ proteins (KCNAB1, KCNAB2, KCNAB3) and their transcripts are alternatively spliced to generate additional variants. Early studies suggesting the functional importance of Kvβ proteins discovered that a leg shaking phenotype in *Drosophila melanogaster* (i.e., 'hyperkinetic') was the result of a mutation in a homologue of the mammalian Kvβ peptides. Subsequent sequence analyses led to the unexpected finding that the Kvβ subunits shared significant homology (15-30% amino acid identity) with members of the AKR superfamily.[104,116] Upon crystallization of Kv1.2-Kvβ2, it was found that Kvβ proteins possess a conserved C-terminal β-barrel structural fold with tightly bound nicotinamide cofactor and, consistent with findings from sequence alignments, the active site had all characteristic features of a catalytically active AKR, including a well-conserved cofactor binding site and a distinct substrate binding pocket.[100,117] Indeed, in these earlier reports on the X-ray crystal structure of a Kv channel complex, and more recently in a study demonstrating the single-particle cryo-electron microscopic structure of Kv1.2-Kvβ2 expressed in lipid nanodiscs [118], electron density could be resolved from $NADP^+$ that was bound to the β subunits.

The active site structure of the AKR6 family is unique in that the α_8/β_8 motif has an additional helix attached to a long loop between β9 and α7 near the cofactor binding pocket.[95] The functional significance of this modification that is shared among AKR6 members is not presently clear. At the quaternary level, the β1 and β2, which are perpendicular to the central axis of the barrel, along with the α2-β5-α3 region, form the intersubunit interface region that participates in β tetramerization, while the α5-α6 region interacts with the T1 docking domains of the Kvα proteins.[100,101] Thus, via the T1 domain, the active site of Kvβ can influence the conformation of the voltage sensing apparatus and thereby impact gating properties because of catalytic activity and/or pyridine nucleotide cofactor binding.

Enzymology and channel biophysics

A prerequisite for investigating and understanding the potential physiological or pathological roles of the Kvβ proteins is a thorough understanding of their catalytic properties and the identification of potentially relevant endogenous or xenobiotic carbonyl substrates. The Kvβ proteins bind pyridine nucleotides, with binding affinities in the low micromolar range (i.e., 0.1-4 μM). The proteins display a ~10-fold greater affinity for NADP(H) compared to NAD(H) cofactors.[119] Considering that in most metabolically active cells, the NADPH:$NADP^+$ ratio is substantially higher than that of NADH:NAD^+, while the absolute concentration of NADP(H) is much lower than that of NAD(H) [120,121], the cofactor predominantly used by Kvβ proteins *in vivo* is not clear and likely varies with respect to cell type. Kvβ2 catalyzes the reduction of a wide range of aldehydes and ketones, although preferential binding and reduction of aldehydes versus ketones, and higher catalytic efficiency for aromatic aldehydes was reported for this subunit.[122] For example, Kvβ2 shows higher catalytic activity with aromatic carbonyls such as phenanthrenequinone than

with straight chain aldehydes such as acrolein or 4-oxo-nonenal.[122] Little or no activity was observed with steroids such as cortisone. Significantly, the protein was also found to be active with products of lipid peroxidation, such as 1-palmitoyl, 2-oxovaleroyl, phosphatidyl choline (POVPC). Given that POVPC and related aldehydes are generated during the oxidation of unsaturated fatty acids in the plasma membrane and that Kvβ is tethered within close proximity to the membrane, it appears plausible that the catalytic function of Kvβ may be to detoxify lipid peroxidation products and thereby protect Kv channels from oxidative damage. Alternatively, binding to lipid peroxidation products could be a potential regulatory mechanism that could alter Kv kinetics under conditions of oxidative stress (e.g., to trigger apoptosis). Although future studies are required to distinguish between these possibilities and to identify other endogenous substrates, the catalytic reactivity of the protein with aldehydes could represent an important link that would regulate Kv channel activity as a function of Kvβ catalysis (regulation of electrical activity by metabolism) or Kvβ catalysis by Kv activity (regulation of metabolism by electrical activity). In either scenario, the link between metabolism and excitability could represent a regulatory mode with profound implications for neural, cardiac, and muscle excitability.

The catalytic activity of Kvβ2 has been found to be sensitive to both pH and ionic strength. Measurements of the enzyme activity at various pH and ionic concentrations found that enzyme activity is maximal between pH 7.2-7.4 and relatively insensitive to varied phosphate concentrations between 100 mM and 250 mM. Yet, at low phosphate concentrations (≤ 50 mM), enzymatic activity is significantly decreased [122-124] and is not impacted by the addition of NADH and or NAD^+, suggesting that the enzyme functions most effectively at a specific ionic strength. As with other AKRs such as aldose reductase and aldehyde reductase, the mechanism of Kvβ catalysis is consistent with an ordered bi-bi rapid equilibrium reaction in which the nucleotide cofactor is the first to bind and the last

to dissociate. Consistent with this, the binding affinities for NADPH and $NADP^+$ by Kvβ2 are significantly different, as NAD(P)H binds with 4-times greater affinity than $NADP^+$. The sequence of cofactor and substrate binding was confirmed using variable concentrations of 4-NB and NADPH to establish the initial velocity, the starting rate of enzymatic activity. When plotting initial velocity against the different NADPH concentrations, a rapid equilibrium mechanism was predominant, indicative of NADPH binding prior to substrate.[122]

The cofactor binding kinetics for Kvβ2, determined by monitoring the reduction in fluorescence of the Kvβ2 reporter fluorescence by addition of each respective cofactor, provided insight into the phasic behavior and rate limitation of catalysis.[123] By measuring the dependence of observed k_{fast} and k_{slow} of kinetic traces on NADP(H) concentration, it was suggested that the binding of NAD(P)H to Kvβ could be described as a three-step process consisting of rapid formation of a loose enzyme-cofactor association, a slow conformational change that securely seats the cofactor in the active site of the enzyme, and further stabilization of the NADPH cofactor to its binding site.[122,123] The binding of $NADP^+$, however, follows the two step model of binding affinity, suggesting that the second conformational change observed with NADPH binding that prevents nucleotide exchange is absent in the binding of oxidized nucleotide.[123] Moreover, studies performed using mutant Kvβ2 in which the catalytic site tyrosine (Y90) is replaced with phenylalanine ($Kv\beta2^{Y90F}$) suggest that high affinity nucleotide binding is not significantly impacted by loss of catalytic function.[122]

By binding of cofactors to the Kvβ subunits, Kv channel activation and inactivation are sensitive to changes in intracellular pyridine nucleotide redox state, which is reflected in the ratio of intracellular $NAD(P)H/NAD(P)^+$.[124] Modulation of Kv activity by oxidized and reduced pyridine nucleotides is determined by the identity of Kvβ subunits present.[125,126]

Unlike Kvβ1 and Kvβ3, the Kvβ2 subunit lacks an inactivating N-terminus tail-like structure.[127,128] Despite this structural difference, Kvβ2 has a common binding affinity to the α subunit T1 domain.[100] Consistent with this, the C-terminal domain of the Kvα subunits is critical for proper association between the Kvα and β subunits.[126,129] The underlying region of importance in the Kv1α C-terminal domain lies between Arg-543 and Val-583 of Kv1.5, a region with differential affinities for NAD(P)H-bound versus NAD(P)+-bound Kvβ. Thus, nucleotide-dependent modification in subunit binding affinity and associated conformational changes within the Kvα transmembrane region may represent a potential mechanism whereby Kvβ redox sensing could alter channel biophysical properties.

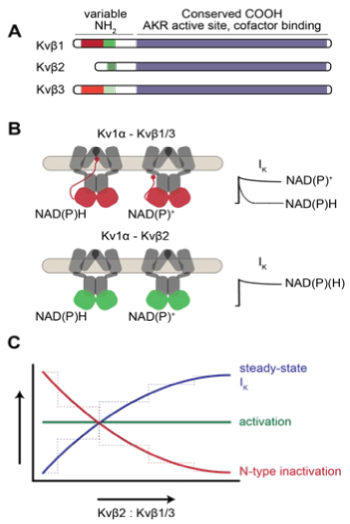
The mechanism and biochemical role of cofactor binding in Kvβ-mediated catalysis are like that observed with other AKRs, such as aldose reductase. As with other AKRs, NAD(P)H binding promotes a change in protein conformation that stabilizes the cofactor within the catalytic pocket.[123] The affinity of this interaction produces a large change in free energy that drives catalysis. As little energy is derived from substrate binding to achieve the transition state, the protein can bind a range of carbonyl substrates. This mode of high affinity binding to pyridine coenzyme seems well-suited for ion channel regulatory functions of Kvβ, as it reduces the constraints of aldehyde binding and renders the cofactor binding pocket an effective sensor of intracellular pyridine nucleotides. Thus, physiological changes in intracellular NAD(P)H:$NAD(P)^+$ could readily impact Kv gating and membrane potential regulation. Under conditions of high intracellular NAD(P)H:$NAD(P)^+$ ratio, binding of reduced cofactor generally enhances the degree and rate of channel inactivation.[124,126] However, this effect can be effectively 'turned off' upon completion of a catalytic cycle resulting in substrate reduction and cofactor oxidation. Accordingly, the net effect of AKR enzymatic function on Kv channel activity likely reflects dynamic homeostatic balance between pyridine nucleotide redox potential as well as the concentrations and molecular

identities of local aldehyde and ketone substrates, which collectively reflect cellular and subcellular metabolic activity.

Physiological roles

Regulation of membrane excitability by cellular metabolism: Considering that Kv channel gating is modulated by intracellular pyridine nucleotides via the Kvβ complex as stated above, these proteins have been proposed as a link between cellular function and metabolic activity. However, a clear view of how the Kvβ proteins operate and modify channel gating behavior in their native cellular and tissue environments has not yet emerged. Complicating this issue, native Kv channels likely consist of heteromeric assemblies of multiple gene products and splice variants, which have not yet been functionally characterized. Moreover, the expression levels of Kvα and β proteins and how their stoichiometry within a given population of functional channels is determined may be cell specific. It is also conceivable that the molecular identity of predominant subunits utilized by a cell may be modified because of changing metabolic cues. Considering the functional diversity imparted by variable N-termini of the

Schematic 1



Schematic 1: Balanced redox regulation of whole cell I_{Kv} by differential incorporation of Kvβ proteins in heteromultimeric channels. (**A**) Schematic of Kvβ1, Kvβ2, and Kvβ3 amino acids showing conserved COOH terminal region (blue) and variable N-termini (green/red). Several splice variants which differ in the N-terminal domain have been found for Kvβ1 (1.1-1.3 and Kvβ2 (2.1, 2.2). Ball-and-chain inactivation domain is shown in red. *Adapted from* [68]. (**B**) Differential regulation of Kv channel inactivation by Kvβ1/3 (red) and Kvβ2 (green) in the presence of oxidized and reduced pyridine nucleotides. *Adapted from* [101]. Kv channels expressed in membranes of excitable cells represent heterogeneous populations of structures with varying β subunit compositions. Channels that assemble with Kvβ1/Kvβ3 proteins demonstrate enhanced inactivation upon binding to reduced pyridine nucleotides. Conversely, sensitivity of channel inactivation to pyridine nucleotide redox is absent in channels that associate with Kvβ2, which does not contain the ball-and-chain. (**C**) Graph showing proposed regulation of Kv channel activation, inactivation, and single channel and whole-cell I_{Kv} as the ratio of expressed Kvβ2:Kvβ1 or Kvβ3 is increased. A reduction in channel inactivation with increased Kvβ2:Kvβ1/3 in native Kv channels could produce an increase in single channel Kv activity and elevation in steady-state I_K, which may lead to significant alteration in membrane excitability and responsiveness to changes in cellular metabolism. Solid lines represent predicted observed effects on whole cell Kv activation, inactivation, and I_K. Dashed lines show plausible graded effects of Kvβ2:Kvβ1/3 ratio on inactivation and I_K.

Kvβ subtypes, the ratio of Kvβ2:Kvβ1/3 present in the Kv auxiliary complex could have a significant impact on inactivation (**Schematic 1**). For example, in channels with non-inactivating Kvα pore subunits (e.g., Kv1.5) and predominantly Kvβ2 subunits, channel inactivation may be slower, as C-type inactivation would be the primary mode of inactivation.[127] Conversely, in channels consisting of variants of Kvβ1 or 3, channel inactivation likely occurs within a faster time frame, as these subunits would contribute to rapid N-type inactivation. In addition to interaction and regulation of Kvα function by the Kvβ proteins, interaction between multiple types of Kvβ can influence the net function of the Kvβ complex on channel gating. For example, incorporation of Kvβ2 can lead to significant inhibition of N-type inactivation imposed by Kvβ1 subunits within the same channel complex.[130] Nonetheless, the extent to which these subunits impact channel activation and inactivation would also be dependent upon pyridine nucleotide redox ratios in the submembrane compartment, as discussed above. Thus, it is plausible that the cell could dynamically fine-tune the regulatory properties of membrane potential to changing metabolic conditions by altering the ratio of Kvβ subunits within the population of functional membrane-inserted channels. In the remainder of this section, I will provide a brief overview of the importance of Kv channels to the cardiovascular, nervous, endocrine, and immune systems, and how the functional expression of Kvβ may influence physiological processes of excitable cells types within each.

Cardiovascular system: In the mammalian heart, multiple types of Kv channels mediate outward K^+ currents with variable activation and inactivation properties that collectively shape the cardiac action potential.[131,132] Attesting to the importance of Kv channel function to cardiac physiology is the robust association between cardiac arrhythmias with mutations in Kv channel subunit genes [133,134], as well as defective ventricular action

potential repolarization in mice lacking Kv proteins.[135-137] The murine heart is known to express Kvβ1.1, Kvβ1.2, and Kvβ2 proteins.[138] Kvβ1 associates primarily with proteins of the Kv4 family and loss of Kvβ1 reduces the abundance of Kv4.3 in the sarcolemma, blunts transient outward K^+ current, and prevents modulation of action potential duration by changes in pyridine nucleotide redox state.[138,139] The physiological role of Kvβ2 remains unclear. Considering that the heart expresses multiple Kvβ subtypes, it is plausible that the promiscuous association between both Kvβ1 and Kvβ2 proteins with Kv1 and Kv4 channels contributes to priming cardiac Kv channels for modulation of channel inactivation. Under conditions of altered nucleotide redox (e.g., altered cardiac workload stress, ischemia), this influences the duration of the early and intermediate phases of repolarization of the action potential.

The Kv channels expressed by vascular smooth muscle are a predominant regulator of vascular tone, and therefore control blood flow and organ perfusion.[140] Kv1 expression and function has been reported in a number of vascular beds, including coronary, pulmonary, mesenteric, and cerebral arteries, among others.[141] Inhibition of Kv1 channels induces vasoconstriction, suggesting that Kv1 channels are tonically active in vascular smooth muscle to oppose vascular tone development.[142] However, little is known regarding the expression and function of Kvβ subunits in the vasculature. Our laboratory recently reported that murine coronary arterial myocytes express heteromeric assemblies of Kvβ complexes in association with Kv1.5 alpha subunits, and that genetic deletion of Kvβ2 reduces the membrane expression of Kv1.5 [143], similar to that reported in neurons and heterologous expression systems.[144,145] Although we and others have speculated that these subunits may play an important regulatory role in coupling tissue oxygen demand with vasodilatory function in various vascular beds [12,146,147], further research is needed to

increase our understanding of how the Kvβ proteins operate in the vasculature and how these may participate in functional or metabolic hyperemic responses.

Unlike most peripheral arteries and arterioles, hypoxia causes rapid and profound vasoconstriction of pulmonary arteries.[148] This phenomenon, referred to as "hypoxic pulmonary vasoconstriction" (HPV), is thought to be an important physiological response of the pulmonary circulation that shunts blood away from under-ventilated lung tissue.[149] However, excessive HPV can lead to pulmonary hypertension, right ventricular hypertrophy, and heart failure.[150] Kv1 channels regulate pulmonary vascular smooth muscle membrane potential and mediate the HPV response.[151,152] HPV is significantly impaired after genetic deletion of redox sensitive Kv1.5 channels, and *in vivo* gene transfer of Kv1.5 normalizes HPV in a model of chronic pulmonary hypertension.[153,154] The association of the Kv1 channels of the pulmonary vasculature with Kvβ proteins may be integral to the HPV response. In support of this, bovine pulmonary arteries exhibit a significant increase in Kvβ1.1 expression with further progression towards higher order pulmonary arteries and arterioles.[155] Higher expression of Kvβ1.1 may impart enhanced inactivation to Kv channels in small vessels in which HPV is apparent by allowing sensing of increases in NADH:NAD ratio upon a decrease in mitochondrial oxidative metabolism during periods of hypoxia.[156] Nonetheless, the precise role of the Kvβ1 subunits in the HPV response has not been directly tested.

Nervous system: Multiple types of Kv channels expressed in the central nervous system control membrane potential and excitability of neurons, and coordinate diverse processes such as action potential propagation and back propagation, neurotransmitter release, and apoptosis.[157] The altered activity or expression of Kv channel proteins in the nervous

system has been associated with human pathological conditions such as epilepsy, multiple sclerosis, and Alzheimer's disease.[158-161] Neurons express multiple Kv alpha subunits that form functional channels, confer A-type K^+ currents, and likely associate with Kvβ proteins, including dendritic Kv4.1, Kv4.2 and Kv4.3 subunits [162-164] and presynaptic Kv1.4 subunits.[162] Variants of all three Kvβ gene products have been found in the brain, with Kvβ2 being the predominant form [165-171], suggesting that Kv1 and Kv4 channels may assemble into heteromers with considerable functional diversity that may participate in the determination of neuronal phenotype. In addition to modulation of channel activation and inactivation characteristics, Kvβ proteins may play a chaperone role and regulate the subcellular targeting of specific populations of Kv channels to distinct neuronal regions (i.e., axonal versus dendritic targeting).[144,145] Genetic deletion of Kvβ2 in mice increases mortality, reduces body weight and results in defects in thermoregulatory processes [172], whereas mice lacking Kvβ1.1 have reduced Kv current inactivation, frequency-dependent spike broadening, and slower afterhyperpolarization compared with wild type mice. These changes in neuronal electrical signaling are associated with impaired learning and memory in water maze and social transmission tasks.[173] Although definitive evidence is lacking, it is possible that changes in brain electrical activity could be strongly modulated by Kvβ-dependent regulation of Kv1 and Kv4 activity during periods of altered neuronal cytosolic redox potential, for example, changes in glucose metabolism.

Endocrine and immune systems: Multiple Kv channel subtypes also participate in the physiological regulation of membrane potential in several cell types outside of the cardiovascular and nervous systems. Studies have suggested that these channels, via regulation of Ca^{2+} influx, also control hormonal secretion in cells of the endocrine system. For example, in pancreatic beta cells, an increase in Ca^{2+} influx following increased

cellular ATP:ADP ratio and inhibition of ATP-sensitive K^+ channels (K_{ATP}) stimulates the release of insulin.[161,174] Repolarization of the cell back to resting potential and cessation of the secretory process, is mediated, in part, by Kv-mediated outward K^+ currents, which are likely mediated by a variety of Kv channel subtypes, including Kv1, Kv2, Kv4.[175] Although the expression profile of associated Kvβ proteins in pancreatic islets is not known, modulation of Kv activity by these subunits may be essential to proper electrical signaling following a glucose-induced rise in NADPH:$NADP^+$ ratio in beta cells.[176] In addition, Kv1 channels have also been shown to be expressed by cells of the immune system.[57] Kv1.5 and Kv1.3 are the predominant Kvα proteins in macrophages and inhibition of Kv1 channels can prevent macrophage activation and proliferation.[177-179] Bone marrow-derived macrophages express all known variants of Kvβ1, and Kvβ2.1 proteins [180]; LPS- and TNF-α induced activation differentially impact the abundance of these proteins and modify channel inactivation. This suggests that modification of Kv1 channel pore and auxiliary subunit composition may reflect an adaptive mechanism that could alter the functional properties of cells in the immune system.

Therapeutic implications

Based on current knowledge of the physiological roles of Kvβ proteins, it is plausible that these proteins and their functional properties may represent an advantageous therapeutic target over conventional pharmacological ion channel blockers for several conditions. Classical inhibitors of AKRs show little inhibition of Kvβ-mediated catalysis, and currently, only a few pharmacological agents are known to impact Kvβ function; these act primarily as inhibitors of catalytic activity or by disrupting the association between the Kvβ and Kvα T1 docking domain. A recent study identified the

acidic dopamine metabolite 3,4-dihydroxphenylacetic acid (DOPAC) as an effective inhibitor of Kvβ2-mediated reduction of 4-nitrobenzaldehyde, inhibiting the production of 4-nitrobenzyl alcohol by ~40% [181], albeit at supraphysiological concentrations. Additional nonendogenous inhibitors such as the cardioprotective drug resveratrol and plant derived flavonoid rutin, only slightly inhibit Kvβ2 catalytic activity by ~38% each. Alternatively, corticosteroids, such as cortisone, directly interact with Kvβ to increase Kv1 channel activity through binding near the cofactor binding pocket and the inter-subunit interface, resulting in dissociation of the Kvβ from the channel.[182] There are currently no known pharmacological agonists that can selectively enhance Kvβ catalytic function. Further elucidation of compounds that can selectively modulate the function of the proteins may be valuable as novel therapeutics for the treatment of multiple disorders. Although the possibility for using compounds identified by these initial studies as Kvβ modulators as therapeutics is unlikely, they provide a useful foundation for further research into more beneficial chemical analogues that may possess more specific biological actions resulting from altering Kvβ function while avoiding off target effects.

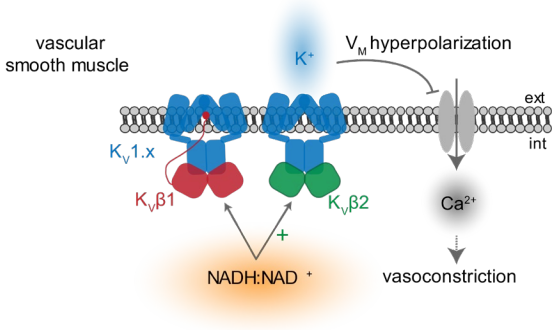
HYPOTHESIS

The regulation of vasomotor control has been studied for over 100 years, however this system in an altered metabolic state has not been well established. Debate over what regulates vasomotor control to constrict or dilate vessels has continued since the first observations of endothelial and sarcolemmal mediated vasomotion.[67] Over the last several decades mediators of vasomotor control during altered metabolism have been proposed and disproved or relegated as weak regulators. However, based on the literature and previous studies conducted by our laboratory and others, I have developed my present hypothesis to begin to investigate this mystery, it states: $K_V\beta$ subunits functionally regulate resistance artery diameter. To address this hypothesis, we developed three key questions or sub-hypothesis; 1) Does deletion of either $K_V\beta$ subunit alter vasoconstriction; 2) Does deletion of either $K_V\beta$ subunit affect vasodilation to stimuli that reflect an altered metabolic state; and finally, 3) Does catalytic activity (of the $K_V\beta2$ subunit) impact vasodilatory function?

INTRODUCTION

The imbalance between myocardial oxygen supply and demand is a salient pathophysiological feature of heart disease, which remains the leading cause of death worldwide.[183] Insufficient myocardial perfusion is associated with cardiac dysfunction in patients with heart failure, hypertension, diabetes, and coronary artery disease.[184-187] A discrepancy between oxygen supply and demand is commonly observed in patients with suppressed coronary vasodilator reserve—even in the absence of stenoses in large coronary arteries—likely due to the inability of small arteries and arterioles to respond to metabolic demand.[188,189] Despite the vital importance of oxygen delivery to the preservation of cardiac function, the fundamental mechanisms by which the coronary vasculature responds to fluctuations in metabolic demand of the myocardium remain poorly understood.

Schematic 2



Schematic 2: Proposed mechanism for $K_V\beta$ mediated modification of K_V channels in the presence of altered pyridine nucleotide redox ratio. The proposed differential response of $K_V\beta$-subunits to an altered pyridine nucleotide redox ratio. For example, in the presence of increased NADH the tail of the $K_V\beta1$ complex inhibits the efflux of K^+ via $K_V1.x$ channels; however, $K_V\beta2$ increases the efflux of K^+ in the presence of increased NADH, inducing membrane hyperpolarization and inhibiting the inward flow of Ca^{+2} thus mediating vasodilation. By contrast, an increase in NAD^+ would result in opposition of the example.

In a healthy heart, the coronary arteries and arterioles operate in a partially constricted state of tone, such that dilation or constriction occurs depending on the need for oxygen

and nutrient delivery.[8,190] As myocardial oxygen consumption increases (e.g., increased heart rate, myocardial contractility, or afterload), the heart requires instantaneous increases in oxygen supply to sustain oxidative energy production by the cardiomyocytes. With little reserve for increased oxygen extraction, sustained cardiac function relies on the intimate link between local and regional metabolic activity and vasodilation of the coronary vascular bed to deliver adequate blood flow to the active myocardium (i.e., metabolic hyperemia).[191] In searching for the molecular entities coupling vascular function with myocardial metabolism, recent studies from our group[56,192] and others[193] have indicated that acute increases in cardiac work promote coronary vasodilation and hyperemia via activation of smooth muscle voltage-gated K^+ channels belonging to the Kv1 family. Nonetheless, how vascular Kv1 channels sense changes in myocardial oxygen demand to regulate blood flow to the heart is unknown. Furthermore, the molecular contributions of the Kv1 channel subunits complex composition and whether these functionally contribute to metabolic hyperemia in the heart has not yet been determined. Based on the literature, and our own data presented here, we posit a physiological role for the K_V1 channels and their β-subunits as mediators of the arterial hyperemic response.

In this study, we tested the hypothesis that vascular function is regulated by the intracellular auxiliary Kvβ proteins. As discussed in c. part 2, the Kvβ proteins are members of the aldo-keto reductase (AKR) superfamily and differentially regulate Kv1 function upon changes in cellular redox status, thus representing a plausible link between metabolic activity and membrane excitability that can influence vasoreactivity. The mammalian genome contains three genes encoding Kvβ proteins, which are expressed in coronary arterial myocytes of humans[194] and rodents.[143] Our previous work indicated the presence of Kvβ heteromers of Kvβ1.1 and Kvβ2 in native Kv1.5 channels of coronary arterial myocytes.[143] Using a combination of genetically engineered animals with *ex vivo*

and *in vivo* approaches, we report that vasoreactivity is controlled by the contrasting regulatory functions of Kvβ1.1 and Kvβ2 proteins.

MATERIALS AND METHODS

Animals

All animal procedures were conducted as approved by Institutional Animal Care and Use Committees at the University of Louisville. Experiments were conducted using age-matched mice and littermates when possible. Additionally, Kvβ1.1$^{-/-}$ and Kvβ2$^{-/-}$ mice [114,173] and strain-matched wild type (C57Bl/6N and 129/SvEv, respectively) mice (25-30 g body mass) were bred in house and fed *ad libitum* normal rodent chow. Transgenic animals were generated (Cyagen) with mouse *Kcnab1* (NM_01059734) as the control of the tetracycline responsive element (TRE, 2nd generation) promoter (TRE-Kcnab1.1). Hemizygous TRE-Kcnab1.1 mice were bred to transgenic mice with the reverse tetracycline transactivator under the control of the murine SM22-alpha (SM22α or transgelin) promotor (SM22α-rtTA; Jackson Laboratories, stock no. 006875, FVB/N-Tg (Tagln-rtTA)E1Jwst/J)[195] to yield double hemizygous SM22α-rtTA:TRE-Kcnab1.1 and littermate single transgenic SM22α-rtTA controls. Male mice (aged 3-6 months) were used for the study. All animals were housed in a temperature-controlled room on a 12:12 light:dark cycle with *ad libitum* access to food and water. Mice were euthanized by intraperitoneal injection of sodium pentobarbital (150 mg·kg^{-1}) and thoracotomy and tissues were excised immediately for ex vivo functional measurements and biochemical assessments.

Chemicals

U46619 (CAS#: 56985-40-1), psora 4 (CAS#: 724709-68-6) was purchased from Tocris Bioscience (Tocris Bioscience, Bristol, United Kingdom). Chemicals purchased

from Sigma-Aldrich (St. Louis, Missouri, US) include: D-glucose (CAS#: 50-99-7), $NaHCO_3$ (CAS#: 144-55-8), sodium chloride (CAS#: 7647-14-5), calcium chloride (CAS#: 10043-52-4), magnesium chloride (CAS#: 7786-30-3), HEPES (CAS#: 7365-45-9), potassium chloride (CAS#: 7447-40-7), potassium phosphate monobasic (CAS#: 7778-77-0), sodium lactate (CAS#: 867-56-1), nifidepine (CAS#: 21829-25-4), and forskolin (CAS#: 66575-29-9). We used a Sartorius Arium mini water system (Sartorius Lab Instruments GmbH & Co., Goettingen, Germany).

Arterial diameter measurements

Third and fourth order branches of mesenteric and first and second order left anterior descending coronary arteries were dissected and kept in ice-cold isolation buffer consisting of (in mM): 134 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 7 D-glucose, pH 7.4. Isolated arteries were used for arterial diameter measurements within 8 h after dissection. Isolated arteries were cleaned of connective tissue and cannulated on glass micropipettes mounted in a linear alignment single vessel myograph chamber (Living Systems Instrumentation, St. Albans, VT, USA). For a set of experiments, the vascular endothelium was functionally ablated by passage of air through the lumen (~30 s) during the cannulation procedure. After cannulation, the chamber was placed on an inverted microscope and arteries were equilibrated at 37°C and intravascular pressure of 20 mmHg, maintained with a pressure servo control unit (Living Systems Instrumentation, St. Albans, VT, USA) under continuous perfusion (3-5 $ml \cdot min^{-1}$) of physiological saline solution (PSS) consisting of (in mM): 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 7 D-glucose, 24 $NaHCO_3$, 2 $CaCl_2$, maintained at pH 7.35-7.45 by aerating with 5% CO_2, 20% O_2, balance N_2.

Following an equilibration period (45-60 min), lumenal diameter was continuously monitored and recorded with a charge coupled device (CCD) camera and edge detection software (IonOptix, Milton, MA, USA). Experiments were performed to examine effects of step-wise increases in intravascular pressure (20-100 mmHg), elevated $[K^+]_o$ (via isosmotic replacement of KCl for NaCl), the synthetic thromboxane A_2 analogue U46619 (Tocris Bioscience, Minneapolis, MN, USA), adenosine (Sigma Aldrich, St. Louis, MO, USA), or L-lactate (Sigma Aldrich, St. Louis, MO, USA). For a set of experiments, hypoxic bath conditions were generated by perfusion of 1 mM $Na_2S_2O_4$-containing PSS that was aerated with 5% CO_2 (balance N_2). Bath O_2 levels were measured using a dissolved oxygen meter (World Precision Instruments, Sarasota, FL, USA). At the end of each experiment, the maximum passive diameter was measured in the presence of Ca^{2+}-free PSS containing the L-type Ca2+ channel inhibitor nifedipine (1 μM) and adenylyl cyclase activator forskolin (0.5 μM), as described previously.[196,197] Vasoconstriction is expressed as a decrease in arterial diameter relative to the maximum diameter at a given intravascular pressure. Changes in diameter (e.g., vasodilation) are normalized to differences from baseline and maximum passive diameters for each experiment.

Statistics

Data are presented as mean ± SEM. Data were analyzed using GraphPad Prism software using paired or unpaired Student's t-tests, and Mann Whitney U and Wilcoxon signed rank non-parametric tests for comparisons of two experimental groups. One-way and two-way analysis of variance or non-parametric tests with Tukey post-hoc tests were used for comparisons of multiple groups and repeated measures datasets, as indicated in Figure Legends. P-value less than 0.05 was considered statistically significant.

RESULTS

Lactate induced vasodilation in mesenteric and coronary arteries

The vasoreactivity to lactate (a byproduct of increased cellular metabolism that is converted by the lactate dehydrogenase enzyme to pyruvate and increases the NADH to NAD^+ ratio) was compared between mesenteric and left anterior descending coronary arteries from WT and $K_V\beta2^{-/-}$ mice **Figure 1** (**A, B**). As indicated in the representative traces **Figure 1** (**A, B**), increasing concentrations of lactate (5-20 mM) induced dilation. This response is summarized in **Figure 1** (**G, H**). I investigated the role of the K_V1 channel in mediating vasodilation in response to vasoactive compounds (e.g., lactate), we first perfused the vasoactive compound (lactate) at several concentrations (5-20 mM) in the absence and presence of known general K_V1 channel inhibitor Psora-4. As indicated in **Figure 1** (**A, B**) 15 mM lactate induced vasodilation in both mesenteric and coronary arteries and addition of Psora-4 (**C, D**) ablated the vasodilation response. To assess the potential role of $K_V\beta$-subunits in K_V1 channel mediated vasodilation in response to lactate, measurements of intralumenal diameter were obtained from freshly isolated mesenteric arterioles pressurized at 80 mmHg. Arteries were preconstricted (100 nM U46619) and perfused increasing concentrations of lactate (5-20 mM). In mesenteric and coronary arteries isolated from WT mice we observed that when 20 mM lactate was perfused into the bath arteries dilated and this was significantly reduced with addition of the K_v1-channel inhibitor Psora-4 (100 nM) (**Figure 1A, B and C, D**, respectively). However, in arteries

from $K_V\beta2^{-/-}$ mice demonstrates lactate (15 and 20 mM) induced vasodilation was significantly ablated **Figure 1** (**E, F**) compared to their respective wildtype.

Figure 1

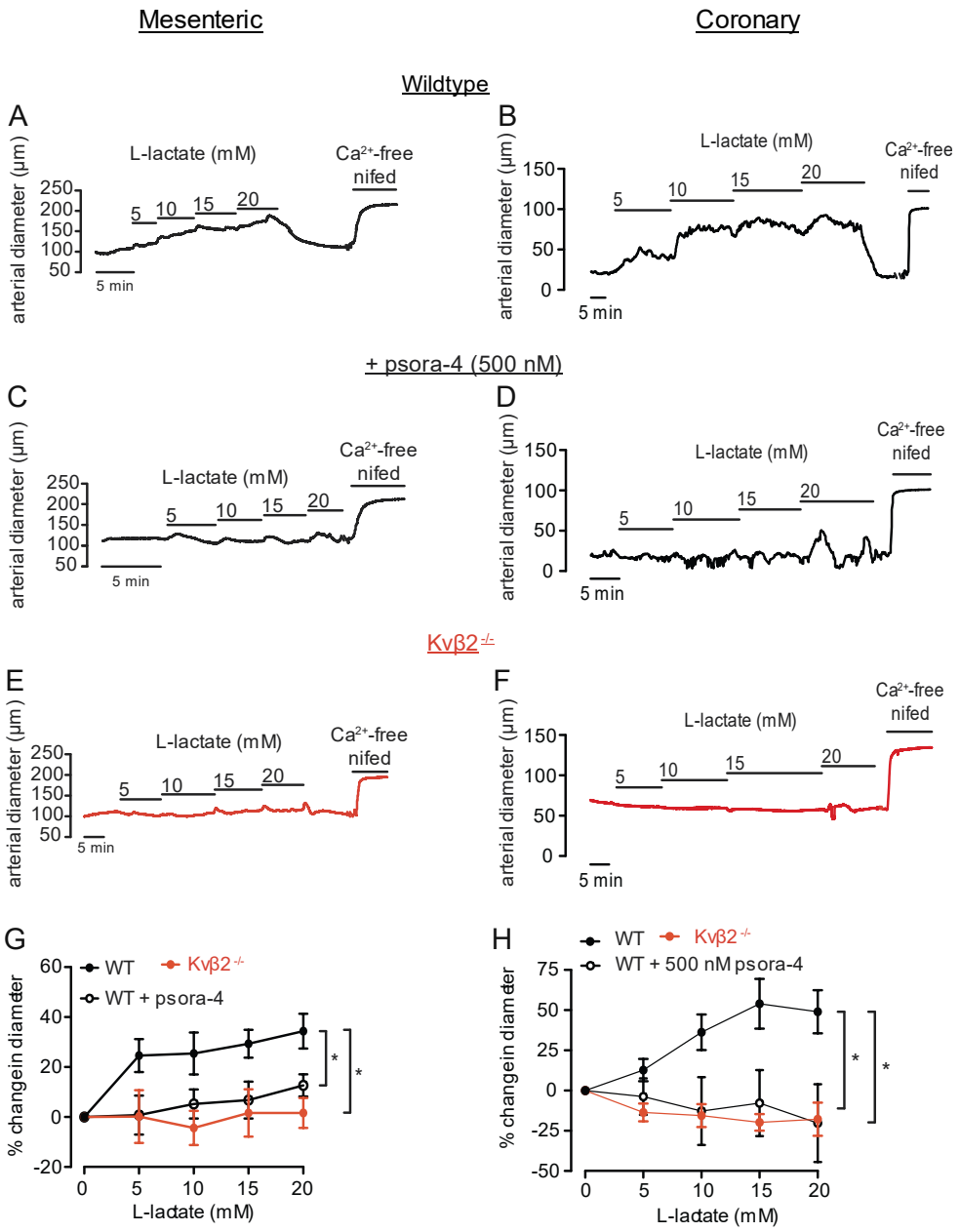


Figure 1. K_V1 channels mediate lactate induced vasodilation in small resistance arteries. (**A, B, C, D, E, F**) Representative traces of arterial diameter recordings of pressurized (80 mmHg) thromboxane A_2 analogue preconstricted (100 nM U4661) mesenteric and left anterior descending (LAD) coronary arteries from WT (± psora4), and $K_V\beta2^{-/-}$ mice before and after perfusion of sodium lactate (5-20 mM). Each experiment was concluded with the addition of Calcium free PSS (Ca^{2+}-free), 1 µM nifedipine (nifed), and 500 nM forskolin (fsk) (n = 5 and 4 arteries mesenteric and coronary respectively; N = 5 and 4 mice, mesenteric and coronary, respectively; * = P<0.05). (**G, H**) Summary of lactate induced dilation for arteries from WT (± psora4), and $K_V\beta2^{-/-}$mice (* = P-value <0.05).

Myogenic tone development is independent of changes to the $K_V\beta$-subunit complex composition.

Regulation of blood flow is tightly controlled by the ability of small resistance arteries (~100-300 μm)[8] to develop myogenic tone and thus regulate vascular resistance.[8] Myogenic tone development is an autoregulatory mechanism meant to maintain a constant flow of blood through resistance arteries. We stepwise increased intravascular pressure through the physiological range (i.e., 60-100 mmHg). Representative traces of arteries from $K_V\beta1$-null ($K_V\beta1^{-/-}$), $K_V\beta2$-null ($K_V\beta2^{-/-}$) and their respective wildtypes show that ablation of either $K_V\beta$-subunit did not impair myogenic tone development (**Figure 1A**). For example, at 80 mmHg, arteries from $K_V\beta1^{-/-}$ mice (**Figure 2B**) and $K_V\beta2^{-/-}$ and constricted like their respective wildtypes (**Figure 2C**). Summary graphs show at each pressure the passive diameters, relative to 0 mmHg, are not different between $K_V\beta1^{-/-}$, $K_V\beta2^{-/-}$ and respective matched wild types (**Figure 2D**). These data support the conclusion that ablation of either $K_V\beta$-subunits does not significantly impact the mechanisms that mediate the development of myogenic tone. Furthermore, these data also verify that the method of knocking out of the $K_V\beta$-subunits does not impact this mechanism of vascular tone regulation.

Figure 2

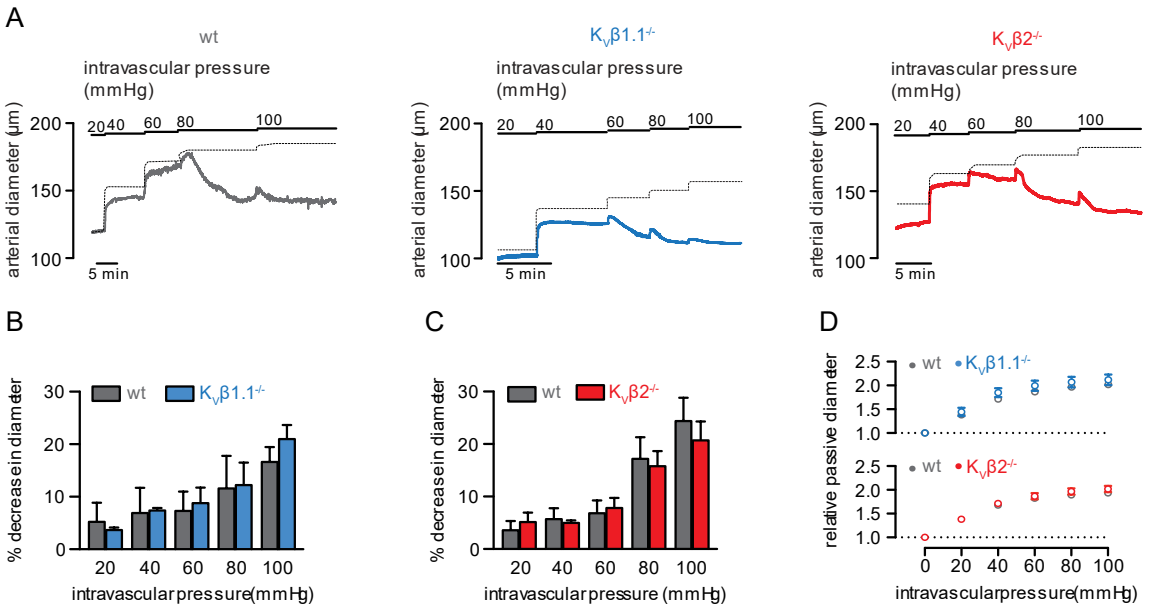


Figure 2. Myogenic tone development is independent from changes to the $K_V\beta$-subunit complex composition. (**A**) Representative arterial diameter recordings obtained from arteries isolated from WT (129SvEv), Kvβ1.1$^{-/-}$, and Kvβ2$^{-/-}$ mice across a range of intravascular pressures, as indicated. Maximum passive diameters, obtained in Ca^{2+}-free bath solution containing 100 nM nifedipine, are shown for each (*dashed traces*). (**B, C**) Bar graphs showing summarized % decrease in diameter at each intravascular pressure tested for arteries isolated from WT (n = 4 arteries from 4 mice) and Kvβ1.1$^{-/-}$ (*D*, n = 5 arteries from 5 mice) mice (*D*), and WT (n = 6 arteries from 5 mice) and Kvβ2$^{-/-}$ (n = 6 arteries from 5 mice) (P>0.05 at each pressure, respectively (Mann-Whitney U). (**D**) Summary of passive diameters relative to diameters at 0 mmHg across the range of intravascular pressures tested for arteries isolated from Kvβ1.1$^{-/-}$ (n = 5 arteries from 5 mice), Kvβ2$^{-/-}$ (n = 6 arteries from 5 mice), and corresponding WT controls (n = 4 arteries from 4 mice; n = 6 arteries from 5 mice) (P-value >0.05 at each pressure, respectively (Mann-Whitney U).

Vasoconstriction response in resistance arteries is not impacted by loss of $K_V\beta$ subunits.

Changes in membrane potential regulate the flow of ions across the membrane, as in the case of Ca^{2+} increased inward flow modulates greater vasoconstriction.[198] The influx of Ca^{2+} is tightly regulated by efflux of K^+ ions which mediate hyperpolarization of the cell membrane and prevent opening of voltage gated Ca^{2+} channels (**Schematic 2**). To assess whether ablation of $K_V\beta$-subunits, $K_V\beta1^{-/-}$ or $K_V\beta2^{-/-}$, modifies the 60 mM K^+ induced vasoconstriction we utilized freshly isolated small resistance arterioles from $K_V\beta1^{-/-}$ and $K_V\beta2^{-/-}$ mice and their respective wildtypes. Introduction of high potassium ($\geq$60 mM potassium) inhibits outward potassium flow effectively clamping membrane potential to the Nernst potential depolarizing the membrane, opening voltage gated calcium channels, and inducing vasoconstriction. In response to 60 mM K^+, arteries isolated from $K_V\beta1^{-/-}$ and WT constricted, respectively (**Figure 3A, B**). Similarly, in $K_V\beta2^{-/-}$ and WT, arteries constricted, respectively (**Figure 3C, D**). We also examined whether the thromboxane A_2 mediated vasoconstrictive response was affected by ablation of either $K_V\beta1^{-/-}$ or $K_V\beta2^{-/-}$. Representative traces from arteries perfused with the thromboxane A_2 analogue U46619 (100 nM) constricted similarly between $K_V\beta1^{-/-}$ and $K_V\beta2^{-/-}$ and their respective strain matched wildtypes (**Figure 3C, D**). From these data, we can conclude that ablation of individual $K_V\beta$-subunits ($K_V\beta1^{-/-}$ and $K_V\beta2^{-/-}$) does not significantly impact mechanisms of vasoconstriction in resistance arteries.

Figure 3

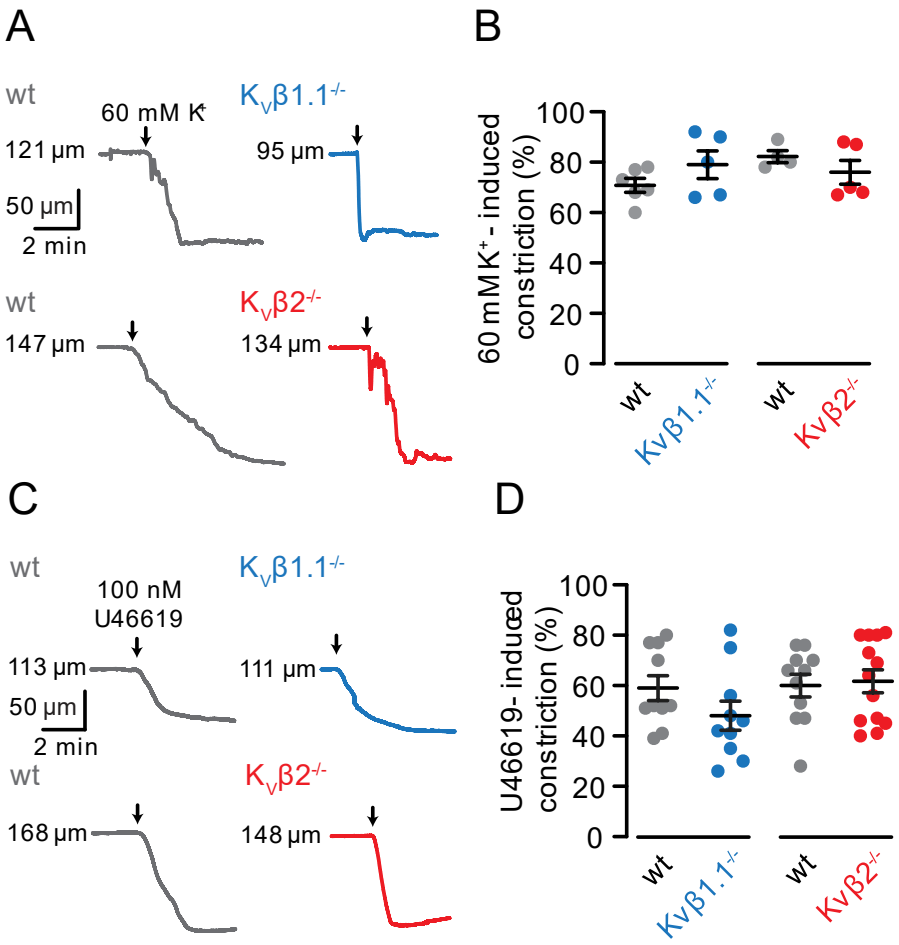


Figure 3. Vasoconstriction response in resistance arteries is not impacted by loss of either Kvβ subunits. (**A, B**) Representative arterial diameter recordings obtained from pressurized (20 mmHg) mesenteric arteries before and after application of 60 mM external K^+ (indicated by arrows; *A*), and summary of 60 mM K^+-induced constriction (% decrease in diameter, *B*) in arteries isolated from Kvβ1.1$^{-/-}$ (n = 5 arteries from 5 mice), Kvβ2$^{-/-}$ (n = 5 arteries from 4 mice), and strain-matched WT controls (C57Bl6N for Kvβ1.1$^{-/-}$, n = 4 arteries from 4 mice; 129SvEv for Kvβ2$^{-/-}$; n = 6 arteries from 5 mice). P=0.41 and. 0.43, respectively; Mann-Whitney U test. (**C, D**) Representative arterial diameter recordings from arteries (80 mmHg) before and after application of 100 nM U46619 (indicated by arrows, *C*), and summary of U46619-induced constriction (% decrease in diameter, *D*), from groups as described in *B*. WT (C57Bl6N): n = 11 arteries from 10 mice; Kvβ1.1$^{-/-}$: n = 11 arteries from 11 mice, WT (129SvEv): n = 10 arteries from 9 mice; Kvβ2$^{-/-}$: n = 10 arteries from 10 mice. P-value >0.05 respectively (Mann-Whitney U).

Adenosine-induced vasodilation is not affected by knockout of K_Vβ-subunits

Adenosine is a potent vasodilator, which acts via $A_{2A/2B}$ to activate the G_S protein to stimulate adenylate cyclase and increase intracellular cyclic adenosine monophosphate (cAMP).[199] Therefore, in order to assess adenosine induced dilation in the absence of specific K_Vβ-subunits, we perfused increasing concentrations of adenosine onto isolated arteries from K_Vβ-subunit null ($K_V\beta1^{-/-}$ or $K_V\beta2^{-/-}$) and wildtype mice. Our data show that at 10^{-5}M, adenosine-induced vasodilation is not significantly different between $K_V\beta1^{-/-}$ or WT (**Figure 4A, B**). Similarly, adenosine induced similar vasodilation in isolated arteries from $K_V\beta2^{-/-}$ and respective WT mice (**Figure 4A, B**).

Figure 4

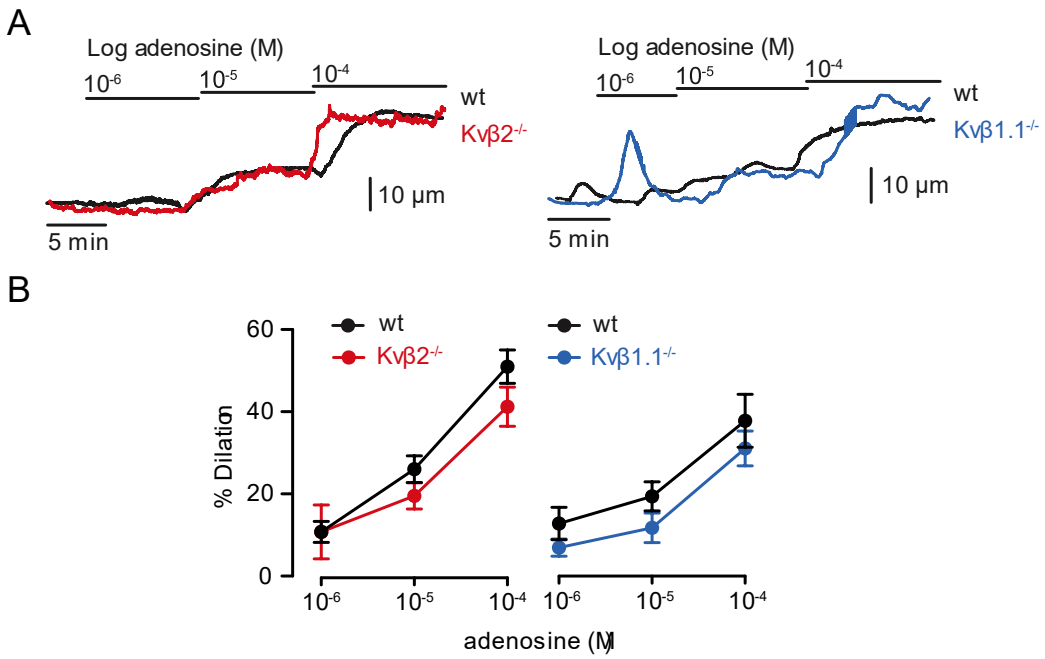


Figure 4. Adenosine-induced vasodilation is not affected by knockout of K_Vβ-subunits. Adenosine induced similar vasodilation in K_Vβ-null and respective wildtypes. At 10 µM adenosine induced vasodilation is not significantly different between $K_V\beta1^{-/-}$ or wildtype (n = 6, 5; N = 6, 4). Similarly, adenosine induced similar vasodilation in $K_V\beta2^{-/-}$ and wildtype (n = 4, 4; N = 3, 3). P-values > 0.05 (Mann-Whitney U test).

Lactate-induces vasodilation independent of endothelial function

The endothelium is considered a key mediator of vasodilation. Lactate could elicit vasodilation via production of endothelium-derived relaxation factors (e.g., H_2O_2). However, we investigated whether the endothelium mediates vasodilation in response to lactate by comparing endothelial intact and denuded vessels. To confirm inactivation (i.e., denudation) of the endothelium we used the SK_{Ca}/IK_{Ca} opener NS309, which induces endothelial dependent vasodilation. **Figure 5** (**A**) shows representative traces of intact and endothelium-denuded (-endo) mesenteric arteries in the presence of the SK_{Ca}/IK_{Ca} opener NS309 (1 μM) to confirm loss of function in denuded vessels. The summary data of these experiments (**Figure 5B**) display loss of NS309 induced dilation. **Figure 5** (**C**) shows representative traces of intact and denuded vessels that both dilated to approximately 20% in the presence of 10 mM lactate. These summary data (**Figure 5D**) reveal that lactate induced similar levels of vasodilation in both intact and denuded arteries indicating that the mechanism of lactate-induced vasodilation is independent of endothelial function.

Figure 5

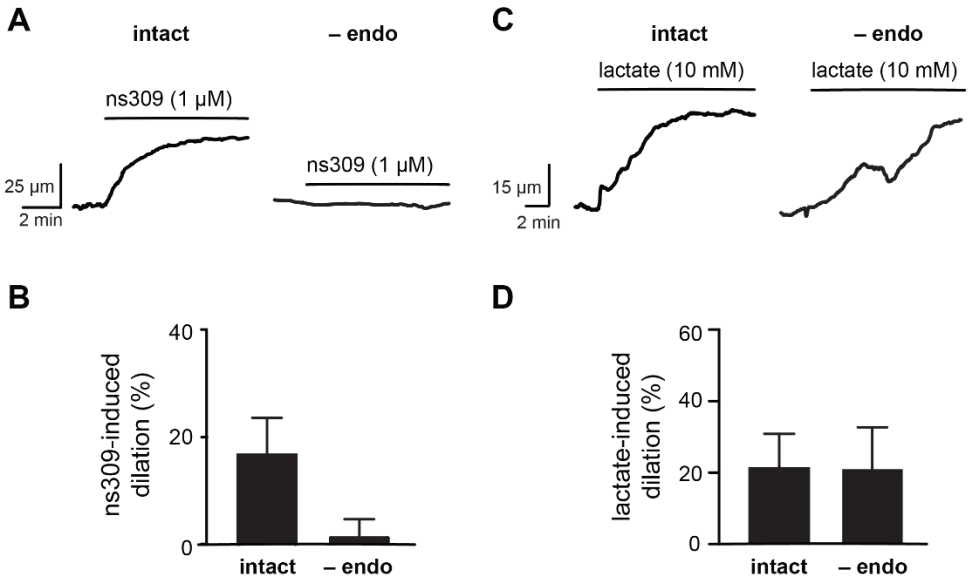


Figure 5: Lactate induced vasodilation independent of endothelial function. (**A**) Arterial diameter recordings from preconstricted (100 nM U46619) intact and endothelium-denuded (- endo) in the absence and presence of the SK_{Ca}/IK_{Ca} opener NS309 (1 µM). (**B**) Summary graph showing 1 uM NS309 induced dilation (%) ± SEM in WT arteries with either an intact or denuded endothelium. n= 5 and 7 arteries from 5 and 4 mice. P = 0.056 (Mann-Whitney U test)**.** (**C**) Representative recordings of arterial diameter from preconstricted (100 nM U46619) WT intact and - endo arteries in the presence of 10 mM lactate. (**D**) Summary bar graph of lactate induced dilation (%) ± SEM in WT intact and - endo arteries. n = 5 and 6 arteries from 5 and 3 mice. P-values > 0.99 (Mann-Whitney U test).

Overexpression of $K_V\beta1.1$ impairs lactate induced vasodilation

Ablation of $K_V\beta1.1$ did not impact lactate induced vasodilation as compared to the respective wildtype **Figure 6 A**. However, vasodilation was significantly blunted in isolated arteries in response to extracellular L-lactate when compared with arteries from single transgenic control mice (**Fig. 6 C, D**). The overexpression of Kvβ1 emulates the observed response to lactate in arteries from $Kv\beta2^{-/-}$ mice (**Fig. 1 E, F**). These data indicate a dynamic difference in the role of the Kvβ-subunits in responding to stimuli (e.g., lactate) where Kvβ1 opposes, and Kvβ2 enhances, vasodilation.

Figure 6

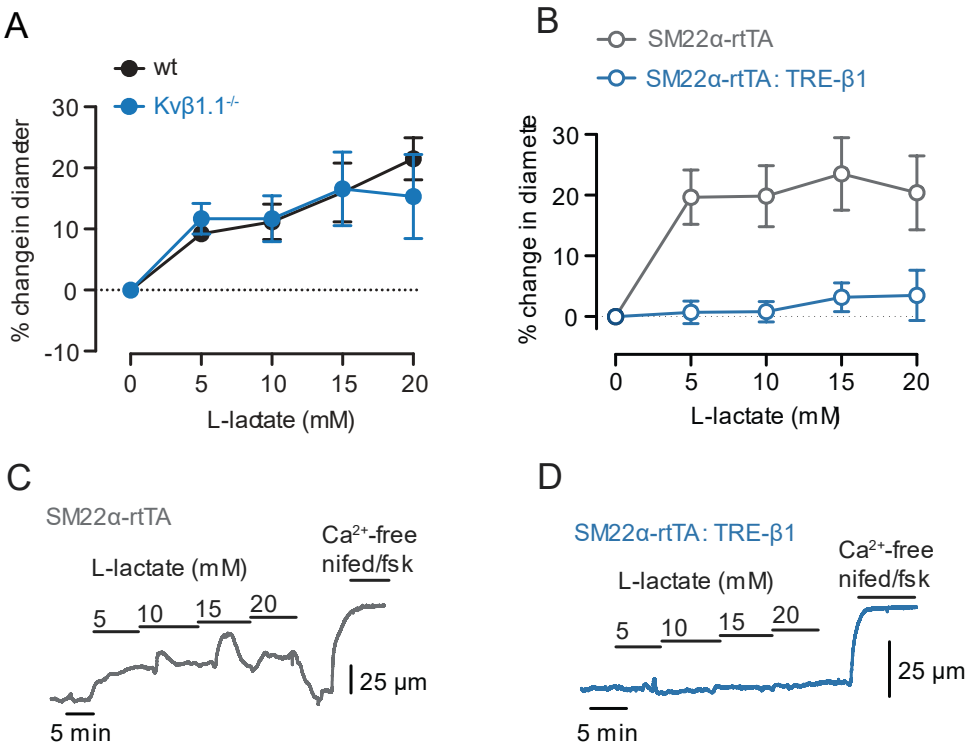


Figure 6. Increasing the ratio of Kvβ1.1:Kvβ2 subunits in smooth muscle , not knockout of Kvβ1.1 (Kvβ1.1$^{-/-}$), inhibit L-lactate-induced vasodilation. (A, B) Summarized data of Kvβ1.1$^{-/-}$ and SM22α-rtTA:TRE-β1 arteries perfused with increasing concentrations of L-lactate (5-20 mM) as compared to the respective wildtypes. (C, D) Representative arterial diameter recordings from 100 nM U46619-preconstricted mesenteric arteries isolated from SM22α-rtTA and SM22α-rtTA:TRE-β1 mice in the absence and presence of L-lactate (5-20 mM), as in Figure 5 B. Passive dilation in the presence of Ca^{2+}-free solution + nifedipine (1 µM) and forskolin (fsk; 0.5 µM) is shown for each recording. Kvβ1$^{-/-}$: n = 7 arteries from 7 mice; SM22α-rtTA: n = 6 arteries from 5 mice; SM22α-rtTA:TRE-β1: n = 10 arteries from 6 mice; * = P-value <0.05 (two-way ANOVA).

Association and expression of K_V1.2, K_V1.5, K_Vβ1 and K_Vβ2 are similar in vascular smooth muscle cells isolated from coronary and mesenteric arteries

The distribution of ion channels, and other proteins expressed in the vasculature are not consistently distributed across vascular beds. It is essential to show the expression and colocalization of the K_V complex composition is consistent across resistance vasculature beds. In **Figure 7** (**A**) Both, coronary and mesenteric vascular smooth muscle cells have similar colocalization of the vascular K_V channel complexes (i.e., K_V1.2, K_V1.5, K_Vβ1 and K_Vβ2). These data are summarized in **Figure 7** (**B**), indicating no significant difference between coronary and mesenteric myocytes colocalization of K_V1.2, K_V1.5, K_Vβ1 and K_Vβ2.

Figure 7

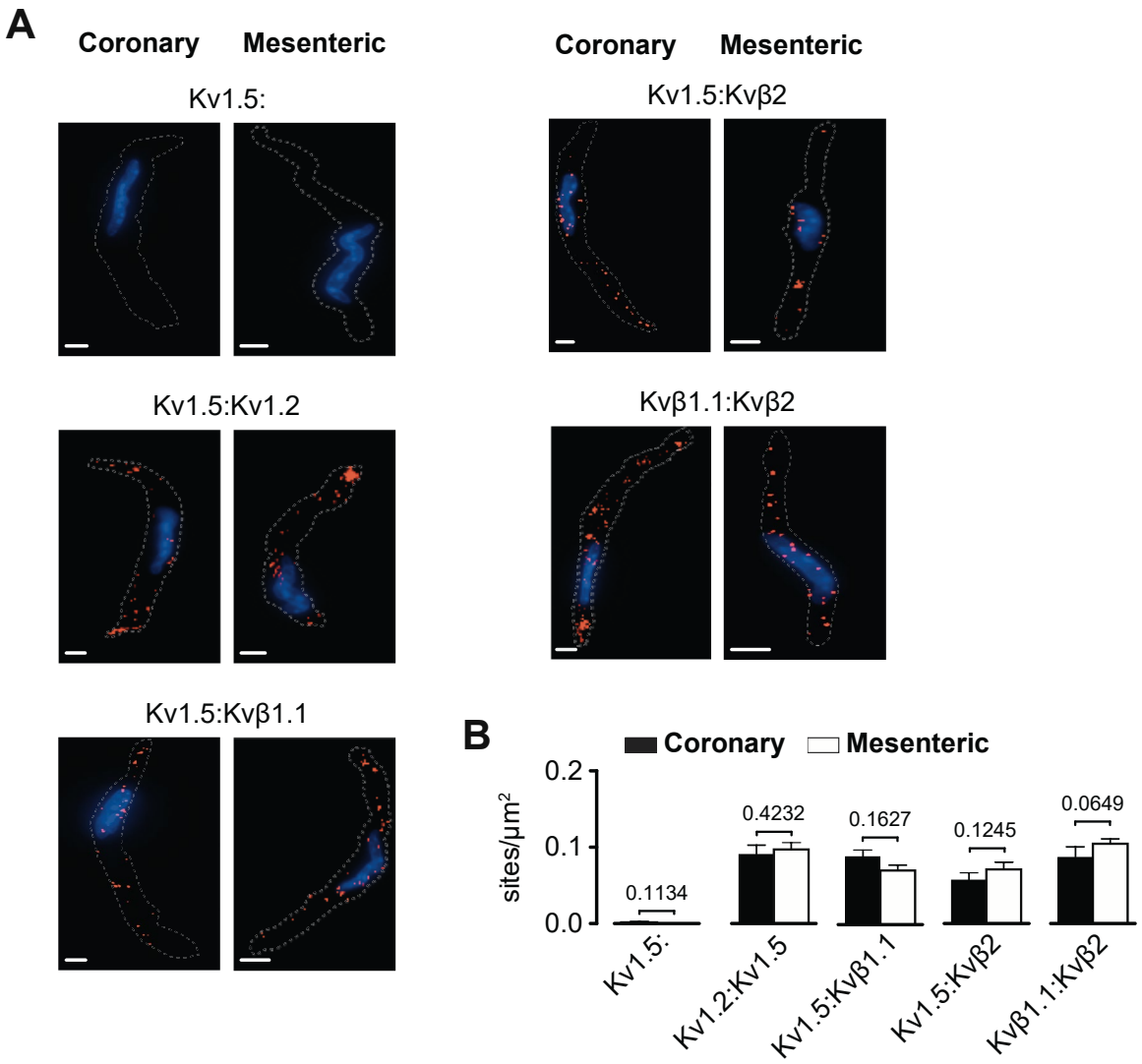


Figure 7. Coronary and mesenteric arterial myocytes colocalization of K_V1.2, K_V1.5, K_Vβ1 and K_Vβ2. (A) Proximity ligation (PLA) experiments show similar localization of K_V1.2, K_V1.5, K_Vβ1 and K_Vβ2 (red foci) in isolated myocytes (membrane surface outlined with dots) from both coronary (left) and mesenteric (right) arteries (nucleus visualized in blue using DAPI) (all scale bars represent 5 μm reference). (B) Summarized data of PLA (n = 3) experiments showing no significant difference in K_V1.2, K_V1.5, K_Vβ1 and K_Vβ2 colocalizations in between coronary or mesenteric myocytes.

Ablation of $K_V\beta1.1$ did not significantly impact hypoxia induced vasodilation

Hypoxia occurs when O_2 saturation drops below 20% and induces the vasodilatory response. Based on the different responses observed in $K_V\beta1^{-/-}$ and $K_V\beta2^{-/-}$ arteries, we proposed that hypoxia induced vasodilation response would be abolished in the absence of the $K_V\beta2$ subunit. Using the knockout mice, described above, we examined the effect of $K_V\beta$-subunit ablation on the hypoxia response in coronary arteries. Representative traces (**Figure 8 A**) show dilation in response to hypoxia in $K_V\beta1^{-/-}$ and their respective wildtype. However, in **Figure 8** (**C**) hypoxia induced dilation does not occur in $K_V\beta2^{-/-}$ arteries, but it does in the respective wildtype. These experiments are graphically summarized in **Figure 8** (**B, D**).

Figure 8

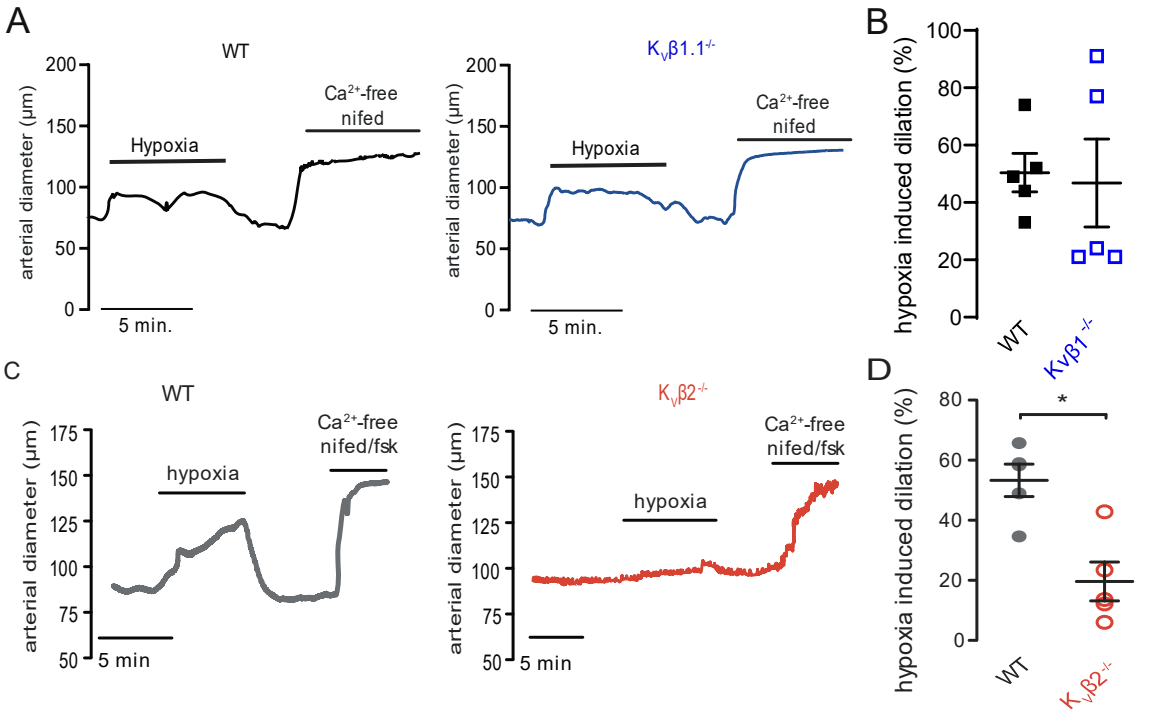


Figure 8. Ablation of $K_V\beta2$ not $K_V\beta1.1$ significantly diminishes hypoxia induced vasodilation. (A) Representative myography trace of hypoxia induced vasodilation in arteries from WT (left, black) and $K_V\beta1.1^{-/-}$ (right, blue) mice. (B) Summary graph of hypoxia induced dilation in WT and $K_V\beta1.1^{-/-}$ arteries, respectively (P-value >0.5). (C) Ablation of $K_V\beta2$ (right, red) significantly abolished hypoxia induced vasodilation as compared to wildtype (grey, left). (D) Summary graph of hypoxia induced dilation in wildtype and $K_V\beta2^{-/-}$.

K_Vβ2 catalytic function is necessary for lactate induced vasodilation

The K_Vβ subunits are members of the aldo-keto reductase (AKR) family and is important in the reduction of carbonyls. The K_Vβ2 subunit retains pyridine nucleotide binding pockets that are conserved throughout the AKR family.[95] The point mutation of the K_Vβ2 subunit at the tyrosine in the 90th position to a phenylalanine abolishes the acid-base catalytic functionality of the subunit.[122] The Y90F point mutation abolishes the K_Vβ2 mediated lactate induced vasodilation response **Figure 9** (**A**).

Figure 9

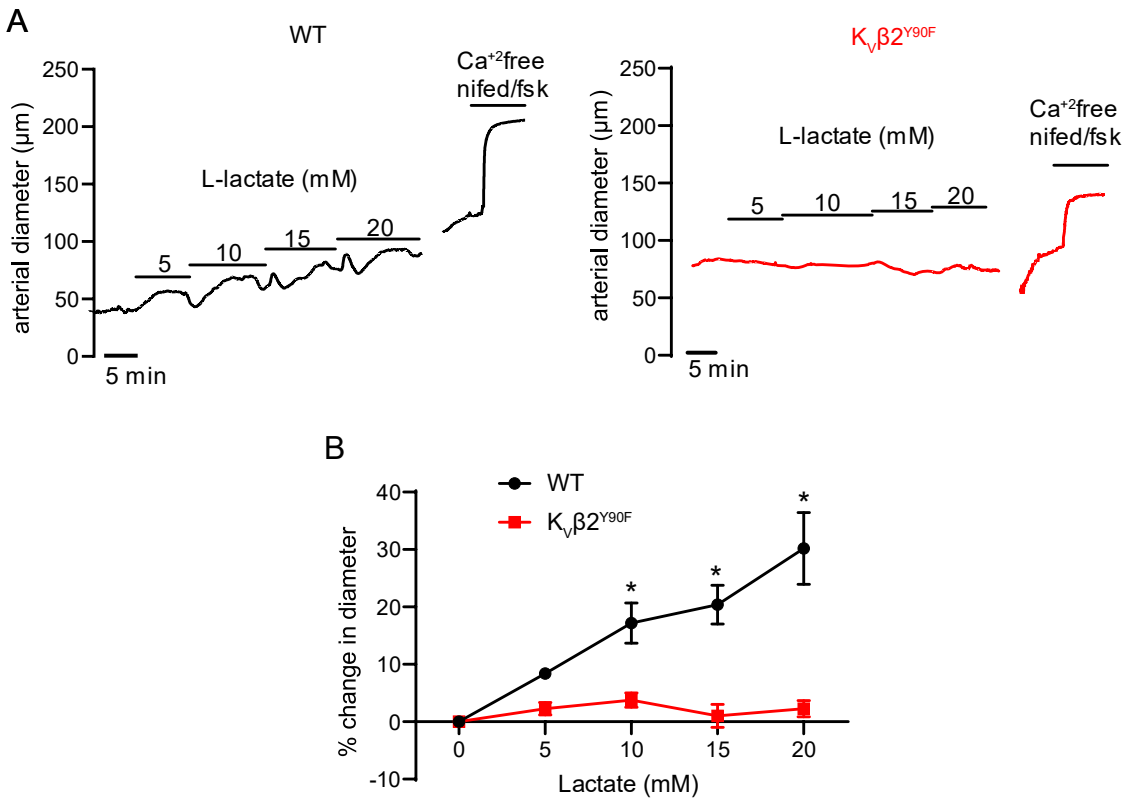


Figure 9. Catalytically inactive $K_V\beta2$ point mutant ($K_V\beta2^{Y90F}$) abolishes lactate induced vasodilation. (A) Representative trace of arterial diameter from mesenteric arteries from wild type and $K_V\beta2^{Y90F}$ mice in the presence of lactate (5-20 mM) and passive dilation in the presence of Ca^{2+} free PSS with 1uM nifedipine and 500 nM forskolin (n = 5 and 4, respectively). (B) Summary graph of percent (%) change in diameter comparing wildtype and $K_V\beta2^{Y90F}$ dilation to L-lactate (5-20 mM) (* = P-value < 0.05).

Figure 10

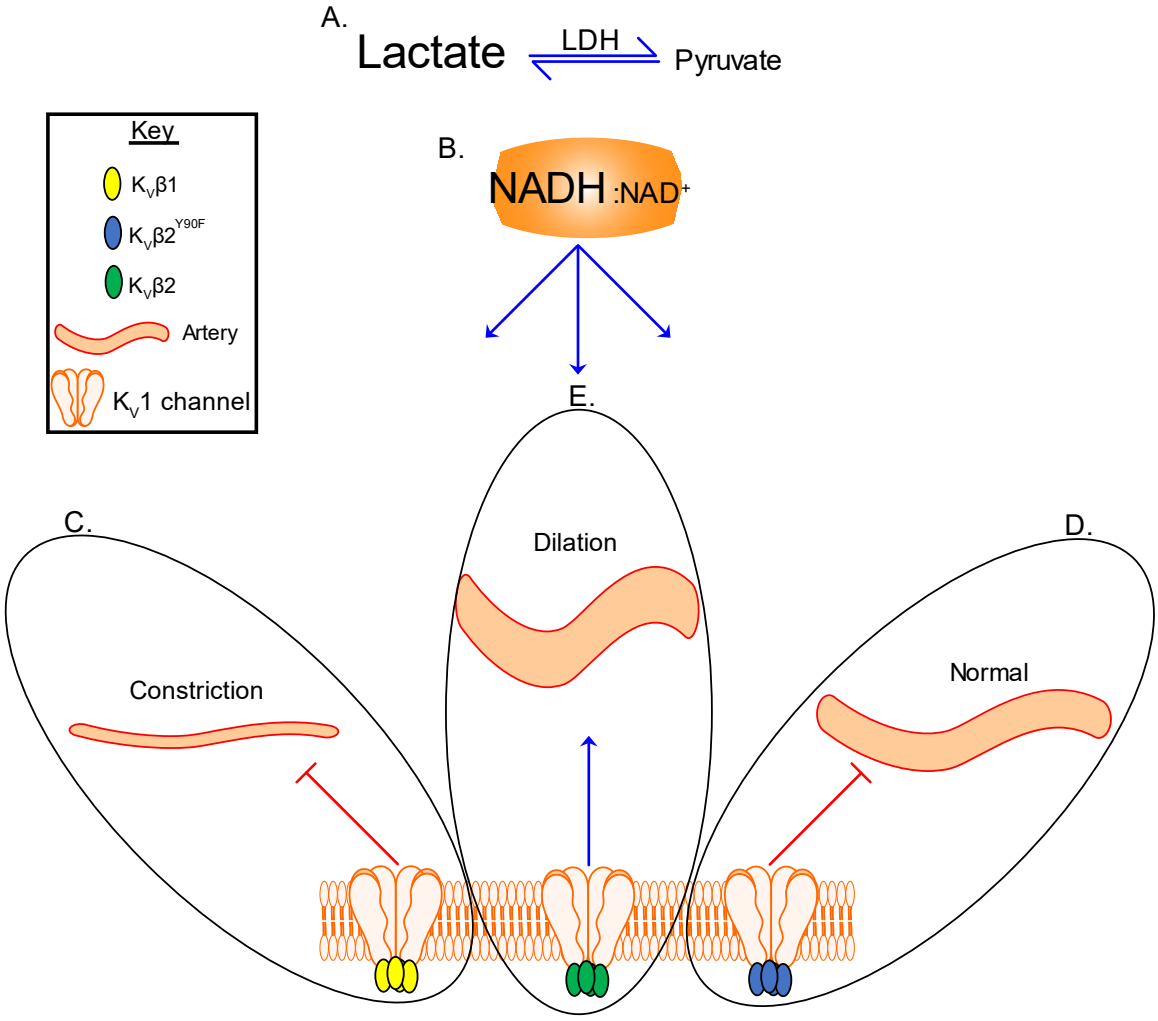


Figure 10. K_V1 mediated vasodilation in response to elevated L-lactate requires catalytically functional $K_V\beta2$. (A) Increased lactate is converted to pyruvate and $NADH^+$ (B) via the lactate dehydrogenase enzyme. (C) In the absence of $K_V\beta2$ (green), the $K_V\beta1$ (yellow) subunit predominates and in the presence of lactate and vasoconstriction occurs. (D) The catalytically inactive $K_V\beta^{Y90F}$ (blue) abolishes lactate induced vasodilation. (E) Catalytically functional $K_V\beta2$ (green) is required for arterial dilation in response to the vasodilator lactate.

DISCUSSION

Here we have identified the Kvβ proteins as functional regulators of vascular tone and further implicate divergent functional roles for Kvβ1 and Kvβ2 in the regulation of metabolic coronary hyperemia. The following key findings support these conclusions: 1) resistance arteries from Kvβ-null (Kvβ1.1$^{-/-}$, Kvβ2$^{-/-}$) mice exhibit normal vasoreactivity to increases in intravascular pressure, elevated external K^+, and thromboxane receptor agonism; 2) vasodilation in response to L-lactate is abolished upon treatment with Kv1-selective inhibitors and point mutation to or genetic ablation of Kvβ2 (K_Vβ2^{Y90F} and K_Vβ2$^{-/-}$, respectively), but not Kvβ1.1 proteins; and 3) that the K_Vβ-subunits function similarly in varying resistance vasculature beds (e.g., mesenteric and coronary) (**Figure 10**). Thus, our results collectively support the concept that the predominant Kv1 auxiliary subunits, Kvβ1.1 and Kvβ2, function to cooperatively control vasodilation upon changes in metabolic demand.

Kvβ proteins of the AKR6 family of aldo-keto reductases are catalytically active hydrophilic proteins that form heterotetrameric complexes at the cytosolic domain of native voltage-gated potassium channels in excitable cell types throughout the cardiovascular, nervous, endocrine, and immune systems.[114] By the non-selective binding of a wide range of carbonyl substrates, these proteins catalyze the NAD(P)H-dependent reduction of a variety of endogenous aldehydes and ketones to primary and secondary alcohols.[139,156] Through differential regulation of channel activation and inactivation properties as a function of pyridine nucleotide redox status[115], these proteins may participate in numerous

physiological processes by coupling outward K^+ current and membrane excitability with intermediary metabolism.[126] Despite nearly two decades of research on these intriguing proteins, further experimentation is necessary to fully elucidate how distinct Kvβ proteins perform unique and cell-specific roles in different organ systems.

The tight regulation of blood flow is crucial to ensure blood pressure, and organ perfusion are maintained. Here, we report a physiologically relevant mechanism of vasodilatory control in small resistance arteries mediated via the K_V1 channel β-subunit complex composition in response to altered metabolic demand that shifts substrates (e.g., NADH etc.) redox ratio.[115] Our data demonstrate that the K_V1-$K_V\beta$-subunit complex is sensitive to lactate, which in turn induces a physiological response i.e., vasodilation. This effect is abolished in the absence of the $K_V\beta2$ subunit or with loss of its catalytic function ($K_V\beta2^{Y90F}$). Additionally, our present work demonstrates that vasoconstriction (e.g., 60 mM K^+, and myogenic tone development) are not affected by changes to the $K_V\beta$-subunit complex composition. Moreover, our data establish that lactate-induced vasodilation is a response that acts independent of endothelial function and requires changes in the vascular smooth muscle cell membrane potential. My data show that the $K_V\beta2$ subunit mediating K_V1 directed vasodilation.

Voltage-gated K^+ channels expressed in excitable cells assemble as heteromeric structures, with mixed compositions of pore-forming Kv1.x α subunits forming functional channels with varied auxiliary subunit complexes. This ‘mix-and-match’ capability of Kv channels may contribute to the considerable heterogeneity of K^+ currents that enable diverse physiological functions across a range of cell types. While our previous work found that Kv1.5 channels in murine coronary arterial myocytes interact with Kvβ1.1/Kvβ2 heteromers,[143] a key finding of the current study is the divergent functional regulation of vascular tone by these proteins. Ablation of only Kvβ2 suppressed vasodilatory function,

while ablation of Kvβ1.1 had little impact on vasodilation. These findings thus lead to the question of the specific physiological role of Kvβ1.1 in the vasculature. Our data suggest that the function of Kvβ2 is predominant. While the reasons for this discrepancy require further inquiry, previous work has shown that Kvβ2 can inhibit the N-terminal inactivation function of Kvβ1,[130] either by competing for binding with pore-forming Shaker subunits, or through biophysical modification of Kvβ1 function via Kvβ-β interactions. It should also be noted that although Kvβ-subunit proteins are expressed in native Kv1 channels in the vasculature, we cannot infer absolute subunit ratios in native Kv1 populations from our data. Thus, considering that these proteins are present in Kv1 channels in smooth muscle cells of wild type mice under normal conditions, it is plausible that the prevalence of Kvβ2 in Kv1 channels underlies the predominance of its functional importance under physiological conditions. Nonetheless, our data also support the notion that conditions that promote adaptive K^+ channel remodeling towards elevated functional expression of Kvβ1.1:Kvβ2 may likely have profound effects on vascular reactivity and blood flow regulation.

Although our study indicates the involvement of Kvβ proteins in the regulation of vasodilation, the upstream endogenous signals that contribute to this phenomenon are unclear. Intriguingly, the Kvβ proteins were discovered as members of the aldo-keto reductase superfamily of oxidoreductases and are functional enzymes that catalyze the reduction of carbonyl compounds to alcohols.[104,122] Hence, the Kvβ proteins each consist of a conserved AKR active site with nucleotide binding domain for nicotinamide cofactor hydride transfer. Later studies from our group and others demonstrating that the redox state of bound pyridine nucleotide differentially modifies channel activation and inactivation properties lead to the proposal that the Kvβs might serve as a critical link between cellular metabolic state and membrane excitability to control diverse functions in

excitable cells types.[125,126,200] Based on the results of these studies and data presented here, further studies are needed to determine whether changes in pyridine nucleotide redox status in arterial myocytes underlie Kvβ-mediated control of blood flow in the heart upon changes in metabolic demand.

Based on the data presented here we can conclude that: 1) lactate induced vasodilation is mediated by the K_V1 channel and requires the catalytically functional $K_V\beta2$ subunit; 2) ablation of either $K_V\beta$-subunit does not impact pressure induced myogenic tone development; 3) ablation of either subunit does not significantly impair the vasoconstrictor response to membrane depolarization or agonist induced constriction; 4) neither $K_V\beta1$ or $K_V\beta2$ appear to enhance or impair adenosine induced vasodilation; 5) lactate induced vasodilation is mediated independent of the endothelium; 6) overexpression of the Kvβ1 subunit emulates the $Kv\beta2^{-/-}$ condition; 7) coronary and mesenteric myocyte K_V-channels have similar quantities of colocalized K_V-α and K_V-β components. These data support crucial functional roles for $K_V\beta1$ and Kvβ2 in modifying K_V1 mediated arterial diameter in response to vasodilators. I have also provided evidence for the $K_V\beta2$ subunit, and its catalytic activity, as a sensor of altered metabolic demand that alters K_V1 mediated vasodilation. Additionally, recently acquired unpublished data using electrophysiological patch clamp, conducted by graduate student Marc Dwenger, shows whole cell outward potassium currents increase significantly in the presence of lactate, and this is inhibited in the presence of Psora-4. Altogether, these data support a functional role for $K_V\beta2$ as a sensor, triggered by altered metabolic demand, that modifies K_V1 mediated vasodilation.

The present study has several key limitations. One limitation is potential sex-based differences that may exist and differently affect the phenomenon we observe.[201,202] To address this, future studies will conduct many of these experiments in age matched female mice to determine any potential sex-based differences. Additionally, the use of global

knockout murine models presents potential issues in other organ systems which may impact the resultant observed phenomenon. To address this issue, I will use tissue-specific doxycycline inducible expression models, as described in the materials and methods section. When these mice are given water containing doxycycline (*ad libitum*) for ten days the reverse tetracycline trans-activator, driven by the SM22α promoter, increases smooth muscle specific expression of the associated β-subunit. Using these models should mitigate any affects observed in the global K_Vβ-knockout murine models. Additionally, the effects of aging on the cardiovascular system are not well understood and could result in a variable response to the phenomenon observed. To address this, future studies will compare differences in K_Vβ subunit expression and function in mice of different age categories (e.g., young versus old).

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