

ROLE OF VOLTAGE-DEPENDENT K⁺ AND Ca²⁺ CHANNELS IN
CORONARY ELECTROMECHANICAL COUPLING:
EFFECTS OF METABOLIC SYNDROME

Zachary C. Berwick

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Cellular & Integrative Physiology,
Indiana University

June 2012

Accepted by the Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Johnathan D. Tune, Ph.D., Chair

David P. Basile, Ph.D.

Doctoral Committee

Kieren J. Mather, M.D.

Alexander G. Obukhov, Ph.D.

April 19, 2012

Michael Sturek, Ph.D.

ACKNOWLEDGEMENTS

The author would like to express their deepest gratitude to Dr. Johnathan D. Tune for providing the outstanding leadership and guidance that made this dissertation possible. The author is also grateful to the distinguished research committee members, Drs. David P. Basile, Kieren J. Mather, Alexander G. Obukhov, and Michael Sturek for their invaluable direction and counsel. This work was supported by AHA grants 10PRE4230035 (ZCB) and NIH grants HL092245 (JDT) and HL062552 (MS).

ABSTRACT

Zachary C. Berwick

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Regulation of coronary blood flow is a highly dynamic process that maintains the delicate balance between oxygen delivery and metabolism in order to preserve cardiac function. Evidence to date support the finding that K_V and $Ca_V1.2$ channels are critical end-effectors in modulating vasomotor tone and blood flow. Yet the role for these channels in the coronary circulation in addition to their interdependent relationship remains largely unknown. Importantly, there is a growing body of evidence that suggests obesity and its pathologic components, i.e. metabolic syndrome (MetS), may alter coronary ion channel function. Accordingly, the overall goal of this investigation was to examine the contribution coronary K_V and $Ca_V1.2$ channels to the control of coronary blood flow in response to various physiologic conditions. Findings from this study also evaluated the potential for interaction between these channels, i.e. electromechanical coupling, and the impact obesity/MetS has on this mechanism. Using a highly integrative experimental approach, results from this investigation indicate K_V and $Ca_V1.2$ channels significantly contribute to the control of coronary blood flow in response to alterations in coronary perfusion pressure, cardiac ischemia, and during increases in myocardial metabolism. In addition, we have identified that impaired functional expression and electromechanical coupling of K_V and $Ca_V1.2$ channels represents a critical mechanism underlying coronary dysfunction in the metabolic syndrome. Thus, findings from this investigation provide novel mechanistic insight into the patho-physiologic regulation of

K_v and $Ca_v1.2$ channels and significantly improve our understanding of obesity-related cardiovascular disease.

Johnathan D. Tune, Ph.D., Chair

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Chapter 1: Introduction

Historical Perspective

Often viewed as the first experimental physiologist, Galen (200 A.D.) initially identified that arteries contain blood and not air (235). His views that blood traverses from the left to right side of the heart and filled with “vital spirit” by the lungs were not dispelled until later works by Vesalius and Servetus in the early 1500s. At the same time the first accurate description of arteries on the heart was recorded pictorially by Leonardo da Vinci (**Fig. 1-1**, (169)). Anatomists termed these arteries coronary from the Latin word coronarius meaning “of a crown” for the way the arteries encircled the heart. However, the greatest hallmark arises from William Harvey who originally described modern fundamentals of the heart and circulation in 1628, thus establishing the basis for investigations into blood flow regulation (4). Studies performed at the beginning of the 20th century by Bayliss and Starling identified unique biophysical properties intrinsic to the vasculature and myocardium and gave a brief glimpse into the complexity of cardiovascular physiology (21; 274). Advances in cell biochemistry and biophysics in the 1950s helped to identify the delicate balance between cardiac function, metabolism, and coronary blood flow as the heart is the only organ to control its own perfusion and resistance to perform work. Thus, regulation of coronary blood flow occurring in elegant coordination with the mechanics of the heart, as demonstrated by the Wiggers diagram (309), represents one of the most dynamic processes in human physiology. Yet despite the best efforts of modern science, we are far from a complete understanding of how coronary blood flow is regulated.

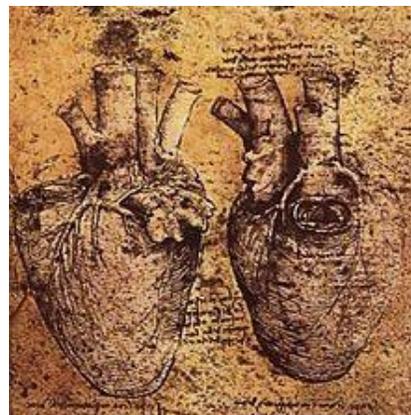


Figure 1-1 *The coronary circulation by Leonardo da Vinci. First anatomical recording of the origin of the coronary arteries “in a bullock’s heart” (~1513, Quaderni d’ Anatomia).*

Regulation of Coronary Blood Flow

The heart is unique in that it requires more energy in relation to its size than any other organ in the body. Operating primarily under oxidative metabolism, the heart can reach ~40% efficiency during ejection with respect to external work performed per oxygen consumed, compared to ~30% for most man-made machines (166; 282). The heart also extracts ~70% of the available oxygen delivered at rest (vs. 30% in skeletal muscle), thus a constant supply of oxygen is required to meet the metabolic requirements of the myocardium. Accordingly, any increase in myocardial metabolism that arises from elevations in heart rate, contractility, or systolic wall tension must be compensated by acute increases in oxygen delivery (86; 294; 296).

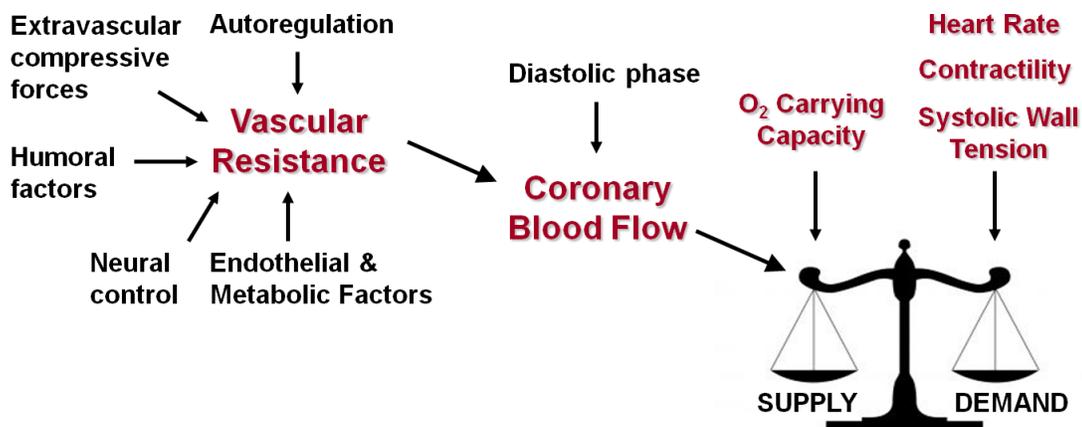


Figure 1-2 Myocardial O₂ supply/demand balance. Schematic representation of the factors which maintain the balance between oxygen delivery and myocardial metabolism. Adapted from Ardehali and Ports (13).

The degree of oxygen delivery to the myocardium is determined by the amount of coronary blood flow and the oxygen-carrying capacity of the blood (13). Although oxygen-carrying capacity is important under clinical conditions of anemia and hypoxemia, in all other circumstances the magnitude of coronary blood flow is the predominant determinant of oxygen delivery. Therefore, regulation of coronary blood flow is an essential process required to match oxygen delivery with myocardial metabolism in order to maintain adequate cardiac performance. The mechanisms that

regulate coronary blood flow do so via alterations in coronary microvascular resistance. Putative factors that function in parallel to control vascular resistance include endothelial and metabolic, aortic pressure/autoregulation, myocardial extravascular compression, as well as neural and humoral mechanisms (**Fig. 1-2** (82; 99)).

Balance between coronary blood flow and myocardial metabolism. Although many factors contribute to the regulation of coronary blood flow, the primary determinant is myocardial metabolism. Thus, local metabolic control of coronary blood flow is the most important mechanism for matching increases in coronary blood flow with myocardial oxygen consumption (MVO_2), i.e. metabolic demand of the heart (235). **Figure 1-3A** depicts this linear relationship and strict coupling between MVO_2 and coronary blood flow. As outlined above, high resting O_2 extraction significantly limits the degree to which increases in O_2 extraction can be utilized to meet increases in MVO_2 . This point is best evidenced by the close proximity of the normal operating relationship (black line) relative to the condition of maximal (100%) O_2 extraction (red line, **Fig. 1-3B**). To this extent, evaluating coronary venous PO_2 ($CvPO_2$), an index of myocardial tissue PO_2 , relative to MVO_2 provides a more sensitive method for detecting changes in the balance between coronary blood flow and metabolism (294; 331). The consistency of $CvPO_2$ with increases in MVO_2 further demonstrates the tight balance between metabolism and coronary blood flow (**Fig. 1-3C**). Importantly, any reduction in the relationship between $CvPO_2$ and MVO_2 indicates that the magnitude of coronary blood flow is insufficient, i.e. the heart is forced to utilize the limited O_2 extraction reserve to meet the oxidative requirements of the myocardium. If increases in coronary blood flow are completely abolished (red line, **Fig. 1-3D**), elevations in MVO_2 are strictly limited to increases in myocardial O_2 extraction (i.e. ~15-20% increase from rest). Thus, assessing the relationship between $CvPO_2$ and MVO_2 is a sensitive method to evaluate the overall

balance between coronary blood flow and myocardial metabolism under physiologic and pathophysiologic conditions.

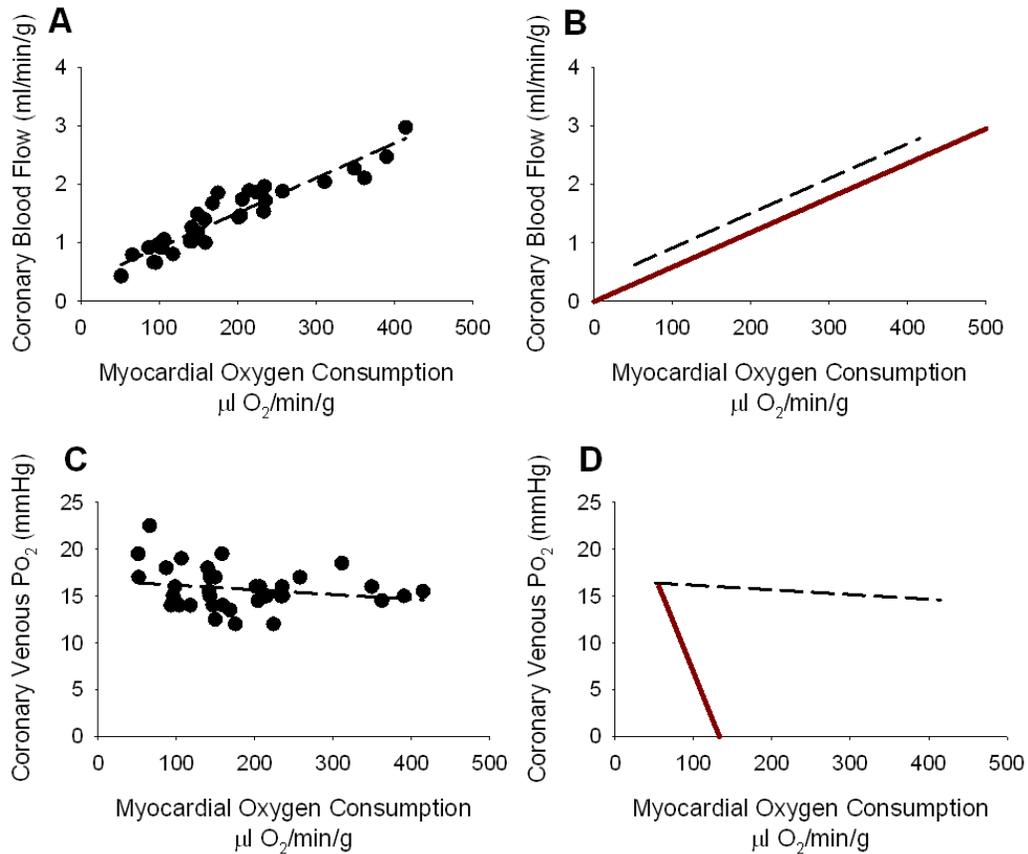


Figure 1-3 Relationship between myocardial oxygen delivery and consumption. (A) Coronary blood flow is associated with myocardial metabolism as indicated by the linear relationship between coronary blood flow and MVO₂. (B) The relationship between coronary blood flow and MVO₂ operates at near maximal oxygen extraction (red line). (C) CvPO₂ as an index of myocardial tissue PO₂ remains constant at a given MVO₂ due to changes in coronary blood flow with metabolism. (D) Supply-demand imbalance is evidenced by alterations in the relationship between CvPO₂ vs. MVO₂ where increases in MVO₂ can be limited by available oxygen extraction reserve (red line).

Extravascular compression and coronary perfusion pressure. In all circulations, perfusion pressure is dependent on the arterial-venous pressure gradient. The heart however is unique in that it generates the pressure for its own perfusion with aortic pressure serving as the driving force for coronary blood flow. Unlike peripheral vascular beds, the heart is also a constantly contracting muscle. During systole, tissue pressure exceeds venous pressure due to extravascular compression and therefore determines the magnitude of coronary blood flow in this phase. Consequently, release of

compressive forces during diastole re-establishes the arterial-venous gradient resulting in high diastolic coronary blood flows; a process termed “vascular waterfall” (**Fig. 1-4A**, (82; 99)). Therefore, alterations in the chronotropic, inotropic or lusitropic state of the heart can have significant effects on phasic coronary blood flow and is particularly important with regard to subendocardial perfusion (99). These influences not only affect tissue pressure, but can also alter MVO_2 .

Another important distinction of the coronary circulation is that it perfuses the organ which provides pressure to the entire circulation (99). Physiologic interventions that change peripheral vascular resistance also influence aortic pressure and contribute to coronary perfusion. As a result, it is often necessary to calculate the conductance of the coronary circulation (flow/pressure) to determine changes in coronary blood flow. Moreover, alterations in peripheral resistance that are met with parallel adjustments in the contractile state of the heart, as described by Anrep (302), significantly affect myocardial perfusion directly and secondary to changes in metabolism (99). Importantly, adequate coronary blood flow permits the generation of pressure sufficient to overcome afterload of the left ventricle and represents the interdependent relationship of coronary perfusion with aortic pressure and the peripheral circulation.

Coronary pressure-flow autoregulation. Coronary perfusion pressure and myocardial metabolism are highly integrated into the control of coronary blood flow. This can be evidenced by examining mechanisms of coronary autoregulation. By definition, coronary pressure-flow autoregulation refers to the intrinsic ability of the coronary circulation to maintain coronary blood flow constant in the presence of changes in perfusion pressure. The autoregulatory phenomenon is classically found within pressures ranges of 60-120 mmHg, beyond which coronary blood flow becomes largely pressure dependent (**Fig. 1-4B**, (99; 100; 160)). Coronary autoregulation is hypothesized to consist of myogenic and metabolic components. The metabolic

component of autoregulation poses that decreases in nutrient delivery during reductions in coronary perfusion pressure activate a local metabolic feedback mechanism to decrease vascular resistance in order to maintain coronary blood flow constant (157). The myogenic postulate of autoregulation rests on findings from Bayliss in that alterations in the degree of pressure or stretch of vascular smooth muscle evoke compensatory adjustments in vascular tone (21). However, a definitive role for either of the proposed components of coronary pressure-flow autoregulation has not been resolved. Determining potential mechanisms remains critical given the ability for perfusion pressure-mediated alterations in MVO_2 to occur both in the presence and absence of steady-state flow conditions (99; 124). Termed the “Gregg effect”, studies identified that MVO_2 increases with elevations in coronary perfusion pressure; an effect observed at higher perfusion pressures and in poorly autoregulating beds (99; 100). Theories explaining the observation that perfusion pressure can alter MVO_2 include coronary pressure-induced increases in contraction and vascular-volume mediated distention of the myocardium (17). Regardless, pressure-flow autoregulation represents a critical mechanism by which coronary blood flow is regulated and how alterations in perfusion pressure can affect the metabolic demands of the myocardium.

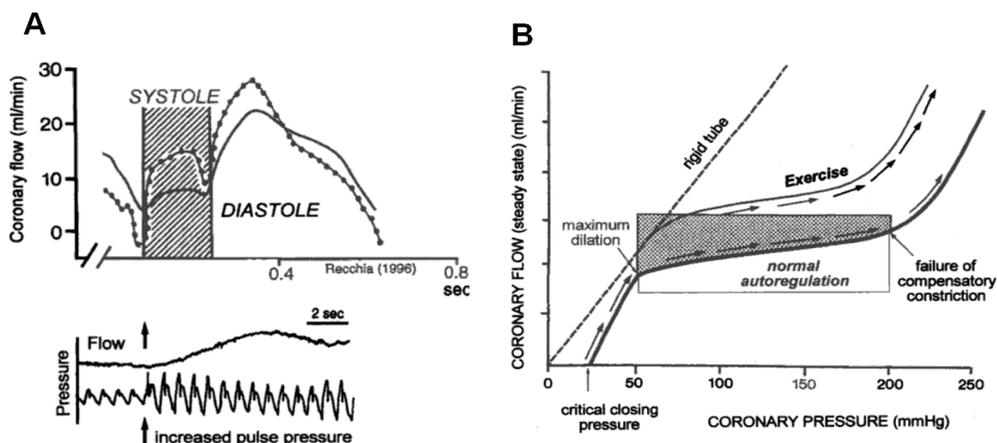


Figure 1-4 Effects of coronary perfusion pressure on coronary blood flow. (A) Dotted line demonstrates the effect of increases in pulse pressure (bottom panel) as may occur during exercise on phasic tracings and elevations in coronary blood flow. (B) Coronary pressure-flow autoregulation maintains blood flow constant within a specific range of perfusion pressures, beyond which the magnitude of coronary blood flow becomes pressure dependent (235).

Neural control of coronary blood flow. Although autoregulation maintains coronary blood flow constant over a wide range of pressures, many physiologic conditions require activation of mechanisms that increase blood flow to sustain cardiac function. Neural modulation of coronary vascular resistance is one such mechanism as coronary smooth muscle is innervated by both the parasympathetic and sympathetic divisions of the autonomic nervous system (99). Dually innervated coronary vessels undergo vagal-cholinergic vasodilation as well as both constriction and dilation via sympathetic activation of α and β adrenoceptors, respectively (**Fig. 1-5**, (99)). Distinguishing the direct neural contribution to blood flow regulation is difficult given confounding effects on metabolism. For example, in order to observe parasympathetic coronary vasodilation, vagal bradycardia must be prevented (99). A similar trend holds true for sympathetic stimulation. Overall sympathetic stimulation increases coronary blood flow due to positive inotropic-induced increases in myocardial metabolism via activation of cardiac β adrenoceptors (102). More recent studies demonstrate that direct sympathetic activation of coronary β receptors causes marked increases in coronary blood flow (82; 84; 99; 102; 116). In addition, a greater microvascular distribution of β receptors relative to α -adrenoceptors suggests that β -mediated coronary vasodilation plays a prominent role in the cardiovascular response to sympathetic activation. Accordingly, β -mediated coronary vasodilation is particularly important during exercise and has been proposed to contribute ~30% to adrenergic-induced increases in coronary blood flow (82; 88; 122; 123). Interestingly, α -mediated constriction limits local metabolic coronary vasodilation ~30% and decreases CvPO₂ (99). Moreover, variable transmural distribution of α -adrenoceptors enables enhanced vasoconstriction via α_2 activation in arterioles (particularly during hypoperfusion and ischemia) as compared to α_1 in larger arteries (56; 57; 141; 180). Therefore, elevations in α -adrenergic control of coronary

vascular tone can significantly impair O₂ delivery and is in direct competition with local metabolic/ β -mediated coronary vasodilation.

Humoral control of coronary blood flow. Regulation of coronary blood flow also occurs by many neural independent mechanisms. Both circulating and local release of vasoactive humoral factors play a role in determining coronary microvascular resistance. Particular peptide hormones implicated include antidiuretic (106; 238; 243), natriuretic (79; 92; 163; 187; 192), vasoactive intestinal (104; 137), substance P (64; 283) calcitonin gene-related (162; 164; 229) and neuropeptide Y (128; 285). With the exception of antidiuretic hormone and neuropeptide Y, exogenous administration of these hormones significantly dilates coronary vessels. However, the extent to which these factors influence coronary vascular resistance at physiologic concentrations has not been fully characterized. Other more investigated candidates include components of the renin-angiotensin-aldosterone system (RAAS). Notwithstanding the systemic and renal influences of these hormones, angiotensin II (AngII) and aldosterone causes vasoconstriction in coronary arterioles via activation of AT_{1,2} and mineralcorticoid receptors (**Fig. 1-5**, (155; 186)). Although endogenous AngII has modest effects on the coronary circulation under normal physiologic conditions, exogenous administration dose-dependently reduces coronary blood flow (329) by enhancing Ca²⁺ influx, stimulating the release of endothelin, and inhibiting bradykinin dilation (81; 261; 322). Aldosterone also produces dose-dependent vasoconstriction *in vivo* in open-chest dogs (114), *in vitro* in isolated perfused rat hearts (220), and in isolated coronary arterioles (186). Binding of intracoronary aldosterone via mineralcorticoid receptors has also been shown to decrease coronary blood flow in ischemic and non-ischemic hearts in addition to modulating cardiovascular function through regulating renal Na⁺ and K⁺ homeostasis (114). More recent evidence suggests that aldosterone may be capable of potentiating AngII constriction by increasing AT₁ receptor expression and/or impairing K⁺ channel

function (10; 318). Thus, changes in circulating hormone levels or functional expression of aldosterone and/or AngII receptors in disease states may significantly influence the regulation of coronary blood flow.

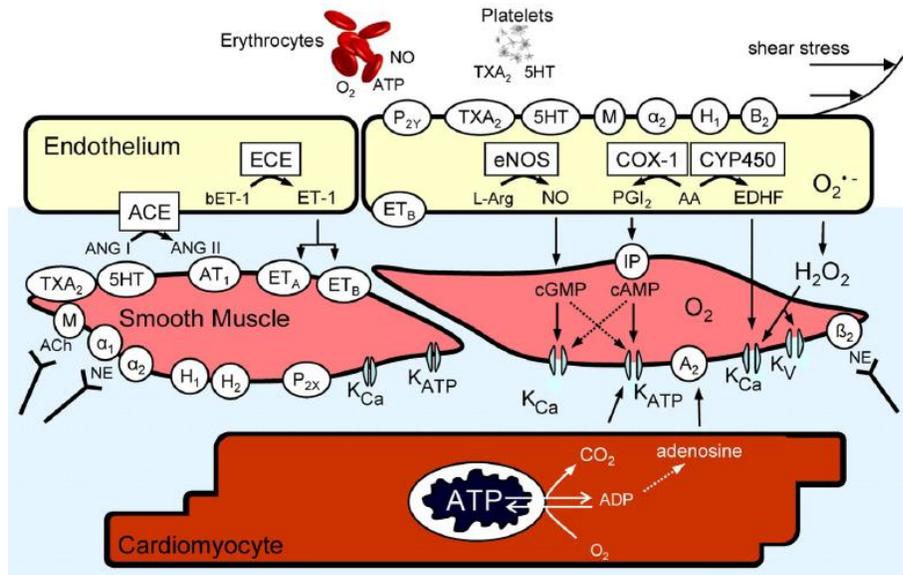


Figure 1-5 Various factors that determine coronary vasomotor tone. Mechanisms discussed in the control of coronary arterial diameter include: PO_2 , oxygen tension; ACh, acetylcholine; Ang II, angiotensin II; AT_1 , angiotensin II receptor subtype 1; A_2 , adenosine receptor subtype 2; β_2 , β 2-adrenergic receptor; α_1 and α_2 , α -adrenergic receptors; K_{Ca} , calcium-sensitive K^+ channel; K_{ATP} , ATP-sensitive K^+ channel; K_V , voltage-sensitive K^+ channel. Receptors, enzymes, and channels are indicated by an oval or rectangle, respectively (82). See text for further explanation.

Adenosine in feedback control of coronary blood flow. Investigations to date indicate that alterations in coronary vascular tone occur predominantly via local feedback mechanisms. Under this premise, alterations in tissue PO_2 release metabolites producing an error signal in proportion to the deviation in myocardial metabolic homeostasis. The metabolite produced or other downstream error signal effectors respond by adjusting coronary blood flow to regain the normal metabolic balance. Therefore, most changes in coronary blood flow occur secondary to a change in the metabolic rate (99). Only two exceptions to this statement have been documented and include feedforward (no error signal) adrenergic and H_2O_2 -mediated coronary vasodilation (122; 217; 264). However, feedback control mechanisms and/or metabolites implicated vary with physiological conditions, i.e. exercise vs. ischemia.

One classic metabolite widely investigated in both of these conditions is adenosine, whereby increases in cardiac metabolism or reduced oxygen delivery lead to increases in cardiac interstitial adenosine from catabolic breakdown of ATP (98). Originally suggested as the primary metabolite for local metabolic control of coronary blood flow under normal conditions by Berne in 1963, the adenosine hypothesis has since received extensive scrutiny. The postulate of a prominent role for adenosine was in part attributed to the large molar ratio of ATP to adenosine (~1000:1). Thus, small reductions in ATP were predicted to significantly increase production of this potent dilator and consequently coronary blood flow. However, more recent investigations fail to find a significant contribution of adenosine to exercise and ischemic-induced hyperemia; albeit many of these conclusions were derived from the use of the non-selective adenosine receptor antagonist 8-Phenyltheophylline (73; 82; 87; 297). As a result, only more recently has the role for individual adenosine receptors subtypes in the coronary circulation been investigated. Of the four adenosine receptors expressed in vascular smooth muscle, data indicate that the A_{2A} and A_{2B} receptor subtypes mediate vasodilation in response to adenosine (**Fig. 1-5**, (25; 136; 170; 172; 221; 284; 293)). However, the extent to which these receptors contribute to coronary vasodilation *in vivo* has not been characterized and remains as an important question given the ability for exogenous adenosine administration to cause marked coronary vasodilation in addition to its various clinical applications.

Studies from the Feigl laboratory affirm that levels of myocardial adenosine production during exercise are not sufficient to be vasoactive (297). Yet coronary venous adenosine concentration progressively increases above 166 nM when coronary perfusion pressure is < 70 mmHg (277). Such levels reported are well within the vasoactive range for endogenous adenosine ($ED_{50} = 77$ nM, (279)) and suggests that adenosine may still play a vasodilatory role in the transition to ischemia (277). Although

previous reactive hyperemia experiments would argue against this statement (73), pharmacologic limitations of non-selective antagonists may underscore the importance of adenosine in ischemic coronary vasodilation, particularly with regard to adenosine receptor subtypes. Thus, the functional contribution of individual adenosine receptors to adenosine-mediated ischemic coronary vasodilation requires further investigation.

Coronary Ion Channels in Vasomotor Control

Adenosine along with many aforementioned mechanisms involved in regulating coronary blood flow achieve their effect via subsequent modulation of end-effector ion channels. Various ion channels regulate the cellular membrane potential (E_M) of coronary artery smooth muscle (CASM) consequently determining the level of vasomotor tone and blood flow. The resting membrane potential (E_M) of CASM is closer to the Nernst potential for K^+ (~ -83 mV) than it is to the equilibrium potential of most other ions (54). Thus, K^+ channels are largely responsible for determining the E_M . However, in CASM the E_M ranges from -60 to -40 mV due to cation permeability through other channels with more positive reversal potentials (74). Several ion channels maintain activation thresholds close to the E_M for CASM cells and often operate in a narrow electrical range of one another. Therefore, small changes in E_M can have dramatic effects on the type and magnitude of ion channel conductance; a process that is central to the control of coronary vascular resistance. For example, small reductions in E_M will promote extracellular Ca^{2+} influx and CASM contraction (**Fig. 1-6A**). Evidence indicates that the predominant ion channels involved in this response are voltage-gated Ca^{2+} channels, i.e. $Ca_v1.2$. Although activation of $Ca_v1.2$ channels contributes to vasoconstriction, many channels modulate $Ca_v1.2$ activity through hyperpolarization and increases in smooth muscle E_M . Since the resting E_M is determined by intracellular K^+ efflux, channels that conduct K^+ hyperpolarize the membrane, prevent activation of

Ca_v1.2 channels and attenuate vasoconstriction (**Fig. 1-6B**, (74)). The degree to which K⁺ channels are activated directly contributes to decreases in vascular resistance and allows for significant increases in coronary blood flow. Moreover, because vasomotor tone varies greatly with small changes in smooth muscle E_M, voltage-sensitive K⁺ channels maintain a large contribution to the overall control of blood flow. This dynamic relationship between voltage-sensitive coronary K⁺ and Ca²⁺ channels is termed “electromechanical coupling” and represents a proposed critical mechanism for regulating coronary vascular resistance.

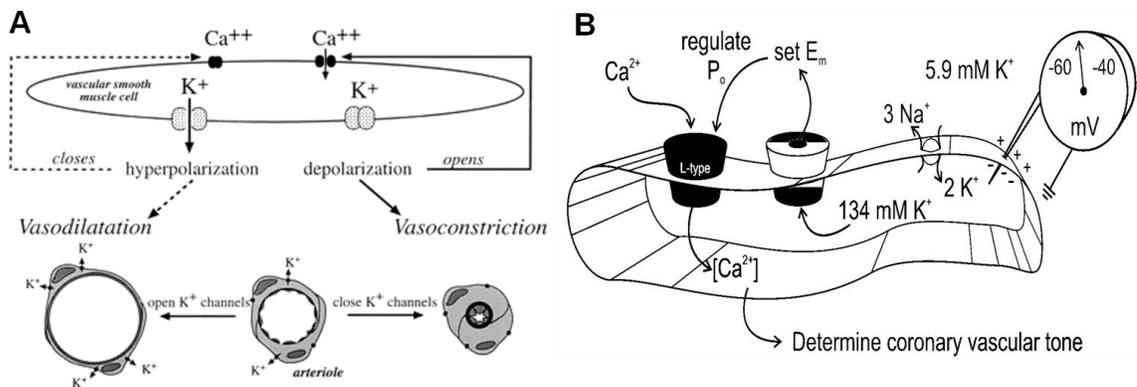


Figure 1-6 Electromechanical coupling of voltage-dependent Ca²⁺ and K⁺ channels. (A) Contribution of interactions between potassium and calcium channels to the control of coronary diameter (154). (B) Interdependent modulation of coronary E_M by voltage-sensitive Ca²⁺ and K⁺ channels (74).

Voltage-gated K⁺ Channels

Of the many K⁺ channels expressed in the coronary circulation, i.e. Ca²⁺-activated (K_{Ca}), ATP-sensitive (K_{ATP}), and inwardly rectifying (Kir) K⁺ channels, voltage-gated K_V channels (K_V) contribute the most to outward K⁺ current at physiologic membrane potentials (73; 74). K_V channels maintain a tetrameric structure of pore-forming α subunits with more than 40 different subunits that provide a broad range of K_V channel activity (74). Members of the same K_V family co-assemble in a homo or heterotetrameric manner to form functional channels (292). Auxiliary β subunits further contribute to the diversity of K_V channels as they co-assemble with α subunits and

regulate both biophysical properties and channel trafficking to the membrane (**Fig. 1-7**, (292)). The number of K_V channels per cell varies with different β -dependent trafficking within vascular beds and across species. Estimating the number of channels with $N = I/iP_o$ (where I is whole-cell current, i is single channel current, and P_o is the open-state probability) indicates there are ~5,000 channels in porcine coronary vs. 750

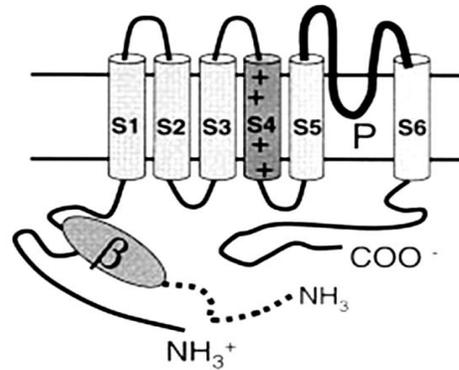


Figure 1-7 Structural characteristics of smooth muscle K_V channels. Schematic representation of voltage-sensing S4 linker, K^+ selective P-loop, and biophysical modulating β -subunit which comprise functional K_V channels (270).

in rabbit cerebral arteries (226). Although only K_V1 and K_V3 families have been identified in CASM, there are likely others that display similar properties of delayed rectification with little time-dependent inactivation (74). Voltage-sensitivity of K_V channels is provided by positively charged amino acids (lysine or arginine) in S4 transmembrane region and a tripeptide sequence motif located in P-loop of the S5-S6 linker represents the K^+ selectivity filter for the pore (270). The combination of these two structural characteristics allows for an activation threshold for K^+ conductance within the range of basal membrane potentials reported for CASM. Since K_V channels in the coronary circulation are delayed rectifiers and have noninactivating properties, coronary K_V channels provide a tonic hyperpolarization of the smooth muscle E_m (226; 255).

The two primary components for delayed rectifier K_V channel classification are that they are both 4-aminopyridine (4AP) sensitive and tetraethylammonium insensitive (292). K_V channels can also be classified according to their unitary conductance and fall broadly into two groups. In the porcine coronary circulation, a small conductance of 7.3 pS for K_V channels has been reported using 4-6 mM extracellular K^+ concentrations ($[K^+]_o$) in cell-attached patch-clamp configurations (300). In contrast, larger single channel conductance of 70 pS has also been demonstrated in rabbit coronary artery at

140 mM $[K^+]_o$ (150; 152). These findings are attributable to the voltage-dependence of K_V channels where although tonic activation occurs at normal physiologic ion concentrations, single channel current increases with depolarization of CASM over the physiological range of membrane potentials (226). Single K_V channel voltage-dependence is hypothetically illustrated below based on experimental data of 0.07, 0.17, and 0.50 pA at -60, -40 and 0 mV, respectively (**Fig. 1-8A**, (255; 299; 300)). In addition, the open probability (NP_o) of K_V channels also increases with depolarization (**Fig. 1-8B**). Increases in NP_o also represents the voltage-dependent activation/inactivation kinetics of the channel as NP_o increases steeply with membrane depolarization until a steady-state NP_o is reached and inactivation kinetics becomes significant. If this were not the case, such as that which is observed in K_{ATP} channel experiments, then whole cell K_V current recordings (**Fig. 1-8C**) would be graphically similar to single channel current voltage relationships and be directly dependent on the number of channels present and basal NP_o (225; 226). Thus, small changes in CASM E_M has marked effects on NP_o and the overall magnitude of whole cell K_V channel current. This sensitivity of K_V channels to changes in E_M is central to their physiologic role in controlling arterial diameter and consequently coronary blood flow.

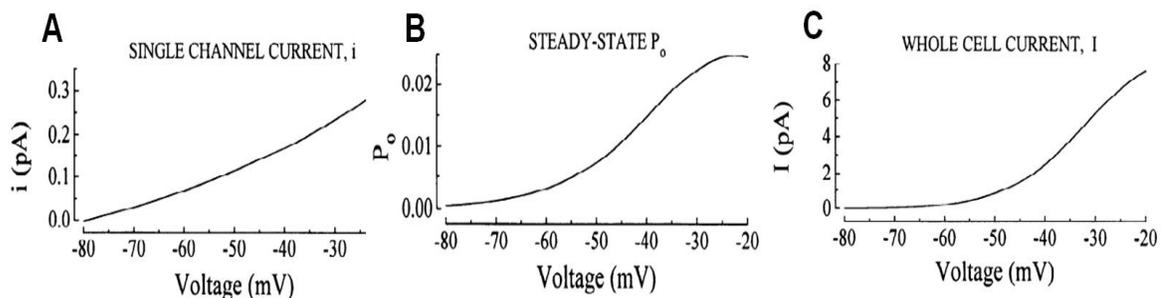


Figure 1-8 Determination of whole cell K^+ currents by K_V channel biophysical properties. (A) Experimentally based theoretical representation of increases in single channel K_V current with membrane depolarization. (B) Open probability of K_V channels increases with reductions in CASM membrane potential. (C) Potential-dependent modulation of K_V channels contributes to whole cell K^+ currents (226).

K_V channels are highly implicated in local feedback control of coronary blood flow as several important metabolites have been shown to activate coronary K_V channels. Patch clamp studies indicate that nitric oxide, prostacyclin, and H₂O₂ increase outward K_V current (3; 74; 196; 256). The intracellular mechanisms by which this

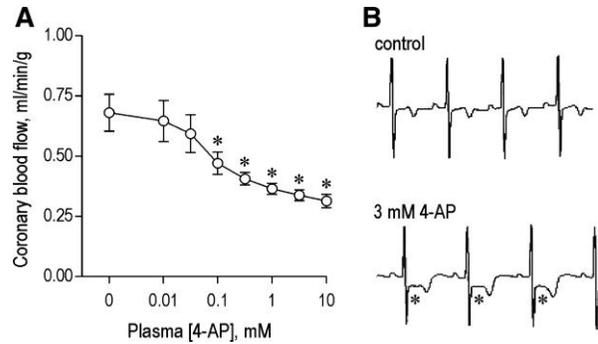


Figure 1-9 Contribution of K_V channels to coronary blood regulation. **(A)** Inhibition of coronary K_V channels dose-dependently reduces coronary blood flow. **(B)** Reductions in coronary blood flow in response to 4AP are sufficient to cause subendocardial ischemia as supported by significant ST segment depression (73).

occurs has not been determined, but evidence suggests that a cAMP/GMP-dependent protein kinase pathway is likely involved (74). Because previous investigations show that adenosine activates both cAMP and K_V channels, it is quite possible that cAMP is a common pathway for K_V activation (134; 135; 172). In contrast, few electrophysiological studies have identified factors that directly inhibit coronary K_V channels, although data from other vascular beds demonstrate that endothelin, Ang II, and thromboxane A₂ attenuate K_V channel current via subsequent activation of protein kinase C (74). Although these findings at the cellular level are important, the functional contribution of coronary K_V channels is clearly evident *in vivo*. Inhibition of coronary K_V channels by 4AP dose-dependently reduces coronary blood flow (**Fig. 1-9A**, (73)). These reductions in coronary blood flow are sufficient to cause subendocardial ischemia as demonstrated by significant ST segment depression (**Fig. 1-9B**). In addition, blockade of K_V channels markedly attenuates coronary reactive hyperemia by ~30%, thus implicating these channels in ischemic coronary vasodilation (73). Moreover, the vasodilatory response to pacing and norepinephrine induced increase in MVO₂ are significantly attenuated by 4AP (264). Therefore, K_V channels play an important role in the regulation of coronary

blood flow. However, the contribution of coronary K_V channels to physiologic-induced increases in coronary blood flow has not been determined. Moreover, whether K_V channels regulate coronary blood flow solely through changes in CASM E_M or via subsequent modulation of coronary $Ca_V1.2$, i.e. electromechanical coupling (**Fig. 1-6**), channels requires further investigation.

Voltage-gated Ca^{2+} Channels

Ca_V1 belongs to a group of at least ten members that comprise the gene superfamily class of voltage-dependent Ca^{2+} channels (161; 292). $Ca_V1.2$ channels represent the high-voltage activated dihydropyridine-sensitive subclass and consist of four 6-transmembrane pore-forming α subunits. Similar to K_V channels, $Ca_V1.2$ contains a voltage-sensitive S4 segment (118). The S5 and S6 segments line the pore along with pore loop that connects them (**Fig. 1-10**). Ca^{2+} selectivity is conferred by a pair of glutamate residues within the pore loop (161). These channels are different in topology compared to K_V channels in that only 1 pore-forming subunit is required to form a $Ca_V1.2$ channel pore whereas K_V channels require the entire tetramer. Like many ion channels, the biophysical properties of $Ca_V1.2$ are also altered by auxiliary subunits. These include $\alpha_2\delta_1$ and β units; each arise from 4 different genes and are implicated in the modulation of channel kinetics and membrane targeting of the α pore (118). In smooth muscle, 3 different β subunits have been identified which are disulfide linked to multiple $\alpha_2\delta_1$ splice variants. When coexpressed, these two subunits enhance the level of expression and enable normal gating properties of the channel (55). In addition, these channels are slow inactivating, i.e. "Long" lasting (L-type) with activation thresholds that fall in the same range of resting CASM E_M .

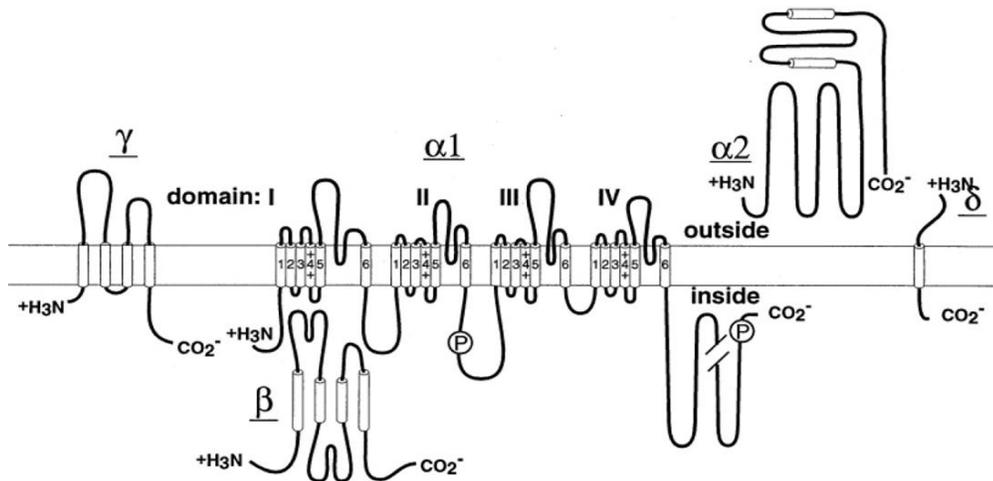


Figure 1-10 Structure of $Ca_v1.2$ Ca^{2+} channels. Six hexameric subunits with voltage-sensing and Ca^{2+} selective regions form the functional channel. Auxiliary $\alpha_2\delta_1$ and β subunits modify biophysical properties and membrane targeting of the α_1c channel pore (55).

In the presence of physiologically large transmembrane Ca^{2+} gradients, opening of only a few $Ca_v1.2$ channels during depolarization can cause large (~10-fold) increases in $[Ca^{2+}]_i$ (161). Thus, modest alterations in CASM E_M can have large effects on $Ca_v1.2$ -mediated Ca^{2+} conductance and vascular tone as it has been reported that a change of even 3 mV can yield a 2-fold increase/decrease in $[Ca^{2+}]_i$ (225; 227). The magnitude of Ca^{2+} influx through $Ca_v1.2$ channels depends on the number of channels, rate of Ca^{2+} entry, and NP_o of the channel. In smooth muscle, Ca^{2+} channels are abundantly expressed with ~5000 channels at a density of $4 \mu m^2$ (118). These channels have a unitary conductance of 3.5-5.5 pS and 5.5-11.0 pS using using 2.0 mM Ca^{2+} and Ba^{2+} as charge carriers, respectively (117; 119; 259). Single channel currents for $Ca_v1.2$ channels in physiologic solutions have been reported at ~0.17 pA and increase with membrane depolarization as normal amplitudes of 0.23, 0.42 and 0.79 pA are observed at -40, -30 and -20 mV, respectively (**Fig. 1-11A**, (118)). Under E_M conditions (-50 mV), this suggests a remarkable 1.04 million ions/s can permeate through a single Ca^{2+} channel (118; 225). Therefore, opening of only one $Ca_v1.2$ channel can raise the $[Ca^{2+}]_i$ 2.3 $\mu M/s$ assuming there is no buffering or extrusion (36; 117; 118). This means that even with a low NP_o and only ~1-10 channels open at more negative potentials of -60 mV, $Ca_v1.2$

channels are constitutively active and likely contribute to basal vascular tone (118). Interestingly, single channel experiments demonstrate long inactivation periods that would serve to limit increases in NP_o . Moreover, single channel recordings do not support steep increases in P_o with depolarization (**Fig. 1-11B**) or the final steady-state values for NP_o with 2 mM Ca^{2+} . Therefore, it has been proposed that an additional slow inactivating component over physiologic ranges of membrane potentials shifts the voltage-dependence of NP_o to more positive potentials by a constant factor of ~ 6 . This supports not only the steep voltage dependence of NP_o , but also and most importantly the marked increases in current and $[Ca^{2+}]_i$ with membrane depolarization (**Fig. 1-11C**, (36; 55; 117; 118; 161; 225; 292)).

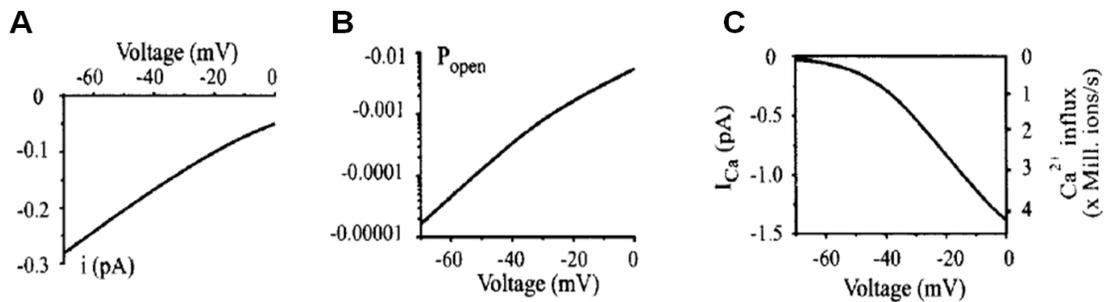


Figure 1-11 Contribution of $Ca_v1.2$ channel properties to whole cell Ca^{2+} current. Single $Ca_v1.2$ channel current (**A**) and open probability (**B**) increases with membrane depolarization. (**C**) Elevations in single channel activity with reductions in membrane potential alters the I-V relationship to significantly increase intracellular calcium levels (118).

Alterations in the regulation of steady-state Ca^{2+} entry by $Ca_v1.2$ channels have marked effects on coronary vasomotor tone. Modulation of $Ca_v1.2$ channels can occur in response to multiple mechanical, metabolic, and signaling mechanisms. It has been demonstrated that increases in intraluminal pressure of resistance arteries cause graded depolarization and Ca^{2+} influx (68; 191). Studies also indicate that $Ca_v1.2$ channels can also be activated by stretch (67; 69). However, more recent findings by Davis *et al* indicate that this response is secondary to stretch activation of nonselective cation channels (292). Regardless, activation of $Ca_v1.2$ channels in response to such mechanical stimuli is a critical component underlying coronary myogenic tone. Moreover,

recent investigations also indicate that protein kinase C (PKC) also contributes to the coronary myogenic response through alterations in $Ca_v1.2$ (214). In addition to PKC, protein kinase A and protein kinase G are also prominent activators of smooth muscle $Ca_v1.2$ channels and represent important pathways by which receptor-dependent activation of kinases can alter $Ca_v1.2$ channel activity (168). Thus, $Ca_v1.2$ channels serve as critical end-effectors to a wide array of factors.

Given the significant contribution of coronary $Ca_v1.2$ channels to regulating Ca^{2+} influx in response to various mechanical and intracellular activation pathways, resulting alterations in $Ca_v1.2$ channel activity has marked effects on the regulation of coronary blood flow. This statement is evidenced by significant increases in coronary microvascular vasoconstriction in response to the $Ca_v1.2$ agonist Bay K 8644 (**Fig. 1-12A**). More importantly, inhibition of coronary $Ca_v1.2$ channels relaxes coronary arteries and arterioles producing marked dose-dependent increases in coronary blood flow (**Fig. 1-12B**). Because the degree of $Ca_v1.2$ channel activity has such dramatic effects on the magnitude of coronary blood flow, any change in the channel themselves or alterations in upstream modulators under pathological conditions may significantly impair coronary blood flow regulation.

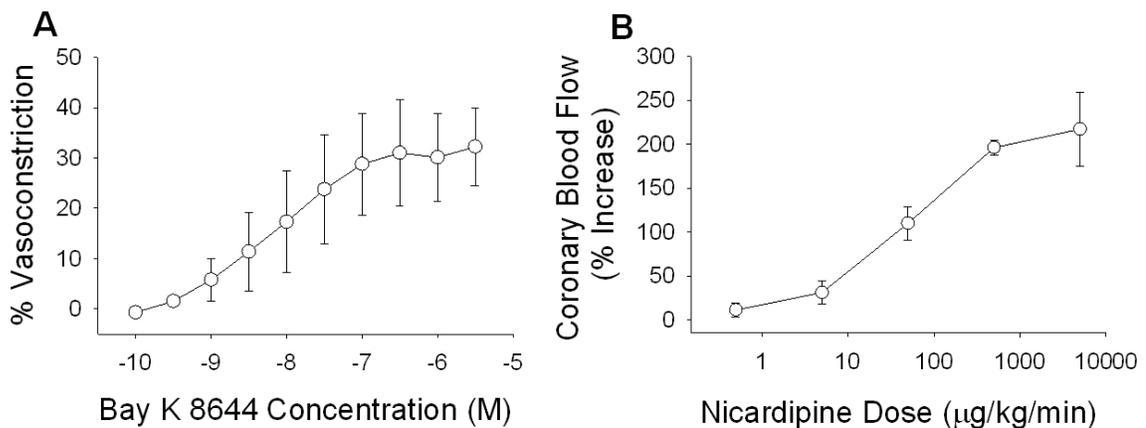


Figure 1-12 Contribution of $Ca_v1.2$ channels to coronary blood flow regulation. **(A)** Activation of $Ca_v1.2$ channels with Bay K 8644 produces significant coronary vasoconstriction of isolated, pressurized arterioles. **(B)** Inhibition of $Ca_v1.2$ channels with nifedipine dose-dependently increases coronary blood flow. Adapted from Knudson *et al* (175).

In summary, there is a significant and well documented role for coronary K_v and $Ca_v1.2$ channels in regulating smooth muscle E_M , vascular tone and coronary blood flow. However, the functional contribution of these channels to normal physiologic responses such as exercise-induced coronary vasodilation and pressure-flow autoregulation has not been determined. Such findings are critical to our understanding of coronary physiology as diminished control of coronary blood flow is associated with the development of cardiovascular disease and an increased incidence of mortality in many patient populations. Importantly and as outlined below, many disease states also impair ion channel function. Specifically, vascular K^+ and Ca^{2+} channel dysfunction has been identified in hypertension, hyperglycemia, and dyslipidemia; conditions which are often associated with obesity (38; 41; 45; 132; 133; 175; 195; 199; 200; 310). Thus it is not surprising given the growing rate of obesity and its associated co-morbidities that the incidence of cardiovascular disease and mortality is also rising. Accordingly, determining the role for K_v and $Ca_v1.2$ channels in the regulation of coronary blood flow under physiologic and pathophysiologic conditions may provide novel mechanistic insight into coronary dysfunction and obesity-related cardiovascular disease.

Epidemic of Obesity and Metabolic Syndrome

With an estimated 100 million Americans being obese or overweight, obesity in Western Society has now reached epidemic proportions (127). In addition, global estimates indicate that there are ~1 billion persons worldwide who are overweight (body mass index 25-30 kg/m^2) (311). Moreover, many of these individuals display other clinical disorders such as dyslipidemia, insulin resistance/impaired glucose tolerance, and/or hypertension which is often accompanied by pro-inflammatory and thrombotic states (126). The combination of these factors with general obesity is classified into the disorder termed metabolic syndrome (MetS). The incidence of this disorder has reached

pandemic levels, as ~20-30% of adults in most developed countries can be classified as having MetS (126; 230). In addition, individual components of this prediabetic syndrome are independent risk factors for cardiovascular disease (126). The increased prevalence of MetS is associated with a 2-fold increased risk for cardiovascular disease, 5-fold increased risk for type 2 diabetes mellitus, and 1.5-fold increase in all-cause mortality (115; 222; 228). As a result MetS patients have significantly elevated morbidity and mortality to many cardiovascular-related diseases including: stroke, coronary artery disease, cardiomyopathies, myocardial infarction, congestive heart failure, and sudden cardiac death (127; 138; 189). Given that heart disease remains a leading cause of death around the world (126), elucidating mechanisms by which MetS increases cardiovascular risk is essential for developing future treatments and preventing this global epidemic.

Alterations in the control of coronary blood flow could underlie increased cardiovascular morbidity and mortality in the MetS. As previously indicated, regulation of myocardial oxygen delivery is critical to overall cardiac function as the heart has limited anaerobic capacity and maintains a very high rate of oxygen extraction at rest (70-80%) (20; 82; 99; 294). Thus, the myocardium is highly dependent on a continuous supply of oxygen to maintain normal cardiac output and blood pressure. MetS impairs the ability of the coronary circulation to regulate vascular resistance and balance myocardial oxygen supply and demand (39; 269; 329). Coronary microvascular dysfunction in MetS is evidenced by reduced coronary venous PO_2 (39; 269; 329), diminished vasodilation to endothelial-dependent and independent agonists (i.e. flow reserve) (23; 179; 246; 265; 289; 290), and altered functional and reactive hyperemia (22; 37; 39; 269; 329). Importantly, these changes occur prior to overt atherosclerotic disease and have been associated with left ventricular systolic and diastolic contractile dysfunction in humans (95; 120; 245; 301; 313) and animal models of MetS (7; 39; 75; 248).

Coronary blood flow in Metabolic Syndrome

Resting flow and vasodilator responses. There is little change in baseline coronary blood flow in either animals (25; 37; 38; 76; 176; 201; 269; 329) or humans (179; 244; 246; 265; 289) with MetS. While myocardial perfusion is equivalent, myocardial oxygen consumption (MVO_2) is elevated in proportion to increases in stroke volume, cardiac output, and blood pressure; i.e. characteristic “hyperdynamic circulation” (39; 59; 75; 244; 248). Basal coronary venous PO_2 is reduced in MetS, indicating an imbalance between coronary blood flow and myocardial metabolism (39; 269; 329). These findings suggest that the MetS forces the heart to utilize its limited oxygen extraction reserve by affecting one or more primary determinants of coronary flow, including: 1) myocardial metabolism; 2) arterial pressure; 3) neuro-humoral, paracrine and endocrine influences; and 4) myocardial extravascular compression (82; 99). Additionally, MetS increases sympathetic output (190; 204; 280; 288) and activates the RAAS (12; 60; 307; 308; 329), increasing blood pressure, myocardial oxygen demand, and coronary vascular resistance. The determinants of coronary flow in MetS are also influenced by diminished nitric oxide (NO) bioavailability (35; 49; 129; 211; 275) and augmented endothelial-dependent vasoconstriction (146; 176; 206-208; 316). However, despite these changes it is not surprising that basal coronary flow is largely unaffected by MetS, as it is well established that inhibition of NO synthesis (9; 29; 51; 83; 295) or endothelin-1 receptors (121; 207; 212; 213) does not alter myocardial perfusion in normal, lean subjects. To date, no studies have specifically examined the effects of MetS on myocardial compressive forces.

MetS attenuates coronary flow responses to pharmacologic vasodilator compounds such as acetylcholine, adenosine, papaverine, and dipyridamole (179; 201; 218; 246; 265; 289; 290). Decreases in coronary flow reserve directly correlate with waist-to-hip ratio (171), body mass index (265), blood pressure (290), degree of insulin

resistance (179; 290), and the clinical diagnosis of MetS (246). Interestingly, our data indicate that specific receptor subtypes and downstream K^+ channels involved in coronary microvascular dilation are altered in Ossabaw swine with early-stage MetS, prior to any absolute change in coronary flow reserve (25). In contrast, decreased coronary flow reserve is evident in swine with later-stage MetS (38; 44; 218) and worsens with the onset of type 2 diabetes (179; 265). Exact mechanisms underlying impaired pharmacologic coronary vasodilation in MetS have not been clearly defined, but are likely related to altered functional expression of receptors and ion channels (25; 38; 74; 201; 218; 219), endothelial and vascular smooth muscle function (35; 50; 74; 275), paracrine and neuro-endocrine influences (174; 175; 190; 240; 280; 288; 308), structural remodeling of coronary arterioles (110; 276; 278), and/or microvascular rarefaction (111; 112; 273).

Coronary response to increases in cardiac metabolism. Energy production of the heart is almost entirely dependent on oxidative phosphorylation for contraction in relation to ventricular wall tension, myocyte shortening, heart rate, and contractility (82). Since the heart maintains a very high rate of O_2 extraction at rest, increases in myocardial energy production must be met by parallel increases in myocardial O_2 delivery (82; 99; 294; 296). Exercise is the most important physiologic stimulus for increases in coronary blood flow, as many of the primary determinants of myocardial O_2 demand are elevated by β -adrenoceptor signaling (82; 296). Data from our laboratory indicate that MetS impairs the ability of the coronary circulation to adequately balance myocardial O_2 delivery with myocardial metabolism at rest and during exercise-induced increases in MVO_2 . In particular, coronary vasodilation in response to exercise is attenuated in Ossabaw swine with MetS. This effect is evidenced by reduction of the slope between coronary blood flow and aortic pressure, which supports that exercise-mediated increases in vascular conductance are attenuated in MetS (**Fig. 1-13A**). Diminished local

metabolic control of the coronary circulation is also evidenced by decreased coronary blood flow at a given coronary venous PO₂ (**Fig. 1-13B**), an index of myocardial tissue PO₂ which is hypothesized to be a primary stimulus for metabolic coronary vasodilation (82; 99; 294). Importantly, coronary venous PO₂ is also depressed by MetS relative to alterations in MVO₂ (the primary determinant of myocardial perfusion) both at rest and during exercise (**Fig. 1-13C**). Together, these findings demonstrate coronary microvascular dysfunction in MetS leads to an imbalance between coronary blood flow and myocardial metabolism that could contribute to the increased incidence of cardiac contractile dysfunction and myocardial ischemia in obese patients (115; 126; 147; 189). This point is supported by an ~25% reduction in baseline cardiac index (cardiac output normalized to body weight) and a marked increase in myocardial lactate production (onset of anaerobic glycolysis) in swine with the MetS (39).

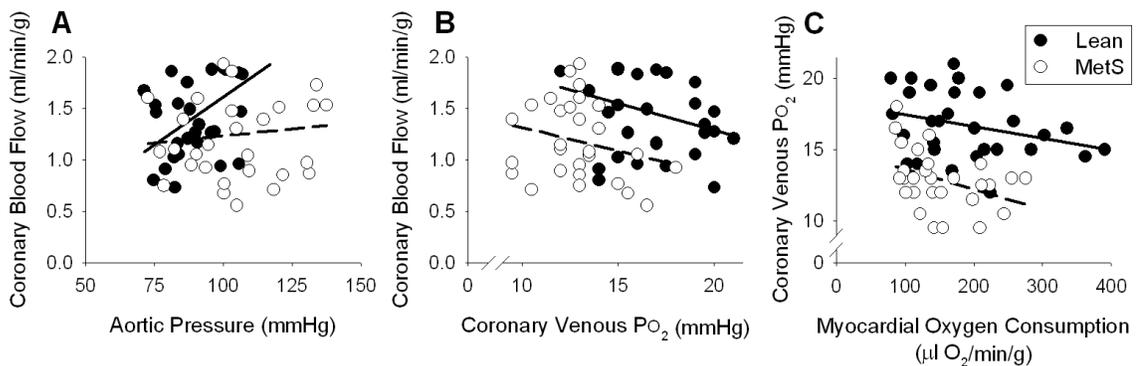


Figure 1-13 Effects of metabolic syndrome on coronary blood flow at rest and during exercise. (A) Reduction of the slope between coronary blood flow and aortic pressure indicates that exercise-mediated increases in vascular conductance are significantly attenuated by the MetS. (B) Diminished local metabolic control is also evidenced by decreased coronary blood flow at a given coronary venous PO₂. (C) Imbalance between myocardial oxygen supply and demand in MetS is evidenced by the reduction of coronary venous PO₂ relative to alterations in MVO₂ (the primary determinant of myocardial perfusion) both at rest and during exercise.

Coronary response to myocardial ischemia. Coronary vasodilation in response to myocardial ischemia is a critical mechanism increasing O₂ delivery to the heart to mitigate ischemic injury and infarction (232; 260). To address the effects of MetS on ischemic vasodilation, we examined coronary flow responses to a 15 sec occlusion in anesthetized, open-chest lean and MetS Ossabaw swine (37). Representative tracings

illustrating reactive hyperemia in lean vs. MetS swine are shown in **Figure 1-14A**. Because coronary reactive hyperemia varies directly with baseline blood flow, estimating overall repayment of incurred oxygen debt is critical for analyzing ischemic dilator responses (232; 260). Our finding that vasodilation in response to cardiac ischemia is impaired by MetS, relative to the deficit in coronary blood flow (i.e. repayment/debt ratio; **Fig. 1-14B**), is consistent with decreased reactive hyperemia of peripheral vascular beds in obese humans (70; 149). We propose that impaired ischemic dilation in MetS could exacerbate myocardial injury in patients with flow-limiting atherosclerotic lesions or acute coronary thrombosis.

In summary, microvascular dysfunction in MetS upsets the balance between coronary blood flow and myocardial metabolism as well as impairs blood flow responses to pharmacologic vasodilator compounds (coronary flow reserve), exercise-induced increases in MVO_2 (physiologic stimuli), and cardiac ischemia (pathophysiologic stimuli). Potential ion channels implicated in the impaired control of coronary blood flow are explored below.

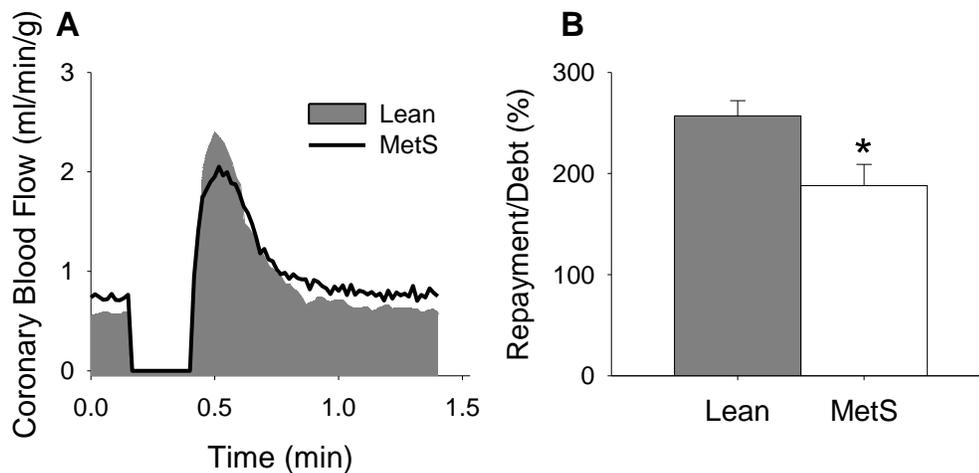


Figure 1-14 Effect of the metabolic syndrome on coronary vasodilation in response to cardiac ischemia. (A) Representative tracings illustrating reactive hyperemic responses in lean and MetS swine. (B) Coronary vasodilation in response to cardiac ischemia is impaired by metabolic syndrome as evidenced by the significant reduction in percent repayment of incurred coronary flow debt (i.e. repayment/debt ratio). * $P < 0.05$ vs. lean-control.

Metabolic Syndrome and Coronary Ion Channels

As previously indicated, coronary smooth muscle cells express a variety of ion channels which regulate E_M and vascular tone (74). Major types include voltage-dependent K^+ (K_V) and Ca^{2+} ($Ca_V1.2$) channels. However, several other important K^+ channels are functionally expressed in the coronary circulation including large conductance, Ca^{2+} -activated (BK_{Ca}), ATP-sensitive (K_{ATP}), and inwardly rectifying (Kir) K^+ channels. With the exception of BK_{Ca} , the affect of metabolic syndrome on the function of these prominent channels has not been characterized.

BK_{Ca} channels activate at a more depolarized E_M (38), but also respond to local Ca^{2+} signaling (218). Recently, we found that the MetS significantly attenuates coronary BK_{Ca} channel function, as evidenced by a reduction in vasodilation to the BK_{Ca} channel agonist NS1619 (**Fig. 1-15B**, (38)). This decrease in vasodilation corresponded with reductions in coronary vascular smooth muscle BK_{Ca} current (**Fig. 1-15A**) and a paradoxical increase in BK_{Ca} channel α and β_1 subunit expression (38). Decreases in total K^+ current and spontaneous transient outward currents, which are elicited by Ca^{2+} sparks and indicative of BK_{Ca} channel activation, have also been reported in coronary microvessels of diabetic dyslipidemic swine (218; 324). Studies in obese, insulin resistant rat models also support these findings and suggest that the reductions in BK_{Ca} current are related to alterations in the regulatory β_1 subunit (203; 324). Although diminished BK_{Ca} channel function in obesity/MetS is well established, data fail to support a significant role for BK_{Ca} channels in the control of coronary blood flow at rest, during increases in MVO_2 or during cardiac ischemia in lean or MetS animal models (37). However, BK_{Ca} channels have been shown to modulate coronary endothelial-dependent vasodilation in normal-lean subjects (215; 216). Thus, we propose that decreases in BK_{Ca} channel function likely contribute to coronary endothelial dysfunction observed in

the setting of the MetS (24), but play little role in the overall impairment of coronary vascular function.

Coronary K_V channels in metabolic syndrome. In contrast to BK_{Ca} , K_V channels are activated in the physiological range of E_M and thus have been implicated in the control of coronary blood flow (74). In particular, our laboratory previously demonstrated that K_V channels regulate coronary blood flow at rest, during ischemia, and with increasing MVO_2 in normal-lean animals (30; 73; 256; 257; 264). More recent data indicates that metabolic coronary vasodilatation is also reduced in $K_V1.5$ knockout mice (231). Importantly, individual components of MetS have been associated with K_V channel dysfunction. Although the specific mechanisms underlying the impairment of coronary K_V channels are unclear, there is evidence that dyslipidemia, hyperglycemia, hypertension, and/or oxidative stress may contribute (43; 45; 132; 133; 195; 198; 199). Activation of the sympathetic nervous system, RAAS, and PLC-PKC signaling pathways could also be involved (26; 63; 74). However, no study to date has determined the extent to which MetS alters coronary K_V channel function and the potential effects this may have on coronary blood flow regulation.

Coronary $Ca_V1.2$ channels in metabolic syndrome. $Ca_V1.2$ Ca^{2+} channels are the predominant voltage-dependent Ca^{2+} channel expressed in coronary smooth muscle (168). Ca^{2+} regulates contraction and gene expression; therefore, alterations in $Ca_V1.2$ channel function by MetS could have many consequences (118; 272; 303). In particular, increased activation of vasoconstrictor pathways (e.g. α_1 adrenoceptors, AT_1 receptors) along with decreased function of smooth muscle K^+ channels (e.g. BK_{Ca} channels, K_V channels) would serve to augment $Ca_V1.2$ channel activity and vasoconstriction (74). Data from our laboratory support this hypothesis as we previously demonstrated that the MetS increases intracellular Ca^{2+} concentration (38), $Ca_V1.2$ channel current (**Fig. 15C**) and arteriolar vasoconstriction to the $Ca_V1.2$ channel agonist Bay K 8644 (175) (**Fig.**

15D). We also found that coronary vasodilation in response to the $Ca_v1.2$ channel antagonist nicardipine is markedly elevated in obese dogs with the MetS (175). These findings are in contrast with earlier studies which documented reductions in $Ca_v1.2$ Ca^{2+} channel current in hypercholesterolemic and/or diabetic dyslipidemic swine (41; 310). Taken together, these data indicate that the entire MetS milieu is critical in determining the overall functional expression of $Ca_v1.2$ channels in the coronary circulation. Whether increases in $Ca_v1.2$ channel activation contribute to the impaired control of coronary blood flow at rest or during increases in MVO_2 in the MetS merits further investigation.

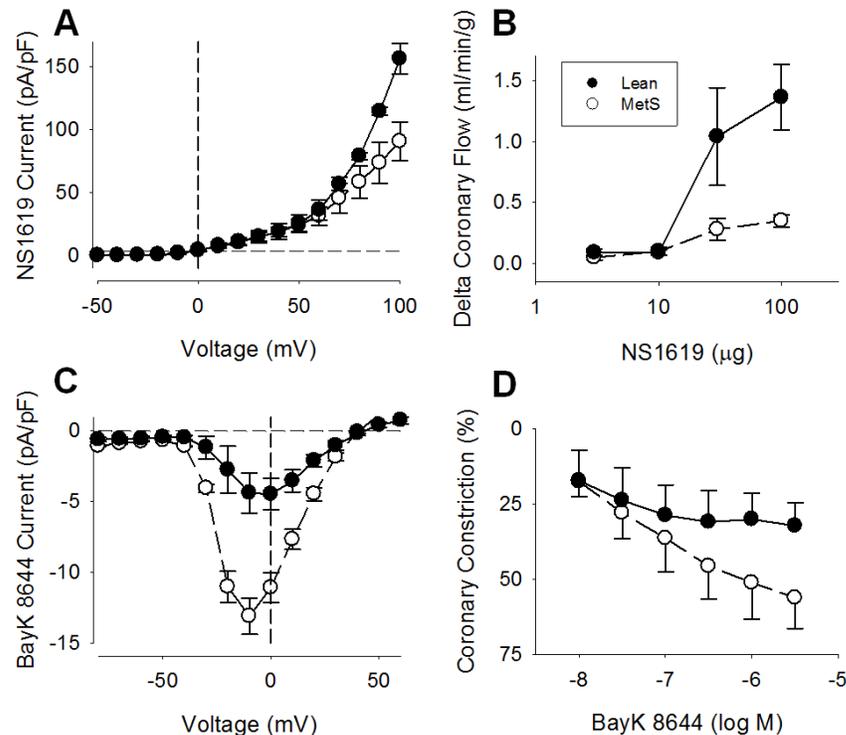


Figure 1-15 Effect of metabolic syndrome on coronary vasoconstriction and ion channel function. (A) Reductions in coronary vascular smooth muscle BK_{Ca} current in response to the BK_{Ca} channel agonist NS1619 directly correspond with (B) diminished coronary vasodilation to NS1619 in MetS swine. Data from reference (175). (C) Increases in coronary vascular smooth muscle L-type Ca^{2+} channel current activation in response to Bay K 8644 are associated with (D) augmented coronary arteriolar vasoconstriction to Bay K 8644 in obese dogs.

In summary, coronary dysfunction is a central contributor to increased mortality in MetS patients. However, the mechanisms underlying impaired regulation of coronary blood flow remain poorly understood. Investigations to date demonstrate that the MetS

significantly attenuates the balance between coronary blood flow and myocardial metabolism. Data obtained from our laboratory and others attribute this imbalance to elevated constrictor/diminished dilator pathways observed in MetS (see schematic diagram in **Fig. 1-16**). Yet our ability to target these pathways has been only modestly effective in attenuating adverse cardiovascular complications associated with the MetS. Importantly, many of the targeted pathways in MetS modulate coronary vasomotor tone via activation of downstream ion channels. However, little is known of the influence MetS has on particular key ion channels, namely K^+ and Ca^{2+} channels. Thus, investigating the contribution of K_v and $Ca_v1.2$ channels to coronary microvascular dysfunction in MetS will greatly improve our understanding of the patho-physiologic regulation of coronary blood flow and poor cardiovascular outcomes in patients with the MetS.

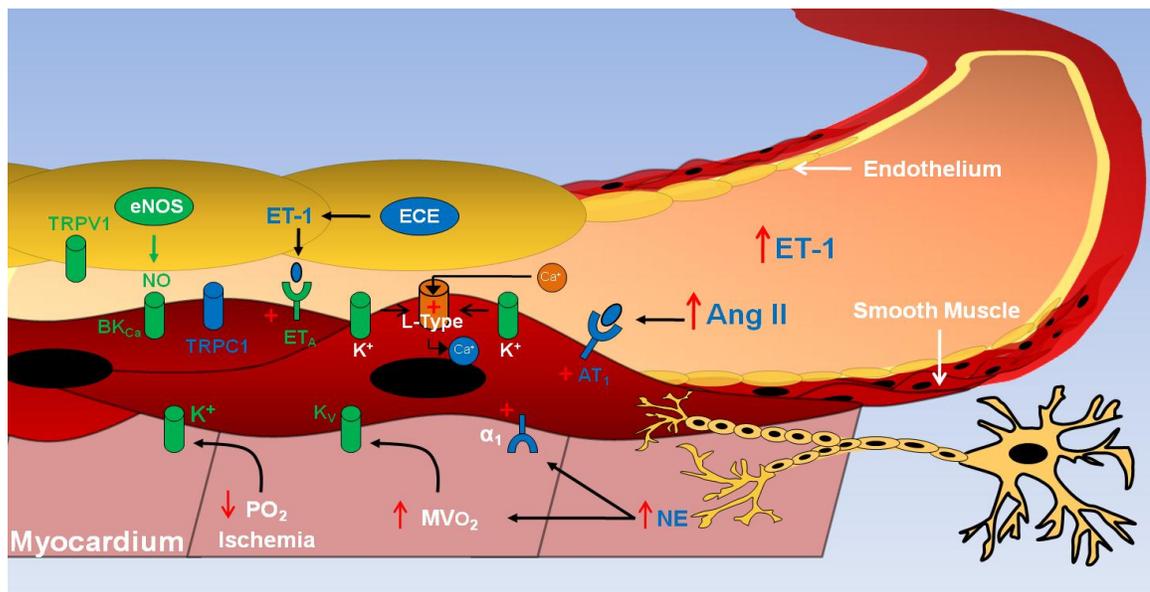


Figure 1-16 Schematic diagram illustrating mechanisms by which the metabolic syndrome impairs control of coronary blood flow. Factors, receptors and ion channels that are downregulated in metabolic syndrome are depicted in green. Factors, receptors and pathways that are upregulated in metabolic syndrome are depicted in blue and/or with + symbol. ET-1 (endothelin-1); Ang II (angiotensin II); AT₁ (angiotensin II type 1 receptor); α_1 (α_1 adrenoceptor); NE (norepinephrine); MVO₂ (myocardial oxygen consumption); TRP (transient receptor potential channel); BK_{Ca} (large conductance, Ca^{2+} activated K^+ channel); ET_A (endothelin type A receptor). eNOS (endothelial nitric oxide synthase); ECE (endothelin converting enzyme).

Hypothesis and Investigative Aims

Evidence to date support the finding that K_V and $Ca_V1.2$ channels are critical end-effectors in modulating coronary vasomotor tone and blood flow. Yet the role for these channels under physiologic conditions remains largely unexplored. In addition, the pathways/mechanisms that regulate channel activity are not fully understood and may involve a strong interdependent relationship, i.e. electromechanical coupling, due to the biophysical properties of K_V and $Ca_V1.2$ channels. Importantly, there is a growing body of evidence that suggests the MetS and its pathologic components may alter coronary ion channel function. Therefore, we propose that determining the contribution of these channels to the physiologic regulation of coronary blood flow and the degree to which K_V and $Ca_V1.2$ channels are altered by MetS may elucidate underlying mechanisms of coronary dysfunction in patients with MetS. Accordingly, the overall goal of this investigation was to: 1) delineate the functional role for coronary K_V and $Ca_V1.2$ channels in the coronary response to ischemia, exercise, and pressure-flow autoregulation; 2) determine potential electromechanical coupling between these channels; and 3) examine the contribution of K_V and $Ca_V1.2$ channels to coronary microvascular dysfunction in MetS. Findings from this investigation stand to offer novel mechanistic insight into the patho-physiologic regulation of K_V and $Ca_V1.2$ channels and significantly improve our understanding of obesity-related coronary vascular disease.

Aim 1 was designed to elucidate the contribution of adenosine A_{2A} and A_{2B} receptors to coronary reactive hyperemia and downstream K^+ channels involved.

The rationale for this study derives from investigations that determined coronary vasodilation in response to adenosine occurs primarily through the activation of A_{2A} and A_{2B} receptor subtypes (25; 136; 170; 172; 221; 284; 293). However, the relative contribution of these subtypes to ischemic coronary vasodilation has not been clearly

defined. Earlier studies demonstrate that both A_{2A} and A_{2B} receptor subtypes converge on downstream K_{ATP} to induce coronary vasodilation (58; 65; 66; 134-136; 148; 172; 298). More recent data from our laboratory also indicate that K_V channels play a significant role in the coronary vascular response to adenosine (73). Importantly, both K_{ATP} and K_V channels have been shown to modulate coronary vasodilation in response to cardiac ischemia (58; 73; 165; 328). However, the extent to which A_{2A} and/or A_{2B} receptor activation contributes to this effect of K^+ channels has not been investigated.

Aim 2 was designed to examine the contribution of K_V and $Ca_v1.2$ channels to coronary pressure-flow autoregulation *in vivo*. The rationale for this aim is obtained from previous studies implicating K_V channels in coronary vasodilation during reductions in coronary perfusion pressure (73) and $Ca_v1.2$ channels in the myogenic response to elevations in pressure; both proposed components of coronary pressure-flow autoregulation. In contrast to other ion channels investigated in coronary autoregulation (i.e. K_{ATP} (277)), K_V channels may play a more prominent role given their contribution to the control of coronary blood flow under a variety of physiologic conditions (30; 32; 73; 256; 257; 264). In addition, a myogenic component of coronary autoregulation is likely critical for mitigating pressure-induced increases in coronary blood flow (184; 210). Increases in intraluminal pressure and stretching of vascular smooth muscle cells results in graded decreases in smooth muscle membrane potential and increases in intracellular $[Ca^{2+}]$ that has been attributed to extracellular influx via voltage-gated ($Ca_v1.2$) Ca^{2+} channels (68; 69; 76; 142). Importantly, the extent to which K_V and $Ca_v1.2$ channels contribute to changes in coronary vasomotor tone in response to alterations in coronary perfusion pressure *in vivo* has not been determined.

Aim 3 was designed to determine the role for K_V channels in metabolic control of coronary blood flow and to test the hypothesis that decreases in K_V channel function and/or expression significantly attenuate myocardial oxygen supply-demand balance in the MetS. The rationale for this aim stems from previous investigations demonstrating that K_V channels represent a critical end-effector mechanism that modulates coronary blood flow at rest (73; 264), during cardiac pacing or catecholamine-induced increases in myocardial oxygen consumption (MVO_2) (264), following brief periods of cardiac ischemia (73), and endothelial-dependent and independent vasodilation (25; 32; 73). However, the functional contribution of K_V channels to metabolic control of coronary blood flow during physiologic increases in MVO_2 , as occur during exercise, has not been examined. In addition, decreases in K_V channel activity have been associated with key components of the MetS, including hypercholesterolemia (132; 133), hypertension (45), and hyperglycemia (195; 199; 200). We hypothesize that such reductions in the functional expression of K_V channels contribute to the impaired control of coronary blood flow in the setting of the MetS.

Aim 4 was designed to delineate the relationship between coronary K_V and $Ca_V1.2$ channels and to evaluate the contribution of $Ca_V1.2$ channels to coronary microvascular dysfunction in MetS. The overall rationale for performing experiments in this study is based on previous investigations implicating $Ca_V1.2$ channels as a predominant regulator of extracellular Ca^{2+} influx and coronary vascular resistance. In addition, depolarizations resulting from K_V channel inhibition increase cytosolic $[Ca^{2+}]$ and are abolished by removal of extracellular Ca^{2+} (205). However, the extent to which $Ca_V1.2$ channels regulate coronary blood flow as a consequence of alterations K_V channel function and the functional importance of this potential interaction is unknown. Importantly, data from our laboratory demonstrate that the MetS increases intracellular

Ca²⁺ concentration (38), Ca_v1.2 Ca²⁺ channel current and arteriolar vasoconstriction to the Ca_v1.2 channel agonist Bay K 8644 (175). We also found that coronary vasodilation in response to the Ca_v1.2 channel antagonist nicardipine is markedly elevated in obese dogs with the MetS (175). Whether increases in Ca_v1.2 channel activation contribute to the impaired control of coronary blood flow at rest or during increases in MVO₂ in the MetS has not been determined.

The significance of the proposed research is that coronary dysfunction is an important contributor to cardiovascular morbidity and mortality in patients with the MetS. The experimental design of these studies utilizes an integrative approach of *in vitro* (e.g. patch-clamp electrophysiology, western blot, flow cytometry, immunohistochemistry) and *in vivo* (acute and chronically instrumented swine) experimental techniques to examine the aims of this investigation. Experiments were conducted in lean canines and swine (**Aim 1, 2**) in addition to our Ossabaw swine model of the MetS (**Aims 3, 4**) fed either a normal maintenance or a high calorie atherogenic diet for 16 weeks. This excess atherogenic diet consistently produces clinical phenotypes of MetS including obesity, insulin resistance, impaired glucose tolerance, dyslipidemia, hypertension, hyperleptinemia, and coronary atherosclerotic disease (38; 44; 281). Taken together, results from this investigation improve our knowledge of obesity-related coronary vascular disease and may afford novel therapeutic strategies to treat a developing national epidemic.

Chapter 2

Contribution of Adenosine A_{2A} and A_{2B} Receptors to Ischemic Coronary Vasodilation: Role of K_V and K_{ATP} Channels

Microcirculation

Volume 17 (8), November, 2010

Zachary C. Berwick¹, Gregory A. Payne¹, Brandon Lynch¹, Gregory M. Dick²,
Michael Sturek¹, and Johnathan D. Tune¹

¹Department of Cellular and Integrative Physiology, Indiana University School of
Medicine, Indianapolis, IN 46202

²Department of Exercise Physiology Center for Cardiovascular & Respiratory Sciences
West Virginia University School of Medicine, Morgantown, WV 26506

Abstract

This study was designed to elucidate the contribution of adenosine A_{2A} and A_{2B} receptors to coronary reactive hyperemia and downstream K⁺ channels involved. Coronary blood flow was measured in open-chest anesthetized dogs. Adenosine dose-dependently increased coronary flow from 0.72 ± 0.1 to 2.6 ± 0.5 ml/min/g under control conditions. Inhibition of A_{2A} receptors with SCH58261 (1 μM) attenuated adenosine-induced dilation by ~50%, while combined administration with the A_{2B} receptor antagonist alloxazine (3 μM) produced no additional effect. SCH58261 significantly reduced reactive hyperemia in response to a transient 15 sec occlusion; debt/repayment ratio decreased from 343 ± 63 to 232 ± 44%. Alloxazine alone attenuated adenosine-induced increases in coronary blood flow ~30% but failed to alter reactive hyperemia. A_{2A} receptor agonist CGS21680 (10 μg bolus) increased coronary blood flow by 3.08 ± 0.31 ml/min/g. This dilator response was attenuated to 0.76 ± 0.14 ml/min/g by inhibition of K_v channels with 4-aminopyridine (0.3 mM) and to 0.11 ± 0.31 ml/min/g by inhibition of K_{ATP} channels with glibenclamide (3 mg/kg). Combined administration abolished vasodilation to CGS21680. These data indicate that A_{2A} receptors contribute to coronary vasodilation in response to cardiac ischemia via activation of K_v and K_{ATP} channels.

KEYWORDS: Reactive hyperemia, coronary vasodilation, adenosine receptor, potassium channel, canine.

Introduction

Since adenosine was first proposed as a constituent of coronary vasomotor regulation by Berne in 1963 (28), the role of this local purinergic metabolite has evolved considerably. Although studies have demonstrated that adenosine is not required for the regulation of coronary blood flow at rest or during increases in myocardial metabolism (15; 87; 297), adenosine has been shown to contribute to coronary vasodilation when myocardial oxygen requirements are not sufficiently met during episodes of ischemia (85; 101; 193). In particular, coronary vasodilation in response to exercise is diminished by combined enzymatic degradation and non-selective inhibition of adenosine receptors in dogs with a coronary stenosis (coronary perfusion pressure = 40 mmHg) (193). This finding is in agreement with data from Stepp and Feigl who demonstrated that cardiac adenosine production is markedly elevated by reductions in coronary perfusion pressure below 60 mmHg (277). Other studies indicate that adenosine contributes to coronary vasodilation in response to brief episodes of cardiac ischemia, i.e. coronary reactive hyperemia (15; 263), however, this is not a consistent finding (33; 73).

The cardiovascular effects of adenosine are mediated via four extracellular receptor subtypes; A_1 , A_{2A} , A_{2B} and A_3 (223). Although coronary microvascular vasodilation in response to adenosine occurs primarily through the activation of A_{2A} and A_{2B} receptor subtypes (25; 136; 170; 172; 221; 284; 293), the relative contribution of these subtypes to ischemic coronary vasodilation has not been clearly defined. Recently, Zatta and Headrick (328) found that selective inhibition of adenosine A_{2A} receptors reduced coronary reactive hyperemia in isolated, buffer-perfused mouse hearts by ~20-30%. The contribution of A_{2A} receptors to microvascular regulation was also demonstrated by Frobert et al. who found that the selective A_{2A} antagonist ZM241385 attenuated hypoxic dilation of isolated porcine coronary arteries by ~30% (113). A_{2B} receptors have also been shown to contribute to coronary vasodilation in response to

adenosine (170; 221; 224), although, not in response to hypoxia (113). At present, it is unclear whether A_{2A} and/or A_{2B} receptors regulate coronary blood flow during cardiac ischemia *in vivo*.

Earlier studies demonstrate that both A_{2A} and A_{2B} receptor subtypes converge on downstream ATP-dependent K^+ channels (K_{ATP}) to induce coronary vasodilation (58; 65; 66; 134-136; 148; 172; 298). More recent data from our laboratory also indicate that voltage-dependent K^+ channels (K_v) play a significant role in the coronary vascular response to adenosine (73). Importantly, both K_{ATP} and K_v channels have been shown to modulate coronary vasodilation in response to cardiac ischemia (58; 73; 165; 328). However, the extent to which A_{2A} and/or A_{2B} receptor activation contributes to this effect of K^+ channels has not been investigated. Accordingly, the purpose of this investigation was to examine the hypothesis that adenosine A_{2A} and/or A_{2B} receptors mediate coronary vasodilation in response to cardiac ischemia via activation of downstream K_v and/or K_{ATP} channels. Our findings provide important new data on the functional contribution of adenosine receptor subtypes to increases in coronary blood flow in response to endogenously produced ischemic metabolites *in vivo*.

Methods

All protocols were approved by the Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996). Male mongrel dogs ($n = 16$) weighing between 20-30 kilograms were administered morphine (3 mg/kg, s.c.) as a sedative, pre-anesthetic before inducing anesthesia with α -chloralose (100 mg/kg, iv). Following completion of experimental protocols, hearts were fibrillated and excised as recommended by the American Veterinary Medical Association Guide on Euthanasia (June 2007).

Surgical preparation. Following induction of anesthesia, dogs were intubated and ventilated with room air supplemented with O₂. A catheter was placed into the thoracic aorta via the right femoral artery to measure aortic blood pressure and heart rate. The left femoral artery was catheterized to supply blood to an extracorporeal perfusion system used to perfuse the left anterior descending coronary artery (LAD). A catheter was also inserted into the right femoral vein for injection of supplemental anesthetic, heparin and sodium bicarbonate. Arterial blood gases were analyzed periodically throughout the experimental protocol and adjustments made as needed to maintain blood gas parameters within normal physiological limits. Following a left lateral thoracotomy, a proximal portion of the LAD was isolated distal to its first major diagonal branch. Following heparin administration (500 U/kg), the LAD was cannulated with a stainless steel cannula connected to an extracorporeal perfusion system. Coronary perfusion pressure was maintained at 100 mmHg throughout the experimental protocol by a servo-controlled roller pump. Hemodynamic parameters were allowed to stabilize for ~30 min before initiation of the experimental protocol.

Experimental Protocol. Adenosine (3 - 30 µg/min) was infused at a constant rate into the LAD perfusion circuit before and during administration of the selective A_{2A} receptor antagonist SCH58261 (1 µM, i.c.), followed by the subsequent inhibition of A_{2B} receptors with alloxazine (3 µM, i.c.). Coronary reactive hyperemia was assessed by a 15 sec occlusion of the LAD before and during administration of SCH58261 (1 µM, i.c.) followed by alloxazine (3 µM, i.c.). Adenosine dose-response and reactive hyperemia studies were also performed in the absence and presence of the A_{2B} antagonist alloxazine alone (i.e. without SCH58261 administration). Additional studies were also conducted with the selective A_{2A} agonist CGS 21680 (10 µg bolus, i.c.), before and after administration of the K_v channel antagonist 4-aminopyridine (4-AP, 0.3 mM, i.c.) and/or inhibition of K_{ATP} channels with glibenclamide (3 mg/kg, i.v.). CGS 21680 infusions were

separated by at least 45 min as time control studies revealed similar coronary vasodilation in response to CGS 21680 following a 45 min washout period. Coronary reactive hyperemia studies were also conducted in the presence of 4-AP, glibenclamide, and 4-AP + glibenclamide. All drugs, with the exception of glibenclamide (dissolved in equal parts of ethanol, propylene glycol, 1N NaOH) and 4-AP (in saline) were dissolved in DMSO, diluted 1:10 with saline and infused i.c. at a constant rate (~300 μ l/min) for ~5 min prior to measurements in order to achieve the desired coronary plasma concentration. LAD perfusion territory was estimated as previously described by Feigl *et al.* (103).

Statistical analyses. Data are presented as mean \pm SE from n dogs. Reactive hyperemic volumes were calculated as area under the curve using Prism software (GraphPad, San Diego, CA). Duration parameters were evaluated at 35 seconds post occlusion and the point at which hyperemic flow had returned to within 5% of baseline. Statistical comparisons were made by t-test, one-way or two-way repeated measures analysis of variance (ANOVA) as appropriate. If statistical differences ($P < 0.05$) in these analyses were noted, a Student-Newman-Keuls multiple comparison test was performed.

Results

Contribution of A_{2A} and A_{2B} receptors to adenosine-mediated coronary vasodilation. Effects of A_{2A} and A_{2B} receptor blockade on baseline hemodynamic variables are listed in **Table 2-1**. Treatment with vehicle, SCH58261 and/or alloxazine did not significantly affect arterial blood pressure or coronary blood flow under baseline-resting conditions. Heart rate was modestly elevated during combined blockade of A_{2A} and A_{2B} receptors. Under control conditions, exogenous adenosine administration dose-dependently increased coronary blood flow from 0.81 ± 0.07 at rest to 2.53 ± 0.51

ml/min/g at the highest dose of adenosine (30 $\mu\text{g}/\text{min}$, i.c.). Administration of the A_{2A} receptor antagonist SCH58261 significantly reduced coronary vasodilation in response to adenosine ~70% (**Fig. 2-1**, $n = 4$). Subsequent administration of the A_{2B} receptor antagonist alloxazine had no additional effect on adenosine dilation. In separate studies, alloxazine alone attenuated adenosine-induced increases in coronary blood flow ~30% (**Fig. 2-3A**, $n = 3$).

Table 2-1 Effect of A_{2A} and A_{2B} receptor blockade on baseline hemodynamics.

Condition	Systolic Pressure, mmHg	Diastolic Pressure, mmHg	Mean Arterial Pressure, mmHg	Heart Rate, bpm	Coronary Pressure, mmHg	Coronary Flow, ml/min/g
Control	125 \pm 11	79 \pm 7	98 \pm 8	75 \pm 12	100 \pm 1	0.81 \pm 0.07
Vehicle	123 \pm 11	79 \pm 6	97 \pm 7	77 \pm 12	100 \pm 1	0.91 \pm 0.07
SCH 58261	123 \pm 7	87 \pm 7	102 \pm 7	97 \pm 13	99 \pm 1	0.96 \pm 0.09
SCH + Alloxazine	115 \pm 7	82 \pm 3	96 \pm 4	105 \pm 13*	100 \pm 1	1.00 \pm 0.09

Values are means \pm SE for $n=7$ dogs. * $P < 0.05$ vs. respective vehicle control.

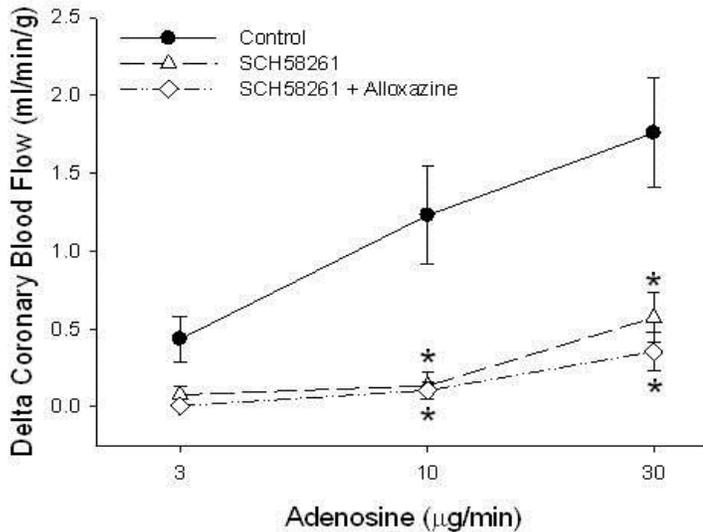


Figure 2-1 Effect of A_{2A} and A_{2B} receptor inhibition on adenosine-induced coronary vasodilation. Inhibition of A_{2A} receptors with SCH58261 (1 μM , ic) significantly attenuated coronary vasodilation in response to exogenously infused adenosine. Combined administration of SCH58261 with the A_{2B} receptor antagonist alloxazine (3 μM , ic) produced no additional effect. * $P < 0.05$ control.

Contribution of A_{2A} and A_{2B} receptors to coronary reactive hyperemia. **Table 2-2** shows the effects of vehicle, A_{2A} and subsequent A_{2B} receptor blockade on key coronary reactive hyperemia variables. Importantly, drug-vehicle had no significant effect on the coronary reactive hyperemic response. Inhibition of A_{2A} receptors with SCH58261 significantly reduced coronary vasodilation in response to brief, 15 sec coronary artery occlusion (**Fig. 2-2A**, $n = 7$). In particular, SCH58261 diminished the duration of the

hyperemic response and the overall volume of repayment (**Table 2-2**). This reduced the repayment of coronary flow debt from $343 \pm 63\%$ in vehicle treated hearts to $232 \pm 44\%$ following SCH58261 administration (**Fig. 2-2C**). Successive blockade of A_{2B} receptors with alloxazine had no additional effect on the coronary reactive hyperemic response (**Fig. 2-2B, Table 2-2**). Sole administration of alloxazine also failed to significantly alter the hyperemic response (**Fig. 2-3B**; $n = 3$).

Table 2-2 Effect of A_{2A} and A_{2B} receptor blockade on coronary reactive hyperemia.

Condition	Peak Flow, ml/min/g	Debt Area, ml/g	Repayment Area, ml/g	Δ flow at 35 seconds, ml/min/g	Duration, seconds
Control	3.15 ± 0.2	0.20 ± 0.02	0.71 ± 0.07	0.92 ± 0.14	83.14 ± 6.24
Vehicle	3.26 ± 0.2	0.23 ± 0.02	0.74 ± 0.11	0.92 ± 0.12	83.57 ± 5.75
SCH 58261	3.05 ± 0.1	0.24 ± 0.02	$0.51 \pm 0.06^*$	$0.57 \pm 0.10^*$	$65.00 \pm 4.43^*$
SCH + Alloxazine	3.29 ± 0.1	0.25 ± 0.02	$0.57 \pm 0.09^*$	$0.69 \pm 0.17^*$	73.29 ± 8.07

Values are means \pm SE for $n = 7$ dogs. * $P < 0.05$ vs. respective vehicle control.

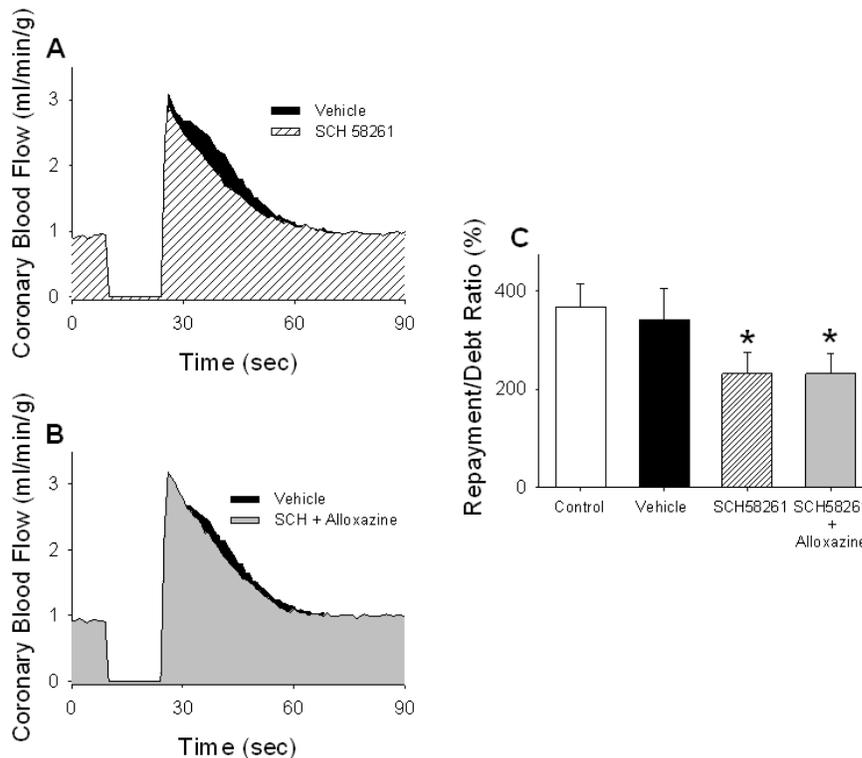


Figure 2-2 Role of adenosine A_{2A} and A_{2B} receptors in ischemic coronary vasodilation. **(A)** Inhibition of A_{2A} receptors with SCH58261 ($1 \mu\text{M}$, ic) significantly reduced coronary vasodilation in response to a 15 sec transient coronary artery occlusion. **(B)** The coronary reactive hyperemic response was unaffected by additional blockade of A_{2B} receptors with alloxazine ($3 \mu\text{M}$). **(C)** Effects of A_{2A} and A_{2B} receptor blockade on the repayment of coronary flow debt. A_{2A} receptor blockade significantly reduced the debt-repayment ratio relative to vehicle-control. Addition of the A_{2B} receptor antagonist alloxazine produced no additional effect. Figures show grouped average traces for $n = 7$ dogs. * $P < 0.05$ vs. vehicle.

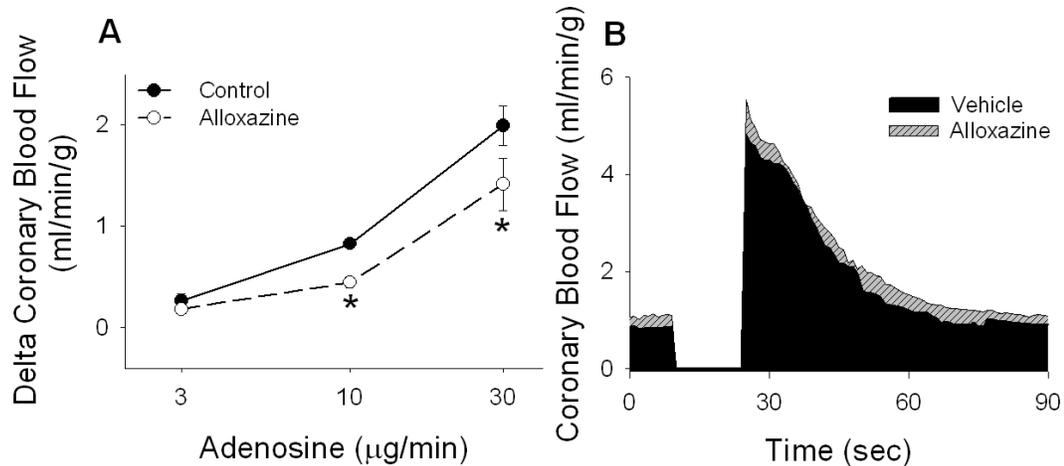


Figure 2-3 Contribution of A_{2B} receptors to exogenous adenosine and ischemia induced vasodilation. (A) Alloxazine (3 μ M, i.c.) significantly reduced coronary blood flow during cumulative adenosine infusions. (B) No effect of A_{2B} receptor blockade on reactive hyperemia. * $P < 0.05$ vs. control.

A_{2A} receptor activation of coronary K^+ channels. Experiments were conducted to assess the contribution of K_V and K_{ATP} channels to coronary vasodilation mediated by A_{2A} receptor activation. Coronary blood flow increased from 0.75 ± 0.09 to 3.32 ± 0.17 ml/min/g following intracoronary administration of the selective A_{2A} agonist CGS 21680 (10 μ g, $n = 4$). A_{2A} -mediated vasodilation was significantly attenuated by inhibition of either K_V (~70%, $n = 3$) or K_{ATP} channels (~96%, $n = 3$) alone (**Fig. 2-4**). Combined administration of 4-AP and glibenclamide essentially abolished the response to CGS 21680 (**Fig. 2-4**, $n = 4$).

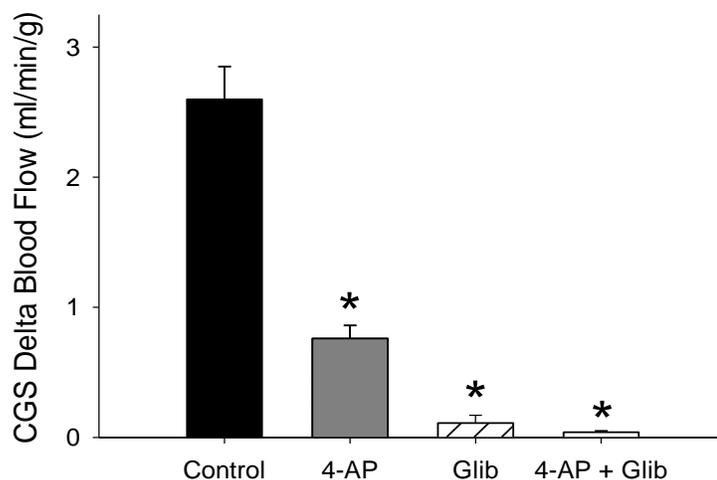


Figure 2-4 Effect of selective K^+ channel blockade on A_{2A} receptor-mediated dilation. Coronary vasodilation in response to A_{2A} receptor activation with CGS 21680 (10 μ g bolus, ic) was markedly attenuated by inhibition of K_V channels with 4-AP (0.3 mM, i.c.) and K_{ATP} channels with glibenclamide (3.0 mg/kg, i.v.). Combined administration of 4-AP and glibenclamide essentially abolished A_{2A} receptor-induced coronary vasodilation. * $P < 0.01$ vs. control.

As recently demonstrated by our laboratory (37; 73), inhibition of K_V ($n = 5$) and/or K_{ATP} ($n = 3$) channels markedly reduced the coronary reactive hyperemic response (**Fig. 2-5**). In particular, the decrease of both peak flow and the duration of the hyperemic response following combined K^+ channel blockade significantly reduced the repayment of coronary flow debt from $422 \pm 51\%$ to $162 \pm 40\%$.

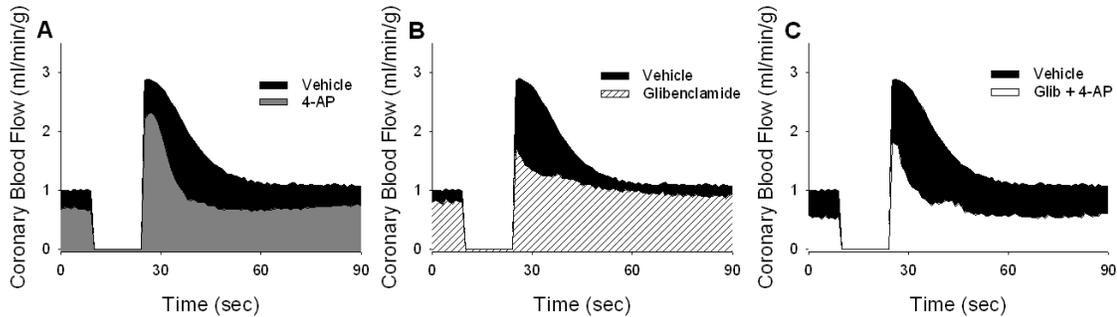


Figure 2-5 Effect of K^+ channel blockade on ischemic coronary vasodilation. Averaged group tracings showing impaired coronary ischemic vasodilation in response to A: K_V channel blockade with 4-AP ($n = 5$). B: K_{ATP} channel blockade with glibenclamide ($n = 3$). C: Combined inhibition of both K_V and K_{ATP} channels ($n = 3$).

DISCUSSION

This study examined the hypothesis that adenosine A_{2A} and/or A_{2B} receptors significantly contribute to coronary vasodilation in response to cardiac ischemia via activation of downstream K_V and K_{ATP} channels. To test this hypothesis, we measured changes in coronary blood flow in open-chest anesthetized canines in response to selective adenosine A_{2A} and A_{2B} receptor blockade during both exogenous adenosine infusion and following a brief coronary artery occlusion. In addition, the coupling of A_{2A} receptors to K_V and K_{ATP} channels was also assessed. The major findings of this study are: 1) A_{2A} receptors contribute to coronary vasodilation in response to exogenous adenosine administration and cardiac ischemia; 2) A_{2A} receptor-induced coronary vasodilation is mediated via signaling pathways that converge on K_V and K_{ATP} channels; 3) A_{2A} and A_{2B} receptors do not contribute to the regulation of coronary blood flow under

baseline-resting conditions; and 4) A_{2B} receptors contribute to coronary vasodilation in response to exogenous adenosine, but are not required for dilation in response to cardiac ischemia. These data indicate that A_{2A} receptors contribute to coronary vasodilation in response to cardiac ischemia via activation of K_V and K_{ATP} channels.

Contribution of A_{2A} and A_{2B} receptors to the control of coronary blood flow.

Findings from this investigation indicate that A_{2A} and A_{2B} receptors contribute to coronary vasodilation in response to exogenous adenosine *in vivo*, with the A_{2A} receptor pathway playing the predominant role. In particular, inhibition of A_{2A} receptors significantly reduced the increase in coronary blood flow to the highest dose of adenosine (30 $\mu\text{g}/\text{min}$) ~40% beyond that of A_{2B} receptor blockade alone (**Figs. 2-1** and **2-3**). Earlier *in vitro* studies using selective antagonists and/or genetic knockout of A_2 receptor subtypes in isolated coronary arteries (25; 113; 170) and buffer-perfused mouse hearts (221; 284; 328) support this finding. In addition, recent *in vivo* studies have also documented marked coronary vasodilation in response to selective A_{2A} receptor agonists (144; 293). Although A_2 receptors are expressed and capable of mediating vasodilation in the canine coronary circulation, it is important to recognize that inhibition of A_{2A} and/or A_{2B} receptors failed to significantly diminish resting coronary flow (**Table 2-1**). Therefore, our data do not support a role for either A_{2A} or A_{2B} receptors in the regulation of baseline coronary vasomotor tone *in vivo*. This conclusion is supported by numerous previous studies utilizing enzymatic and/or non-selective adenosine receptor antagonists (15; 87; 297; 320).

A_{2A} and A_{2B} receptors in coronary reactive hyperemia. A primary goal of this investigation was to assess the functional contribution of A_{2A} and A_{2B} receptors to coronary vasodilation in response to endogenous metabolites produced during cardiac ischemia. We show, for the first time *in vivo*, that A_{2A} receptors contribute to coronary reactive hyperemia, as inhibition of A_{2A} receptors with SCH58261 reduced repayment of

coronary flow debt ~32% (**Fig. 2-2**). Although earlier studies indicate that the mechanisms of coronary reactive hyperemia are dependent on species, experimental preparation and the duration of coronary occlusion (109; 131; 328), our finding is consistent with data from Zatta and Headrick who reported that SCH58261 (100 nM) resulted in an ~20-30% reduction in the coronary reactive hyperemic response in isolated mouse hearts (328). In addition, data from Frobert *et al.* demonstrate that the selective A_{2A} blockade attenuates hypoxic dilation of isolated porcine coronary arteries by ~30% (113). The effect of A_{2A} receptor inhibition on coronary reactive hyperemia is in contrast with our recent study which found little/no effect of the non-selective adenosine receptor antagonist 8-phenyltheophylline on ischemic vasodilation in the canine coronary circulation (73). We hypothesize these discrepant findings are related to the opposing effects A_1 vs. A_2 receptors on coronary microvascular resistance (65; 221; 284; 287).

Although A_{2B} receptor blockade reduced coronary vasodilation in response to exogenous adenosine (**Fig. 2-3A**; see **Limitations of the study**), alloxazine did not significantly affect the coronary reactive hyperemic response (**Fig. 2-3B**). Similarly, hypoxic coronary vasodilation of isolated coronary artery rings was unaffected by selective A_{2B} receptor inhibition with MRS1754 (113). This lack of effect of A_{2B} receptor blockade is consistent with the lower affinity of A_{2B} vs. A_{2A} receptors for adenosine (K_a ranges 1-20 nM for A_{2A} vs. 5-20 μ M for A_{2B}) (105; 234) as well as the differential expression of A_{2A} receptors in the coronary microcirculation vs. A_{2B} receptor expression in larger coronary arteries (136). Taken together, these findings argue against a prominent role for A_{2B} receptors in the modulation of coronary blood flow in response to endogenously produced adenosine.

A_{2A} activation of coronary K^+ channels. Based on our initial findings, the next goal of this investigation was to determine the relative contribution of K^+ channels to A_{2A} receptor mediated coronary vasodilation *in vivo*. This question is important because

although numerous earlier studies have demonstrated that adenosine and A_2 receptor signaling converge on K_{ATP} channels (14; 58; 65; 134-136; 148; 172; 182; 319), more recent data from our laboratory indicate that K_V channels also play a significant role (25; 73). In addition, both K_{ATP} and K_V channels have been shown to modulate coronary vasodilation in response to cardiac ischemia (58; 73; 165; 328). Accordingly, we conducted experiments to examine the effects of K_V and K_{ATP} channel inhibition on the coronary vasodilatory response to the selective A_{2A} agonist CGS 21680 (136; 172; 317). We found that coronary vasodilation in response to A_{2A} receptor activation is mediated via signaling pathways that converge on both K_{ATP} and K_V channels as inhibition of either K_V channels or K_{ATP} channels markedly attenuated vasodilation in response to CGS 21680 (**Fig. 2-4**). Importantly, the decrease in the coronary reactive hyperemia following inhibition of K_V and K_{ATP} channels (~60% reduction; **Fig. 2-5**) was much greater than the relatively modest effect A_{2A} receptor blockade alone (~30% reduction; **Fig. 2-2A**). These data support previous studies implicating a prominent role for K^+ channels in ischemic coronary vasodilation (37; 73; 89) and indicate that while A_{2A} receptor signaling is a component of K_V and K_{ATP} channel activation in response to cardiac ischemia, numerous other pathways must also converge on these channels to mediate the marked increase in coronary blood flow following a brief episode of cardiac ischemia.

Limitations of the study. We fully acknowledge that conclusions derived from this study regarding the relative contribution of A_{2A} vs. A_{2B} receptors to the regulation of coronary blood flow are dependent on the selectivity of the antagonists used. SCH58261 is an A_{2A} receptor antagonist that effectively inhibits A_{2A} mediated coronary vasodilation at 1 μ M (253), the concentration used in the present study. Alloxazine is a non-xanthine A_2 antagonist with a K_B value of 2.3 μ M for A_{2B} receptors (284); a coronary plasma concentration of 3 μ M was used for these experiments. Our studies with these antagonists revealed somewhat paradoxical results in that SCH58261 reduced coronary

vasodilation in response to adenosine (~70%), with no additional effect of alloxazine. However, alloxazine alone (when administered without SCH58261) also reduced adenosine-induced increases in coronary blood flow by ~30%. Since SCH58261 is ~1000-fold more selective for A_{2A} over A_{2B} receptors (233; 234; 323), while alloxazine has much more limited selectivity (~10-fold greater selectivity for A_{2B} vs. A_{2A}; (109)), we propose the most likely explanation for our paradoxical results is that alloxazine also impaired A_{2A} signaling as opposed to an SCH58261-induced impairment of A_{2B} signaling. However, the extent to which SCH58261 and alloxazine selectively inhibit A_{2A} and A_{2B} induced coronary vasodilation *in vivo* requires further investigation.

Equally important in this study is the selectivity of the K⁺ channel antagonists used. We recently demonstrated that 0.3 mM 4-AP, the same concentration used in the present study, significantly attenuates coronary vasodilation in response adenosine, but does not affect increases in coronary blood flow to the K_{ATP} channel agonist pinacidil (73). However, glibenclamide has been shown to attenuate voltage-gated K current in rabbit kidney (325). This could explain the similar effect of glibenclamide alone vs. glibenclamide + 4-AP on CGS 21860 dilation (**Fig. 2-4**) and the coronary reactive hyperemic response (**Fig. 2-5**). Regardless, the reduction of the coronary reactive hyperemic response following K⁺ channel inhibition was much greater than the relatively modest effect of A_{2A} receptor blockade alone.

In conclusion, findings from this investigation indicate that adenosine A_{2A} receptors contribute to coronary vasodilation in response to exogenous adenosine infusion and cardiac ischemia. Importantly, these findings are consistent with earlier studies in A_{2A} and A_{2B} receptor knockout mice (247; 253; 254; 258). We have also identified that A_{2A}-induced coronary vasodilation *in vivo* is mediated via activation of K_V and K_{ATP} channels. Although A_{2B} receptors are functionally expressed, our data do not support an active role for A_{2B} receptors in the endogenous regulation of coronary blood

flow in healthy control subjects. However, it is important to note that Bender *et al.* recently found that the obese-metabolic syndrome augments the contribution of A_{2B} receptors to adenosine-induced dilation *in vitro* (25). Interestingly, this increase was accompanied by a decrease in coronary A_{2B} receptor protein expression as well as a loss of downstream K_{ATP} channel activation. Other studies in human atrial appendage microvessels (170) support that disease states may significantly alter the functional contribution of A_2 receptor subtypes to the control of coronary microvascular tone. The significance of these changes along with the role of alternative adenosine receptor subtypes (A_1 and A_3) remains to be elucidated.

ACKNOWLEDGEMENTS

This work was supported by NIH grants HL092245 (JDT) and HL062552 (MS).

Chapter 3

Contribution of Voltage-Dependent K⁺ and Ca²⁺ Channels to Coronary Pressure- Flow Autoregulation

Basic Research in Cardiology

Volume 107 (3), May, 2012

Zachary C. Berwick¹, Steven P. Moberly¹, Meredith C. Kohr¹, Ethan B. Morriscal¹,
Michelle M. Kurian¹, Gregory M. Dick² and Johnathan D. Tune¹

¹Department of Cellular and Integrative Physiology, Indiana University School of
Medicine, Indianapolis, IN 46202

²Department of Exercise Physiology Center for Cardiovascular & Respiratory Sciences
West Virginia University School of Medicine, Morgantown, WV 26506

Abstract

The mechanisms responsible for coronary pressure-flow autoregulation, a critical physiologic phenomenon that maintains coronary blood flow relatively constant in the presence of changes in perfusion pressure, remain poorly understood. This investigation tested the hypothesis that voltage-sensitive K^+ (K_V) and Ca^{2+} ($Ca_V1.2$) channels play a critical role in coronary pressure-flow autoregulation *in vivo*. Experiments were performed in open-chest, anaesthetized Ossabaw swine during step changes in coronary perfusion pressure (CPP) from 40-140 mmHg before and during inhibition of K_V channels with 4-aminopyridine (4AP, 0.3 mM, ic) or $Ca_V1.2$ channels with diltiazem (10 μ g/min, ic). 4AP significantly decreased vasodilatory responses to H_2O_2 (0.3 - 10 μ M, ic) and coronary flow at CPPs = 60-140 mmHg. This decrease in coronary flow was associated with diminished ventricular contractile function (dP/dT) and myocardial oxygen consumption. However, the overall sensitivity to changes in CPP from 60 to 100 mmHg (i.e. autoregulatory gain; G_c) was unaltered by 4-AP administration ($G_c = 0.46 \pm 0.11$ control vs. 0.46 ± 0.06 4-AP). In contrast, inhibition of $Ca_V1.2$ channels progressively increased coronary blood flow at CPPs > 80 mmHg and substantially diminished coronary G_c to -0.20 ± 0.11 ($P < 0.01$), with no effect on contractile function or oxygen consumption. Taken together, these findings demonstrate that: 1) K_V channels tonically contribute to the control of microvascular resistance over a wide range of CPPs, but do not contribute to coronary responses to changes in pressure; 2) progressive activation of $Ca_V1.2$ channels with increases in CPP represents a critical mechanism of coronary pressure-flow autoregulation.

KEYWORDS: autoregulation, coronary blood flow, potassium channel, calcium channel, swine.

Introduction

Coronary pressure-flow autoregulation is an essential mechanism by which the coronary circulation maintains constant blood flow in the presence of alterations in perfusion pressure. The autoregulatory capacity of the coronary vascular bed is particularly important during flow-limiting stenosis where if absent, hypoperfusion can rapidly diminish cardiac function (100; 304). Alternatively, lack of vascular responses to elevations in perfusion pressure can lead to increases in coronary vascular volume, myocardial stiffness and oxygen consumption (MVO_2), i.e. Gregg Phenomenon (17; 124). However, despite the importance of coronary pressure-flow autoregulation, the mechanisms underlying this phenomenon remain poorly understood.

Previous investigations of coronary autoregulation have focused primarily on the contribution of myocardial tissue pressure, local metabolic and myogenic mechanisms (71; 77; 99; 100; 157). Although support for tissue pressure can be found in encapsulated organs such as the kidney (143; 158), evidence in the heart is limited as similar increases in intramyocardial pressure occur in the presence and absence of pressure-flow autoregulation (80; 99). In contrast, more prominent implications for local metabolic control have been identified as studies by the Feigl laboratory support that ~23% of the changes in coronary conductance that occur with alterations in perfusion pressure are mediated by the synergistic effects of CO_2 and O_2 (46). Other studies suggest that additional metabolites such as nitric oxide (NO), hydrogen peroxide (H_2O_2) and adenosine could also be involved, albeit at lower perfusion pressures (52; 267; 271; 321). However, inhibition or catalytic degradation of these metabolites has failed to significantly alter coronary responses to changes in perfusion pressure (i.e. autoregulatory closed-loop gain). Subsequent data show that blockade of end effector K_{ATP} channels, which contribute to vasodilation in response to adenosine, also does not influence coronary autoregulatory capacity (277). We propose that voltage-sensitive K^+

(K_v) channels may play a more prominent role as these channels contribute to the control of coronary blood flow at rest, in response to brief episodes of cardiac ischemia and during increases in MVO₂ (30; 32; 73; 256; 257; 264). However, the contribution of K_v channels to coronary pressure-flow autoregulation has not been investigated.

In addition to metabolic mechanisms, myogenic vasoconstriction is likely critical for mitigating pressure-induced increases in coronary blood flow (184; 210). Data from isolated vessel preparations indicate that coronary responses to increases in intraluminal pressure activate an endothelium-independent (183), mechanosensitive mechanism that results in graded decreases in smooth muscle membrane potential (68; 142). Stretching vascular smooth muscle cells also induces an ~50% increase in intracellular [Ca²⁺] that has been attributed to extracellular influx via voltage-gated (Ca_v1.2) Ca²⁺ channels (69). Importantly, the extent to which Ca_v1.2 channels contribute to changes in coronary vasomotor tone in response to alterations in coronary perfusion pressure *in vivo* has not been determined.

Accordingly, the purpose of this investigation was to test the following hypotheses: 1) vasoactive metabolites produced in response to changes in perfusion pressure modulate coronary vascular resistance and autoregulatory capacity via a K_v channel-dependent mechanism; 2) progressive activation of Ca_v1.2 channels in response to elevations in perfusion pressure is critical for pressure-flow autoregulation in the coronary circulation.

Methods

This investigation was approved by the Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 2011). Animals utilized for this study were Ossabaw swine (n = 12) weighing 30-60 kg. Following completion of experimental protocols,

hearts were fibrillated and excised as recommended by the American Veterinary Medical Association Guide on Euthanasia (June 2007).

Surgical preparation. Swine were initially sedated with telazol (5 mg/kg, sc), zylazine (2.2 mg/kg, sc) and ketamine (3.0 mg/kg, sc). Following endotracheal intubation and venous access, anesthesia was maintained with morphine (3.0 mg/kg, sc) and α -chloralose (100 mg/kg, iv). The animals were mechanically ventilated (Harvard respirator) with room air supplemented with oxygen. Catheters were placed into the right femoral artery and vein for systemic hemodynamic measurements and administration of supplemental anesthetic, heparin, and sodium bicarbonate, respectively. The left femoral artery was catheterized to supply blood to an extracorporeal perfusion system used to perfuse the left anterior descending (LAD) coronary artery at controlled pressures. Arterial blood gases were analyzed periodically throughout the experimental protocol and adjustments made as needed to maintain blood gas parameters within normal physiological limits. A left lateral thoracotomy was performed to expose the heart, and the LAD was isolated and cannulated distal to its first major diagonal branch following heparin administration (500 U/kg, iv). Coronary perfusion pressure (CPP) was regulated by a servo-controlled roller pump and coronary blood flow was continuously measured by an in-line Transonic Systems flow transducer (Ithaca, New York, USA). A catheter was also inserted into the interventricular coronary vein for venous sampling of blood draining the LAD perfusion territory. Left ventricular contractile function was measured with a Millar Mikro-Tip manometer (Millar Instruments, Inc. Houston, TX). Data were continuously recorded on IOX data acquisition software from Emka Technologies (Falls Church, VA).

Experimental protocol. Following a stabilization period (~20 min post cannulation), H_2O_2 (0.3 - 10 μ M) was infused into the LAD perfusion circuit before and during administration of the K_v channel antagonist 4-aminopyridine (4AP; 0.3 mM, i.c.)

with CPP held constant at 100 mmHg (n = 5). Pressure-flow autoregulation was assessed by 10 mmHg increment changes in CPP from 140 mmHg to 40 mmHg before or during intracoronary infusion of 4AP (0.3mM, n = 7) or the Ca_v1.2 channel antagonist diltiazem (10 µg/min, n = 5). Arterial and coronary venous blood samples were collected simultaneously once hemodynamic variables were stable at each CPP. Blood samples were analyzed with an Instrumentation Laboratories automatic blood gas analyzer (GEM Premier 3000) and CO-oximeter (682) system. MVO₂ (µl O₂/min/g) was calculated by multiplying coronary blood flow by the arterial coronary venous difference in oxygen content. As previously reported (17), closed-loop autoregulatory gain (G_c) was calculated from the following formula: $G_c = 1 - [(\Delta F/F)/(\Delta P/P)]$. Changes in flow and pressure were assessed relative to control responses at CPP = 100 mmHg. All drugs were dissolved in saline, adjusted to physiologic pH, and infused at a constant, continuous rate.

Statistical analyses. Data are presented as mean ± SE for n swine. Statistical comparisons were made using a one-way or two-way (Factor A: drug treatment; Factor B: pressure) repeated measures analysis of variance (ANOVA) as appropriate (Sigma Stat 11.0 Software). If statistical differences ($P < 0.05$) in these analyses were noted, a Student-Newman-Keuls multiple comparison test was performed.

Results

Contribution of K_v channels to H₂O₂-mediated coronary vasodilation. Consistent with previous studies (257), intracoronary administration of H₂O₂ (0.3-10 µM) dose-dependently increased coronary blood flow from 0.52 ± 0.03 ml/min/g, under control conditions, to 1.40 ± 0.04 ml/min/g at the highest dose of H₂O₂ ($P < 0.001$, n = 5). Inhibition of coronary K_v channels with 4AP (0.3 mM) at CPP = 100 mmHg decreased coronary blood flow, indices of cardiac contractile function (dP/dt_{max} and dP/dt_{min}), and

MVO₂. Mean aortic pressure and heart rate were unaffected by 4AP (**Table 3-1**). Coronary vasodilation in response to exogenous H₂O₂ was markedly depressed by the administration of 4AP (**Fig. 3-1**, $P < 0.05$).

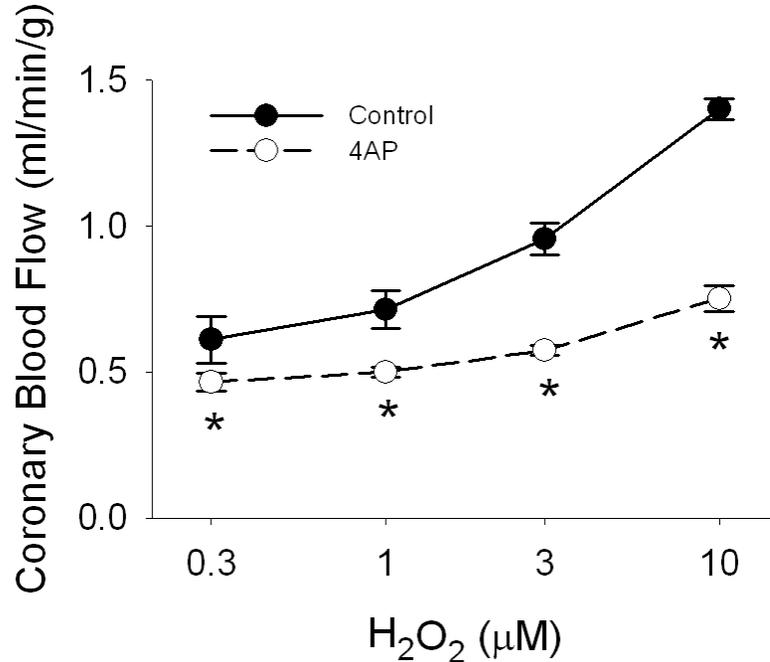


Figure 3-1 Role of K_v channels in H₂O₂-mediated coronary vasodilation. Intracoronary administration of H₂O₂ dose-dependently increased coronary blood flow. Coronary vasodilation in response to exogenous H₂O₂ was markedly depressed by the administration of 4AP. Figure shows grouped average traces for n = 5 swine; * $P < 0.05$ vs. control, same concentration of H₂O₂.

Coronary vascular response to changes in perfusion pressure. Effects of alterations in CPP on systemic hemodynamics are listed in **Table 3-1**. Modest changes in coronary blood flow (0.11 ± 0.02 ml/min/g) were noted over a CPP range of 60-100 mmHg, without significant effects on cardiac contractile function or MVO₂ (n = 7). Determination of G_c indicated an autoregulatory capacity of 0.46 ± 0.11 in untreated hearts over a CPP range of 60-100 mmHg (G_c = 1 being perfect). However, G_c was significantly reduced to -0.43 ± 0.29 at CPPs ranging from 100-140 mmHg and -0.52 ± 0.42 at CPPs ranging from 40-60 mmHg (**Fig. 3-2D**). Reductions in coronary blood flow below CPP 60 mmHg (**Fig. 3-2A**) were associated with diminished dP/dt_{max}, dP/dt_{min}, MVO₂ and mean aortic pressure (**Table 3-1**).

CPP	140	130	120	110	100	90	80	70	60	50	40
Systolic Blood Pressure (mmHg)											
Control	134 ± 9	133 ± 9	132 ± 8	132 ± 8	132 ± 8	131 ± 8	130 ± 8	129 ± 8	128 ± 8	125 ± 7	119 ± 6
4AP	132 ± 9	134 ± 9	134 ± 9	130 ± 10	133 ± 10	131 ± 11	128 ± 11	128 ± 12	125 ± 11	120 ± 9	115 ± 8
Diltiazem	103 ± 8*†	100 ± 8*†	99 ± 8*†	97 ± 7*†	95 ± 7*†	97 ± 8*†	97 ± 9*†	95 ± 9*†	93 ± 8*†	93 ± 9*†	82 ± 6*†
Diastolic Blood Pressure (mmHg)											
Control	83 ± 6	82 ± 6	82 ± 7	82 ± 7	82 ± 7	81 ± 7	81 ± 7	81 ± 6	80 ± 6	81 ± 5	78 ± 5
4AP	77 ± 4	77 ± 5	84 ± 5	79 ± 4	83 ± 5	80 ± 5	79 ± 5	79 ± 6	76 ± 5	76 ± 4	74 ± 4
Diltiazem	69 ± 5	66 ± 5	66 ± 5†	65 ± 4†	63 ± 4†	63 ± 4†	63 ± 4†	62 ± 4†	61 ± 4†	60 ± 4†	54 ± 4†
Mean Aortic Pressure (mmHg)											
Control	108 ± 8	107 ± 8	106 ± 7	106 ± 7	106 ± 7	105 ± 7	105 ± 7	104 ± 7	103 ± 6	102 ± 6	97 ± 5
4AP	101 ± 5	104 ± 7	107 ± 6	104 ± 7	106 ± 7	104 ± 7	102 ± 8	101 ± 8	98 ± 7	96 ± 6	93 ± 6
Diltiazem	86 ± 6*	83 ± 7*†	82 ± 6*†	81 ± 6*†	79 ± 5*†	80 ± 6*†	80 ± 6*†	78 ± 6*†	76 ± 6*†	76 ± 7*†	74 ± 7*†
Heart Rate (beats/min)											
Control	50 ± 5	51 ± 5	51 ± 6	51 ± 6	52 ± 6	53 ± 7	61 ± 12	53 ± 7	53 ± 7	56 ± 6	61 ± 5
4AP	49 ± 7	46 ± 7	46 ± 6	48 ± 7	48 ± 6	49 ± 7	48 ± 7*	48 ± 7	48 ± 7	49 ± 6	52 ± 6*
Diltiazem	79 ± 8*†	80 ± 7*†	80 ± 6*†	81 ± 6*†	81 ± 7*†	77 ± 6*†	76 ± 6*†	77 ± 6*†	79 ± 6*†	76 ± 6*†	81 ± 6*†
Coronary Blood Flow (ml/min/g)											
Control	0.76 ± 0.05	0.64 ± 0.04	0.58 ± 0.03	0.53 ± 0.03	0.50 ± 0.03	0.48 ± 0.03	0.45 ± 0.03	0.43 ± 0.03	0.39 ± 0.03	0.32 ± 0.04	0.23 ± 0.04
4AP	0.55 ± 0.03*	0.43 ± 0.03*	0.44 ± 0.04*	0.43 ± 0.03*	0.39 ± 0.04*	0.38 ± 0.05*	0.37 ± 0.05*	0.34 ± 0.04*	0.31 ± 0.03*	0.26 ± 0.02*	0.17 ± 0.03*
Diltiazem	1.48 ± 0.18*†	1.30 ± 0.12*†	1.15 ± 0.09*†	0.99 ± 0.08*†	0.86 ± 0.06*†	0.70 ± 0.05*†	0.60 ± 0.03*†	0.51 ± 0.03*†	0.44 ± 0.03	0.37 ± 0.03	0.22 ± 0.03
Myocardial Oxygen Consumption (μl O₂/min/g)											
Control	69 ± 8	68 ± 8	66 ± 8	64 ± 7	64 ± 7	60 ± 7	58 ± 6	56 ± 6	51 ± 5	44 ± 5	32 ± 5
4AP	59 ± 5*	54 ± 4*	51 ± 5*	52 ± 5*	48 ± 4*	48 ± 6*	47 ± 6*	44 ± 5*	41 ± 4*	34 ± 3*	26 ± 4
Diltiazem	72 ± 17	68 ± 16	63 ± 16	64 ± 13	62 ± 11	56 ± 9	53 ± 7	50 ± 7	50 ± 6	40 ± 7	28 ± 5
Coronary Venous PO₂											
Control	27 ± 3.0	24 ± 2.3	21 ± 2.3	19 ± 2.6	18 ± 2.0	18 ± 2.0	17 ± 2.0	17 ± 1.2	16 ± 1.3	15 ± 0.7	15 ± 1.0
4AP	23 ± 2.2*	22 ± 1.7	21 ± 1.9	21 ± 1.5	19 ± 1.3	19 ± 1.4	19 ± 1.3	18 ± 1.5	17 ± 1.1	14 ± 0.9	14 ± 0.9
Diltiazem	44 ± 5.3*†	42 ± 5.3*†	40 ± 5.1*†	37 ± 4.5*†	33 ± 3.9*†	31 ± 3.0*†	29 ± 2.6*†	26 ± 1.7*†	24 ± 1.6*†	21 ± 1.0†	19 ± 1.2
dP/dt_{max} (mmHg/s)											
Control	1501 ± 114	1501 ± 115	1527 ± 121	1532 ± 115	1569 ± 113	1551 ± 111	1561 ± 106	1559 ± 111	1534 ± 115	1393 ± 130	1135 ± 99
4AP	1362 ± 115	1334 ± 121*	1351 ± 128*	1349 ± 125*	1258 ± 127*	1254 ± 110*	1228 ± 123*	1242 ± 127*	1200 ± 127*	1144 ± 135*	999 ± 82
Diltiazem	1476 ± 239	1491 ± 264	1473 ± 265	1477 ± 270	1454 ± 251	1456 ± 265	1425 ± 270	1426 ± 293	1389 ± 279	1365 ± 267	1065 ± 130
dP/dt_{min} (mmHg/s)											
Control	-1448 ± 159	-1455 ± 148	-1429 ± 148	-1359 ± 167	-1359 ± 150	-1338 ± 137	-1306 ± 130	-1276 ± 127	-1136 ± 134	-1016 ± 155	-922 ± 146
4AP	-1177 ± 132*	-1148 ± 135*	-1129 ± 133*	-1086 ± 133*	-1124 ± 155*	-1079 ± 156*	-1058 ± 147*	-1058 ± 140*	-987 ± 131	-879 ± 127	-744 ± 114
Diltiazem	-1330 ± 117	-1271 ± 110	-1292 ± 116	-1290 ± 125	-1278 ± 127	-1305 ± 156	-1265 ± 171	-1212 ± 154	-1185 ± 139	-1138 ± 169	-786 ± 165

Table 3-1 Hemodynamic, cardiac and blood gas parameters during variable coronary perfusion pressure with and without 4AP or Diltiazem. Values are mean ± SE for Control, 4AP (n = 7), and diltiazem (n = 5). * P < 0.05 vs. control same CPP; † P < 0.05 vs. 4AP.

K_V channels in coronary pressure-flow autoregulation. Relative to untreated control conditions, inhibition of coronary *K_V* channels with 4AP significantly attenuated coronary blood flow ~20% at CPPs ranging from 60-140 mmHg (**Fig. 3-2A**, *P* < 0.05). Intracoronary 4AP administration had no effect on mean aortic pressure at CPPs > 50 mmHg (**Table 3-1**, n = 7). The reductions in coronary blood flow were associated with diminished cardiac contractile function as evidenced by the ~10-20% decrease in dP/dt_{max} and dP/dt_{min} over a wide range of CPPs (**Table 3-1**, *P* < 0.05). These changes in contractile function were also accompanied by decreases in MVO₂ at CPPs ranging from 50-140 mmHg (**Table 3-1**, *P* < 0.05). However, 4AP administration did not alter the relationship between coronary blood flow and MVO₂ (**Fig. 3-2C**). Importantly, inhibition of *K_V* channels did not affect the overall change in coronary blood flow at CPPs < 120 mmHg (**Fig. 3-2B**) as flow only varied 0.08 ± 0.01 ml/min/g over CPPs of 60-100 mmHg (*P* = 0.53 vs. untreated control). Calculation of G_c over this range of CPPs also revealed

essentially identical Gc's for both control (0.46 ± 0.11) and 4AP (0.46 ± 0.06) treated conditions (**Fig. 3-2D**, $P = 0.99$). Administration of 4AP also failed to significantly influence Gc over CPPs ranging from 40-60 mmHg ($P = 0.78$) or 100-140 mmHg ($P = 0.46$). Alternatively, pressure-induced increases in coronary blood flow were attenuated by 4AP at CPPs of 130-140 mmHg (**Fig. 3-2B**), while coronary zero flow pressure (Pzf) was unaffected (average = 23 ± 2 mmHg).

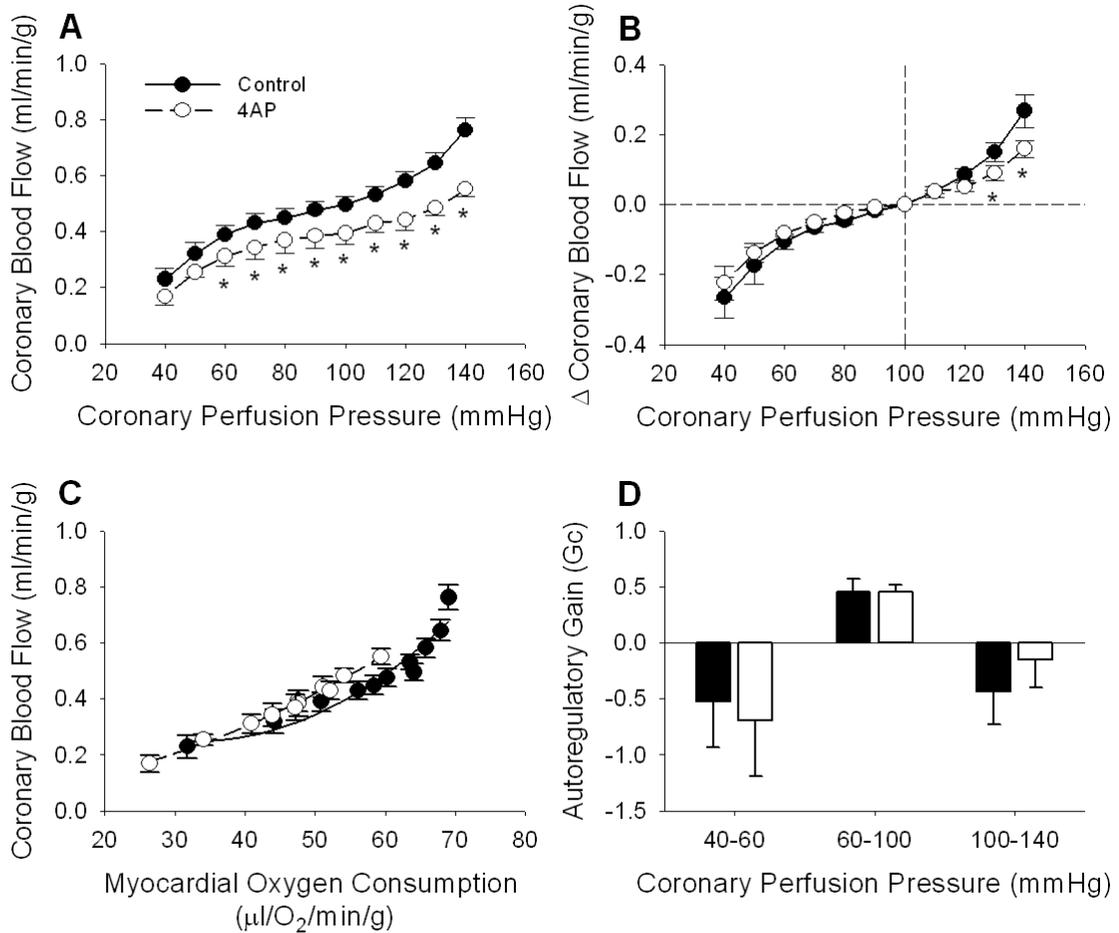


Figure 3-2 Role of K_V channels in coronary pressure-flow autoregulation. (A) Inhibition of coronary K_V channels with 4AP significantly attenuated coronary blood flow at CPPs from 60-140 mmHg. 4AP administration did not affect the change in coronary blood flow at CPPs < 120 mmHg (B), the balance between coronary blood flow and MVO_2 (C) or autoregulatory gain (D). Figures show grouped average traces for $n = 7$ swine. * $P < 0.05$ vs. control, same CPP.

Ca_v1.2 channels in coronary pressure-flow autoregulation. Inhibition of coronary $Ca_{v1.2}$ channels with diltiazem (10 $\mu\text{g}/\text{min}$) progressively increased coronary blood flow (**Fig. 3-3A**, $P < 0.05$) and significantly increased the change in blood flow to 0.42 ± 0.06 ml/min/g over CPPs ranging from 60-100 mmHg (**Fig. 3-3B**; $P < 0.01$ vs. untreated

control). Diltiazem administration reduced mean aortic pressure and increased heart rate at all CPPs (**Table 3-1**, $P < 0.05$). However, this vasodilatory effect was not associated with alterations in indices of cardiac contractile function or MVO_2 (**Table 3-1**). Thus, diltiazem-mediated increases in coronary blood flow were independent of changes in MVO_2 (**Fig. 3-3C**). Importantly, inhibition of coronary $Ca_v1.2$ channels markedly reduced Gc to -0.20 ± 0.11 over a CPP range of 60-100 mmHg, i.e. essentially abolished pressure-flow autoregulation (**Fig. 3-3D**, $P < 0.01$). Diltiazem did not affect Gc at CPPs ranging from 40-60 mmHg ($P = 0.79$), 100-140 mmHg ($P = 0.47$) or significantly alter Pzf relative to untreated controls (average = 20 ± 1 mmHg).

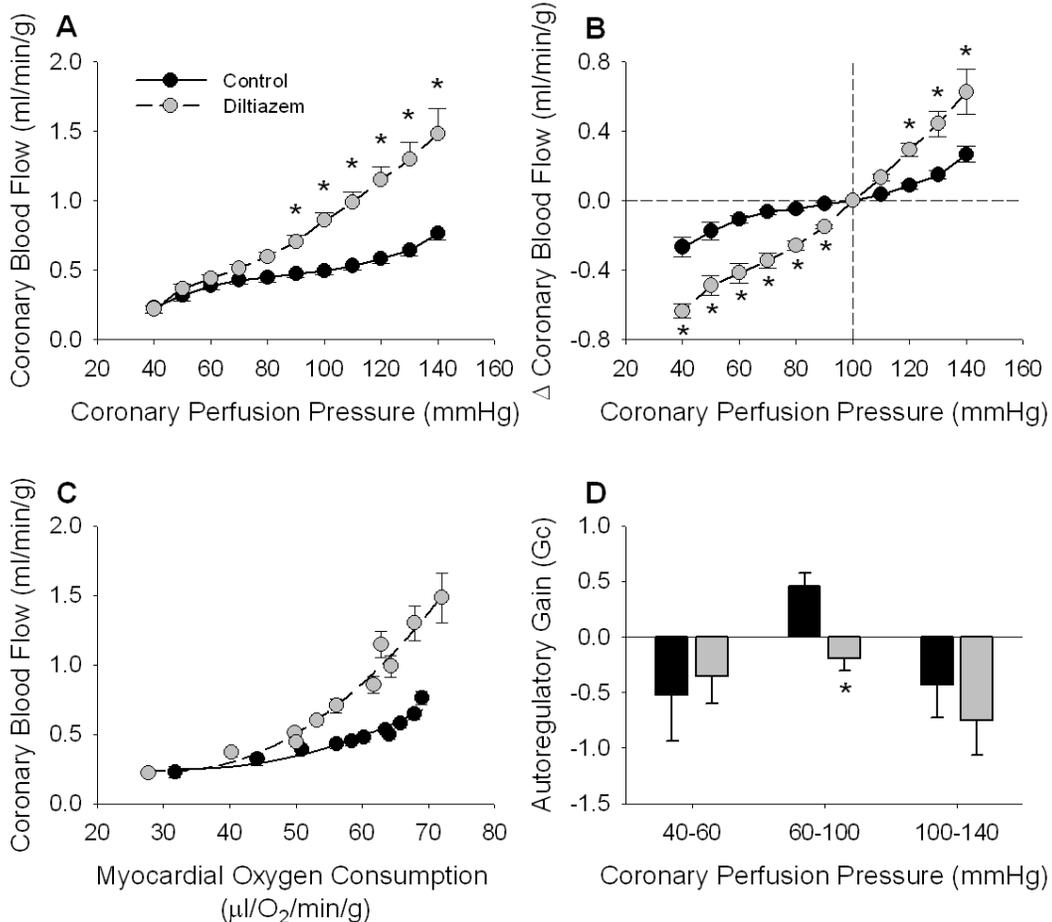


Figure 3-3 Role of $Ca_v1.2$ channels in coronary pressure-flow autoregulation. Inhibition of coronary $Ca_v1.2$ channels with diltiazem ($10 \mu\text{g}/\text{min}$) significantly increased coronary blood flow at CPPs > 80 mmHg (**A**) and the change in blood flow to over a wide range of CPPs (**B**). Coronary vasodilation in response to diltiazem was independent of MVO_2 (**C**) and abolished pressure-flow autoregulation within CPPs ranging from 60-100 mmHg (**D**). Figures show grouped average traces for $n = 5$ swine. $*P < 0.05$ vs. control.

Discussion

First identified in the coronary circulation by Eckel in 1949 (77), coronary autoregulation refers to the intrinsic ability of the heart to maintain constant blood flow despite changes in arterial perfusion pressure (158). Although numerous studies have focused on delineating the interdependent relationship between coronary blood flow and CPP (103; 236; 305), the underlying mechanisms responsible for coronary pressure-flow autoregulation have not been clearly defined. Given the important role for voltage-dependent K_V and $Ca_V1.2$ channels in the control of smooth muscle membrane potential and coronary vascular resistance (225; 292; 312), we hypothesized that these specific channels may also serve as critical end-effectors in modulating the vascular responses to alterations in CPP. Findings from this investigation demonstrate that K_V channels tonically contribute to the control of microvascular resistance over a wide range of CPPs, but do not contribute to coronary responses to changes in perfusion pressure within the autoregulatory range. In contrast, progressive activation of $Ca_V1.2$ channels with increases in CPP represents a critical mechanism of coronary pressure-flow autoregulation.

Role of H_2O_2 and K_V channels in coronary pressure-flow autoregulation. Experiments performed in this study were designed to test the hypothesis that vasoactive metabolites produced in response to changes in perfusion pressure modulate coronary vascular resistance and autoregulatory capacity via a K_V channel-dependent mechanism. The rationale for this hypothesis is based on earlier studies by our group which demonstrated that activation of K_V channels is critical to the regulation of coronary blood flow (30; 37; 73) and that several key vasoactive metabolites (e.g. adenosine, NO, H_2O_2) mediate coronary vasodilation predominantly via K_V channels (32; 73; 256; 257). Consistent with prior studies in dogs (257), the present findings indicate that H_2O_2 induces marked coronary vasodilation via activation of K_V channels in swine (**Fig. 3-1**).

We propose this dilator effect is due to direct activation of smooth muscle K_V channels as Rogers *et al.* found that H_2O_2 dose-dependently activates 4AP sensitive K^+ current in coronary smooth muscle cells (256) and that denudation has no effect on H_2O_2 -induced dilation of isolated coronary arterioles (257). These data are in contrast with alternative studies that documented H_2O_2 is a key endothelium-derived hyperpolarizing factor that mediates coronary vasodilation via a BK_{Ca} channel-dependent mechanism (197; 330).

To examine the role of K_V channels in coronary pressure-flow autoregulation, we performed experiments in anesthetized, open-chest swine in which CPP was varied from 40 mmHg to 140 mmHg via a servo-controlled, extracorporeal perfusion circuit. In these studies, we demonstrated that the inhibition of K_V channels significantly reduced coronary blood flow over a wide range of CPPs (**Fig. 3-2A**). However, 4AP administration did not affect the change in coronary blood flow with alterations in perfusion pressure at CPPs < 120 mmHg (**Fig. 3-2B**) or influence the overall autoregulatory capacity (G_c) of the coronary circulation (**Fig. 3-2D**). Thus, given that 4AP abolished H_2O_2 -mediated coronary vasodilation, our findings do not support a prominent role for K_V channel-dependent pathways, such as H_2O_2 , in modulating coronary vascular responses to changes in perfusion pressure within the autoregulatory range. This conclusion differs from that of Yada *et al.* who suggested that H_2O_2 , in cooperation with NO and adenosine, plays an important role in coronary pressure-flow autoregulation *in vivo* in dogs (321). However, other investigations fail to support a prominent role for NO or adenosine in coronary autoregulation (178; 180; 271; 277). Closer examination of the data presented by Yada *et al.* also indicates that the significant reductions in coronary blood flow at lower CPPs in the presence of L-NAME and/or catalase did not significantly alter the coronary pressure-flow relationship; i.e. closed-loop autoregulatory gain. Interestingly, our findings with K_V channel inhibition are similar with those of Stepp *et al.* who determined that blockade of K_{ATP} channels with

glibenclamide produced a tonic decrease in coronary blood flow, but did not significantly influence the autoregulatory capability of the coronary circulation (277). Taken together, these data indicate that neither K_V channels, K_{ATP} channels, nor the upstream vasodilatory factors known to converge on these channels, are necessary to support a metabolic component of coronary pressure-flow autoregulation (188). In contrast, the limitation of pressure-induced increases in coronary flow by 4AP supports earlier studies implicating K_V channels as an important negative-feedback mechanism that limits myogenic constriction, especially at high perfusion pressures (CPP > 120 mmHg) (2; 78; 315).

Role of $Ca_V1.2$ channels in coronary pressure-flow autoregulation. Functioning as a predominant mediator of extracellular Ca^{2+} influx, $Ca_V1.2$ channels constitutively contribute to the control of coronary microvascular resistance. This is evidenced *in vivo* by the marked, dose-dependent increases in coronary blood flow observed in response to $Ca_V1.2$ channel blockade (175). Findings from the present study are the first to demonstrate that inhibition of coronary $Ca_V1.2$ channels results in a progressive increase in coronary blood flow as CPP is elevated (**Fig. 3-3A**). Thus, administration of diltiazem significantly augmented pressure-induced changes in coronary blood flow (**Fig. 3-3B**), resulting in responses that would be predicted in a maximally dilated bed; i.e. passive vasculature. Importantly, the dose of diltiazem used (10 μ g/min) did not produce maximal dilation (coronary flow = 0.86 ± 0.06 ml/min/g at CPP = 100 mmHg) but did markedly diminish coronary autoregulatory capacity as Gc was reduced to -0.20 ± 0.11 within the autoregulatory range of 60-100 mmHg (**Fig. 3D**), i.e. inhibition of $Ca_V1.2$ channels essentially abolished pressure-flow autoregulation (see **Limitations of the study**). This observed decrement of pressure-flow autoregulation supports that increasing activation of $Ca_V1.2$ channels with elevations in CPP is a central mechanism underlying the intrinsic ability of the coronary circulation to maintain blood flow constant

with changes in perfusion pressure. Our findings are consistent with earlier *in vitro* myogenic studies demonstrating pressure-induced increases in membrane potential and arteriolar wall $[Ca^{2+}]$ are dependent on $Ca_v1.2$ channels (68; 69). Experiments in isolated, pressurized arterioles indicate a functional myogenic component also exists in the human and porcine coronary microcirculation (159; 185; 214). However, evidence for the involvement of other mechanosensitive, nonselective cation channels in myogenic vasoconstriction has also been reported (68; 142). Consequently, it is important to point out that the present data cannot distinguish between a strictly myogenic vs. metabolic-induced activation of $Ca_v1.2$ channels. Regardless, the present data demonstrate that $Ca_v1.2$ channel-dependent pathways represent a critical mechanism of coronary pressure-flow autoregulation *in vivo*.

Relationship between coronary blood flow, CPP, and MVO_2 . Coronary blood flow is dependent on CPP and MVO_2 (269), and coronary pressure can arguably influence metabolism via the “Gregg Phenomenon” (124; 268). Although the Gregg effect is more pronounced in poorly autoregulating hearts (~55% increase in MVO_2 over CPP range of 60-120 mmHg (17)), modest changes in MVO_2 are detected in hearts with effective pressure-flow autoregulation (~20% increase in MVO_2 over CPP range 60-120 mmHg (17; 78)). Thus, changes in arterial pressure and/or myocardial metabolism can influence the overall level of myocardial perfusion. In this study, administration of both 4AP and diltiazem altered these determinants of coronary blood flow. In particular, 4AP-mediated reductions in coronary flow were accompanied by reductions in cardiac function and MVO_2 , while diltiazem decreased arterial pressure and reflexively increased heart rate (**Table 3-1**). Changes in arterial pressure did not directly influence CPP as these experiments were conducted in a cannulated, extracorporeal perfused preparation. However, to account for these drug-induced alterations, we plotted coronary blood flow relative to its respective MVO_2 at CPPs ranging from 40-140 mmHg. These plots indicate

that 4AP did not significantly affect the balance between coronary blood flow and MVO_2 as CPP was changed over this range of perfusion pressures (**Fig. 3-2C**). Alternatively, blockade of coronary $Ca_v1.2$ channels resulted in a progressive increase in coronary blood flow at a given level of MVO_2 (**Fig. 3-3C**). Thus, the increases in coronary blood flow induced by diltiazem were not mediated by significant increases in MVO_2 ; i.e. reflect pressure-mediated increases in coronary flow, not metabolic vasodilation. Regardless of the experimental condition, our findings support a strong interdependent relationship between CPP, MVO_2 , and coronary blood flow. To evaluate the individual contribution of each of these factors, additional examination of the interrelationship between coronary blood flow, MVO_2 and CPP was assessed by 3-dimensional analysis. These data further support that inhibition of K_v channels did not significantly affect the relationship between coronary blood flow, MVO_2 and CPP (**Fig. 3-4A**). In contrast, increases in coronary blood flow observed with elevations in CPP and MVO_2 were significantly augmented following diltiazem administration (**Fig. 3-4B**), further supporting a prominent role of $Ca_v1.2$ channels in coronary pressure-flow autoregulation.

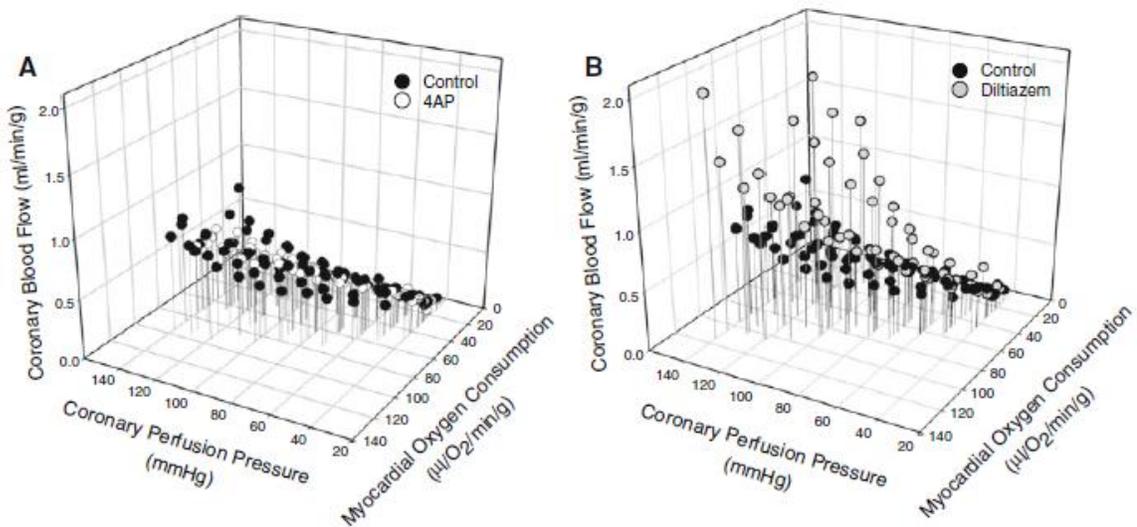


Figure 3-4 Effects of K_v and $Ca_v1.2$ channel inhibition on 3-dimensional analysis of coronary pressure-flow autoregulation. Inhibition of K_v channels reduced coronary blood flow and MVO_2 but did not affect pressure-induced changes in coronary flow (**A**). Blockade of coronary $Ca_v1.2$ channels significantly increased coronary blood flow as CPP and MVO_2 were elevated (**B**). Figures show individual data for $n = 7$ (control, 4AP) or $n = 5$ (diltiazem) swine.

Limitations of the study. Conclusions regarding the role of K_v and $Ca_v1.2$ channels in coronary pressure-flow autoregulation are confounded by specific effects of both 4AP and diltiazem on cardiac function and MVO_2 . In the case of 4AP, we found that inhibition of K_v channels significantly decreased coronary blood flow, and that these reductions in flow were accompanied by decreases in left ventricular dP/dt and MVO_2 (**Table 3-1**). Such data pose a circular argument as to whether 4AP-mediated decreases in coronary flow resulted in diminished contractile performance and subsequent decreases in MVO_2 , or alternatively if 4AP initially decreased contractile function which then led to reductions in MVO_2 and coronary blood flow. Importantly, when 4AP administration is initiated, we typically find that coronary blood flow falls within a matter of seconds, which contrasts with reductions in left ventricular dP/dt that typically occur within ~ 2 min of 4AP administration. Accordingly, we conclude that 4AP resulted in rapid (tonic) coronary vasoconstriction that was followed by commensurate reductions in contractile function and MVO_2 ; an effect consistent with characteristics of myocardial hibernation (53). The present findings are in contrast with earlier studies (264) regarding the effect of 4AP on the balance between coronary blood flow and myocardial metabolism as 4AP failed to significantly decrease coronary venous PO_2 at CPPs <140 mmHg. We propose this discrepancy is related to the higher levels of MVO_2 's reported in these studies at rest (70-100 $\mu l O_2/min/g$) and during increases in metabolism (up to ~400 $\mu l O_2/min/g$), relative to the much lower levels of MVO_2 reported in our current preparation (~50 $\mu l O_2/min/g$ at rest). This point is supported by the significant reduction in coronary venous PO_2 in the presence of 4AP when MVO_2 was elevated to ~60 $\mu l O_2/min/g$ at CPP = 140 mmHg (**Table 3-1**).

Earlier studies have demonstrated that administration of coronary vasodilator agents significantly impair coronary pressure-flow autoregulation (77; 140). In the present study, inhibition of $Ca_v1.2$ channels resulted in an ~70% increase in baseline

coronary flow at CPP = 100 mmHg. This effect of diltiazem significantly complicates interpretation as to whether decreased coronary autoregulatory capacity following diltiazem administration was due to the inhibition of Ca_v1.2 channels alone or simply to its vasodilator influence. This matter is further complicated by the fact that compounds induce vasodilation via activation of coronary K⁺ channels (167), which hyperpolarizes smooth muscle and inhibits Ca_v1.2 channels (42; 91; 139; 151; 286). Thus, studies which demonstrate reductions in coronary autoregulation in the presence of vasodilators actually support our present conclusion regarding the role of Ca_v1.2 channels in pressure-flow autoregulation. Importantly, the dose of diltiazem used in the present study is selective for Ca_v1.2 channels and increased coronary blood flow from 0.50 to 0.86 ml/min/g; i.e. an average flow that is within the “normal range” of baseline coronary blood flow (82). Despite these confounding effects, inhibition of Ca_v1.2 channels has been shown to produce marked, dose-dependent increases in baseline coronary blood flow (175), which supports a prominent role for these channels in the regulation of coronary vasomotor tone.

Summary and Implications. We have identified a tonically active role for K_v channels in the control of coronary microvascular resistance over wide range of CPPs (60-140 mmHg). However, our data indicate that these channels, and the vasodilatory pathways known to converge on them (adenosine, NO, H₂O₂), are not required for coronary responses to changes in perfusion pressure within the autoregulatory range. Although K_v channels are not necessary for coronary pressure-flow autoregulation, it is possible that alternative pathways/channels are activated to compensate for K_v channel inhibition. Alternatively, our findings do support that K_v channels serve as a negative-feedback mechanism that limits myogenic constriction, especially at high perfusion pressures (CPP > 120 mmHg). These findings are important in light of recent studies

implicating impaired K_v channel activity in a variety of disease states including hypercholesterolemia, hypertension, and hyperglycemia (45; 132; 133; 195; 199; 200).

Data from this study are the first to demonstrate that inhibition of $Ca_v1.2$ channels with diltiazem essentially abolishes the ability of the coronary circulation to maintain blood flow constant with alterations in perfusion pressure. Although our findings support a critical role for $Ca_v1.2$ channels in pressure-flow autoregulation, the mechanism by which $Ca_v1.2$ channels are activated (myogenic vs. metabolic) requires further investigation. Delineating mechanisms of coronary $Ca_v1.2$ channel activation is important given findings supporting elevated $Ca_v1.2$ channel function and associated activators (e.g. PKC) during hypertension and metabolic syndrome (175; 214; 242; 249). We propose that administration of $Ca_v1.2$ channel blockers to such patients would likely prove beneficial, despite effects on autoregulatory capacity, as these agents would act to increase coronary blood flow, reduce left ventricular afterload, and improve the balance between myocardial oxygen delivery and metabolism.

ACKNOWLEDGEMENTS

This work was supported by AHA/NIH grants 10PRE4230035 (ZCB) and HL092245 (JDT).

Chapter 4

Contribution of Voltage-Dependent K⁺ Channels to Metabolic Control of Coronary Blood Flow

Journal of Molecular and Cellular Cardiology

Volume 52 (4), April, 2012

**Zachary C. Berwick¹, Gregory M. Dick², Steven P. Moberly¹, Meredith C. Kohr¹,
Michael Sturek¹, Johnathan D. Tune¹**

¹Department of Cellular and Integrative Physiology, Indiana University School of
Medicine, Indianapolis, IN 46202

²Department of Exercise Physiology Center for Cardiovascular & Respiratory Sciences
West Virginia University School of Medicine, Morgantown, WV 26506

Abstract

The purpose of this investigation was to test the hypothesis that K_V channels contribute to metabolic control of coronary blood flow and that decreases in K_V channel function and/or expression significantly attenuate myocardial oxygen supply-demand balance in the metabolic syndrome (MetS). Experiments were conducted in conscious, chronically instrumented Ossabaw swine fed either a normal maintenance diet or an excess calorie atherogenic diet that produces the clinical phenotype of early MetS. Data were obtained under resting conditions and during graded treadmill exercise before and after inhibition of K_V channels with 4-aminopyridine (4-AP, 0.3 mg/kg, i.v.). In lean-control swine, 4-AP reduced coronary blood flow ~15% at rest and ~20% during exercise. Inhibition of K_V channels also increased aortic pressure ($P < 0.01$) while reducing coronary venous PO_2 ($P < 0.01$) at a given level of myocardial oxygen consumption (MVO_2). Administration of 4-AP had no effect on coronary blood flow, aortic pressure, or coronary venous PO_2 in swine with MetS. The lack of response to 4-AP in MetS swine was associated with a ~20% reduction in coronary K_V current ($P < 0.01$) and decreased expression of $K_V1.5$ channels in coronary arteries ($P < 0.01$). Together, these data demonstrate that K_V channels play an important role in balancing myocardial oxygen delivery with metabolism at rest and during exercise-induced increases in MVO_2 . Our findings also indicate that decreases in K_V channel current and expression contribute to impaired control of coronary blood flow in the MetS.

Keywords: Coronary, exercise, K_V channels, metabolic syndrome, swine

Introduction

The myocardium is highly dependent on a continuous supply of oxygen and nutrients from the coronary circulation to meet its metabolic requirements and to maintain contractile performance (82; 294). Despite extensive investigation over the past half century, the primary mechanisms responsible for balancing myocardial oxygen delivery with myocardial energy demand have remained elusive. Metabolic control of coronary blood flow is hypothesized to occur via local production of vasoactive substances which regulate microvascular resistance via activation of downstream K^+ channels on vascular smooth muscle (74). Although multiple types of K^+ channels are expressed in coronary smooth muscle, recent data from our investigative team indicate that voltage-dependent K^+ (K_v) channels represent a critical end effector mechanism that modulates coronary blood flow at rest (73; 264), during cardiac pacing or catecholamine-induced increases in myocardial oxygen consumption (MVO_2) (264), following brief periods of cardiac ischemia (73), and endothelial-dependent and independent vasodilation (25; 32; 73). However, the functional contribution of K_v channels to metabolic control of coronary blood flow during physiologic increases in MVO_2 , as occur during exercise, has not been examined.

Earlier studies have demonstrated that disease states such as obesity and the metabolic syndrome (MetS) markedly impair the ability of the heart to adequately balance coronary blood flow with myocardial metabolism (31; 39; 175). Coronary microvascular dysfunction in the MetS is evidenced by reductions in coronary venous PO_2 (39; 269; 329), diminished vasodilatory responses to pharmacologic agonists (i.e. coronary flow reserve) (201; 218; 246; 265), and alterations in functional and reactive coronary hyperemia (37; 290). Decreases in K^+ channel function contribute to this impairment as MetS depresses outward K^+ current in coronary artery smooth muscle cells (38; 48; 202; 218) and diminishes the role of specific K^+ channels in coronary

vasodilatory responses (25; 37). In particular, decreases in K_V channel activity have been associated with key components of the MetS, including hypercholesterolemia (132; 133), hypertension (45), and hyperglycemia (195; 199; 200). We hypothesize that such reductions in the functional expression of K_V channels contribute to the impaired control of coronary blood flow in the setting of the MetS.

Accordingly, the primary goals of the present study were to: 1) examine the contribution of coronary K_V channels to regulation of coronary blood flow at rest and during exercise-induced increases in MVO_2 ; and 2) determine the effects of the MetS on coronary K_V channel activity and expression. Experiments were designed to test the hypothesis that decreases in K_V channel function and/or expression significantly attenuate myocardial oxygen supply-demand balance in MetS. This hypothesis was examined in chronically instrumented Ossabaw swine fed either a normal maintenance diet or an excess calorie, atherogenic diet that produces the common clinical phenotype of early MetS; i.e. obesity, insulin resistance, impaired glucose tolerance, dyslipidemia, hypertension, and atherosclerosis (90; 281). Hemodynamic data and arterial/coronary venous blood samples were obtained before and during inhibition of K_V channels with 4-aminopyridine (4-AP, 0.3 mg/kg, iv) at rest and during graded treadmill exercise. In addition, whole cell K^+ currents were measured in freshly isolated coronary artery smooth muscle cells from lean and MetS swine and expression of coronary $K_V1.5$ and $K_V3.1$ channels determined by Western blot.

Methods

Ossabaw swine model of metabolic syndrome. All experimental procedures and protocols used in this investigation were approved by the Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals*. Lean control swine were fed ~2200 kcal/day of standard chow (5L80, Purina

Test Diet, Richmond, IN) containing 18% kcal from protein, 71% kcal from complex carbohydrates, and 11% kcal from fat. MetS swine were fed an excess ~8000 kcal/day high fat/fructose, atherogenic diet containing 16% kcal from protein, 41% kcal from complex carbohydrates, 19% kcal from fructose, and 43% kcal from fat (mixture of lard, hydrogenated soybean oil, and hydrogenated coconut oil), and supplemented with 2.0% cholesterol and 0.7% sodium cholate by weight (KT324, Purina Test Diet, Richmond, IN). Both lean (n = 7) and MetS (n = 5) castrated male swine were fed their respective diets for 16 weeks prior to surgical instrumentation.

Surgical instrumentation. Following an overnight fast, Ossabaw swine were sedated with telazol (5 mg/kg, sc) and xylazine (2.2 mg/kg, sc). After endotracheal intubation, a surgical plane of anesthesia was maintained by mechanical ventilation with 1-3% isoflurane gas, supplemented with oxygen. Utilizing sterile technique, a left lateral thoracotomy was performed in the fifth intercostal space. A 17 Ga pressure monitoring catheter (Edwards LifeSciences) was implanted in the descending thoracic aorta for blood pressure measurements and arterial blood sampling. A second catheter was placed in the coronary interventricular vein for coronary venous blood sampling and intravenous drug infusions. The left anterior descending coronary artery (LAD) was dissected free and a perivascular flow transducer (Transonic Systems Inc.) was placed around the artery. The pneumothorax was evacuated and the chest was closed in layers. Catheters and the flow transducer wire were tunneled subcutaneously and exteriorized between the scapulae. Antibiotics (exceld, 5 mg/kg, im), rimadyl (4mg/kg, im) and buprenorphine (0.015mg/kg, im) were administered to prevent infection and manage post-operative pain. Externalized wires/catheters were protected by a jacket and an elastomeric balloon pump (MILA International) was connected to the coronary venous catheter for continuous infusion of heparinized saline (5U/ml at 5ml/hr). The aortic catheter was flushed daily and filled with heparinized saline (5,000 U/ml).

Experimental protocol and blood sampling. Following recovery from surgery, experiments were conducted in lean (n = 7) and MetS (n = 5) Ossabaw swine under resting conditions and during graded treadmill exercise before and during inhibition of K_v channels with 4-AP (0.3 mg/kg, iv). Hemodynamics were continuously recorded at baseline and during two levels of treadmill exercise at ~ 2 mph and ~5 mph. Arterial and coronary venous blood samples were collected simultaneously in heparinized syringes when hemodynamic variables were stable at rest and at each level of exercise. Each exercise period was ~2 min in duration, and the animals were allowed to rest sufficiently between each level for hemodynamic variables to return to baseline.

Arterial and coronary venous blood samples were collected, immediately sealed and placed on ice. The samples were analyzed in duplicate for pH, PCO_2 , PO_2 , glucose, hematocrit, and oxygen content with an Instrumentation Laboratories automatic blood gas analyzer (GEM Premier 3000) and CO-oximeter (682) system. LAD perfusion territory was estimated to be 30% of total heart weight, as previously described by Feigl (103). MVO_2 ($\mu l O_2/min/g$) was calculated by multiplying coronary blood flow by the arterial coronary venous difference in oxygen content.

Patch-clamp electrophysiology. Coronary smooth muscle cells were freshly isolated from proximal segments of the LAD as previously described (38). Briefly, patch-clamp recordings were performed within 8 h of cell dispersion. Whole-cell K^+ currents were measured at room temperature with the conventional dialyzed configuration of the patch-clamp technique. Bath solution contained (in mM) 138 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, and 5 Tris (pH 7.4). Pipettes had tip resistances of 2-4 $M\Omega$ when filled with solution containing (in mM) 140 KCl, 3 Mg-ATP, 0.1 Na-GTP, 0.1 EGTA, 10 HEPES, and 5 Tris (pH 7.1). After whole-cell access was established, series resistance and membrane capacitance were compensated. Current voltage relationships

were assessed by 400-ms step pulses from -60 to +20 mV in 10-mV increments from a holding potential of -80 mV.

Western blot analysis. Following excision of hearts, coronary arteries from lean (n = 5) and MetS (n = 5) swine were quickly isolated, cleaned of adventitia, placed in liquid N₂ and stored at -80°C. Arteries were homogenized with lysis buffer and total protein collected and quantified by DC Protein Assay. Equivalent amounts of protein (40 µg) were loaded onto 7.5% acrylamide gels and transferred overnight. Membranes were blocked for 1 h at ambient temperature prior to 24 h incubation at 4°C with rabbit polyclonal antibodies (Alomone Labs) directed against K_v 1.5 (1:100) and K_v 3.1 (1:500) in blocking buffer with 0.1% Tween 20 and mouse anti-actin antibody (MP Biomedicals, 1:15,000). Blots were washed and incubated for 1 h with IRDye 800 donkey anti-rabbit (1:10,000) and IRDye 700 donkey anti-mouse (1:20,000) secondary antibodies. Immunoreactivity for K_v channel subtypes was determined by the Li-Cor Odyssey system (Li-Cor Biosciences) and expressed relative to actin (loading control).

Statistical Analyses. Data are presented as mean ± SE. Statistical comparisons were made by unpaired t-test (phenotype data in **Table 4-1**) or by two-way analysis of variance (ANOVA) for within group analysis (Factor A: drug treatment; Factor B: exercise level) and between group analysis (Factor A: diet with drug treatment; Factor B: exercise level) as appropriate. For all statistical comparisons, *P* < 0.05 was considered statistically significant. When significance was found with ANOVA, a Student-Newman-Keuls multiple comparison test was performed to identify differences between groups and treatment levels. Multiple linear regression analysis was used to compare slopes of response variables (aortic pressure, coronary venous PO₂) plotted vs. MVO₂. If the slopes of the regression lines were not significantly different, an analysis of covariance (ANCOVA) was used to adjust response variables for linear dependence on MVO₂.

Results

Table 4-1 Phenotypic characteristics of lean and metabolic syndrome Ossabaw swine.

	Lean	MetS
Body Weight (kg)	46 ± 3	72 ± 4*
Heart wt. / Body wt. (x 100)	0.36 ± 0.03	0.41 ± 0.03
Glucose (mg/dl)	74 ± 4	85 ± 5
Insulin (μU/ml)	16 ± 6	30 ± 9
HOMA index	2.9 ± 1.1	6.0 ± 1.5
Total cholesterol (mg/dl)	87 ± 5	486 ± 70*
LDL/HDL ratio	1.6 ± 0.1	5.9 ± 1.4*
Triglycerides (mg/dl)	43 ± 5	67 ± 18

Values are mean ± SE for lean (n = 7) and MetS (n = 5) swine. * $P < 0.05$ vs lean.

Phenotype of Ossabaw swine. Phenotypic characteristics of lean and MetS swine are given in **Table 4-1**. Consistent with our recent studies (37-39; 44), we found that the excess calorie, atherogenic diet induced classic features of early MetS in Ossabaw swine. In particular, relative to their lean counterparts MetS swine exhibited a significant 1.6-fold increase in body weight, a 5.6-fold increase in total cholesterol, a 3.7-fold increase in LDL/HDL ratio and a 1.5-fold increase in triglyceride levels. Blood samples obtained from swine at the time of exercise experiment (non-fasted) revealed modest increases in plasma glucose and insulin concentration ($P = 0.10$). Homeostatic model assessment (HOMA) index values were also ~2-fold higher in MetS swine ($P = 0.13$).

Table 4-2 Hemodynamic and blood gas variables at rest and during graded treadmill exercise in lean and metabolic syndrome Ossabaw swine with and without 4AP (0.3mg/kg).

	Exercise		
	Rest	Level 1	Level 2
Systolic Blood Pressure (mmHg)			
Lean	113 ± 5	112 ± 4	121 ± 5
Lean + 4AP	119 ± 6	119 ± 4	125 ± 5
MetS	119 ± 6	131 ± 9†	138 ± 9
MetS + 4AP	126 ± 7	125 ± 6	134 ± 4

Diastolic Blood Pressure (mmHg)

Lean	73 ± 4	70 ± 4	75 ± 4
Lean + 4AP	79 ± 6	76 ± 4	86 ± 6*
MetS	86 ± 3	89 ± 8†	92 ± 6†
MetS + 4AP	89 ± 5	81 ± 7	90 ± 5

Mean Aortic Pressure (mmHg)

Lean	93 ± 4	92 ± 3	98 ± 4
Lean + 4AP	99 ± 6*	99 ± 4*	105 ± 4*
MetS	103 ± 5	108 ± 8†	115 ± 8†
MetS + 4AP	100 ± 6	104 ± 7	114 ± 5

Heart Rate (beats/min)

Lean	120 ± 8	162 ± 11	212 ± 13
Lean + 4AP	129 ± 10	172 ± 9	201 ± 10
MetS	134 ± 19	168 ± 20	183 ± 14
MetS + 4AP	125 ± 6	170 ± 7	184 ± 6

Coronary Blood Flow (ml/min/g)

Lean	1.11 ± 0.09	1.46 ± 0.12	1.92 ± 0.12
Lean + 4AP	0.94 ± 0.13	1.24 ± 0.16*	1.54 ± 0.17*
MetS	0.80 ± 0.08	1.07 ± 0.14†	1.21 ± 0.15†
MetS + 4AP	0.95 ± 0.16	1.07 ± 0.16	1.25 ± 0.17

Coronary Conductance (μl/min/g/mmHg)

Lean	11.9 ± 0.8	15.8 ± 0.8	19.5 ± 0.6
Lean + 4AP	9.3 ± 1.0*	12.4 ± 1.3*	14.6 ± 1.4*
MetS	8.0 ± 1.1†	10.2 ± 1.8†	10.6 ± 1.3†
MetS + 4AP	9.7 ± 1.9	10.3 ± 1.5	11.0 ± 1.4

Myocardial O₂ Consumption (μl O₂/min/g)

Lean	117 ± 14	178 ± 23	244 ± 21
Lean + 4AP	102 ± 18	131 ± 19	166 ± 14*
MetS	102 ± 7	143 ± 19	158 ± 22†
MetS + 4AP	117 ± 23	132 ± 21	151 ± 21

Arterial pH

Lean	7.55 ± 0.01	7.55 ± 0.01	7.54 ± 0.01
Lean + 4AP	7.60 ± 0.03	7.60 ± 0.03	7.56 ± 0.03
MetS	7.53 ± 0.02	7.52 ± 0.01†	7.50 ± 0.02†
MetS + 4AP	7.58 ± 0.02*	7.56 ± 0.01	7.54 ± 0.01

Coronary Venous pH

Lean	7.47 ± 0.01	7.48 ± 0.01	7.47 ± 0.01
Lean + 4AP	7.50 ± 0.02	7.50 ± 0.02	7.49 ± 0.02
MetS	7.45 ± 0.02	7.45 ± 0.01	7.45 ± 0.01
MetS + 4AP	7.50 ± 0.02*	7.49 ± 0.01*	7.48 ± 0.01*

Arterial PCO₂ (mmHg)			
Lean	32 ± 1	31 ± 2	31 ± 1
Lean + 4AP	26 ± 2*	25 ± 2*	27 ± 2
MetS	33 ± 2	31 ± 2	30 ± 1
MetS + 4AP	29 ± 2	27 ± 1	29 ± 1
Coronary Venous PCO₂ (mmHg)			
Lean	49 ± 1	43 ± 3	45 ± 1
Lean + 4AP	41 ± 2*	42 ± 3	40 ± 3
MetS	49 ± 3	46 ± 2	44 ± 3
MetS + 4AP	42 ± 3*	42 ± 3*	44 ± 3
Arterial PO₂ (mmHg)			
Lean	94 ± 3	98 ± 3	95 ± 4
Lean + 4AP	107 ± 3*	108 ± 3	100 ± 8
MetS	89 ± 4	89 ± 3	94 ± 4
MetS + 4AP	98 ± 4	97 ± 2	96 ± 3
Coronary Venous PO₂ (mmHg)			
Lean	18 ± 0.6	18 ± 0.8	17 ± 0.7
Lean + 4AP	15 ± 0.6*	16 ± 0.9*	16 ± 0.8
MetS	14 ± 1.4†	12 ± 1.2†	12 ± 1.0†
MetS + 4AP	13 ± 1.4	13 ± 1.5	13 ± 1.7
Coronary Venous O₂ Saturation (%)			
Lean	16 ± 2	13 ± 3	14 ± 2
Lean + 4AP	13 ± 3	13 ± 3	13 ± 2
MetS	14 ± 2	11 ± 1	10 ± 1
MetS + 4AP	13 ± 2	12 ± 2	12 ± 3
Arterial Hematocrit (%)			
Lean	34 ± 1	37 ± 1	37 ± 1
Lean + 4AP	30 ± 2	30 ± 1*	33 ± 2
MetS	36 ± 3	37 ± 2	37 ± 1
MetS + 4AP	32 ± 2	31 ± 2*	33 ± 2

Values are mean ± SE for lean (n = 7) and MetS (n = 5) swine. * $P < 0.05$ vs. untreated control, same diet/condition; † $P < 0.05$ vs. lean, same treatment.

Coronary and cardiovascular response to exercise: lean vs. MetS swine.

Hemodynamic and blood gas data for lean and MetS Ossabaw swine at rest and during exercise are summarized in **Table 4-2**. Although no changes in systolic blood pressure were observed in untreated MetS vs. lean swine under baseline-resting conditions, MetS

swine tended to have higher diastolic blood pressure (**Table 4-2**; $P = 0.08$). Exercise-induced increases in mean aortic pressure were significantly augmented in MetS swine while no differences in heart rate were noted between groups at rest or during exercise. Given these changes in blood pressure, coronary blood flow was reduced ~30-35% at rest and during exercise (**Table 4-2**). Normalizing coronary blood flow to aortic pressure revealed significant reductions in coronary conductance in MetS vs. lean swine at all levels (**Table 4-2**). MVO_2 was modestly reduced ~15% in MetS swine under baseline conditions ($P = 0.57$), but was significantly depressed ~35% at the highest level of exercise (**Table 4-2**). These changes in coronary blood flow and MVO_2 were associated with a significant decrease in coronary venous PO_2 (index of tissue PO_2) at rest and during exercise. Importantly, the slope of the relationship between coronary venous PO_2 and MVO_2 (**Fig. 4-1A vs. Fig. 4-1B**) was significantly increased (lower PO_2 at given level of MVO_2) in untreated MetS vs. lean swine ($P < 0.02$).

Role of K_v channels in coronary and cardiovascular response to exercise. Effects of K_v channel inhibition with 4-AP (0.3 mg/kg, iv) on hemodynamic and blood gas variables at rest and during exercise are also summarized in **Table 4-2**. Administration of 4-AP significantly increased mean aortic pressure at rest and during exercise in lean, but not MetS swine. Blockade of K_v channels also increased aortic pressure in lean animals to a level closer to that observed in MetS swine (**Table 4-2**). Heart rate was unaffected by 4-AP in either group. Despite increases in blood pressure in lean swine, 4-AP reduced coronary blood flow ~15% at rest ($P = 0.17$) and ~20% at the highest level of exercise ($P < 0.05$) (**Table 4-2**). Coronary blood flow was not significantly altered by the administration of 4-AP in MetS swine at rest or during exercise. Coronary conductance was significantly decreased by 4-AP at rest and during exercise in lean, but not MetS swine (**Table 4-2**). Increases in MVO_2 to exercise were also diminished ~30% by inhibition of K_v channels in lean swine. Regression analysis demonstrated that 4-AP

produced a significant, parallel downward shift in the relationship between coronary venous PO_2 vs. MVO_2 in lean, but not MetS swine (**Fig. 4-1**).

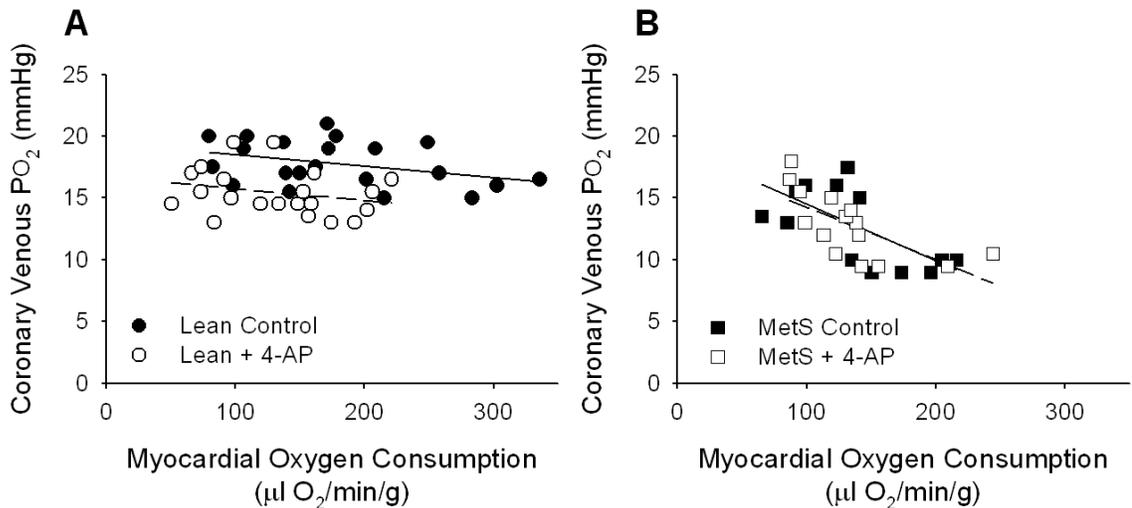


Figure 4-1 Effect of K_V channel inhibition on the relationship between coronary venous PO_2 and myocardial oxygen consumption in lean (**A**) and MetS (**B**) swine. Inhibition of K_V channels with 4-aminopyridine (4-AP) significantly reduced coronary venous PO_2 at a given level of metabolism in lean ($P < 0.01$) but not MetS swine ($P = 0.84$). The slope of this relationship was also significantly decreased in untreated lean vs. MetS swine ($P < 0.02$).

Functional expression of coronary K_V channels in lean vs. MetS swine. Whole cell patch clamp recordings (**Fig. 4-2A**) demonstrate a ~20% reduction in coronary K^+ current at potentials greater than 0 mV, i.e. currents biophysically consistent with K_V channels (**Fig. 4-2B**, $P < 0.01$). Pharmacological characterization of K_V current, including separation from BK_{Ca} current can be found in the supplement. K_V channels produce characteristic tail currents upon repolarization of the membrane (**inset Fig. 4-2A**), the magnitude of which was reduced in cells from MetS pigs (1.4 ± 0.3 vs. 2.0 ± 0.2 pA/pF; $P < 0.05$). This observation supports the idea that the difference in outward current in **Figure 4-2B** is a reduction in K_V current. Importantly, however, other characteristics of the tail currents were not different (voltage of half activation and slope factor of -6 ± 1 mV and 8 ± 1 vs. -8 ± 1 mV and 8 ± 1), suggesting the same types of K_V channels are expressed in cells from lean and MetS swine (**Fig. 4-2C**).

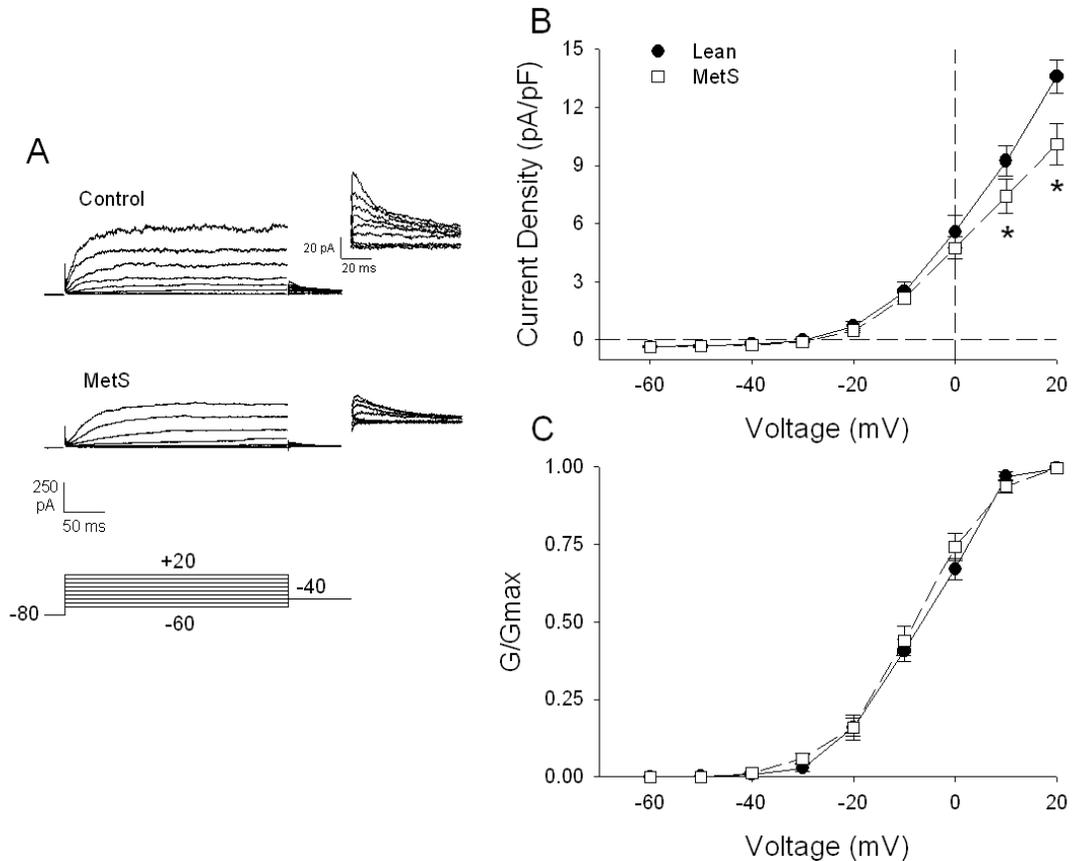


Figure 4-2. Whole-cell voltage-dependent K^+ current in coronary smooth muscle of lean and MetS swine. (A) Families of current traces from representative cells of lean and MetS pigs. Voltage template is 400 ms long. K_V channels produce characteristic tail currents upon repolarization of the membrane (inset), the magnitude of which was reduced in cells from MetS pigs. (B) Group I-V data demonstrate a significant reduction in outward K^+ current at potentials greater than 0 mV, i.e. currents biophysically consistent with K_V channels. (C) Group G-V curves derived from tail currents at -40 mV. The voltage-sensitivity of the currents were not different (voltage of half activation and slope factor of -6 ± 1 mV and 8 ± 1 vs. -8 ± 1 mV and 8 ± 1 in control and MetS pigs, respectively. * $P < 0.01$ vs. lean, same voltage).

Several K_V channel proteins have been proposed to underlie the native current in smooth muscle, including $K_V1.5$ (291) and $K_V3.1$ (132). Protein expression data of $K_V1.5$ and $K_V3.1$ channels in coronary arteries from lean and MetS swine are shown in **Figure 4-3**. Bands for $K_V1.5$ and $K_V3.1$ were 68 and 98 kDa, respectively. Western analysis revealed a significant ~49% reduction in coronary $K_V1.5$ channel expression in arteries from MetS swine (**Fig. 4-3A**; $P < 0.05$). No significant difference in coronary $K_V 3.1$ channel expression was noted in lean vs. MetS swine (**Fig. 4-3B**; $P = 0.36$).

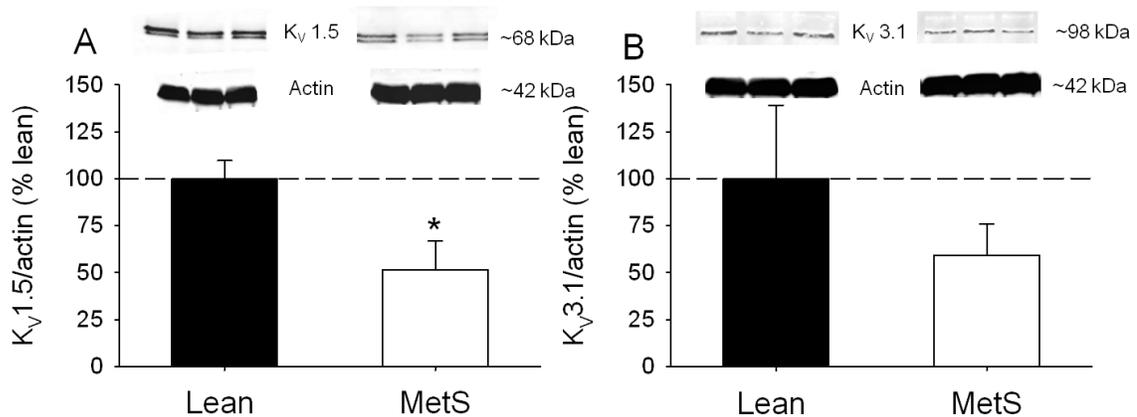


Figure 4-3. Expression of K_V1.5 and K_V3.1 channels in coronary arteries of lean and MetS swine. **(A)** Western blot analysis demonstrated a significant reduction in K_V1.5 channel protein expression in coronary arteries from MetS swine. **(B)** Expression of coronary K_V3.1 channel protein was not significantly affected by induction of MetS ($P = 0.36$). * $P < 0.05$ vs. lean.

Discussion

The primary goal of this investigation was to examine the hypothesis that coronary K_V channels contribute to local metabolic control of coronary blood flow and that reduced functional expression of these channels plays a role in microvascular dysfunction in the setting of the MetS. This hypothesis is supported by earlier studies indicating that K_V channels modulate coronary blood flow *in vivo* (37; 73; 257; 264) and that specific components of the MetS decrease smooth muscle K_V current and their contribution to arteriolar vasodilatory responses (26; 45; 62; 132; 133; 177; 200; 324). The novel findings of this study are: 1) inhibition of K_V channels increases blood pressure at rest and during exercise in lean, but not MetS swine; 2) K_V channels contribute to the regulation of coronary blood flow at rest and during increases in MVO₂ in lean, but not MetS swine; 3) induction of MetS significantly decreases K_V channel current in coronary artery smooth muscle cells; 4) expression of K_V 1.5 channels is diminished in the coronary circulation of MetS swine. Taken together, these data demonstrate that K_V channels play a crucial role in balancing myocardial oxygen delivery

with myocardial oxygen demand at rest and during exercise-induced increases in MVO_2 in normal lean swine. Our findings also indicate that decreases in K_V channel activity and expression contribute to impaired control of coronary blood flow in the MetS.

Role of K_V channels in control of blood pressure and coronary blood flow. K_V channels are widely expressed in both the systemic and coronary circulation (74). Earlier investigations have established an active role for K_V channels in modulating smooth muscle membrane potential in isolated smooth muscle cells, arteries, and arterioles as well as vascular tone in anaesthetized preparations (74). In particular, data from the present study demonstrate that inhibition of K_V channels with 4-AP significantly elevates mean aortic pressure at rest and during exercise in normal lean animals (**Table 4-2**). These data are consistent with previous findings from our laboratory (37) as well as others (1; 26; 326) and implicate a critical role for K_V channels in the control of systemic vascular resistance. We propose that the effects of 4-AP on blood pressure are mediated by effects on vascular smooth muscle and not by direct cardiac effects as intracoronary administration of 4-AP at concentrations ≤ 0.3 mM does not significantly alter arterial pressure (73). In addition, 4-AP has also been shown to augment arterial pressure in the presence of adrenoceptor antagonists in anesthetized cats, arguing against direct sympathetic pressor effects (326).

Consistent with other recent studies (73; 256; 257; 264), the present findings support a prominent role for K_V channels in regulating coronary blood flow. This effect is primarily evidenced by the ~15-20% reduction in coronary blood flow at rest and during exercise (**Table 4-2**) following 4-AP administration in lean swine. It is important to recognize that this decrease in coronary flow occurred in the presence of significant increases in blood pressure, i.e. 4-AP markedly reduced coronary conductance (**Table 4-2**). However, the parallel downward shift in the relationship between coronary venous PO_2 and MVO_2 supports more of a “tonic” role for K_V channels in the control of coronary

blood flow; i.e. similar contribution to coronary vascular resistance at rest and during increases in MVO_2 (**Fig. 4-1A**). Together, these results indicate that vasodilator substances that converge on K_V channels are required for adequate myocardial oxygen supply-demand balance over a wide range of MVO_2 . Although we did not examine the identity of specific factor(s) that mediate coronary vasodilation via K_V channels in this study, previous studies from our investigative team implicate H_2O_2 as a feedforward dilator that couples coronary blood flow with myocardial metabolism, predominantly through 4-AP sensitive K^+ channels (256; 257; 264). Other factors that have been shown to induce coronary vasodilation, at least in part, through K_V channels include adenosine, nitric oxide, prostacyclin, and EDHF (74). However, a prominent role for these factors in local metabolic control is unlikely as inhibition of these pathways has little, if any effect on coronary blood flow at rest or during increases in MVO_2 (294).

Effects of MetS on function and expression of coronary K_V channels. Although K_V channels regulate membrane potential, arteriolar diameter (74; 132), and coronary blood flow (73; 264) in normal lean animals, data from this study importantly demonstrate that the MetS markedly impairs the functional expression of K_V channels in vascular smooth muscle. In particular, while inhibition of K_V channels influenced blood pressure, coronary blood flow and the balance between coronary blood flow and MVO_2 in lean swine, 4-AP had no effect on any of these key variables in obese, MetS swine (**Table 4-2**). Interestingly, MVO_2 was significantly decreased in MetS vs. lean swine during exercise, despite a larger rate-pressure product in MetS swine (**Table 4-2**). The reason for this difference is not apparent but is not clearly associated with alterations in K_V channel function and suggests that the MetS independently abrogates the relationship between coronary blood flow and myocardial metabolism. The absence of any cardiovascular effect of 4-AP in MetS swine, along with the augmented pressor response (**Table 4-2**) and substantial imbalance between coronary blood flow and myocardial metabolism

(**Fig. 4-1B**), indicates that microvascular dysfunction typically observed in the setting of the MetS (31) is directly related to the diminished contribution of K_V channels to overall vascular resistance. It is possible that the lack of an effect of 4-AP on the balance between coronary blood flow and MVO_2 is related to a generalized vasoconstriction of the coronary vasculature in MetS hearts. However, the absence of coronary effects of 4-AP in combination with the reduction in outward K_V current and expression of coronary K_V channels indicates that diminished functional expression of K_V channels contributes to the impairment in the control of coronary blood flow in the MetS. The overall degree to which decreased K_V channel function influences coronary microvascular dysfunction in MetS is unclear, but could be related to alterations in the release of specific vasoregulatory factors that converge on K_V channels, changes in K_V channel activity, specific channel subunit expression and/or a combination of these mechanisms.

To examine potential mechanisms by which MetS impairs the contribution of K_V channels to the control of coronary blood flow, we performed patch-clamp electrophysiology and Western blot studies in order to address functional and molecular expression of the channel proteins. Functional expression of K_V current was reduced in smooth muscle cells from MetS pigs (**Fig. 4-2B**). Importantly, our supplemental data show that the currents we recorded in porcine coronary smooth muscle cells possessed pharmacological properties consistent with those mediated by K_V channels (i.e. largely sensitive to inhibition by 4-AP) and were not contaminated by large conductance, Ca^{2+} -sensitive K^+ (BK_{Ca}) current (i.e. insensitive to penitrem A). This raises the possibility that MetS: a) reduces the expression of K_V channels and/or b) induces a phenotypic switch to other K_V channel types. The latter mechanism, however, seems unlikely, as intrinsic K_V current characteristics including the voltage-dependence of activation were not changed ($V_{1/2}$ and slope factor k ; **Fig. 4-2C**). Thus, we further investigated the possibility that MetS decreases K_V channel protein expression. It is unclear what K_V channel

subtypes underlie the native K_V current in coronary smooth muscle, but candidates include $K_V1.5$ (291) and $K_V3.1$ (132). In particular, $K_V1.5$ has been implicated as redox/oxygen sensing channels (291) while $K_V3.1$ has been interrogated in impaired adenosine-induced dilation in hypercholesterolemic swine (132). Importantly, $K_V3.1b$ channels are also sensitive to oxygen (237) and auxiliary β subunits can confer oxygen sensitivity to subtypes not typically considered to be redox-sensitive (241). We found that expression of $K_V1.5$ protein was reduced in coronary arteries from MetS pigs (**Fig. 4-3A**), while expression of $K_V3.1$ protein was not statistically affected ($P = 0.36$). These data indicate that $K_V1.5$ channels are a component of the native K_V current and that the MetS-induced reduction in K_V current in coronary smooth muscle could be related to reduced molecular expression of $K_V1.5$ channels. Our interpretation is consistent with recent preliminary data indicating that metabolic coronary vasodilatation is reduced in $K_V1.5$ knockout mice (231). The potential contribution of alternative K_V channel subtypes (e.g. K_V 1.2, 1.3, 1.5, and/or 2.1) merits further investigation.

It remains unclear what component(s) of the MetS milieu alters K_V channel function and expression in coronary smooth muscle; however, several of the individual constituents have been investigated previously. These include hyperglycemia, hypercholesterolemia, and increased levels of circulating neurohumoral factors (endothelin, angiotensin II, and catecholamines). In particular, elevated glucose and an associated increase in reactive oxygen species production impaired K_V channel function in smooth muscle cells from rat coronary arteries (195; 200). Whether a similar mechanism is at play in MetS pigs with modestly elevated glucose, insulin, and HOMA scores remains to be determined. Hypercholesterolemia also impairs coronary arteriolar relaxation mediated by K_V channels and reduces K_V current in coronary vascular smooth muscle (132; 133). Our swine MetS model produces profound hypercholesterolemia; therefore, it is possible that this factor contributes to decreased molecular and functional

expression of K_V channels. In addition, MetS is associated with an increase in circulating levels of endothelin (5; 107; 132; 145) and sensitization of endothelin-mediated coronary vasoconstriction in dogs (176) and humans (207). Endothelin also inhibits vascular smooth muscle K_V channels by a pathway involving protein kinase C (252), the activity of which we have recently reported to be increased in our MetS swine (240). Thus, it is possible that elevated endothelin levels alter the functional and molecular expression of K_V channels in MetS swine. Similarly, related signal mechanisms activated by catecholamines (76) and angiotensin II (329) may contribute to impaired K_V channel function and expression in MetS pigs.

Limitations of the study. It is important to point out that systemic administration of 4-AP may confound interpretation of the present findings as increases in arterial pressure (~6 mmHg) influence both coronary blood flow and MVO_2 (82; 294). However, any effect of 4-AP on MVO_2 , the primary determinant of coronary blood flow, is accounted for by plotting key coronary response variables (coronary venous PO_2) relative to MVO_2 (**Fig. 4-1**). Intravenous administration of 4-AP also tended to decrease hematocrit in both lean and MetS swine (**Table 4-2**). The mechanism underlying this effect is unclear but it would likely act to increase (not decrease) coronary blood flow secondary to a reduction in myocardial oxygen delivery. Given these effects, we speculate that the overall contribution of K_V channels to the control of coronary blood flow may be underestimated in this study. This hypothesis is supported by earlier data from our laboratory which showed a much greater reduction in coronary blood flow (~40-50% decrease) in response to intracoronary 4-AP in normal-lean canines (73). Thus, future studies to examine the effects of intracoronary 4-AP on metabolic control of coronary blood flow are warranted.

We also acknowledge the use of conduit coronary arteries for patch-clamp studies and measurement of K_V channel expression as a limitation as changes in conduit

K⁺ channel current and protein expression may not directly reflect alterations at the microvascular level. Whether differences in macro vs. microcirculation account for the disparate ~20% reduction in K_V current (**Fig. 4-2**) relative to the complete loss of an effect of 4-AP on coronary blood flow (**Table 4-2**) is unclear. However, data obtained from the idealized conditions that allow for examination of K⁺ current in isolated coronary vascular smooth muscle cells may not directly associate with overall K⁺ channel function *in vivo*, where other compensatory mechanisms could also be at play (82). Unfortunately we were unable to acquire sufficient measures of whole cell K⁺ current in the presence of 4-AP in smooth muscle cells from lean and MetS swine. Characterization of the currents is provided in the supplement.

Conclusions

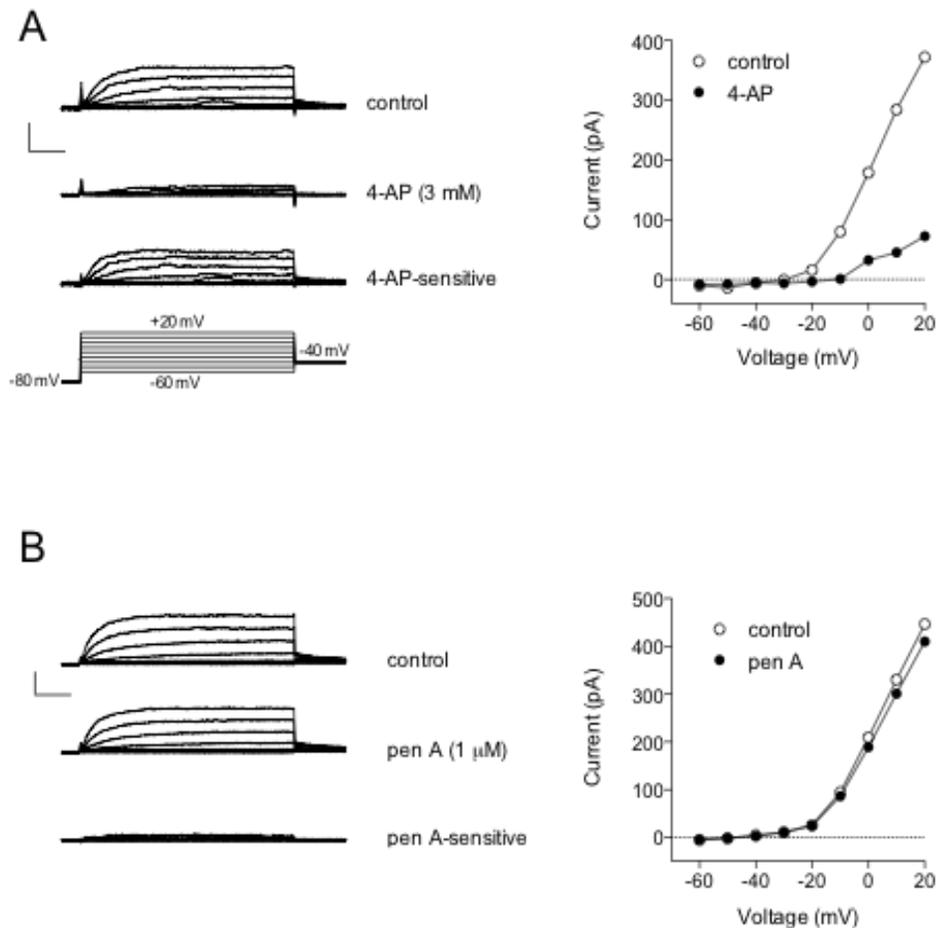
In summary, data from this investigation support that vasodilatory factors that converge on K_V channels play a critical role in the control of systemic vascular resistance and the balance between coronary blood flow with myocardial metabolism at rest and during exercise in conscious, lean swine. In addition, our findings also demonstrate that diminished functional expression of K_V channels significantly contributes to coronary microvascular dysfunction and the imbalance between myocardial oxygen supply-demand observed in the setting of the MetS (31). We hypothesize that therapeutic targeting of MetS components (e.g. hypercholesterolemia, angiotensin II) and/or signaling pathways (e.g. PKC) that are known to alter K_V channel activity and expression could improve cardiovascular outcomes in patients with the MetS.

ACKNOWLEDGEMENTS

This work was supported by AHA grants 10PRE4230035 (ZCB) and NIH grants HL092245 (JDT) and HL062552 (MS).

Supplemental data

Smooth muscle cells from the coronary artery possess a number of K^+ channels (74); therefore, it is important to demonstrate isolation of voltage-dependent K^+ (K_V) current in our recordings. Specifically, we show that the currents we recorded in porcine coronary smooth muscle cells: 1) possessed pharmacological properties consistent with those mediated by K_V channels and 2) were not contaminated by large conductance, Ca^{2+} -sensitive K^+ (BK_{Ca}) current. Currents between -60 and +20 mV were largely sensitive to 4-aminopyridine (4-AP; 3 mM; Supplemental figure, panel A), indicating they were mediated by K_V channels (299). In contrast, currents were largely insensitive to penitrem A (1 μ M; Supplemental figure, panel B), indicating very little contribution of BK_{Ca} channels (173).



Chapter 5

Contribution of K_v and $Ca_v1.2$ Electromechanical Coupling to Coronary Dysfunction in Metabolic Syndrome

Zachary C. Berwick¹, Gregory M. Dick², Heather A. O'Leary³, Sean B. Bender⁴,
Adam G. Goodwill¹, Steven P. Moberly¹, Meredith C. Kohr¹, Alexander Obukhov¹,
Johnathan D. Tune¹

¹Department of Cellular and Integrative Physiology,
Indiana University School of Medicine, Indianapolis, IN 46202

²Department of Exercise Physiology
Center for Cardiovascular and Respiratory Sciences
West Virginia University School of Medicine

³Department of Microbiology and Immunology,
Indiana University School of Medicine, Indianapolis, IN 46202

⁴Department of Internal Medicine,
University of Missouri School of Medicine

Abstract

Previous investigations by our group indicate that diminished functional expression of K_V channels impairs control of coronary blood flow in obesity/metabolic syndrome (MetS). The goal of this investigation was to test the hypothesis that K_V channels are electromechanically coupled to $Ca_V1.2$ channels and that coronary microvascular dysfunction in MetS is related to subsequent increases in $Ca_V1.2$ channel activity. Initial studies revealed that inhibition of K_V channels with 4-aminopyridine (4AP, 0.3 mM) increased intracellular $[Ca^{2+}]_i$, contracted isolated coronary arterioles, and decreased coronary reactive hyperemia, and that these effects were reversed by blockade of $Ca_V1.2$ channels. Further studies in chronically instrumented Ossabaw swine showed that inhibition of $Ca_V1.2$ channels with nifedipine (10 μ g/kg, iv) had no effect on coronary blood flow at rest or during exercise in lean swine. However, blockade of $Ca_V1.2$ channels significantly elevated coronary blood flow, conductance, and the balance between flow and metabolism in swine with the MetS ($P < 0.05$). These changes were associated with a ~50% increase in inward $Ca_V1.2$ current and elevations in α_1c membrane expression in coronary smooth muscle cells from MetS swine. Taken together, the results from this investigation indicate that increased functional expression of coronary $Ca_V1.2$ channels contributes to coronary microvascular dysfunction in the MetS.

Keywords: Coronary, exercise, $Ca_V1.2$ channels, metabolic syndrome, swine

Introduction

Coronary ion channels play a critical role in the regulation of microvascular resistance and consequently coronary blood flow. Multiple channels function in dynamic equilibrium to effect changes in coronary smooth muscle membrane potential (E_M) and vascular tone in response to metabolic needs of the surrounding myocardium. Thus, modulation of key coronary ion channels facilitates the preservation of the delicate balance between myocardial oxygen delivery and consumption (MVO_2). Although the mechanisms by which this balance is regulated are not fully understood, previous investigations implicate voltage-gated $Ca_v1.2$ channels as a predominant mediator of extracellular Ca^{2+} influx and coronary vascular resistance (175; 225). Activation of $Ca_v1.2$ channels occurs in response to various stimuli, including changes in smooth muscle E_M elicited by voltage-dependent K^+ (K_v) channels, which have been shown to contribute to the regulation of coronary blood flow at rest (73), during increases in MVO_2 (30), and following myocardial ischemia (32). However, the extent to which this “electromechanical coupling” between K_v and $Ca_v1.2$ channels modulates coronary microvascular tone and reactivity has not been specifically examined.

Recent studies indicate that obesity and the metabolic syndrome (MetS) markedly attenuate coronary vascular function and the ability of the coronary circulation to adequately balance coronary blood flow with myocardial metabolism (31). We recently documented that this impairment is related, at least in part, to diminished expression and activity of coronary K_v channels (30). These findings are intriguing in relation to earlier studies which documented that inhibition of K_v channels depolarizes smooth muscle to within the activation threshold for $Ca_v1.2$ channels and elevates cytosolic $[Ca^{2+}]$ (239), an effect abolished by removal of extracellular Ca^{2+} (205). Accordingly, we hypothesize that diminished K_v channel function in MetS impairs coronary microvascular function, at least in part, via increases in $Ca_v1.2$ activity. Recent data from our laboratory support this

hypothesis as we have demonstrated that the MetS increases intracellular $[Ca^{2+}]$ in coronary smooth muscle, augments coronary vasoconstriction to the $Ca_v1.2$ channel agonist Bay K 8644 and increases coronary vasodilation in response to the $Ca_v1.2$ channel antagonist nicardipine (38; 175). However, the extent to which alterations in $Ca_v1.2$ channels contributes to the deleterious effects of the MetS on coronary blood flow regulation *in vivo* has not been investigated.

The purpose of the present investigation was to: 1) determine the functional significance of electromechanical coupling between K_v and $Ca_v1.2$ channels in the coronary circulation; 2) evaluate the contribution of $Ca_v1.2$ channels to the regulation of coronary blood flow at rest and during physiologic-induced increases in myocardial metabolism; 3) assess the influence of the MetS on the interaction between coronary K_v and $Ca_v1.2$ channels, the role of $Ca_v1.2$ channels in metabolic control of coronary blood flow as well as $Ca_v1.2$ channel current and subunit expression. In particular, vertically integrative experiments utilizing a wide variety of molecular/cellular and whole-systems approaches were performed to test the hypothesis that diminished K_v channel function and impaired physiologic regulation of coronary blood flow in the setting of the MetS is related to increased activity and/or expression of $Ca_v1.2$ channels.

Methods

Ossabaw swine model of metabolic syndrome. All experimental procedures and protocols used in this investigation were approved by the Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals*. Lean control swine were fed ~2200 kcal/day of standard chow (5L80, Purina Test Diet, Richmond, IN) containing 18% kcal from protein, 71% kcal from complex carbohydrates, and 11% kcal from fat. MetS swine were fed an excess ~8000 kcal/day high fat/fructose, atherogenic diet containing 16% kcal from protein, 41% kcal from

complex carbohydrates, 19% kcal from fructose, and 43% kcal from fat (mixture of lard, hydrogenated soybean oil, and hydrogenated coconut oil), and supplemented with 2.0% cholesterol and 0.7% sodium cholate by weight (KT324, Purina Test Diet, Richmond, IN). Both lean and MetS castrated male swine were fed their respective diets for 16 weeks prior to surgical instrumentation. Additional lean animals were also utilized for acute open-chest procedures. Coronary arteries, microvessels and myocardium were isolated immediately following heart excision for subsequent functional/molecular experiments.

Isolated Vessels. Epicardial coronary arteries were isolated, cleaned of adventitia, cut into 3mm rings, and mounted in organ baths containing Krebs buffer (37°C) for isometric tension studies as previously described (240). Coronary segments were adjusted to optimal length (~3.5 g tension) as determined by <10% change in active tension in response to 60 mM KCl before administration of either BayK 8644 (10 μ M, n = 6 lean) or KCl (20 mM, n = 4 lean/MetS) directly to the organ bath. Following a wash, administration of respective drugs was then repeated in the presence of the selective K_v channel antagonist 4-aminopyridine (4AP, 0.3 mM). Active tension development was measured using Emka software (Falls Church, VA). Remaining coronary arteries not used for tension studies were enzymatically digested to disperse smooth muscle cells or frozen for subsequent molecular experiments.

Subepicardial coronary arterioles (n = 3; 50- to 150- μ m diameter) were also isolated from the left ventricular apex of lean swine, cannulated, and pressurized to 60 cmH₂O, as described previously (25). Intraluminal diameter was measured continuously with videomicrometers and recorded on a MacLab workstation. Arterioles free from leaks were allowed to equilibrate for ~1 h at 37°C with the bath solution changed every 15 min. Arterioles that did not develop at least 20% spontaneous tone were excluded. Following development of tone, arterioles were treated with 4AP (0.3mM). Once a stable

diameter was achieved with 4AP, the $Ca_v1.2$ Ca^{2+} channel antagonist nifedipine (10 μ M) was added to the vessel bath. Diameter responses were normalized to maximal arteriolar diameter determined at the end of each experiment by changing the bath solution to Ca^{2+} -free physiological salt solution.

Cell Dispersions and Molecular Expression. Coronary myocytes were isolated as described previously (38; 310) for microfluorimetry, patch-clamp, and flow cytometry. Coronary arteries from lean animals were loaded with Fura-2 (0.1 mM, n = 59 cells from 3 animals), placed in a superfusion chamber and observed with a monochromator-based imaging system (TILL-Photonics, Martinsreid, Germany) equipped with a DU885 charge-coupled device camera (Andor Technology plc, South Windsor, CT) used to monitor Fura-2 wavelengths. Fura-2 fluorescence was excited at 345 and 380 nm. Emitted light was collected with a 510-nm long-pass filter. Data were analyzed using TILLvision software and reported as the change in baseline F345/380 in response to KCl (120 mM) or KCl + 4AP (0.3mM).

Coronary smooth muscle cells from lean and MetS swine were isolated from proximal segments of the LAD and patch-clamp recordings were performed within 8 h of cell dispersion. Whole-cell $Ca_v1.2$ currents were measured at room temperature with the conventional dialyzed configuration of the patch-clamp technique. Bath solution contained (in mM) 138 NaCl, 5 KCl, 2 BaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, and 5 Tris (pH 7.4). Pipettes had tip resistances of 2-4 M Ω when filled with solution containing (in mM) 140 CsCl, 3 Mg-ATP, 0.1 Na-GTP, 0.1 EGTA, 10 HEPES, and 5 Tris (pH 7.1). After whole-cell access was established, series resistance and membrane capacitance were compensated. Current voltage relationships were assessed by 400-ms step pulses from -60 to +60 mV in 10-mV increments from a holding potential of -80 mV.

Dispersed coronary myocytes from lean (n = 3) and MetS (n = 3) swine were fixed and permeabilized using the cytofix/cytoperm kit (BD). Cells were blocked in

permwash containing 10mg/ml BSA and stained for smooth muscle actin (R&D) and Ca_v1.2 α_1c (Santa Cruz Biotech) or concentration matched isotype controls (R&D, Calbiochem respectively). Samples were run using Cellquest Pro software on a FACSCalibur (BD) and analyzed with flow cytometry software. Membrane expression was determined by gating against isotype control with cells that were positive for smooth muscle actin and α_1c positive staining.

Coronary arteries from lean (n = 5) and MetS (n = 5) swine were homogenized with lysis buffer and total protein collected and quantified by DC Protein Assay. Equivalent amounts of protein (40 μ g) were loaded onto 7.5% acrylamide gels and transferred. Membranes were blocked for 1 h at ambient temperature prior to 24 h incubation at 4°C with rabbit polyclonal antibodies (Santa Cruz Biotech) directed against Ca_v1.2 α_1c (1:150), β (1:200) and $\alpha_2\delta_1$ (1:200) subunits. Primary antibodies were added to blocking buffer with 0.1% Tween 20 and mouse anti-actin antibody (MP Biomedicals, 1:20,000). Blots were washed and incubated for 1 h with IRDye 800 donkey anti-rabbit/goat (1:10,000) and IRDye 700 donkey anti-mouse (1:20,000) secondary antibodies. Immunoreactivity for Ca_v1.2 channel subunits was determined by the Li-Cor Odyssey system (Li-Cor Biosciences) and expressed relative to actin (loading control).

In vivo coronary blood flow studies. Lean Ossabaw swine (n = 5) utilized for reactive hyperemia experiments were sedated as described previously (37). Blood pressure was monitored via catheters in the right femoral artery. Arterial blood samples were analyzed to maintain blood gas parameters within physiologic limits via ventilatory adjustments and/or intravenous sodium bicarbonate administration. Following a thoracotomy, the LAD was isolated to measure coronary blood flow using a perivascular flow transducer (Transonic Systems Inc.) and snare was used to occlude the LAD. Once hemodynamics were stabilized (15-30 min) and baseline measurements acquired, reactive hyperemia was assessed via 15 second coronary occlusions in the

presence/absence of diltiazem (0.3 mg/kg, iv) to inhibit Ca_v1.2 channels and/or 4-aminopyridine (0.3 mg/kg iv) to block K_v channels.

Lean and MetS Ossabaw swine were instrumented as previously reported for exercise experiments (30). Briefly, utilizing a sterile technique, a left lateral thoracotomy was performed in the fifth intercostal space. Pressure monitoring catheters (Edwards LifeSciences) were implanted in the descending thoracic aorta for blood pressure measurements and arterial blood sampling. Another catheter was placed in the coronary interventricular vein for coronary venous blood sampling and intravenous drug infusions. A perivascular flow transducer (Transonic Systems Inc.) was placed around the left anterior descending (LAD) coronary artery to measure coronary blood flow. Catheters/wires were externalized, the chest closed in layers, and appropriate post-operative pain/antibiotics administered. Following recovery from surgery, experiments were conducted in lean (n = 7) and MetS (n = 6) Ossabaw swine under resting conditions and during graded treadmill exercise (~ 2 mph and ~5 mph) before and during inhibition of Ca_v1.2 channels with nifedipine (10 µg/kg, iv). Arterial and coronary venous blood samples were collected simultaneously in heparinized syringes when hemodynamic variables were stable and analyzed in duplicate with an automatic blood gas analyzer (IL GEM Premier 3000) and CO-oximeter (682) system. Each exercise period was ~2 min in duration, and the animals were allowed to rest sufficiently between each level for hemodynamic variables to return to baseline. LAD perfusion territory was estimated as previously described by Feigl (x).

Statistical Analyses. Data are presented as mean ± SE. Statistical comparisons were made by unpaired t-test (phenotype data) or by two-way analysis of variance (ANOVA) for within group analysis (Factor A: drug treatment; Factor B: exercise level) and between group analysis (Factor A: diet with drug treatment; Factor B: exercise level) as appropriate. When significance was found with ANOVA, a Student-Newman-Keuls

multiple comparison test was performed to identify differences between groups and treatment levels. Multiple linear regression analysis was used to compare slopes of coronary blood flow plotted vs. MVO_2 . If the slopes of the regression lines were not significantly different, an analysis of covariance (ANCOVA) was used to adjust response variables for linear dependence on MVO_2 . Hyperemic volume was determined by calculating the area under the curve using Prism software (GraphPad). For all statistical comparisons, $P < 0.05$ was considered statistically significant.

Results

Table 5-1 Phenotypic characteristics of lean and metabolic syndrome Ossabaw swine.

	Lean	MetS
Body Weight (kg)	47 ± 2	72 ± 3*
Heart wt. / Body wt. (x 100)	0.37 ± 0.02	0.40 ± 0.03
Glucose (mg/dl)	75 ± 3	87 ± 5*
Insulin (μU/ml)	21 ± 4	30 ± 8
HOMA index	3.9 ± 0.8	6.2 ± 1.2
Total cholesterol (mg/dl)	85 ± 6	439 ± 74*
LDL/HDL ratio	1.6 ± 0.1	4.5 ± 0.4*
Triglycerides (mg/dl)	41 ± 5	70 ± 15

Values are mean ± SE for lean (n = 7) and MetS (n = 5) swine. * $P < 0.05$ vs lean.

Phenotype of Ossabaw swine. Phenotypic characteristics of lean and MetS swine are given in **Table 5-1**. Consistent with our recent studies (37-39; 44), we found that the excess calorie, atherogenic diet induced classic features of early MetS in Ossabaw swine. In particular, relative to their lean counterparts MetS swine exhibited significant increases in body weight, glucose, total cholesterol, and LDL/HDL ratio. Blood samples obtained from swine at the time of exercise experiment (non-fasted) revealed modest increases in triglyceride levels and insulin concentrations. Homeostatic model assessment (HOMA) index values were also 1.6-fold higher in MetS swine ($P = 0.13$).

K_V-dependent modulation of coronary Ca_V1.2 channels

Activation of Ca_V1.2 channels by KCl (120 mM) increased baseline F_{345/380} ~15% in coronary smooth muscle myocytes from lean animals ($P < 0.05$). This response was elevated ~2-fold by subsequent addition of 4AP (**Fig. 5-1A**, $P < 0.01$). Isometric tension experiments in epicardial coronary artery segments support that inhibition of K_V channels increases Ca_V1.2-mediated constriction as active tension development in response to the selective Ca_V1.2 channel agonist BayK 8644 (10 μM) was also increased ~2-fold following the administration of 4AP (**Fig. 5-1B**, $P < 0.01$). The contribution of functional coupling between coronary K_V and Ca_V1.2 channels to the control of coronary microvascular resistance is demonstrated in **Figure 5-2**. Inhibition of K_V channels in isolated coronary microvessels (pressurized diameter = 102 ± 8 μM) reduced arteriolar diameter (**Fig 5-2A**) as evidenced by a significant ~2-fold increase in arteriolar tone (**Fig. 5-2B**, $P < 0.01$). Subsequent administration of the Ca_V1.2 channel antagonist nifedipine (10 μM) reversed arteriolar constriction to 4AP, i.e. inhibition of Ca_V1.2 channels abolished reductions in coronary arteriolar diameter in response to 4AP (**Fig. 5-2B**, $P < 0.01$).

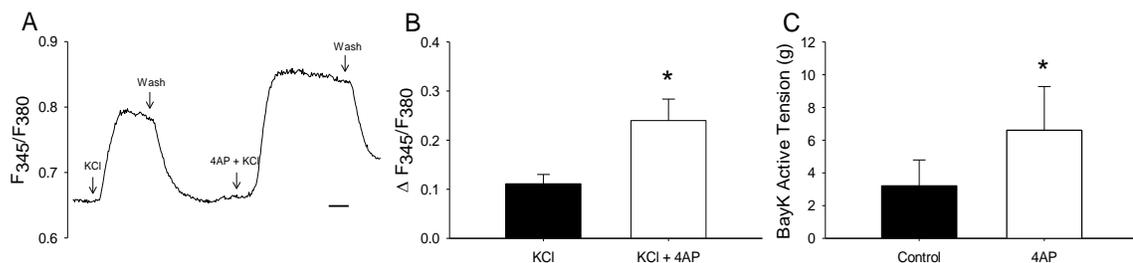


Figure 5-1 *K_V channel inhibition increases intracellular Ca²⁺ and coronary active tension.* (A) Time course tracing of Fura-2 experiments in isolated coronary myocytes showing effect of 4AP on F_{345/380} in response to KCl (scale bar = 30 sec). (B) 4AP (0.3mM) significantly increased KCl-mediated elevations in intracellular Ca²⁺ ($P < 0.05$). (C) Active tension development in isolated coronary artery segments in response to the Ca_V1.2 channel agonist BayK 8644 (10 μM) was significantly increased by 4AP ($P < 0.01$).

To examine the potential for electromechanical coupling between K_V and $Ca_V1.2$ channels *in vivo*, coronary reactive hyperemia experiments were performed before and after the inhibition of K_V channels with 4AP and/or $Ca_V1.2$ channels with diltiazem. A representative tracing demonstrating the marked reduction in coronary reactive hyperemia following 4AP administration is shown in **Figure 5-2C**. We found that diltiazem alone did not significantly alter the reactive hyperemic response relative to untreated-control hearts (tracing not shown; P value for repayment to debt ratio = 0.24). In agreement with data from isolated coronary microvessels (**Fig. 5-2B**), we importantly found that administration of diltiazem prior to the inhibition K_V channels completely prevented 4AP-mediated reductions in coronary reactive hyperemia (**Fig. 5-2C**). More specifically, diltiazem abolished 4AP-induced reductions in both the peak hyperemic response and the overall repayment of coronary flow debt (**Fig. 5-2D**).

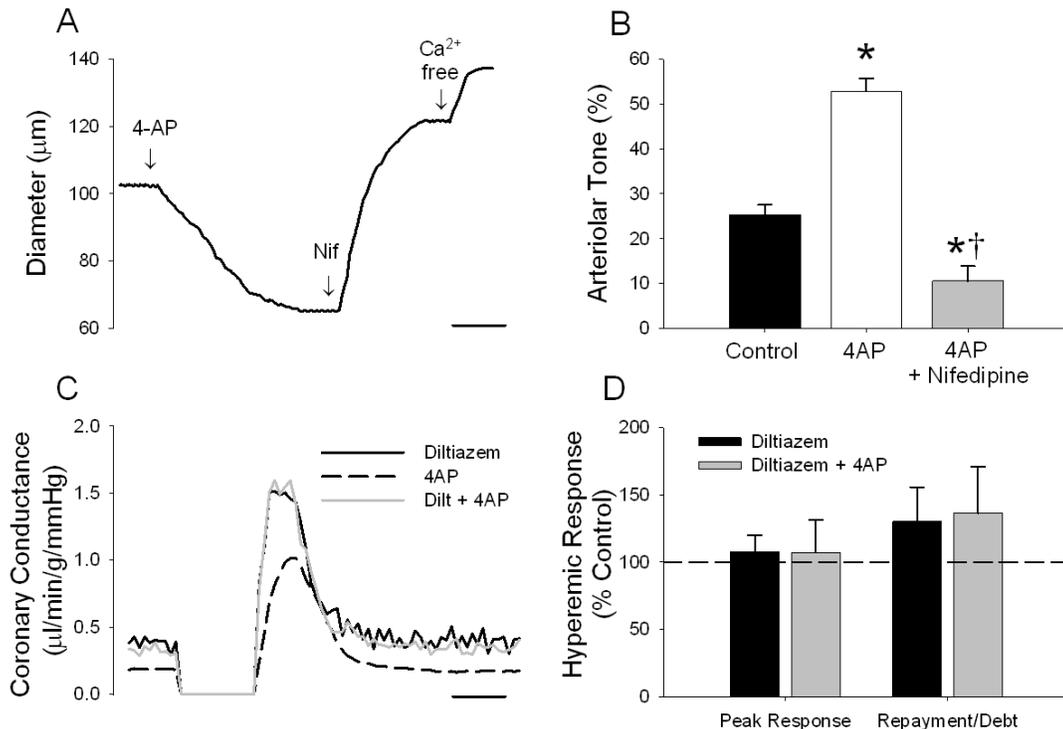


Figure 5-2. Functional coupling between coronary K_V and $Ca_V1.2$ channels. **(A)** Protocol tracing of isolated supracardial coronary microvessels during 4AP and nifedipine treatments (scale bar = 2 min). **(B)** Elevations in microvascular tone by 4AP were abolished by inhibition of $Ca_V1.2$ channels with nifedipine ($P < 0.01$). **(C)** Representative tracing from past and present reactive hyperemia experiments in open-chest anesthetized swine showing effects of additive $Ca_V1.2$ and K_V channel blockade with diltiazem and 4AP on ischemic vasodilation (scale bar = 20 sec). **(D)** Addition of 4AP to diltiazem did not alter the hyperemia peak or repayment to debt responses relative to diltiazem alone or untreated controls.

Alterations in K_V and $Ca_V1.2$ channels in Metabolic Syndrome. Additional isometric tension studies were performed on isolated coronary arteries from lean and MetS swine. Active tension development in response to KCl (20 mM) was elevated ~2-fold in arteries from MetS relative to lean swine under untreated-control conditions ($P = 0.02$). The response to 20 mM KCl in lean swine was augmented ~40% by the administration of 4AP (**Fig. 5-3A**, $P < 0.05$). Importantly, inhibition of K_V channels had no effect on active tension development to KCl in arteries from MetS swine (**Fig. 5-3A**, $P = 0.83$). In agreement with these results, we found that 4AP significantly decreased baseline coronary blood flow in conscious, instrumented lean but not MetS swine (**Fig. 5-3B**, $P = 0.05$). Furthermore, inhibition of $Ca_V1.2$ channels with nifedipine (10 $\mu\text{g}/\text{kg}$, iv) had no effect on baseline coronary flow in lean animals, but significantly increased coronary flow in MetS swine (**Fig. 5-3B**, $P < 0.02$).

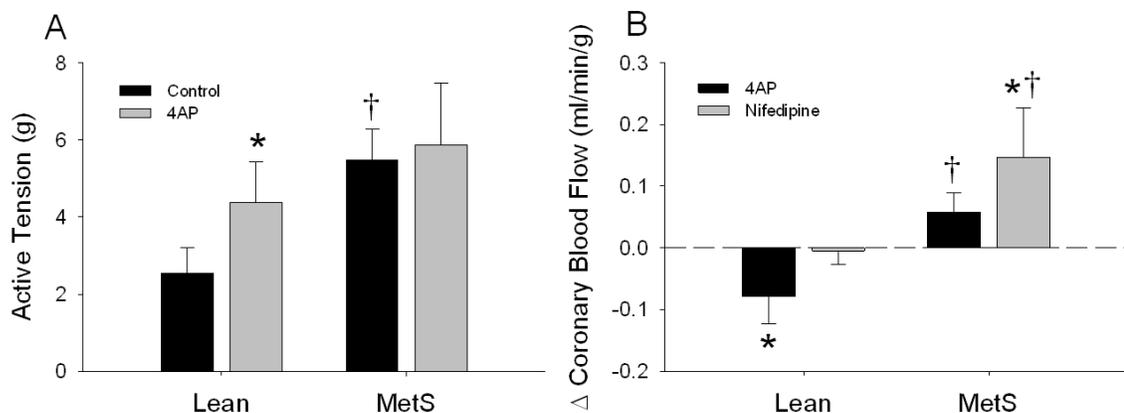


Figure 5-3 K_V and $Ca_V1.2$ coupling in lean and MetS swine. (A) Active tension development in response to KCl (20mM) is significantly elevated in arteries from MetS relative to lean swine ($P = 0.02$). 4AP increased this response in arteries from lean ($P < 0.05$) but not MetS swine ($P = 0.83$). (B) Baseline coronary blood flow is reduced by 4AP in lean swine ($P = 0.05$) while the change in coronary blood flow in response to nifedipine is markedly elevated in swine with the MetS ($P < 0.02$).

Coronary and cardiovascular response to exercise. Hemodynamic and blood gas data for lean and MetS Ossabaw swine at rest and during exercise are summarized in **Table 5-2**. Systolic, diastolic and mean aortic pressure were not significantly different in

MetS vs. lean swine under baseline/resting conditions in either treated or untreated groups. However, the systemic pressure response to exercise was elevated in MetS swine as both systolic and mean aortic pressure were significantly increased during exercise relative to their lean counterparts; a finding that occurred in the absence of differences in heart rate. Despite this increase in arterial pressure, coronary blood flow was modestly reduced ~5-15% in MetS swine and was associated with significant decreases in coronary venous PO₂ (index of myocardial tissue PO₂) at rest and during exercise (**Table 5-2**). MetS also reduced coronary conductance (flow/pressure) ~25% at the highest level of exercise. Evaluation of coronary blood flow in lean and MetS swine at a given level of MVO₂ revealed a modest reduction in the slope of the relationship between coronary blood flow and MVO₂ under untreated-control conditions (**Fig. 5-4A vs. 5-4B**). However, taking into account the effects of blood pressure revealed a significant reduction in the slope of the relationship between coronary conductance and MVO₂ in MetS relative to lean swine ($P < 0.02$, **Fig. 5-4C vs. 5-4D**); indicating a marked impairment of exercise-induced coronary vasodilation in MetS swine.

Table 5-2 Hemodynamic and blood gas variables at rest and during graded treadmill exercise in lean and metabolic syndrome Ossabaw swine with and without Nifedipine.

	Exercise		
	Rest	Level 1	Level 2
Systolic Blood Pressure (mmHg)			
Lean	116 ± 7	114 ± 3	126 ± 4
Lean + Nifedipine	109 ± 4	113 ± 4	119 ± 4
MetS	126 ± 6	136 ± 7†	151 ± 1†
MetS + Nifedipine	116 ± 4	125 ± 4†	133 ± 4*†
Diastolic Blood Pressure (mmHg)			
Lean	76 ± 5	76 ± 2	84 ± 4
Lean + Nifedipine	73 ± 4	74 ± 3	78 ± 3
MetS	88 ± 11	82 ± 4	92 ± 5
MetS + Nifedipine	76 ± 3	76 ± 3	80 ± 6
Mean Aortic Pressure (mmHg)			
Lean	98 ± 6	97 ± 3	106 ± 3
Lean + Nifedipine	91 ± 3	95 ± 3	100 ± 3
MetS	102 ± 3	109 ± 5	125 ± 10†

MetS + Nifedipine	97 ± 4	101 ± 3	107 ± 5*
Heart Rate (beats/min)			
Lean	148 ± 11	187 ± 8	201 ± 18
Lean + Nifedipine	145 ± 11	183 ± 8	205 ± 7
MetS	149 ± 6	201 ± 19	194 ± 9
MetS + Nifedipine	145 ± 5	186 ± 8	203 ± 8
Coronary Blood Flow (ml/min/g)			
Lean	1.01 ± 0.11	1.35 ± 0.11	1.64 ± 0.13
Lean + Nifedipine	1.01 ± 0.12	1.39 ± 0.14	1.60 ± 0.15
MetS	0.96 ± 0.07	1.25 ± 0.10	1.41 ± 0.10
MetS + Nifedipine	1.11 ± 0.11	1.39 ± 0.11	1.62 ± 0.15*
Coronary Conductance (μl/min/g/mmHg)			
Lean	10.3 ± 1.0	13.9 ± 0.9	15.7 ± 1.5
Lean + Nifedipine	11.3 ± 1.4	14.7 ± 1.4	16.1 ± 1.5
MetS	9.5 ± 0.9	11.6 ± 0.9	11.5 ± 1.0†
MetS + Nifedipine	11.5 ± 1.1	13.8 ± 1.1*	15.3 ± 1.4*
Myocardial O₂ Consumption (μl O₂/min/g)			
Lean	116 ± 14	176 ± 24	231 ± 24
Lean + Nifedipine	111 ± 13	163 ± 17	201 ± 26*
MetS	126 ± 14	165 ± 14	182 ± 21
MetS + Nifedipine	111 ± 13	178 ± 12	185 ± 11
Arterial pH			
Lean	7.57 ± 0.01	7.55 ± 0.01	7.56 ± 0.02
Lean + Nifedipine	7.59 ± 0.02*	7.60 ± 0.01*	7.57 ± 0.02
MetS	7.54 ± 0.02	7.52 ± 0.01	7.51 ± 0.01†
MetS + Nifedipine	7.52 ± 0.01†	7.52 ± 0.02†	7.50 ± 0.02†
Coronary Venous pH			
Lean	7.47 ± 0.01	7.49 ± 0.01	7.48 ± 0.01
Lean + Nifedipine	7.49 ± 0.01	7.51 ± 0.01	7.50 ± 0.01
MetS	7.44 ± 0.02	7.45 ± 0.01†	7.45 ± 0.01
MetS + Nifedipine	7.47 ± 0.01	7.48 ± 0.02	7.47 ± 0.01
Arterial PCO₂ (mmHg)			
Lean	31 ± 1	31 ± 2	29 ± 2
Lean + Nifedipine	29 ± 3	28 ± 2	30 ± 2
MetS	31 ± 2	30 ± 2	29 ± 2
MetS + Nifedipine	33 ± 2	31 ± 2	30 ± 2
Coronary Venous PCO₂ (mmHg)			
Lean	51 ± 1	43 ± 3	45 ± 2
Lean + Nifedipine	44 ± 2*	42 ± 2	44 ± 2
MetS	46 ± 3	44 ± 2	41 ± 3
MetS + Nifedipine	41 ± 2*	41 ± 3	40 ± 2

Arterial PO₂ (mmHg)			
Lean	97 ± 3	100 ± 3	101 ± 5
Lean + Nifedipine	102 ± 4	105 ± 4	99 ± 5
MetS	92 ± 4	91 ± 3	93 ± 3
MetS + Nifedipine	88 ± 2†	90 ± 3†	94 ± 2
Coronary Venous PO₂ (mmHg)			
Lean	17 ± 0.8	17 ± 1.1	16 ± 1.0
Lean + Nifedipine	18 ± 1.4	16 ± 1.1	16 ± 1.1
MetS	14 ± 1.4†	12 ± 1.3†	12 ± 1.1†
MetS + Nifedipine	15 ± 1.9	14 ± 1.3*	14 ± 1.2*
Coronary Venous O₂ Saturation (%)			
Lean	14 ± 2	11 ± 3	12 ± 2
Lean + Nifedipine	16 ± 2	13 ± 2	12 ± 2
MetS	14 ± 2	11 ± 1	10 ± 1
MetS + Nifedipine	15 ± 2	11 ± 2	11 ± 2
Arterial Hematocrit (%)			
Lean	34 ± 1	38 ± 1	39 ± 1
Lean + Nifedipine	33 ± 2	33 ± 2*	37 ± 1
MetS	36 ± 2	38 ± 2	37 ± 1
MetS + Nifedipine	32 ± 2	35 ± 2	33 ± 1

Values are mean ± SE for lean (n = 7) and MetS (n = 6) swine. * $P < 0.05$ vs. untreated control, same diet/condition; † $P < 0.05$ vs. lean, same treatment.

Role of Ca_v1.2 channels in coronary and cardiovascular response to exercise.

Effects of Ca_v1.2 channel inhibition with nifedipine (10 µg/kg, iv) on hemodynamic and blood gas variables at rest and during exercise are also summarized in **Table 5-2**. Administration of nifedipine significantly reduced systolic and mean aortic pressure during exercise in MetS, but not lean swine. Augmented blood pressure responses to exercise in MetS swine were also abolished by nifedipine (**Table 5-2**). Similar to untreated conditions, heart rate was unaffected by nifedipine in either group. Coronary blood flow and conductance were not significantly altered by nifedipine administration in lean swine at rest or during exercise (**Table 5-2, Fig. 5-4A and 5-4C**). In contrast, nifedipine markedly increased coronary blood flow and conductance during exercise in MetS swine (**Table 5-2, Fig. 5-4B and 5-4D**). Regression analysis demonstrated that nifedipine significantly increased the slope of the relationship between coronary blood

flow ($P = 0.03$) and conductance ($P < 0.01$) vs. MVO_2 in MetS, but not lean swine (**Fig. 5-4**). Importantly, no differences in slope were noted between MetS nifedipine treated vs. lean untreated swine, indicating that inhibition of $Ca_v1.2$ channels in MetS restored the coronary response to exercise to a similar level as that which is observed in lean swine.

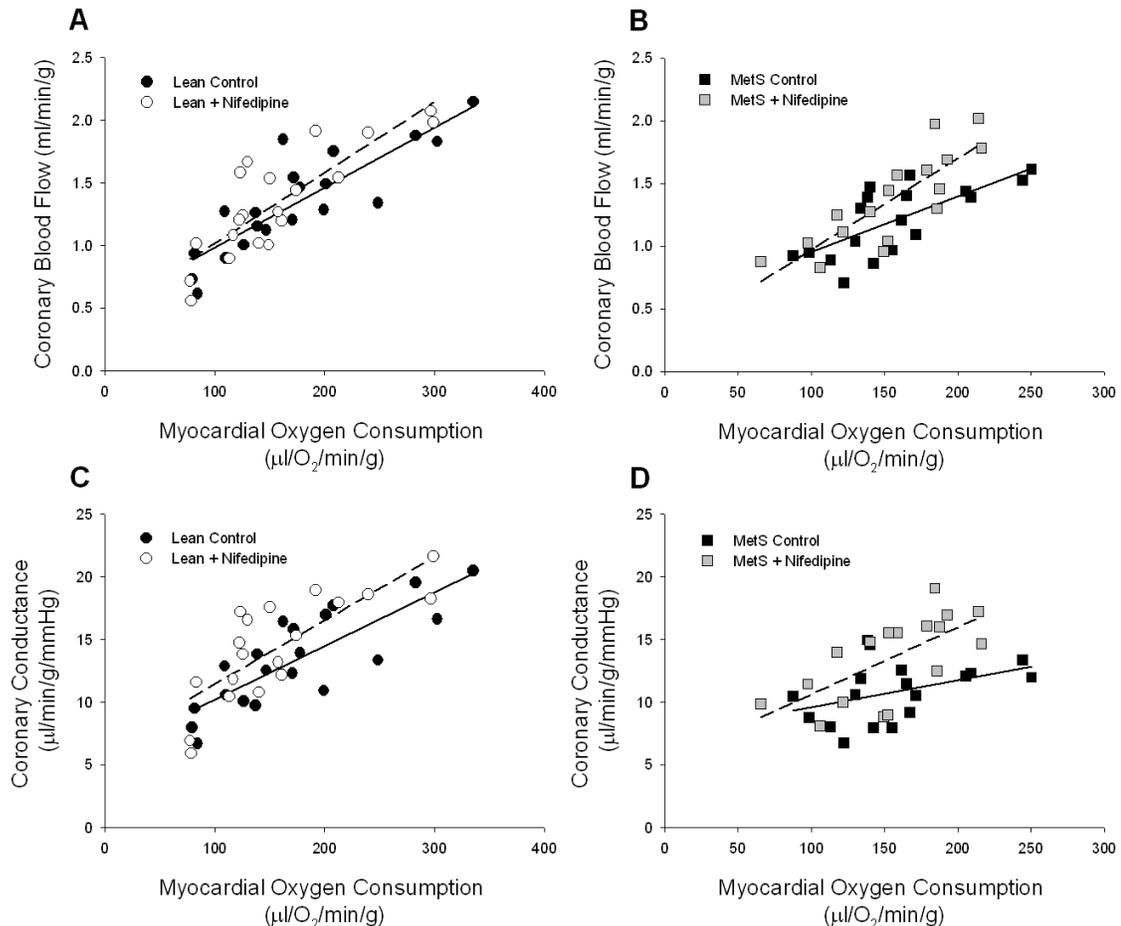


Figure 5-4 Effects of $Ca_v1.2$ channel inhibition on exercise-mediated coronary vasodilation. (A) Inhibition of $Ca_v1.2$ channels did not alter the relationship between coronary blood flow and MVO_2 in lean swine. (B) Nifedipine produced a significant upward shift in the relationship between coronary blood flow and MVO_2 in swine with MetS ($P = 0.03$). (C) No affect of nifedipine on conductance vs. MVO_2 were observed in lean swine. (D) Similar to coronary blood flow, the slope relationship between coronary conductance and MVO_2 was markedly exacerbated in MetS swine ($P < 0.02$).

Functional and molecular expression of coronary $Ca_v1.2$ channels in MetS.

Whole cell patch clamp recordings (**Fig. 5-5A**) demonstrated a significant ~35-60% increase in native coronary $Ca_v1.2$ currents at 0-30 mV potentials (**Fig. 5-5B**, $P < 0.05$) over a voltage range consistent with $Ca_v1.2$ channel activity. Electrophysiologic

recordings did not indicate any alterations in properties of channel activation/inactivation, i.e. changes in current may be related to an increase in the number of $Ca_v1.2$ channels.

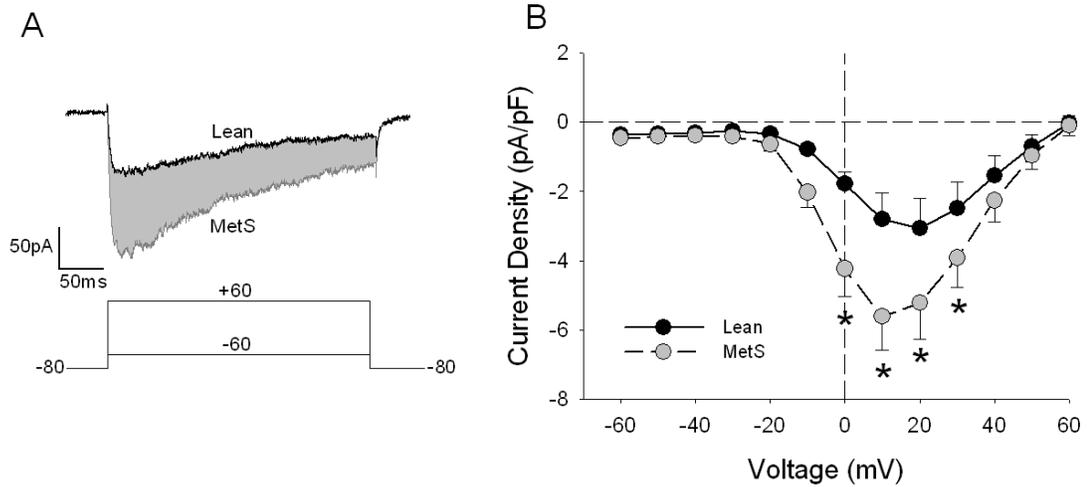


Figure 5-5 Whole-cell voltage-dependent $Ca_v1.2$ current in coronary smooth muscle of lean and MetS swine. (A) Representative current tracings from lean and MetS coronary myocytes. Voltage template is 400 ms long. (B) Group I-V data demonstrate a significant increase in inward $Ca_v1.2$ channel current at potentials greater than -10 mV. * $P < 0.01$ vs. lean, same voltage.

Accordingly, protein expression of regulatory subunits implicated in membrane targeting of the pore-forming α_1c subunit was determined. $Ca_v1.2$ α_1c channel pore protein expression was increased ~70% in MetS coronary arteries (Fig. 5-6B, $P < 0.05$). In contrast, β_1 subunit was decreased ~70% ($P < 0.01$) while $\alpha_2\delta_1$ subunit expression was unchanged ($P = 0.49$). Determination of $Ca_v1.2$ membrane expression with flow cytometry revealed a ~2-fold increase in α_1c membrane expression in MetS relative to lean swine (Fig. 7B, $P < 0.05$).

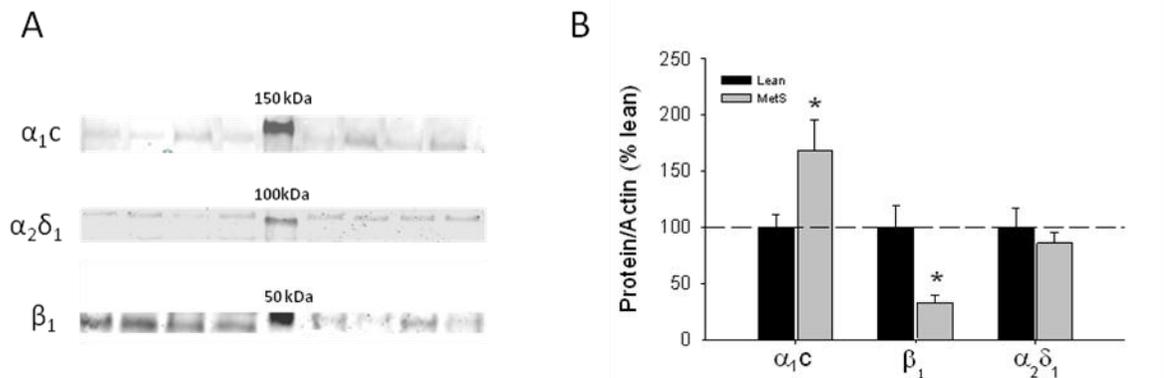


Figure 5-6. Expression of coronary $Ca_v1.2$ channel subunits. (A) Representative westerns blots of whole cell $Ca_v1.2$ subunit expression. (B) Analysis of western blots demonstrated significant increases in α_1c and reductions in β_1 subunit expression in coronary arteries from MetS vs. lean swine. Expression of $\alpha_2\delta_1$ was not affected by MetS. * $P < 0.05$ vs. lean.

Discussion

The goal of this investigation was to determine the functional significance of electromechanical coupling between coronary K_V and $Ca_V1.2$ channels and the contribution of $Ca_V1.2$ channels to the regulation of coronary blood flow at rest and during physiologic-induced increases in myocardial metabolism. In addition, we assessed the influence of the MetS on the interaction between coronary K_V and $Ca_V1.2$ channels, the role of $Ca_V1.2$ channels in metabolic control of coronary blood flow as well as $Ca_V1.2$ channel current and subunit expression. Specifically, we hypothesized that diminished K_V channel functional expression contributes to increased $Ca_V1.2$ channel activity and coronary dysfunction in MetS. This hypothesis is supported by previous investigations by our laboratory demonstrating that the contribution of K_V channels to local metabolic control of coronary blood flow and ischemic coronary vasodilation is impaired by MetS (30; 32). We have also documented that MetS results in an increase in intracellular $[Ca^{2+}]_i$, BayK activated channel current and coronary vasoconstriction (38; 175). However, whether increases in $Ca_V1.2$ channel activity in MetS is attributable to reductions in K_V channel function or the result of alterations in the $Ca_V1.2$ regulatory subunits and channel expression is unclear. Accordingly, the major findings of this study are: 1) increases in coronary smooth muscle $[Ca^{2+}]_i$ and vascular tone in response to K_V channel inhibition are attributable to activation of $Ca_V1.2$ channels; 2) attenuated K_V channel function in MetS is associated with elevated $Ca_V1.2$ channel activity; 3) enhanced $Ca_V1.2$ activity and molecular expression contributes to diminished exercise-induced coronary vasodilation in MetS. Taken together, these data demonstrate electromechanical coupling between K_V and $Ca_V1.2$ channels is critical to the regulation of coronary vasomotor tone and that increases in $Ca_V1.2$ channel activity contributes to coronary microvascular dysfunction in the setting of MetS.

Functional coupling of coronary K_V and $Ca_V1.2$ channels. Evidence to date supports a prominent role for K_V channels in the control of smooth muscle E_M and coronary blood flow (74). In addition, $Ca_V1.2$ channels represent a significant source of extracellular Ca^{2+} influx and coronary smooth muscle contraction (225; 292). Since $Ca_V1.2$ channels are activated within the range for smooth muscle E_M , we hypothesized that alterations in E_M elicited by K_V channels modulate $Ca_V1.2$ channel activity and control coronary blood flow. The potential for functional interactions between these channels is important given the ability for modest alterations in smooth muscle E_M to have large effects on $Ca_V1.2$ -mediated Ca^{2+} conductance and vascular tone. Opening of only a few $Ca_V1.2$ channels during depolarization in the presence of large transmembrane Ca^{2+} gradients can cause significant (~10-fold) increases in $[Ca^{2+}]_i$ (161). Thus, even modest fluctuations in E_M (~3 mV) are capable of producing ~2-fold changes in $[Ca^{2+}]_i$ (225; 227). Accordingly, potential voltage-dependent interactions between K_V and $Ca_V1.2$ channels may have profound consequences on the control of coronary vascular resistance and blood flow.

In agreement with the premise of coupling between K_V and $Ca_V1.2$ channels, administration of 4AP has been shown to markedly increase $[Ca^{2+}]_i$ in various cell types (72; 125; 262). Although we now demonstrate this in coronary smooth muscle (**Fig. 5-1A**), discrepancies exist as to whether increases in $[Ca^{2+}]_i$ from 4AP are the result of voltage-dependent activation of $Ca_V1.2$ channels or IP_3 -mediated mobilization of SR stores and subsequent opening of store operated calcium channels (19; 108; 205; 314). Specifically, previous studies have shown that 4AP can increase $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} in isolated neurons, astrocytes, and skeletal muscle (125). While a parallel role for 4AP-induced SR Ca^{2+} release in smooth muscle cannot be disregarded (96), it is unlikely that this mechanism contributes to coronary vasoconstriction given that SR Ca^{2+} release mainly functions as a negative feedback mechanism to contraction; i.e.

spark-induced activation of calcium-sensitive K^+ channels (156; 306). Importantly, our data demonstrate that the increase in $[Ca^{2+}]_i$ during K_V channel inhibition potentiates $Ca_V1.2$ -mediated coronary vasoconstriction in response to BayK 8644 (**Fig. 5-1B**). In addition, administration of 4AP alone is sufficient to induce marked $Ca_V1.2$ -sensitive vasoconstriction in isolated microvessels (**Fig. 5-2A**) thus supporting that increases in $[Ca^{2+}]_i$ and arteriolar tone are the direct result of K_V -dependent modulation of extracellular Ca^{2+} influx via $Ca_V1.2$ channels, i.e. electromechanical coupling.

Our data are the first to demonstrate that the influence of K_V and $Ca_V1.2$ channel interactions on the regulation of $[Ca^{2+}]_i$ and vascular tone shown *in vitro* also plays an important role in the control of coronary blood flow. Our laboratory has previously demonstrated that inhibition of K_V channels significantly impairs coronary reactive hyperemia as the repayment to debt ratio is reduced ~30% by 4AP (representative tracing **Fig. 5-3C**). Experiments performed in the present study in lean swine show that these reductions in the hyperemic response during K_V channel inhibition are abolished by subsequent blockade of $Ca_V1.2$ channels with diltiazem (**Fig. 5-3D**). These data indicate that reductions in coronary blood flow during K_V channel inhibition are attributable to the direct activation of $Ca_V1.2$ channels and support the functional importance of K_V - $Ca_V1.2$ electromechanical coupling *in vivo*.

Implications of electromechanical coupling between K_V and $Ca_V1.2$ channels shown during ischemic coronary vasodilation is particularly important given the growing body of evidence indicating that K^+ channel function is altered in various disease states (30; 37; 74). Specifically, findings from our laboratory demonstrate that the MetS reduces K_V channel current, molecular expression, and exercise-mediated coronary vasodilation (30). Based on these previous studies and current findings regarding coupling between K_V and $Ca_V1.2$ channels, we hypothesized that decreases in K_V channel function in MetS may impair the control of coronary blood flow via increases in

Ca_v1.2 channel activity. This hypothesis is supported by significant increases in coronary active tension and diminished effects of 4AP in MetS vs. lean coronary artery segments (**Fig. 5-3A**). Likewise, coronary blood flow was not reduced by 4AP and was significantly increased by nifedipine in swine with MetS; results which are opposite to lean counterparts and indicative of impaired K_v and elevated Ca_v1.2 channel function (**Fig. 5-3B**).

Effects of MetS on function and expression of coronary Ca_v1.2 channels.

Findings from the present investigation demonstrate that MetS impairs the regulation of coronary blood flow via a Ca_v1.2-dependent mechanism. These results are consistent with previous studies on individual components of MetS (i.e. diabetes, hypertension, hypercholesteremia) showing that elevations in vascular tone are associated with increases in intracellular [Ca²⁺] and calcium channel current (181; 209; 242; 249). However, the extent to which potential alterations in Ca_v1.2 channel function contributes to impaired coronary vasomotor responses to physiologic stimuli in MetS has not previously studied. In agreement with findings from Bache *et al.* (16), nifedipine did not alter coronary blood flow or conductance in lean animals at rest or during exercise, nor were these variables different from untreated controls at a given level of MVO₂ (**Fig. 5-4A, 5-4C**). Relative to coronary and cardiovascular responses to exercise in lean swine, MetS significantly increased microvascular resistance (i.e. impaired coronary dilation and elevated pressure) as evidenced by decreases in the slope relationship between coronary conductance and MVO₂ under untreated control conditions. Importantly, the dysfunction observed in swine with the MetS was ameliorated by subsequent inhibition of Ca_v1.2 channels with nifedipine as the relationship between both coronary blood flow and conductance vs. MVO₂ were restored to lean untreated levels (**Fig. 5-4B, 5-4C**).

In order to determine whether increases in $Ca_V1.2$ channel function is the result of alterations in $Ca_V1.2$ regulatory pathways (i.e. K_V channels) or intrinsic to changes within the channels themselves, we evaluated the effects of MetS on $Ca_V1.2$ channel current and subunit expression. MetS significantly increased inward $Ca_V1.2$ channel current ~35-60% without significantly affecting thresholds for activation/inactivation (**Fig. 5-5**). Accordingly, we speculated that such marked increases in $Ca_V1.2$ channel current may be the result of elevated membrane expression. Indeed, whole cell expression of the α_{1c} pore-forming subunit was increased ~70% in coronary arteries from swine with MetS (**Fig. 5-6B**). Interestingly, flow cytometry revealed that membrane expression of α_{1c} is further elevated ~2-fold in MetS coronary myocytes suggesting possible alterations in β_1 or $\alpha_2\delta_1$ -dependent membrane targeting of the channel pore (**Fig. 7B**).

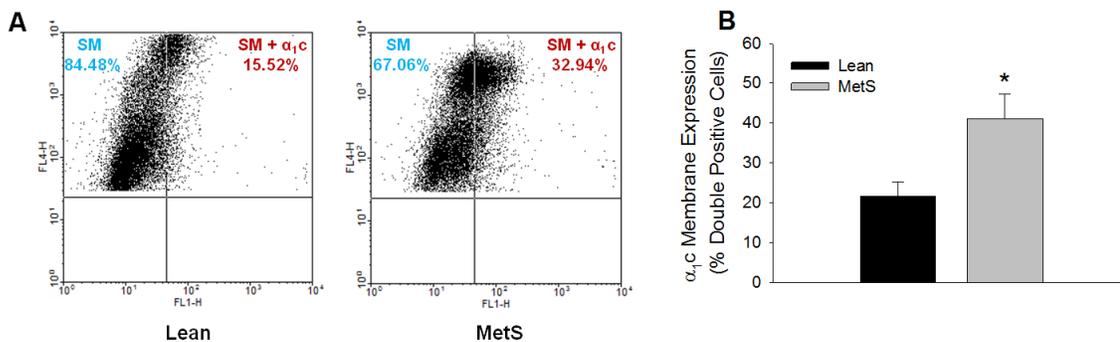


Figure 5-7 Membrane expression of coronary $Ca_V1.2$ channels. (A) Representative scatter showing double positive stained cells for α_{1c} and coronary smooth muscle actin. (B) Membrane expression of $Ca_V1.2$ α_{1c} subunit is significantly elevated in coronary smooth muscle from swine with MetS. * $P < 0.05$ vs. lean.

Although both of these subunits have been implicated in membrane targeting of the α_{1c} channel pore (18; 40; 97), our data do not demonstrate the expected increase in either subunit as $\alpha_2\delta_1$ expression was unchanged while β_1 expression was significantly depressed in MetS. While our findings for reductions in β_1 subunit are paradoxical, this may be attributable to conformational changes/splice variants in α_{1c} subunits. Specifically, previous studies have demonstrated that an ER retention signal exists in the I-II loop of α_{1c} subunits that is masked upon binding with β_1 subunits (40; 97). In

addition, β subunits have also been implicated in proteasomal degradation of $\text{Ca}_v1.2$ channels (8). However, whether our findings are the result of structural changes in the α_1c interaction domain or impaired signaling for degradation is unknown. Additional studies at the transcriptional level are likely needed to clarify this discrepancy.

Implications. Results obtained from this investigation demonstrate that electromechanical coupling between K_v and $\text{Ca}_v1.2$ channels is a critical mechanism by which coronary blood flow is regulated in health and disease. Given the prominent role for K_v channels and the marked effect diminished coronary K_v channel function has on coronary blood flow regulation, establishing the functional consequences of coupling between these channels is vital to our understanding of coronary (patho)physiology. However, our findings also demonstrate that in addition to the contribution of impaired K_v channel function to elevated $\text{Ca}_v1.2$ activity, MetS is also associated with an increase in $\text{Ca}_v1.2$ activity which likely occurs independent of differential modulation by K_v channels. In particular, our data indicate that MetS increases $\text{Ca}_v1.2$ channel current and membrane expression of the pore-forming α_1c subunit. However, the mechanism underlying this increase in $\text{Ca}_v1.2$ molecular expression is unknown and merits further investigation.

Currently, thiazide-like diuretics are the first-line therapy in most subjects with hypertension (93). However, in MetS patients, the use of thiazides has been shown to increase insulin insensitivity, triglyceride levels (327), the incidence of new-onset diabetes (153; 251) and produce overall less favorable metabolic profiles (34). In contrast to thiazides, studies have found that metabolically neutral calcium channel blockers can improve insulin sensitivity and reduce total plasma cholesterol and lipid levels in hypertensive subjects with obesity and glucose intolerance (6; 11; 94; 153). Moreover, findings from several trials indicate that these agents are equally effective at managing blood pressure (47; 130) and are superior in reducing cardiovascular

morbidity and mortality compared to HCTZ (251). This cardioprotective effect is further supported by a recent meta-analysis of ~27 trials which found that long-acting calcium channel blockers reduce the risk of all-cause mortality (61).

Taken together, these trials along with data from the present investigation suggest that administration of calcium channel blockers to MetS subjects without overt hypertension may not only attenuate coronary microvascular dysfunction via $Ca_v1.2$ inhibition, but could possibly improve metabolic profiles and cardiovascular outcomes. In individuals with MetS and hypertension, adjunctive therapy with ACE inhibitors may confer an additional and more favorable combined therapeutic option for managing blood pressure compared to thiazide-like diuretics (327). However, to more clearly define the contribution of increased $Ca_v1.2$ channel activity in MetS to adverse cardiovascular outcomes, a multi-center randomized control trial comparing the efficacy of calcium channel blockers (and possibly ACE inhibitors/ARBs) in MetS vs. MetS hypertensive subjects is required and will likely provide improve our ability to treat this growing patient population.

ACKNOWLEDGEMENTS

This work was supported by AHA grants 10PRE4230035 (ZCB) and NIH grants HL092245 (JDT) and HL062552 (MS).

Chapter 6: Discussion

Major Findings of Investigation

Extensive research into coronary physiology has greatly improved our understanding of how coronary blood flow is regulated. However, despite our best efforts, many key questions remain unanswered. The focus of this investigation was centered on these critical components of coronary physiology that continue to elude us. In particular, no study to date has been able to delineate the primary mechanisms responsible for pressure-flow autoregulation or ischemic and exercise-mediated coronary vasodilation. Given that coronary microvascular dysfunction is a significant contributor to cardiovascular disease, our ability to provide targeted therapies for individual patient populations is absolutely contingent on level of understanding we have for these central components of coronary physiology.

The prevalence of obesity and MetS in western society is growing at an alarming rate and the management of cardiovascular disease in these patients continues to be a challenge for clinicians and healthcare systems. Evidence to date indicates that MetS has profound deleterious effects on coronary microvascular function that precedes the development of overt cardiovascular disease and contributes to the increased incidence of morbidity and mortality in patients with the MetS. Findings from our laboratory and others have identified that coronary dysfunction in MetS is associated with alterations in important vasoregulatory pathways. Critical to note is that these pathways elicit their effect via modulation of end-effector ion channels, namely K^+ and Ca^{2+} channels. However, despite growing evidence that cardiovascular disease alters ion channel function, the physiologic role for more prominent coronary ion channels such as voltage-dependent K^+ and Ca^{2+} has not been characterized.

Accordingly, the overall goal of this investigation was to: 1) delineate the functional role for coronary K_V and $Ca_V1.2$ channels in the coronary response to

upstream converging metabolites (i.e. adenosine, peroxide), cardiac ischemia, exercise, and pressure-flow autoregulation; 2) determine potential electromechanical coupling between these channels; and 3) examine the contribution of K_V and $Ca_v1.2$ channels to coronary microvascular dysfunction in MetS. In order to address these key questions in coronary physiology, the following specific aims were investigated:

Aim 1 was designed to elucidate the contribution of adenosine A_{2A} and A_{2B} receptors to coronary reactive hyperemia and downstream K^+ channels involved.

The major findings of this study are: 1) A_{2A} receptors contribute to coronary vasodilation in response to exogenous adenosine administration and cardiac ischemia; 2) A_{2A} receptor-induced coronary vasodilation is mediated via signaling pathways that converge on K_V and K_{ATP} channels; 3) A_{2A} and A_{2B} receptors do not contribute to the regulation of coronary blood flow under baseline-resting conditions; and 4) A_{2B} receptors contribute to coronary vasodilation in response to exogenous adenosine, but are not required for dilation in response to cardiac ischemia.

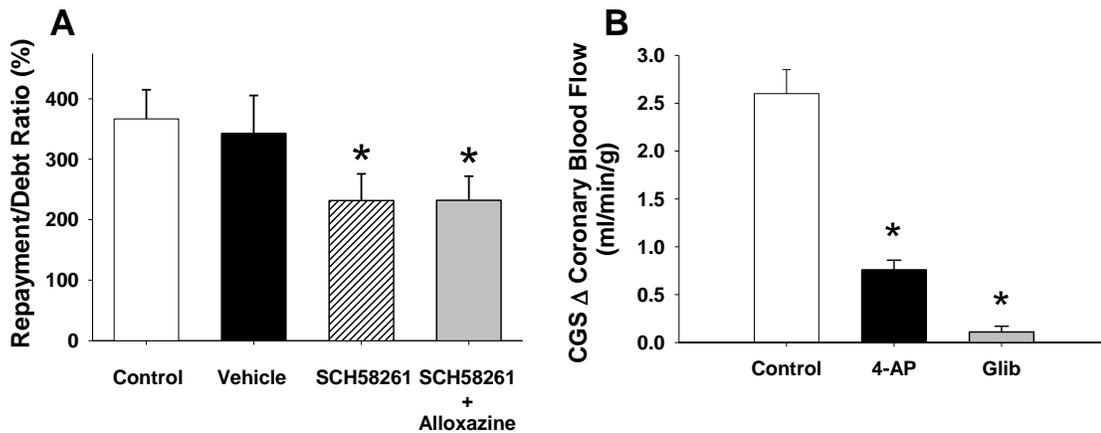


Figure 6-1 Role of adenosine receptors in ischemic coronary vasodilation and K^+ channel activation. (A) Inhibition of A_{2A} receptors significantly attenuates coronary reactive hyperemia while A_{2B} blockade has no effect. (B) Selective A_{2A} activation causes significant K_V and K_{ATP} -dependent coronary vasodilation. * $P < 0.05$

The role of adenosine in the coronary circulation has been extensively scrutinized with most reports failing to support a vasoactive function for endogenous adenosine (27; 73; 297). However, few studies have examined the contribution of

individual adenosine receptor subtypes to the regulation of coronary blood flow, particularly during cardiac ischemia. Thus, findings from investigating aim 1 are novel and demonstrate that in contrast to earlier studies, adenosine A_{2A} receptors significantly contribute to coronary vasodilation in response to exogenous adenosine administration and cardiac ischemia. This conclusion is supported by the ~30% reduction in hyperemia repayment to debt during inhibition of A_{2A} receptors. Subsequent additive blockade of A_{2B} receptors did not further reduce the hyperemic response or dilation to exogenous adenosine indicating that A_{2A} is the predominant adenosine receptor involved in adenosine-mediated coronary vasodilation and reactive hyperemia (**Fig. 6-1A**). We have also identified that A_{2A} -induced coronary vasodilation *in vivo* is mediated via activation of K_V and K_{ATP} channels (**Fig. 6-1B**). Moreover, combined blockade of K_V and K_{ATP} channels abolished coronary reactive hyperemia; a result that has not been previously achieved and greatly extends the critical nature of these ion channels in coronary physiology.

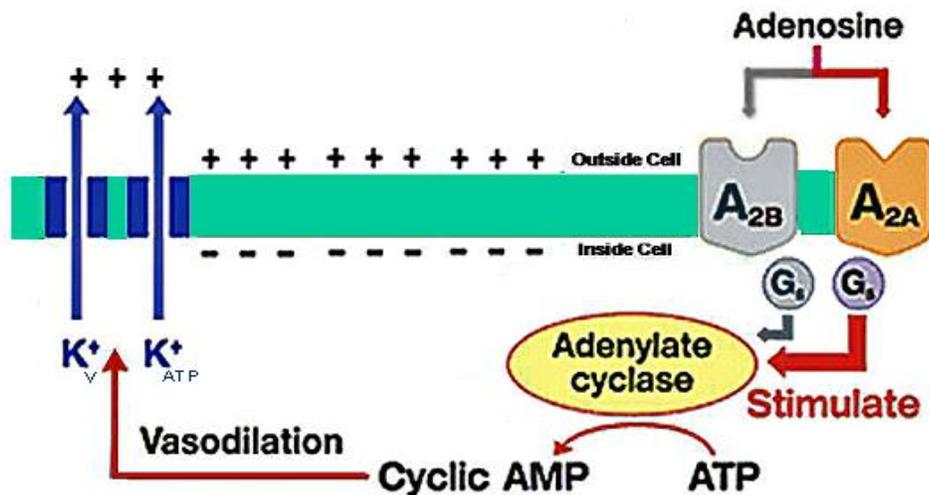


Figure 6-2 Mechanism of A_{2A} -induced coronary vasodilation. Stimulation of A_{2A} receptors by adenosine leads to coronary vasodilation via subsequent cAMP-dependent activation of K_V and K_{ATP} channels.

Aim 2 was designed to examine the contribution of K_V and $Ca_v1.2$ channels to coronary pressure-flow autoregulation *in vivo*. The major findings from investigating aim 2 are: 1) K_V channels exert a tonic contribution to the control of coronary microvascular resistance over wide range of CPPs (60-140 mmHg); 2) K_V channels and vasodilatory pathways known to converge on them (i.e. adenosine, NO, H_2O_2) are not required for coronary responses to changes in perfusion pressure; and 3) progressive activation of coronary $Ca_v1.2$ channels is essential for maintaining blood flow constant with alterations in perfusion pressure.

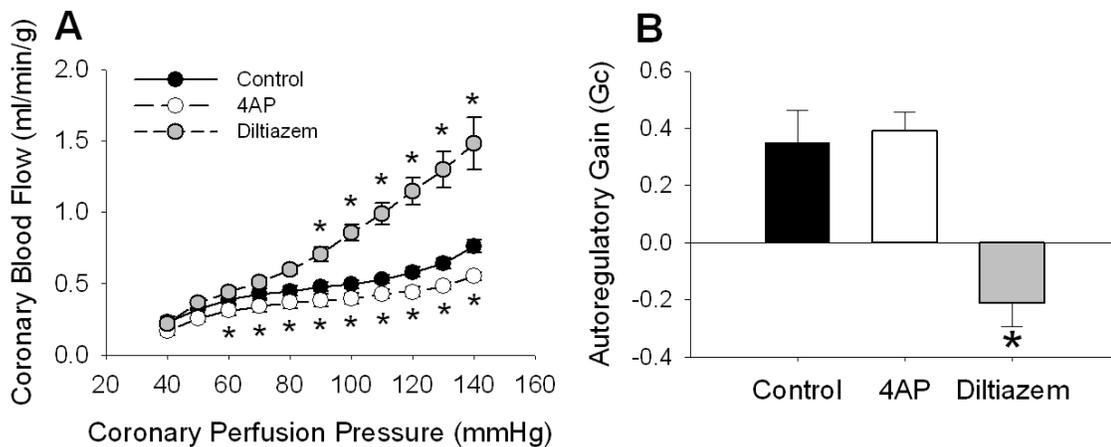


Figure 6-3 Role of K_V and $Ca_v1.2$ channels in coronary pressure-flow autoregulation. (A) Inhibition of K_V and $Ca_v1.2$ channels results in tonic reductions and progressive increases in coronary blood flow in response to increases in perfusion pressure. (B) Coronary pressure-flow autoregulation is abolished by $Ca_v1.2$ channel inhibition while blockade of K_V channels has no effect.

The importance of coronary pressure-flow autoregulation has resulted in decades of investigations attempting to delineate the underlying mechanisms responsible for this phenomenon. However, the possible contribution of K_V and $Ca_v1.2$ channels has not been determined. Consistent with earlier investigations by our laboratory (73; 74), results from investigating aim 2 supports that K_V channels significantly contribute to the control of coronary vascular resistance. In general, we found that inhibition of K_V channels causes a tonic reduction in coronary blood flow over a wide range of pressures (**Fig. 6-3A**). Such marked flow reductions diminished cardiac contractile function while preserving the balance between flow and metabolism, i.e. resulted in myocardial

hibernation. However, despite significant reductions in coronary blood flow at pressures > 50 mmHg, the sensitivity of the coronary circulation to changes in perfusion pressure (autoregulatory gain) was unaffected (**Fig. 6-3B**). Thus, although our data extend the importance of K_V channels in regulating coronary microvascular resistance, we failed to find a prominent role for K_V channels in pressure-flow autoregulation.

To date, no study has been able to identify a primary mechanism responsible for pressure-flow autoregulation. However, data from this study are the first to demonstrate that inhibition of a particular target, $Ca_v1.2$ channels, essentially abolishes the ability of the coronary circulation to maintain blood flow constant with alterations in perfusion pressure. In particular, progressive increases in coronary blood flow with elevations in perfusion pressure in the presence of diltiazem was independent of significant alterations in cardiac metabolism or function, i.e. reflected pressure-dependent changes in coronary blood flow. Most importantly, however, is that Gc was reduced to -0.21 ± 0.08 over the classic autoregulatory range of 60-120 mmHg (**Fig. 6-3B**). These hallmark findings are the first to isolate a single factor responsible for coronary pressure-flow autoregulation *in vivo*.

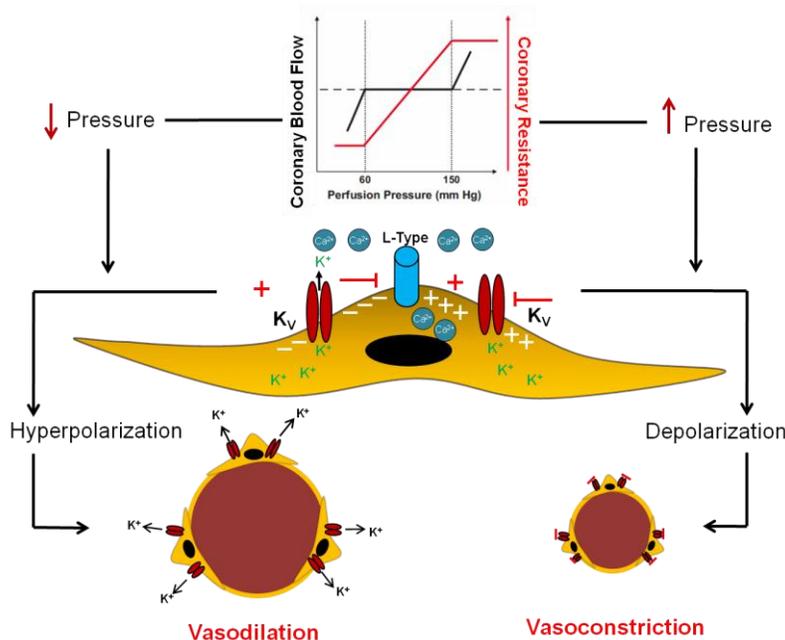


Figure 6-4 Effects of alterations in coronary perfusion pressure on K_V and $Ca_v1.2$ channel activation. Elevations in pressure progressively activate $Ca_v1.2$ channels to increase coronary resistance and maintain blood flow constant. Tonic activation of K_V channels may serve to limit $Ca_v1.2$ -dependent constriction in response to increases in perfusion pressure.

Aim 3 was designed to determine the role for K_V channels in metabolic control of coronary blood flow and to test the hypothesis that decreases in K_V channel function and/or expression significantly attenuate myocardial oxygen supply-demand balance in the MetS. The novel findings of this study are: 1) inhibition of K_V channels increases blood pressure at rest and during exercise in lean, but not MetS swine; 2) K_V channels contribute to the regulation of coronary blood flow at rest and during increases in MVO_2 in lean, but not MetS swine; 3) induction of MetS significantly decreases K_V channel current in coronary artery smooth muscle cells; and 4) expression of K_V 1.5 channels is diminished in the coronary circulation of MetS swine.

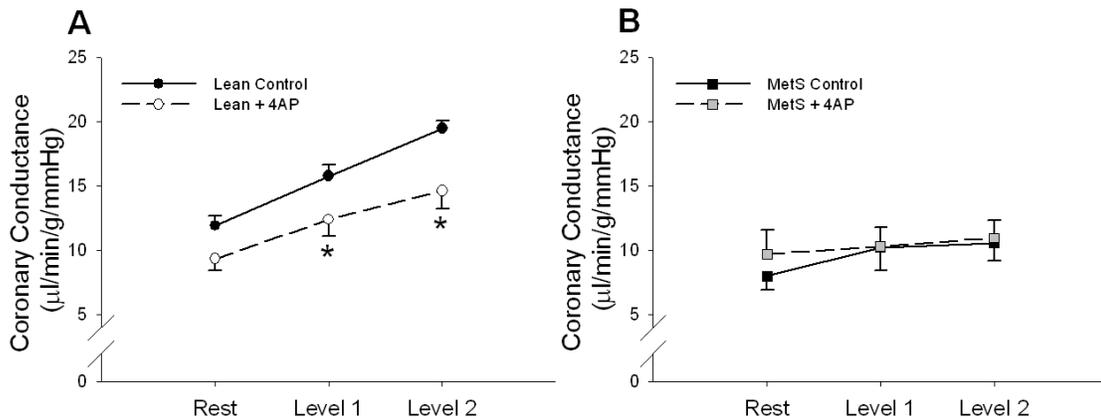


Figure 6-5 Diminished K_V channel function impairs exercise-induced coronary vasodilation in MetS. 4AP significantly reduces coronary conductance in lean (A) but not MetS swine (B).

Findings from this investigation support that vasodilatory factors converging on K_V channels play a critical role in the control of systemic vascular resistance and coronary blood flow at rest and during exercise in conscious lean swine (Fig. 6-5A). In addition, our data demonstrate that diminished functional expression of K_V channels significantly contributes to coronary microvascular dysfunction (Fig. 6-5B) and the imbalance between myocardial oxygen supply-demand observed in the setting of the MetS (31). Our findings also attribute this impairment to decreases in K_V channel current and expression. Thus, results obtained from investigating aim 3 greatly improve our

understanding of exercise-mediated coronary vasodilation and microvascular dysfunction in MetS.

Aim 4 was designed to delineate the relationship between coronary K_V and $Ca_V1.2$ channels and to evaluate the contribution of $Ca_V1.2$ channels to coronary microvascular dysfunction in MetS. The major findings of this study are: 1) increases in coronary smooth muscle $[Ca^{2+}]_i$ and vascular tone in response to K_V channel inhibition are attributable to activation of $Ca_V1.2$ channels; 2) attenuated K_V channel function in MetS is associated with elevated $Ca_V1.2$ channel activity; and 3) enhanced $Ca_V1.2$ functional and molecular expression in MetS impairs exercise-induced coronary vasodilation.

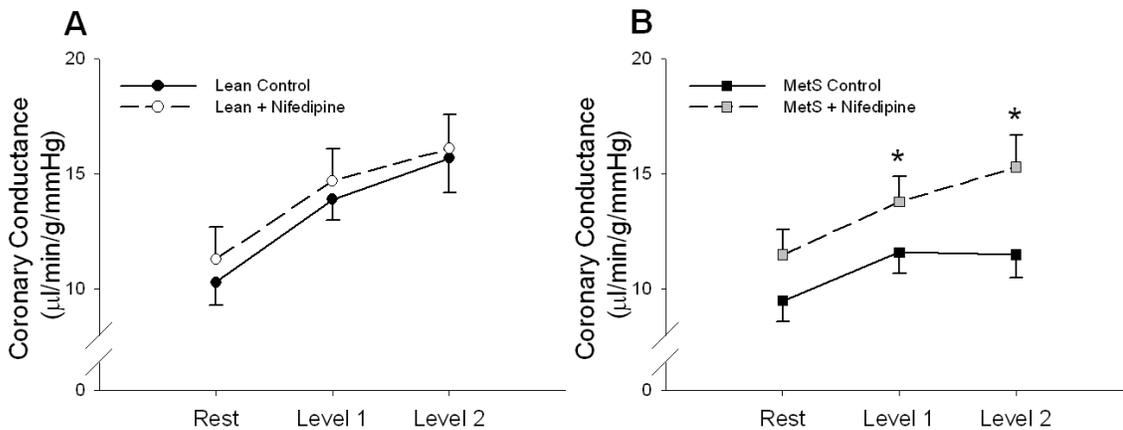


Figure 6-6 Elevated $Ca_V1.2$ channel function impairs exercise-induced coronary vasodilation in MetS. Inhibition of $Ca_V1.2$ channels significantly increases coronary conductance in MetS (B) but not lean swine (A).

Results obtained from this investigation demonstrate that K_V - $Ca_V1.2$ electromechanical coupling is a critical mechanism by which coronary blood flow is regulated. Our data indicate that inhibition of K_V channels increases $[Ca^{2+}]_i$ via voltage-dependent activation of $Ca_V1.2$ channels leading to subsequent reductions in arteriolar diameter and coronary blood flow. Given the prominent role for K_V channels and the marked effect diminished coronary K_V channel function has on coronary blood flow regulation, establishing the functional consequence of coupling between these channels

is vital to our understanding of coronary dysfunction in MetS. Notwithstanding impaired K_V channel function, our data indicate that MetS also increases $Ca_V1.2$ channel current and α_1c membrane expression. Taken together, these data demonstrate that K_V - $Ca_V1.2$ electromechanical coupling plays a significant role in the regulation of coronary vasomotor tone and that increases in $Ca_V1.2$ channel activity contributes to coronary microvascular dysfunction in the setting of MetS (**Fig. 6-6**).

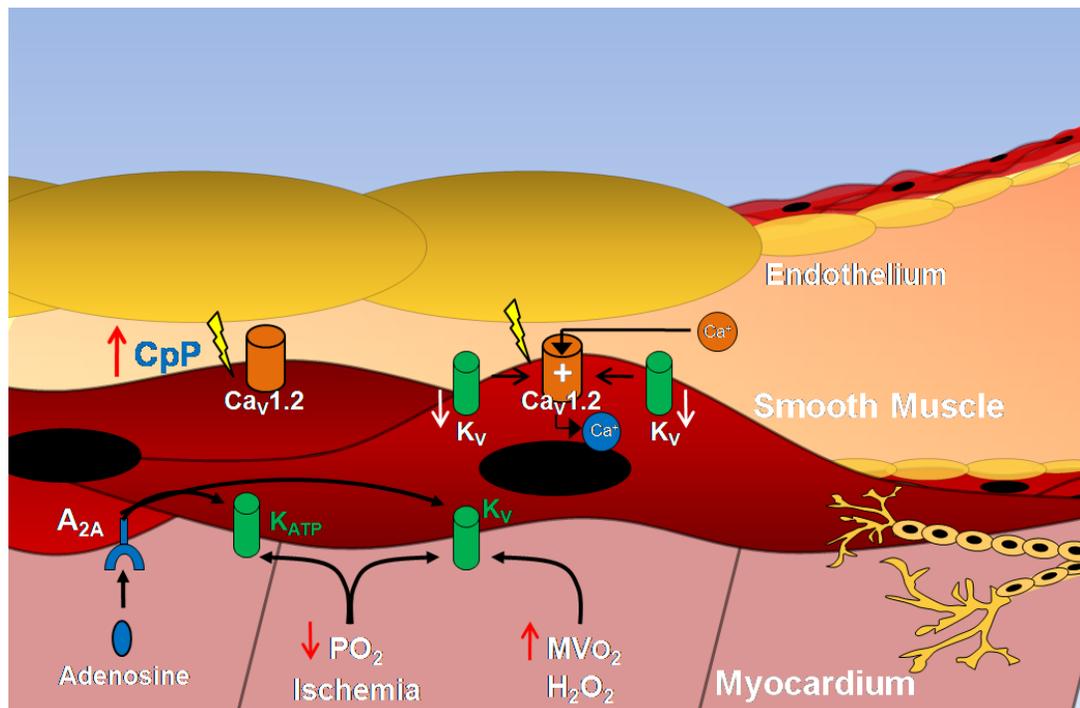


Figure 6-7 Schematic diagram illustrating primary investigative findings.

Implications

In addition to extending our physiologic understanding of coronary physiology, overall findings from this investigation also have significant clinical implications. For example, determining the mechanism for A_{2A} -induced coronary vasodilation is of particular importance given previously documented alterations in A_2 receptor expression (25) along with present findings for impaired K_V channel function in MetS (**Fig. 6-7**). Based on these data and the clinical applications for adenosine (i.e. supraventricular tachycardia, stress testing), we propose that the normal clinical indications may prove to

be ineffective in MetS patients or other populations with altered A_2 receptor and/or K_V channel function.

Although our findings do not support an active role for K_V channels in coronary pressure-flow autoregulation, these findings are of interest given the emphasis on a metabolic component of autoregulation. Specifically, our data indicate that these channels, and the vasodilatory pathways known to converge on them (adenosine, NO, H_2O_2), are not required for coronary responses to changes in perfusion pressure within the autoregulatory range. Additionally, our findings do support that K_V channels serve as a negative-feedback mechanism that limits myogenic constriction, particularly at higher perfusion pressures. Even though a negative finding for K_V channels in coronary autoregulation lacks clinical implications, our results shift the focus from previously emphasized metabolic to myogenic components of pressure-flow autoregulation, particularly when taking into account the role for $Ca_V1.2$ channels.

In contrast to K_V , $Ca_V1.2$ channels do play a prominent role in coronary pressure-flow autoregulation (**Fig. 6-7**). Although the exact mechanisms by which these channels are modulated during alterations in perfusion pressure are unknown, the preponderance of evidence suggests that these channels are end-effectors to myogenic constriction thus further supporting this component of pressure-flow autoregulation. In addition, $Ca_V1.2$ channel blockers are often used clinically in the management of blood pressure. Although our findings indicate that administration of calcium channel blockers would abolish coronary autoregulatory capacity, they would still provide a significant benefit to hypertensive patients given that they function to increase coronary blood flow, reduce left ventricular afterload, and improve the balance between myocardial oxygen delivery and metabolism.

Perhaps the greatest implication of our investigation surrounds the findings for K_V and $Ca_V1.2$ electromechanical coupling and their associated alterations in MetS (**Fig. 6-**

7). Several components of MetS have been shown to impair K_V channel function, namely hypercholesterolemia, hyperglycemia, and elevations in endothelin and reactive oxygen species (5; 107; 132; 133; 145). Thus, we propose that targeting these pathways may attenuate coronary dysfunction through direct effects on K_V channels and indirect reductions in $Ca_V1.2$ channel activity via electromechanical coupling. However, based on our findings, a more targeted approach may be obtained through inhibition of $Ca_V1.2$ channels.

Several concerns have been expressed regarding the indication for calcium channel blockers in blood pressure management (194; 250). Currently, thiazide-like diuretics are the first-line therapy in most subjects with hypertension (93). However, in MetS patients, the use of thiazides and/or β -blockers has been shown to increase insulin insensitivity, triglyceride levels (327), the incidence of new-onset diabetes (153; 251) and produce overall less favorable metabolic profiles (34). Despite these findings, many clinicians still use low-dose thiazides in combination with β -blockers or ACE inhibitors to control blood pressure in obese diabetic patients.

Less frequently administered in MetS patients are calcium channel blockers which have been shown to be as equally effective at managing blood pressure (47; 130). In contrast to thiazide-like diuretics, calcium channel blockers are metabolically neutral and have been shown to reduce insulin insensitivity (as do ACE inhibitors), total plasma cholesterol and lipids in hypertensive subjects with obesity and glucose intolerance (6; 11; 94; 153). Moreover, a recent meta-analysis of ~27 trials suggests that these agents reduce the risk of all-cause mortality (61) to various conditions which supports cardioprotective effects demonstrated by the hypertension optimal treatment trial in diabetic hypertensive patients (327). Further, the combination of ACE inhibitors (which confer additional renal and vascular protection (327)) with calcium channel blockers has been shown to be more advantageous than β -blockers/diuretics in obese hypertensive

patients (266) and is superior in reducing cardiovascular morbidity and mortality compared to hydrochlorothiazide (251).

Based on these adverse findings for thiazide-like diuretics and results from our current investigation, we advocate that calcium channel blockers be indicated as a first-line therapy for patients with MetS as $Ca_v1.2$ inhibition in these patients may not only attenuate coronary microvascular dysfunction, but will likely improve metabolic profiles and cardiovascular outcomes. In individuals with MetS and overt hypertension, adjunctive therapy with angiotensin-converting enzyme inhibitors may provide an additional and more favorable therapeutic option for blood pressure management.

Future Directions

Several issues remain unresolved by this investigation. While data from this study support a critical role for $Ca_v1.2$ channels in pressure-flow autoregulation, the mechanism by which $Ca_v1.2$ channels are activated (i.e. myogenic vs. metabolic) requires further investigation. Although $Ca_v1.2$ channel activation likely represents a myogenic element of pressure-flow autoregulation given that these channels can be directly activated by pressure/stretch-induced membrane deformation, there still remains a diltiazem and nifedipine insensitive component to myogenic constriction (68; 69). Data suggests that such residual myogenic activity may be the result of mechanosensitive nonselective cation channels (67). Thus, other pathways have been implicated. Narayanan et al. showed that IP_3 and DAG are produced in increasing proportions to elevations in intraluminal pressure. Such factors are capable of modulating $Ca_v1.2$ channels via subsequent activation of PKC (214) in addition to mobilization of intracellular stores through IP_3 receptors (68; 142). Therefore, future studies should address the role of non-selective cation channels and metabolically activated intracellular messengers in coronary pressure-flow autoregulation. More importantly,

however, is that no study to date has determined the effects of disease states such as MetS on coronary autoregulation. In addition, the patho-physiologic contribution of increases in Ca_v1.2 channel functional expression to coronary pressure-flow responses remains to be determined.

Additional findings from studies in MetS swine demonstrate a paradoxical decrease in Ca_v1.2 β₁ expression with elevations in pore-forming α_{1c} membrane expression. The preponderance of evidence suggests that Ca_v1.2 β₁ subunits are responsible for trafficking of α_{1c} from the ER to the membrane (97). Thus, we would predict that β₁ subunit expression would be increased. Interestingly, the degree of change between α_{1c} and β₁ subunit expression is proportional (~70%). Based on these observations, it is interesting to speculate that such reductions in β₁ expression may be the result of a feedback mechanism. Specifically, it has been shown that an ER retention signal exists in the I-II loop of α_{1c} subunits that is masked upon binding with β₁ subunits (40; 97). We hypothesize that a potential splice variant in this region may alter the retention signal thus resulting in constitutive α_{1c} membrane targeting. Accordingly, reflexive decreases in β₁ expression may occur to compensate for uninhibited α_{1c} membrane expression. Alternatively, β₁ subunits have also been implicated in proteasomal degradation of Ca_v1.2 channels (8). However, in order to determine the role for these potential mechanisms during increased α_{1c} membrane expression, further analysis of splice variants in α_{1c} and β₁ subunits is required.

Notwithstanding the contribution of altered K_v and Ca_v1.2 channel function to coronary microvascular dysfunction in MetS, the upstream mechanisms by which these changes in channel expression occur has not been determined. Recent findings indicate that MetS is associated with an upregulation of RAAS signaling pathways (31; 308; 329). In addition, increases in circulating levels of angiotensin II and AT₁ receptor expression have been shown to modulate ion channel function (26). Thus, these findings provide a

strong rationale for studies in patients and instrumented MetS swine involving chronic administration of AT_1 and/or mineralocorticoid receptor antagonists. Further, to more clearly define the contribution of increased $Ca_v1.2$ channel activity in MetS to adverse cardiovascular outcomes, a multi-center randomized control trial comparing the efficacy of calcium channel blockers (and possibly ACE inhibitors/ARBs) in MetS vs. MetS hypertensive subjects is needed.

Concluding Remarks

In summary, investigating the aims of this study has concluded in several key findings that greatly advance our understanding of how coronary blood flow is regulated and the mechanisms which contribute to coronary microvascular dysfunction in MetS. Specifically, we have established that adenosine A_{2A} receptors play a significant role in ischemic coronary vasodilation via subsequent activation of coronary K_V and K_{ATP} channels. Moreover, inhibition of these critical end-effectors essentially abolishes the hyperemic response. Although a similar critical finding for K_V channels in pressure-flow autoregulation was not obtained, our findings do identify that $Ca_v1.2$ channel activation may be the single most important contributor to coronary autoregulation. In addition, we have established that electromechanical coupling between K_V and $Ca_v1.2$ channels is a vital mechanism by which coronary blood flow is regulated. The interaction between these channels is of particular importance in MetS whereby reductions in K_V channel activity and expression is associated with elevated $Ca_v1.2$ functional and molecular expression. Importantly, evidence obtained from this investigation demonstrates that K_V and $Ca_v1.2$ channel dysfunction significantly impairs the balance between coronary blood flow and myocardial metabolism in MetS.

The epidemic of MetS in western society is a crisis with fatal and extensive financial consequences. Despite coronary dysfunction being a central contributor to

increased mortality in these patients, many of the mechanisms underlying impaired regulation of coronary blood flow remain poorly understood. Although the focus on MetS is growing, much of what we know today and our therapeutic approach stems from investigations on the individual components which comprise MetS. Thus, given the complexity of the multifaceted MetS, our ability to integrate proposed pathological components into an effective and targeted therapy has been limited. Hence, much work remains to fully understand the etiology of MetS in hopes of abrogating adverse cardiovascular outcomes in this patient population.

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Curriculum Vitae

Zachary C. Berwick

EDUCATION

2008	B.S./A.A.	Biology/Mathematics Indiana University New Albany, Indiana
2008-2012	Ph.D.	Cellular and Integrative Physiology Indiana University Indianapolis, Indiana

RESEARCH EXPERIENCE

2006 - 2008	Research Assistant	Indiana University Department of Chemistry New Albany, Indiana Mentor: Elaine Haub, Ph.D.
2009 - 2012	Research Assistant	IU School of Medicine Cardiovascular Laboratory Indianapolis, Indiana Mentor: Johnathan D. Tune, Ph.D.

FUNDING AWARDS

2010	T32 National Institutes of Health, Indiana University Diabetes and Obesity Training Program. <i>Relinquished for AHA pre-doc award.</i>
2010	Pre-Doctoral Fellowship Award, American Heart Association, <i>Ca_v1.2 channel dysfunction in metabolic syndrome.</i>

PEER REVIEWED PUBLICATIONS

1. Borbouse L, Dick GM, Payne GA, **Berwick ZC**, Neeb ZP, Alloosh M, Bratz IN, Sturek M, Tune JD. Metabolic syndrome reduces the contribution of K⁺ channels to ischemic coronary vasodilation. *Am J Physiol Heart Circ Physiol*. 2010 Apr; 298(4):H1182-9.
2. **Berwick ZC**, Payne GA, Lynch B, Dick GM, Sturek M, Tune JD. Contribution of adenosine A_{2A} and A_{2B} receptors to ischemic coronary dilation: role of K_v and K_{ATP} channels. *Microcirculation*. 2010 Nov;17(8):600-7.
3. Kurian MM, **Berwick ZC**, Tune JD. Contribution of IKCa channels to the control of coronary blood flow. *Exp Biol Med* (Maywood). 2011 May 1;236(5):621-7.

4. **Berwick ZC**, Dick GM, Tune JD. Heart of the Matter: Coronary dysfunction in metabolic syndrome. *J Mol Cell Cardiol.* 2012 Apr;52(4):848-56.
5. **Berwick ZC**, Dick GM, Moberly SP, Kohr MC, Sturek M, Tune JD. Contribution of voltage-dependent K⁺ channels to metabolic control of coronary blood flow. *J Mol Cell Cardiol.* 2012 Apr;52(4):912-9.
6. Chakraborty S, **Berwick ZC**, Bartlett PJ, Kumar S, Thomas AP, Sturek MS, Tune JD, Obukhov AG. Bromoenol Lactone inhibits Ca_v1.2 and TRP channels. *J Pharmacol Exp Ther.* 2011 Nov;339(2):329-40.
7. Bender S, **Berwick ZC**, Laughlin M, and Tune JD. Functional contribution of P2Y1 receptors to the control of coronary blood flow. *J Appl Physiol.* 2011 Dec;111(6):1744-50.
8. Moberly S, **Berwick ZC**, Kohr M, Svendsen M, Mather K, and Tune JD. Intracoronary glucagon-like peptide 1 preferentially augments glucose uptake in ischemic myocardium independent of changes in coronary flow. *Exp Biol Med (Maywood).* *Exp Biol Med.* 2012 Mar 1;237(3):334-42.
9. Svendsen M, Prinzen F, Das MK, **Berwick ZC**, Rybka M, Tune JD, Combs W, Barbari EJ, Kassab GS. Left Ventricular Lateral Wall Pacing Improves Pump Function Only with Adequate Myocardial Perfusion. *In press.*
10. **Berwick ZC**, Moberly SP, Kohr MC, Kurian M, Morrical E, and Tune JD. Contribution of voltage-dependent K⁺ and Ca channels to coronary pressure-flow autoregulation. *Basic Res Cardiol.* 2012 May;107(3):1-11.
11. Asano S, Bratz IN, **Berwick ZC**, Fancher IS, Tune JD, and Dick GM. Penitrem A as a tool to understand the role of BK channels in vascular function. *In Press.*

ABSTRACTS

1. **Berwick ZC**, Kroessel T, Haub E. Reactions of 2,6-Dihydroxybenzoic Acid. Indiana University Undergraduate Research Conference Journal. 2007.
2. **Berwick ZC**, Lynch B, Payne GA, Dick G, Sturek M, Tune JD. Contribution of adenosine A_{2A} and A_{2B} receptor subtypes to coronary reactive hyperemia: Role of K_V and K_{ATP} channels. *FASEB J.* 2010.
3. Bender SB, **Berwick ZC**, Laughlin MH, Tune JD. Functional expression of P2Y1 purinergic receptors in the coronary circulation. *FASEB J.* 2010.
4. **Berwick ZC**, Kurian MM, Kohr MC, Moberly SP, and Tune JD. Contribution of IKCa Channels to the Control of Coronary Blood Flow. *FASEB J.* 2011.
5. Moberly SP, **Berwick ZC**, Kohr M, Svendsen M, Mather KJ, and Tune JD. Intracoronary infusion of Glucagon-like peptide 1 acutely enhances myocardial glucose uptake during ischemia in canines. *FASEB J.* 2011.

6. **Berwick ZC**, Dick GM, Bender SB, Moberly SP, Kohr MC, Goodwill AG, Tune JD. Contribution of Ca_v1.2 channels to coronary microvascular dysfunction in metabolic syndrome. *FASEB J. In press.*
7. **Berwick ZC**, Moberly SP, Kohr MC, Morrical E, Kurian MM, Goodwill AG, Tune JD. Contribution of voltage-dependent potassium and calcium channels to coronary pressure-flow autoregulation. *FASEB J. In press.*
8. Moberly SP, **Berwick ZC**, Kohr MC, Mather KJ, Tune JD. Cardiac responses to intravenous glucagon-like peptide 1 are impaired in metabolic syndrome. *FASEB J. In press.*
9. Kohr MC, Lai X, Moberly SP, **Berwick ZC**, Witzmann FA, Tune JD. Augmented coronary vasoconstriction to epicardial perivascular adipose tissue in metabolic syndrome. *FASEB J. In press.*

PROFESSIONAL MEMBERSHIPS

2005	American Chemical Society
2009	American Physiological Society
2010	American Heart Association
2011	Microcirculatory Society
2011	Society for Experimental Biology and Medicine

ACADEMIC ASSOCIATIONS AND AWARDS

2006	President of IUS Field Biology Club
2007	Pinnacle Honors Society
2007	Student Government Association Senator
2009	Graduate Representative of Cellular and Integrative Physiology
2010	IU Graduate Department Travel Fellowship
2010	President of IU School of Medicine Graduate Student Organization
2011	President of IUPUI Graduate and Professional Student Government
2011	Theodore J.B. Stier Fellowship for Excellence in Research
2011	Indiana Physiological Society Outstanding Abstract Award
2011	Gill Symposium Outstanding Graduate Student Thesis Award honorable mention
2012	Theodore J.B. Stier Fellowship for Excellence in Research
2012	Burton E. Sobel Young Investigator Award