Requisite Role of Kv1.5 Channels in Coronary Metabolic Dilation


Rationale: In the working heart, coronary blood flow is linked to the production of metabolites, which modulate tone of smooth muscle in a redox-dependent manner. Voltage-gated potassium channels (Kv), which play a role in controlling membrane potential in vascular smooth muscle, have certain members that are redox-sensitive.

Objective: To determine the role of redox-sensitive Kv1.5 channels in coronary metabolic flow regulation.

Methods and Results: In mice (wild-type [WT], Kv1.5 null [Kv1.5−/−], and Kv1.5−/− and WT with inducible, smooth muscle–specific expression of Kv1.5 channels), we measured mean arterial pressure, myocardial blood flow, myocardial tissue oxygen tension, and ejection fraction before and after inducing cardiac stress with norepinephrine. Cardiac work was estimated as the product of mean arterial pressure and heart rate. Isolated arteries were studied to establish whether genetic alterations modified vascular reactivity. Despite higher levels of cardiac work in the Kv1.5−/− mice (versus WT mice at baseline and all doses of norepinephrine), myocardial blood flow was lower in Kv1.5−/− mice than in WT mice. At high levels of cardiac work, tissue oxygen tension dropped significantly along with ejection fraction. Expression of Kv1.5 channels in smooth muscle in the null background rescued this phenotype of impaired metabolic dilation. In isolated vessels from Kv1.5−/− mice, relaxation to H2O2 was impaired, but responses to adenosine and acetylcholine were normal compared with those from WT mice.

Conclusions: Kv1.5 channels in vascular smooth muscle play a critical role in coupling myocardial blood flow to cardiac metabolism. Absence of these channels disassociates metabolism from flow, resulting in cardiac pump dysfunction and tissue hypoxia. (Circ Res. 2015;117:612-621. DOI: 10.1161/CIRCRESAHA.115.306642.)

Key Words: cardiac function ■ contrast echocardiography ■ hydrogen peroxide ■ ion channel ■ transgenic mice ■ vasodilation ■ voltage-gated potassium channels

The principal function of the coronary circulation is to deliver oxygen and energetic substrates to the myocardium to match myocardial demand for oxygen and energy with the proper supply under various physiological conditions. Oxygen extraction in the coronary circulation is 75% to 80% under baseline physiological conditions, leaving little oxygen extraction reserve. Because this extraction is near maximum to increase oxygen delivery, further extraction is not a viable option,1,2 which necessitates that an increase in myocardial work be met, nearly instantaneously, by an increase in coronary flow to maintain an adequate oxygen supply. Imbalance of myocardial oxygen supply-to-demand ratio results in a deterioration of myocardial function within a few seconds.3 The tight matching of oxygen supply and demand must be guaranteed by local flow regulatory mechanism in any condition to avoid pump failure.

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Coronary blood flow is dependent on multiple physiological factors that affect force generation by coronary vascular smooth muscle cells (VSMCs). These factors include intrinsic response of VSMCs to intravascular pressure (the vascular myogenic response) and release of vasoactive metabolites...
from cell types, including endothelium, nerves, and cardiomyocytes. The tone of vascular smooth muscle is largely regulated by the membrane potential, which controls the amount of calcium in the sarcoplasm via the voltage-gated calcium channels. Membrane hyperpolarization through the opening of potassium channels in VSMCs reduces activation of voltage-gated calcium channels, leading to reduction of Ca\(^{2+}\) entry and vasodilatation. In contrast, closure of K\(^+\) channels leads to membrane depolarization and causes vasoconstriction.

Four major classes of potassium channels have been identified in VSMCs: adenosine triphosphate (ATP)-sensitive (K\(_{ATP}\)) potassium channels, inward rectifier (Kir) potassium channels, large conductance Ca\(^{2+}\)-activated (BK) potassium channels, and voltage-dependent potassium (Kv) channels. Of these channel families, our previous results have suggested that the Kv family is involved in coronary metabolic flow regulation, in a scheme where production of hydrogen peroxide (H\(_2\)O\(_2\)) from mitochondria produces opening of the channels and, thus, dilation in a feed-forward manner. We also found that H\(_2\)O\(_2\)-induced redox-sensitive coronary vasodilatation is mediated by 4-aminopyridine-sensitive K\(^+\) channels. However, it is important to recognize that the use of a pharmacological antagonist does not irrefutably test for a specific ion channel because drugs, such as 4-aminopyridine, can antagonize other classes of ion channels, for example, K\(_{ATP}\). Moreover, the Kv channel family is large, with 12 families of channels and multiple channels in each family. Thus, the precise ion channel(s) linking the products of metabolism to coronary blood flow is (are) unknown.

Although several types of Kv family channels are expressed in various cells in the heart, we focused on Kv1.5 channels, which are reported to be oxygen- and redox-sensitive and expressed in VSMCs. These results, when taken together with our previous observations, provoked us to hypothesize that Kv1.5 channels play a critical role in the coupling of myocardial blood flow (MBF) to cardiac work (CW). Accordingly, we studied coronary metabolic dilation (changes in MBF in response to increases in CW, i.e., the connection of coronary blood flow to myocardial metabolism) in wild-type (WT) mice, mice null for Kv1.5 channels (Kv1.5\(^{-/-}\)), and mice with inducible, smooth muscle–specific expression of Kv1.5 channels (on Kv1.5\(^{-/-}\) and WT backgrounds). We also measured tissue oxygenation to understand whether the balance between oxygen supply (product of blood flow and oxygen content) and CW, which is a surrogate for myocardial oxygen consumption, was altered in the genetically modified animals. Our results support a new concept, vis-à-vis, that Kv1.5 channels play a critical role in connecting blood flow to metabolism in the myocardium.

### Methods

A detailed description of the methodologies, protocols, and statistical analyses are presented in the Online Data Supplement. The murine models are described later.

All procedures were conducted with the approval of the Institutional Animal Care and Use Committee of the Northeastern Ohio Medical University and Department of Radiology and Medicine, Geisel School of Medicine at Dartmouth College, and in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, revised 1996). Mice were housed in a temperature-controlled room with a 12:12-h light/dark cycle and maintained with access to food and water ad libitum.

### Murine Models

WT mice (C57Bl/6N and S129) and mice of both backgrounds that were null for the KCNA5 gene, which encodes Kv1.5 channels (Kv1.5\(^{-/-}\)), were used in this study. Kv1.5\(^{-/-}\) mice on a S129 background were a gift of Helmut Kettenmann (Max Delbrueck Center for Molecular Medicine, Berlin). These mice were backcrossed into a C57Bl/6N background (>6 generations) to obtain C57Bl/6N null for KCNA5.

### Generation of Inducible Double Transgenic Mouse

**With Smooth Muscle–Specific Expression of Kv1.5 Channels**

Transgenic mice with smooth muscle–specific expression (SM22-\(\tau\)) promoter of the reverse tetracycline transactivator gene (rtTA), SM22-kitTA, were purchased from Jackson Laboratory (Figure 1). Transgenic mice expressing Td-Tomato and Kv1.5 channel with tet-on expression were made by promolecular injection of DNA construct of Tet-On 3G tetracycline-inducible system (Clontech) with insertion of Td-Tomato and mouse Kv1.5 cDNA in an IRES (internal ribosome entry site) construct. After screening, the founders expressing Kv1.5 channels and Td-tomato were crossed with the SM22-rtTA mice to produce double transgenic mice (SM-22-tet-Kv1.5). Double transgenic mice have copies of each transgene in all cells, but without tetracycline, the genes are not expressed. In the presence of doxycycline, the Kv1.5 and Td-Tomato gene are transcribed in smooth muscle. In this model, 7 to 10 days of doxycycline treatment (2 mg/mL in drinking water) will induce expression of Kv1.5 channels and Td-Tomato only in smooth muscle.

**Smooth Muscle–Specific Rescue: Expression of Kv1.5 in Smooth Muscle in Kv1.5\(^{-/-}\)**

To determine whether expression of Kv1.5 channels in smooth muscle is critical for coronary flow regulation during changes in CW, we determined whether expression of Kv1.5 channels in smooth muscle would rescue the phenotype observed in the global Kv1.5 knockout mice. To accomplish this, double transgenic mice (SM-22-tet-Kv1.5) were crossed with Kv1.5 null mice to create a mouse with reconstituted (RC) doxycycline-inducible expression of Kv1.5 channels in smooth muscle in Kv1.5\(^{-/-}\) mice, namely SM-Kv1.5 RC (Figure 1).

We studied 4 groups of 4- to 5-month-old mice: Kv1.5\(^{-/-}\) (N=15), WT (N=15), double transgenic (SM-22-tet-Kv1.5, N=15), and reconstituted smooth muscle–specific Kv1.5 expression on the null background (SM-Kv1.5 RC, N=15).

### Results

#### Cardiac Function and Hemodynamics

Figure 2A shows a typical M-Mode image obtained at mid-papillary muscle level from which ejection fraction (EF) was...
calculated. It is also worth noting that differences in cardiac function (EF and fractional shortening) between WT and Kv1.5−/− were apparent at all doses of norepinephrine (Online Figure I). During norepinephrine infusion, EF was significantly lower at all time points in Kv1.5−/− mice compared with WT mice (Figure 2B). In the double transgenic mice on the Kv1.5−/− background, with 7 to 10 days of doxycycline treatment to induce smooth muscle expression of Kv1.5 channels, cardiac function (EF) was increased by norepinephrine to levels comparable to those of WT mice (P=NS) and significantly greater than those of Kv1.5−/− mice (P<0.05). The time course of changes in systolic arterial pressure in WT (Figure 2C) and Kv1.5−/− (Figure 2D) mice show a striking difference. In WT mice, during the highest dose of norepinephrine, blood pressure increased to a steady-state level and dropped only after the norepinephrine infusion was stopped. In contrast, Kv1.5−/− mice maintained arterial pressure only transiently, for ≈30 s, during infusion of norepinephrine, then
the pressure dropped. If the norepinephrine infusion was not stopped, mortality ensued in the null mice. Figure 2E illustrates the effects of norepinephrine on arterial systolic pressure in the Kv1.5−/−-RC mice treated with doxycycline to induce smooth muscle-specific expression of Kv1.5 channels. These mice, like WT mice, had a steady-state increase in arterial pressure to norepinephrine infusion, which fell only when the infusion was stopped. To obtain measurements of MBF, we used the 30-s period when pressure was elevated as a quasi-steady state to obtain flow and work measurements during high-dose norepinephrine (5 μg/kg per minute). At baseline, mean arterial pressure was significantly higher in Kv1.5−/− mice. After 7 days of treatment with doxycycline, the blood pressure dropped significantly in Kv1.5−/−-RC and WT-RC mice. Mean arterial pressure, heart rate, and CW was not significantly different between WT and Kv1.5−/−-RC mice after treatment with doxycycline (Online Figure I). Left ventricular (LV) mass in Kv1.5−/− mice was significantly higher compared to WT mice, but after doxycycline treatment in Kv1.5−/−-RC mice, LV mass was not significantly different than that of WT mice (Online Figure II). There were no significant differences in body weight among all 4 groups of mice.

The relationships between MBF and the double product (DP) for the 3 groups are shown in Figure 3. MBF in Kv1.5−/− mice was significantly (P<0.05) lower at any given DP than that in either WT or the transgenic (Kv1.5−/−-RC) mice after doxycycline treatment. In Kv1.5−/− mice at baseline and at any given dose of norepinephrine, the mean arterial pressure was higher compared with that in WT and Kv1.5−/−-RC mice, which shifted the DP line to the right. In transgenic mice (Kv1.5−/−-RC), re-expression of Kv1.5 channels in smooth muscle, by administering doxycycline for 7 days, re-established the connection between MBF and oxygen demands as indicated by DP. Treatment of the Kv1.5−/−-RC with doxycycline increased expression of mRNA level of Kv1.5 channel in the aorta ≈8-fold (Online Figure III). Doxycycline alone does not change the Kv1.5 channel expression in null mice or in WT mice, but in WT mice, with the 2 transgenes, increased expression of Kv1.5 channels shifted the relationship between DP and MFB to the left (Online Figure IV). This resulted in the observation that for any workload, MFB in the WT-RC animals was greater than in WT animals (P<0.05). We also analyzed MFB against CW (Online Figure V) to ascertain whether this relationship provided different insights than the plot of MFB versus DP; our conclusions were unchanged in that mice null for Kv1.5 channels had compromised increases in flow during enhanced metabolic demands depicted by either DP or CW.

Sometimes the deletion of a specific gene in one genetic background (in mice) leads to a different result than if the deletion is in another background. To determine whether the background of the mice makes a difference in the relationships between work and flow in the heart, we compared these variables in WT and Kv1.5−/− mice of C57Bl/6N and S129 mice (Online Figure VI). The results indicated that background did not influence our findings. The responses in the WT mice were comparable, and the deletion of Kv1.5 channels showed similar compromised metabolic dilation in both strains. We also would like to add that expression of other ion channels was altered in Kv1.5−/− mice. In particular, we noted that expression of Kv1.2, Kir6.1, and Kir6.2 channels was upregulated (mRNA measured by real-time polymerase chain reaction), Kv1.3 and Kv7.1 appeared to be downregulated, and Kv2.1 was not altered (Online Figure VII).

To determine whether the imbalance between metabolism and flow, that is, insufficient coronary blood flow to meet cardiac metabolic demands, resulted in tissue hypoxia, we measured myocardial tissue oxygenation. Myocardial oxygen tension (PO2) was significantly (P<0.05) lower in Kv1.5−/− mice compared with WT mice at baseline and any time point after high dose of norepinephrine injection (Figure 4). We also used hypoxyprobe-1 to identify hypoxic regions in the myocardium. As shown in Online Figure VIII, augmented metabolic demands induced by norepinephrine infusion increased myocardial tissue hypoxia in Kv1.5−/− (as indicated by higher signal intensities for the fluorescence) more than in WT mice. The average signal intensity from the ischemic zone was significantly higher in Kv1.5−/− mice compared with WT mice (P<0.05 compared with WT). In Kv1.5−/−-RC mice after 7 days of doxycycline treatment, the hypoxic areas were significantly less intense compared with that in Kv1.5−/− mice. It is important to note the re-expression of the Kv1.5 channels in smooth muscle minimized tissue hypoxia, that is, the fluorescent signals were comparable to those of controls.

Figure 5 illustrates vasodilatory responses of small coronary arteries (internal diameter averaged 100–150 μm) to H2O2, adenosine, and acetylcholine. In arterioles isolated from Kv1.5−/− mice, vasodilatation to H2O2 was significantly lower than that in WT mice. Re-expression of Kv1.5 channels in smooth muscle (Kv1.5−/−-RC after 7 days of doxycycline) increased H2O2-induced dilation to levels comparable to WT, and significantly greater than Kv1.5−/− (P<0.05). Dilation to adenosine or to acetylcholine was not different between the WT and Kv1.5−/− mice. Normal dilation to adenosine and to acetylcholine shows that the impaired vasodilation in
Kv1.5−/− mice is not as a result of a nonspecific alteration of smooth muscle or the endothelium in the null mice. We also evaluated the vasoactive effects of norepinephrine, which did not produce constriction in these vessels (data not shown). This observation is similar to what we have observed previously in other species25,26 where coronary resistance vessels do not respond directly to α-adrenergic agonists. This latter observation is critical because it could be argued that the blunted metabolic dilation in Kv1.5−/− mice during the norepinephrine stress test is caused by augmented adrenergic constriction; however, this was not the situation.

Discussion

In this study, we observed that Kv1.5 channels in smooth muscle play a key role in connecting MBF to cardiac metabolism. This observation was based on impaired increases in MBF during increased CW in Kv1.5−/− mice. This inadequate dilation during increased CW was associated with severe tissue hypoxia and decrements in cardiac function, suggesting that oxygen delivery was not being correctly matched to oxygen consumption. We also found that the phenotype of impaired metabolic dilation in Kv1.5−/− mice during the norepinephrine stress test is caused by augmented adrenergic constriction; however, this was not the situation.

Importance of the Coronary Microcirculation in Health and Disease

The microcirculation of the heart comprises the bulk of vascular resistance27 and is the segment most responsive to locally produced vasoactive metabolites.28–30 Because of these attributes, under physiological conditions, the coronary microcirculation is the element of the coronary circulation most responsible for the dilation of blood vessels that occurs during increases in cardiac blood flow—the connection of flow to metabolism. Derangements in this connection can have undesirable consequences on cardiac function in that the myocardium requires a continual supply of oxygen for energy production. Perhaps one of the more important implications of our study is shown in Figure 2C–2E. Figure 2D illustrates the outcome when flow, and thus, oxygen delivery, is uncoupled from CW (oxygen demands). Cardiac pump function and arterial pressure cannot be sustained during the metabolic stress because there is insufficient flow to meet the metabolic requirements of the heart. The decrease in pressure and pump function does not occur instantly when demands are increased.
by norepinephrine, but happens ≈30 s after norepinephrine induced the initial increase in arterial pressure. Note, in WT mice (Figure 2C) or in mice with smooth muscle–specific expression of Kv1.5 channels on the null background (Kv1.5−/−; Figure 2E), arterial pressure was maintained during the entire period of norepinephrine infusion, indicating that the oxygen supply matched oxygen demands. In support of this concept, that in the Kv1.5 null mice, flow was uncoupled from metabolism, tissue hypoxia occurred in the null mice during the norepinephrine stress test (Figure 4; Online Figure VIII), but tissue oxygenation was sustained at basal levels in WT and Kv1.5-RC mice. The maintenance of myocardial PO2 indicates that oxygen consumption was matched to oxygen delivery, whereas a decrease in PO2 suggests that flow and oxygen delivery were inadequate to meet the needs of the working heart. We also will add that the myocardial PO2’s were higher than what we expected. Perhaps this is because of the placement of the crystals, which are injected in the LV free wall. We cannot eliminate the possibility that some portion of the signal arises from the LV lumen (perhaps some crystals are close to the lumen); however, despite this limitation, the Kv1.5 null mice show a different response to metabolic stress with decreases in PO2, which would not happen if a large portion of the signal arose from oxygenated blood in the LV lumen. Another caveat is that we are not claiming that norepinephrine produces tissue hypoxia in a WT animal, which could be inferred from the results in Online Figure VIII. The presence of hypoxic tissue could be an artifact of the tissue harvesting and processing, in which there are brief periods of time when the tissue is not perfused, and such a period could result in the appearance of hypoxia, when in fact during perfusion there was none. Nevertheless, even with this hindrance, we would like to emphasize that this would occur in both samples and would not be an explanation for why myocardial tissue hypoxia was more severe in the Kv1.5−/− mice compared with the WT mice.

We think that our observations facilitate an understanding of microvascular disease in the heart. There have been several clinical indications; for example, the Women’s Ischemia Syndrome Evaluation (WISE) trial has revealed that women, without large-vessel disease, show symptoms consistent with myocardial ischemia when stressed.31,32 Also these women show abnormal coronary vasodilator reserve to adenosine.33 On the surface, results from the WISE trial would seem inconsistent with our observations that adenosine-induced vasodilation was comparable in isolated coronary arterioles from WT and Kv1.5−/− mice. We think that it is difficult to compare in vivo and in vitro responses to an agonist; considering the WISE trial studied patients with ischemic heart disease. In the Global Use of Strategies to Open Occluded Coronary Arteries in Acute Coronary Syndromes IIb (GUSTO IIb) trial, it was reported that in patients with acute coronary syndromes, 30.5% of women with unstable angina and 10.2% of women with STEMI (ST segment elevation myocardial infarction) had normal coronary angiographies.34 Moreover, a recent analysis has further supported the importance of microvascular disease in the heart; specifically, patients without large-vessel disease but with compromised coronary vasodilator reserve had mortality rates equivalent to those with large-vessel disease.35 It is worth emphasizing that we are not concluding that Kv1.5 channels are the basis for microvascular disease in the human heart (also termed nonobstructive coronary disease); rather, we speculate that they may be. More likely, there may be several genetic polymorphisms, perhaps in different combinations, involved in nonobstructive coronary disease, which may be analogous to a condition like Long QT Syndrome, where many known polymorphisms of ion channels are known to cause the condition.36

Previously, polymorphisms in several ion channels and in eNOS (endothelial nitric oxide synthase), but not in Kv1.5 channels, were associated with coronary microvascular disease.37

We would like to speculate about an implication of our results that may also bear on clinical observations in patients with nonobstructive coronary disease. For example, in mice null for Kv1.5 channels, cardiac function as defined by measurements of EF and fractional shortening were less than in WT mice even during basal conditions (Online Figure IX). Perhaps the disassociation of flow from metabolism induces mild cardiac dysfunction under basal conditions, which becomes more evident during a stress test. We speculate that in patients with nonobstructive coronary disease, the higher incidence of cardiovascular complications relates to insufficient blood flow to the myocardium.38

Considerations From the Literature

The factors and the effectors responsible for coronary metabolic dilation have remained elusive. Historically, adenosine was considered to be the metabolite linking coronary blood flow to oxygen consumption,40,41 but this hypothesis was rigorously challenged by estimates of interstitial adenosine concentrations that are insufficient to produce dilation during increased CW.42,43 Although some pharmacological studies show an involvement of adenosine in metabolic dilation when other mechanisms of dilation are blocked,44 this may be more of the result of ischemic dilation as opposed to an aerobic process linking work to metabolism. Other efforts have suggested a role for KATP channels and the KCa channels in coronary metabolic dilation.45,46 but it is important to point out that a limitation of such work is the exquisite reliance on pharmacological approaches to draw conclusions about particular ion channels. Moreover, other work has challenged the role of KATP channels in local coronary metabolic dilation.47,48 More recently, the role of ATP released by red blood cells was postulated,49,50 but this hypothesis requires endothelial production of nitric oxide, which has been found to be not essential for coronary metabolic hyperemia.43 Indeed, our present results bear on these previous findings in that responses to adenosine and to nitric oxide were not affected in the Kv1.5 null mice; yet coronary metabolic dilation was severely compromised in these mice. These observations are in concordance with previous studies, concluding that neither adenosine nor nitric oxide is critically involved in coronary metabolic dilation.

Previously, we proposed that mitochondrial production of H2O2 is a feed-forward link between metabolism and flow in the heart.18 We further established that the vasodilatory actions of H2O2 were redox-dependent19 and mediated by 4-aminopyridine-sensitive ion channels.17 Although it is reasonable to speculate that 4-aminopyridine-sensitive ion channels are Kv channels, it is presumptuous to make this conclusion only on the basis of pharmacological evidence.
The current state of knowledge linking specific ion channels to metabolic dilation in the heart is primarily based on the use of pharmacological agents, for example, tetraethylammonium to block MaxiK channels, glibenclamide to block K<sub>ATP</sub> channels, and 4-aminopyridine to block all Kv channels. In these experiments, the antagonists were used to attenuate coronary metabolic dilation to exercise, administration of inotropes, and pacing in anesthetized dogs. Definitive conclusions are limited, however, because of the relative non-specificity of the ion channel antagonists. For example, tetraethylammonium blocks virtually any K channel, and glibenclamide antagonizes both the sarcolemmal K<sub>ATP</sub> channel and the mitochondrial K<sub>ATP</sub> channel, as well as Kv channels. In addition to blocking Kv channels, 4-aminopyridine also can antagonize K<sub>ATP</sub> channels. Accordingly, we opine that conclusions about a specific ion channel transducing metabolic signals into changes in coronary blood flow is based on experiments using traditional pharmacological responses are premature. What is clear from the literature is the 4-aminopyridine-sensitive ion channels are involved in the coronary metabolic dilation, but the identity of the specific channel is not revealed by these previous studies. Although several types of Kv family channels are expressed by various cells in the heart, we focused on Kv1.5 channels because their oxygen and redox sensitivity makes them likely candidates to mediate metabolic dilation.

**Implications of Kv1.5 Channels in the Control of Coronary Blood Flow and Vascular Tone**

Our results are consistent with the concept that Kv1.5 channels are directly involved in coronary metabolic dilation and play a role in the link between MBF and cardiac metabolism. Before we discuss evidence supporting this conclusion, we would like to emphasize that we do not think this ion channel is the only effector modulated by metabolites that connects flow to metabolism. If it was, then we would expect the knockout to be lethal, but this is not the observation. Although there were modest changes in MBF at rest between the Kv1.5<sup>−/−</sup> mice and WT mice, this difference was insufficient to affect basal cardiac function; however, when the null mice were subjected to a metabolic stress (induced by norepinephrine), the increase in MBF (metabolic dilation) was insufficient to sustain cardiac function and the development of profound tissue hypoxia (Figures 2D, 3, and 4, respectively). In contrast, WT mice subjected to the same metabolic stress maintained cardiac function, and the appropriate metabolic dilation maintained tissue oxygenation during the duration of the increased metabolic demands.

An important implication of our results is that in addition to the Kv1.5 channels, other mechanisms of vasodilation must play a role in the metabolic control of the coronary circulation. There are several observations supporting this statement. First, knockout of Kv1.5 channels was not lethal. If Kv1.5 channels were the only connection between metabolism and flow in the heart, we would expect lethality in a knockout because cardiac function cannot be sustained by anaerobic metabolism. Second, we observed a connection, albeit blunted, between CW and coronary blood flow in the Kv1.5 null mice. Accordingly, there must be other mechanisms involved in coronary metabolic dilation. Third, responses to the coronary metabolic dilator, H<sub>2</sub>O<sub>2</sub>, were not completely abolished in the Kv1.5 null mice. Taken together, these results imply that other mechanisms, eg, different redox-sensitive ion channels, ion channels modulated directly by oxygen, and/or other redox-dependent signaling processes, eg, dimerization of protein kinase G, compensate for the loss of the Kv1.5 channel control mechanism. Finally, the knockout of the Kv1.5 channels was associated with an upregulation of Kv1.2, Kir6.1, and Kir6.2 channels (Online Figure VII), which may compensate for the loss of this membrane ion channel through their control of both smooth muscle membrane potential and vascular tone.

A potential criticism of our study design is that we used mice with global knockout of Kv1.5 channels in the study of coronary metabolic dilation. Because these channels are located in many cell types, for example, cardiac myocytes, endothelial cells, and neurons, it could be argued that the effects observed in the global knockout can be attributed to cell types other than vascular myocytes. Although a cell-specific knockout would have provided cogent information, given our results that the re-expression of the channel in smooth muscle on the null background rescued the phenotype, it is likely that our conclusions would be the same, that is, the importance of the Kv1.5 channel in facilitating the connection between coronary blood flow and myocardial metabolism. It is worth noting that our model of conditional (Tet-On) expression of the channel would not cause developmental compensations as would permanent knockout of the channel—even in a cell-specific manner. These results build on our previous results, suggesting that the H<sub>2</sub>O<sub>2</sub> is a metabolic dilator in the heart, in that small arteries isolated from the null mice showed blunted dilation to this reactive oxygen species (compared with WT mice), and doxycycline-inducible expression of Kv1.5 channels in smooth muscle restored this vasodilation. It is also worth noting that vessels from the null mice did not show any response to norepinephrine, so the blunted metabolic dilation during the norepinephrine stress test should not be interpreted as possible enhanced constriction to this adrenergic agonist.

Another possible explanation is that the deletion of Kv1.5 channels in cardiac myocytes renders them more responsive to the production of vasoconstrictors when stimulated by adrenergic agonists. This possibility is founded on previous work from our laboratory showing that stimulation of adrenergic receptors in cardiac myocytes results in the production of a substance or substances that mediate coronary arterial vasoconstriction. However, we do not think this explanation is plausible given that re-expression of the Kv1.5 channels only in smooth muscle restored MBF during the norepinephrine stress test.

The application of contrast echocardiography to measure MBF was described over 2 decades ago, and since this observation, the technique has been used to measure blood flow in a variety of animal models and in the clinical setting. One advantage of this technique is the ability to measure blood flow in vivo under minimally invasive conditions (intravenous catheter for contrast infusion). Previous measurements of MBF in the mouse heart were confined to buffer-perfused isolated heart models which impact a variety of control mechanisms for coronary blood flow, causing concerns about...
physiological relevance of the observations. In our study, we used contrast echocardiography to measure MBF in anesthetized mice, and these values were 40% higher compared with the flows measured with microspheres. Raher et al. compared microsphere measurements of flow to those obtained with contrast echocardiography and similar to our results found a strong correlation between the two measurements. Although the slope of their linear regression was close to identity, they did not quantify blood flow and left the myocardial contrast echocardiography measurement in units of db/s. Therefore, it is difficult to relate our comparison of blood flows derived from myocardial contrast echocardiography and microspheres to theirs. Magnetic resonance imaging was used to measure myocardial perfusion in a murine model, and these investigators reported baseline flows in the range of 7 mL/min per gram. This range was less than our myocardial contrast echocardiography measurements of 12 mL/min per gram at baseline, but if we corrected based on the microsphere measurements, our values would be comparable. Our values, especially those at the highest dose of norepinephrine, were higher than what we expected, but similar to the highest values in buffer-perfused, isolated hearts (Online Figure X). We have not corrected the blood flow measurements that we report, but wish to emphasize that any adjustment in the measurement would not change the most important aspect of our study—the impairment in metabolic dilation in Kv1.5−/− mice and restoration of flows in the Kv1.5−/−RC mice. After correction, MBF values for all mice would be decreased by a certain factor, and the differences would remain the same.

Conclusions

In the heart, vascular Kv1.5 channels play a critical role in coupling MBF to cardiac metabolism. This coupling is critical in the maintenance of tissue oxygenation during hemodynamic challenges through balancing oxygen delivery via metabolic coronary dilation and oxygen consumption via CW.

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Disclosures

None.

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**Novelty and Significance**

What Is Known?

- The heart is an organ system requiring a continuous supply of oxygen, via myocardial blood flow, to maintain normal cardiac pump function.
- Myocardial blood flow is coupled to cardiac work through a process known as metabolic dilation.
- Metabolic dilation in the heart is mediated, in part, by feed-forward production of H$_2$O$_2$ from mitochondria during aerobic metabolism.
- H$_2$O$_2$-induced vasodilation is, in part, mediated by voltage-gated potassium (Kv) channels.

What New Information Does This Manuscript Contribute?

- Kv1.5 channels in smooth muscle are critical for metabolic dilation in the heart.
- Kv1.5 channels in smooth muscle are critical for maintaining oxygen balance in the heart.

What Is New?

- Absence of Kv1.5 channels uncouples myocardial blood flow from cardiac work, resulting in tissue hypoxia and impaired myocardial function.

This study shows the role of Kv1.5 channels in smooth muscle in the regulation of coronary blood flow. Absence of Kv1.5 channels uncouples myocardial blood flow from cardiac work, resulting in coronary insufficiency, that is, insufficient blood flow to meet the metabolic needs of the myocardium. Insufficient blood flow creates an imbalance in tissue oxygenation (consumption of oxygen exceeds delivery), resulting in tissue hypoxia. Expression of Kv1.5 channels in only smooth muscle in a global knockout completely rescues the abnormality in coronary regulation and restores the balance of tissue oxygenation. Our findings may help explain how patients with nonobstructive coronary disease show impairments in flow regulation that can lead to myocardial ischemia.
Requisite Role of Kv1.5 Channels in Coronary Metabolic Dilation

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Supplemental Material

Data and Methods

**Transthoracic stress echocardiography.** Transthoracic echocardiography was used to evaluate cardiac function. M-mode images were obtained from parasternal short axis view, mid-papillary muscle level using a VEVO 770 High-Resolution echocardiography Imaging System (VisualSonics, Toronto, Ontario, Canada) designed for small animal studies. After induction of anesthesia (3% isoflurane with 100% oxygen, 1 L/min, in small chamber) mice were placed on controlled heating table designed for small animal echocardiography. The anesthetic and oxygen were delivered through the nose cone (1-2% isoflurane, 0.5 L/min with oxygen). After removing hair from the chest, warmed (37°C) Aquasonic 100 gel (Parker Laboratories, Fairfield, NJ) was placed on the chest to optimize visibility of cardiac structures. Cardiac function was measured at baseline, after Hexamethonium (5 mg/kg) and different doses of NE (0.5, 1.0, 2.5 and 5.0 µg/kg.min⁻¹, intravenous, continues for 3 minutes) infusion. Left ventricular volume at end diastole (LVEDV) and end systole (LVESV), as well left ventricular internal diameter at end diastole (LVID,d) and end systole (LVID,s) were measured at steady state after drug infusion. Left ventricular volume was calculated by modified Teichholz formula: LVV=((7.0 / (2.4 + LVID)) * LVID. Left ventricular ejection fraction (LVEF %) was calculated by: (LVEDV-LVESV)/LVEDV. Fractional shortening was calculated by: (LVID,d-LVID,s)/LVID,d (Figure 1A). All echocardiographic calculations and measurements were carried out offline using the Vevo770/3.0.0 software. All measurements were averaged over 5 cycles.

**Hemodynamic measurements and calculation of cardiac work.** After induction of anesthesia (3% isoflurane with 100% oxygen, 1 L/min, in small chamber) mice were placed on controlled heating surgical table designed for small-animal surgeries and echocardiography. Body temperature was maintained at 37°C via rectal probe. Mice were secured in the supine position and placed under a dissecting microscope. The right jugular vein was cannulated with PE-50 polyethylene tubing (Becton Dickinson, Oakville, ON, Canada) containing heparin (50 U/ml in Dulbecco’s PBS) in saline for intravenous drug infusions. The femoral artery was isolated and cannulated with a 1.2F pressure catheter (Scisense Inc, Ontario, Canada) connected to a data acquisition system (PowerLab ML820; ADInstrument, Colorado Springs, CO) through a pressure interface unit (SP200 Pressure System) designed to measure arterial blood pressure and heart rate (HR). Pressure catheter was advanced ~10mm into abdominal aorta. All measured variables were continuously recorded and stored on an iMac computer that used the PowerLab system (AD Instruments; Castle Hill, Australia). The blood pressure data were collected and analyzed using AD Instruments Chart 7 software. All mice were euthanized following the experimental protocol with a lethal dose of pentobarbital sodium (50 µg/kg).

The work that the heart completes per beat is termed stroke work, which is the area within the ventricular pressure-volume loop. Mathematically stroke work can be
calculated as the product of stroke volume (SV) and mean arterial pressure (MAP). Mean arterial pressure was calculated by: MAP = \frac{2}{3} \text{ diastolic pressure} + \frac{1}{3} \text{ systolic pressure}. In the current study we used double product to estimate cardiac work (HR \times MAP), but CW is most accurately represented by the triple product of MAP \times HR \times SV (stroke volume). Because of technological constraints we could not measure SV during acquisition of images to measure MBF. In order to calculate stroke volume changes during NE infusion, we used separate group of mice and by using M-mode tracing pictures we calculated SV changes. We used the same age and body weight mice for this experiments (WT and Kv 1.5\textsuperscript{-/-}). Accordingly we determined how accurately DP reflects cardiac work in mice by comparing DP to the triple product (CW) (Supplement Figure 11). We found a linear relationship between triple and double product (R\textsuperscript{2}=0.951) suggesting DP was a reasonable surrogate for CW. Cardiac work directly reflects myocardial oxygen consumption, and we employed this index as a surrogate for oxygen consumption to decipher the basis of coronary metabolic dilation.

**Myocardial perfusion imaging by contrast echocardiography.** Lipid-shelled microbubbles were prepared by sonication of an aqueous lipid dispersion of polyoxyethylene-40-stearate and distearoyl phosphatidylcholine saturated with decafluorobutane gas. Contrast agent was prepared fresh before the experiments. Microbubble concentration and size distribution were determined by using Zetasizer Nano-ZS90 (Malvern Instruments). Prior to particle measurements, the microbubble suspension was diluted (1:5 v/v) with filtered water (0.22 µm filter, Nalgene International) to ensure that light scattering signals are within the sensitivity of the instrument.\textsuperscript{5}

For myocardial contrast echocardiography (MCE), animals were prepared as above for transthoracic echocardiography. Myocardial perfusion imaging was performed with a linear-array transducer (15L8) interfaced with an ultrasound system (Sequoia 512, Siemens Medical Systems, Mountain View, CA). The right jugular vein was cannulated with PE-50 polyethylene tubing containing heparin (50 U/ml) and was used for drug and contrast infusion. Long axis images were obtained for perfusion imaging. In all mice, the contrast agent was infused intravenously (jugular vein) at 20 µl/min (5 × 10\textsuperscript{5} bubbles min\textsuperscript{-1}). MCE was performed with a multi-pulse contrast-specific pulse sequence designed to detect the non-linear microbubble signal at a low mechanical index (MI 0.2-0.25). Data were acquired after a high-MI (1.9) pulse sequence used to destroy microbubbles in the acoustic field. All settings for processing were adapted and optimized for each animal: penetration depth was ~2–2.5 cm, near field was focused on the middle of the left ventricle (short axis view), and gains were adjusted to obtain images with no signal from the myocardium and then held constant. Regions of interest (ROI) were positioned within the anterolateral in the short axis view. A curve of signal intensity over time was obtained in ROI and fitted to an exponential function: y = A(1 - e\textsuperscript{-βt}), where y is the signal intensity at any given time, A is the signal intensity corresponding to the microvascular cross sectional volume, and β is the initial slope of the curve, which corresponds to the blood volume exchange frequency.\textsuperscript{6,7} Relative blood volume (RBV) was calculated as the ratio of myocardial to cavity signal intensity (RBV=A/A\textsubscript{LV}). A\textsubscript{LV} corresponds to the signal intensity for the LV cavity. Color coded
parametric images were used to outline region of interest (region of the left ventricle). Myocardial blood flow (MBF) was estimated as the product RBV × β.\(^8\) The analysis of nearby regions within the myocardium and the left ventricle is proposed to compensate for regional beam inhomogeneities and contrast shadowing.\(^9\) MBF was calculated from the blood volume pool relative to the surrounding myocardial tissue, the exchange initial slope of curve, and tissue density \(\rho\) \((\rho_T=1.05).\(^8\) MBF was measured in 3-5 different images obtained from the same condition (baseline and treatments). **MCE analyses were performed by readers blinded to the genotype and treatment.** Although MCE has been used in mice previously to measure myocardial blood flow, we felt it was important to correlate flow using MCE to flow measured using microspheres (Supplement Figure 10). The correlations between the two flow measurements showed substantial agreement, although the slope of the line indicated that flows were higher in MCE vs microspheres. We have reported the calculated flows from MCE in all figures in this paper.

**Isolated microvessel studies.** Single arterioles and small coronary arteries were dissected from the epicardial surface of the left ventricle or from the septum. A portion of the left ventricular free wall or septum was removed and small arteries (100-200 µm, internal diameter) were located under a dissecting microscope. The vessel with surrounding ventricular muscle was excised and transferred to a temperature-controlled dissection dish (4°C) containing PSS and dissected free of the muscle tissue under epi-illumination. The arteriole was cannulated at both ends using micropipettes that have been shaped in a microforge. The outside of the arteriole was securely tied to each pipette using 11-0 suture, and then was transferred to the stage of an inverted microscope for study. Arteriolar dimensions (internal diameter) were measured using videomicroscopy during the course of an experiment. Vessels were contracted with the thromboxane mimetic U46619 (1 µM), and subsequent hydrogen peroxide, adenosine and acetylcholine relaxation was determined. Some vessels segments were used for isometric force generation experiments. For these preparations, the vessels were isolated as described, but two small (30 µm diameter) wires were inserted into the vessel and connected to a force transducer (Living Instruments, Inc). After optimization of tension, experiments were conducted to determine constrictor or dilator responses to agonists. For either preparation, pharmacological agents were administered in the bath.

**Determination of tissue oxygenation.** We used two complementary methods to evaluate myocardial tissue oxygen levels during the norepinephrine stress test. First, we analyzed tissue sections using Hypoxyprobe-1, which measures protein modifications induced by tissue \(pO_2\) of less than 10 mmHg.\(^{10}\) Hypoxyprobe was dissolved in 0.9% saline and injected intravenously into mice at a dose of 60 mg/kg body weight. Thirty minutes after the injection of hypoxyprobe, the mice were sacrificed and the heart tissues were harvested and frozen in liquid nitrogen. Frozen tissues were cryosectioned into 10 µm sections and were stored at -80°C. After thawing, the sections were fixed in cold acetone (4°C) for 10 min. The sections were rinsed and incubated overnight at 4°C with mouse monoclonal anti-pimonidazole antibody (clone 4.3.11.3)(MAb1) diluted at 1:50 in PBS containing 0.1% bovine serum albumin and 0.1% Tween 20. The sections were then incubated for 2 hours with Alexa Fluor 594-conjugated anti-mouse antibody at the dilution of 1:500. The slides were mounted with ProLong® Gold Antifade with DAPI
We also determined myocardial tissue pO$_2$ as an index of the balance between oxygen supply and oxygen consumption using L-Band electron paramagnetic resonance (EPR) oximetry.\textsuperscript{11,12} The principle of EPR oximetry is based on molecular oxygen-induced line-width changes in the EPR spectrum of a paramagnetic probe. The technology has been well-established and validated for measurements of pO$_2$ in the heart. The myocardial pO$_2$ measurements in the present study were performed using a modified Varian E-15 EPR spectrometer, equipped with a home-made low-frequency (1.2 GHz, L-band) microwave bridge. Microcrystals of LiNc-BuO were used as oxygen-sensing probes for EPR oximetry. Mice, under inhalation anesthesia (1-2% isoflurane), had the probes implanted in the left-ventricular mid-myocardium during a left thoracotomy. In order to recover from surgical trauma and healing, the animals were allowed to recover for 2-3 weeks before final study of tissue oxygenation. To measure oxygenation, the mice were situated in a right-lateral position with the chest closer to the loop of a surface-coil resonator. EPR spectra were acquired as single 30-second duration scans using the following settings: microwave frequency, 1.2 GHz (L-band), incident microwave power, 8 mW; modulation amplitude, one-third of the EPR line width with scan time of 10 sec., modulation frequency 24 kHz; scan range, 2 Gauss; receiver time constant, 0.2 second. The peak-to-peak width of the EPR spectrum was used to calculate pO$_2$ using a standard calibration curve.\textsuperscript{13}

Statistical analysis. Measurements of left ventricular volumes, diameter, EF and FS in WT, Kv 1.5$^{-/-}$ and Kv 1.5$^{-/-}$-RC mice are presented as mean values ± SD. Difference of LV volumes, diameter, EF and FS were analyzed for statistical significance with one-way ANOVA using GraphPad Prism version 4.0 for Windows (GraphPrism Software Inc, San Diego, CA.). The correlation of cardiac works (between DP and TP), myocardial blood flows (MCE determined and microsphere estimated) where assessed by linier regression analysis. Vasodilation responses to H$_2$O$_2$, adenosine and ACH, relationship between MBF and DP, myocardial oxygen partial pressure changes were also assessed by linier regression analysis. $R^2$ and P values are reported for all regression analysis. A probability of p<0.05 was accepted as statistically significant.

References

models for cardiac MRI in rodents: Comparison of quantification of left ventricular volumes and function by various geometrical models with a full-volume MRI data set in rodents. *American Journal of Physiology. Heart and Circulatory Physiology.* 2012;302:H709-715


Supplement data

Online Figure I. Mean Arterial Pressure (panel A), Heart rate (panel B) and cardiac work (Double Product, panel C) at baseline and during different doses of Norepinephrine injection (0.5-5 ug/kg.min⁻¹). *P<0.05 Kv 1.5⁻/⁻ vs WT mice; *P<0.05 WT-RC vs. Kv 1.5⁻/⁻ mice.
Online Figure II. Left ventricular mass (LV mass, panel A), Body weight (Panel B) and LV mass/body weight ratio (Panel C). LV mass in Kv 1.5^- was higher compared to WT mice, but LV BW ratio was not significant between all groups. •P<0.05 Kv 1.5^- vs WT mice
Online Figure III. Doxycycline-induced SM22-promoter-driven KV1.5-Td-Tomato expression in aorta. A and B illustrate tomato red fluorescence in WT and the mice with doxycycline-inducible, smooth muscle specific expression of Kv1.5 channels (after 7 days of doxycycline). The lower panels represent higher magnification views of the circumscribed areas in the boxes of the top panels. The fluorescent signal in WT is due to autofluorescence of elastic lamina (distinct lines in WT, lower panel). In contrast, note the entire media is fluorescent in the transgenics indicating smooth muscle expression. Panel C shows m-RNA relative fold changes after 7 days of doxycycline treatment. *P<0.05 WT-RC compared to WT mice after DOX treatment. *P<0.05 Kv 1.5^-RC mice compared to WT mice. Without DOX treatment there was no mRNA expression Kv 1.5^-RC mice.
Online Figure IV. Myocardial blood flow and double product relationship in WT, and WT transgenic mice (WT-RC). After 7 days doxycycline treatment to both lines (resulting in increased smooth muscle expression only in WT-RC), the DP-MBF relationship in the WT-RC was shifted to the left of WT, indicating that for every level of cardiac work, flow was greater in the WT-RC compared to WT.
Online Figure V. Relationship between myocardial blood (MBF) flow and triple product (Cardiac Work (CW)). MBF was significantly lower in Kv 1.5−/− at any given level of cardiac work (*P<0.05).
Online Figure VI. Relationship between MBF and DP in WT (S129 and C57BL/6N backgrounds), Kv1.5<sup>-/-</sup> (S129 and C57BL/6N backgrounds). Note, there were no significant differences in the relationship between DP and MBF between the backgrounds in the WT or Kv1.5<sup>-/-</sup> mice.
Online Figure VII. Expression of other ion channels in Kv 1.5−/− mice.
Online Figure VIII. A) Hypoxprobe fluorescent and secondary antibody control signal intensity in myocardial tissue from wild type (WT), Kv1.5⁻/⁻, and Kv1.5⁻/⁻ RC on doxycycline (7 days). B) Fold change of signal intensity in the 3 groups. ⚫P<0.05 Kv 1.5⁻/⁻ vs WT mice; *P<0.05 Kv 1.5⁻/⁻ RC vs. Kv 1.5⁻/⁻ mice.
Online Figure IX. Left ventricular volume (LV Vol) at diastole, systole and stroke volumes in Kv 1.5\(^-\) (panel A) and WT (panel B) mice. Panel C- Ejection fraction (EF) at baseline and after NE treatment. LV internal diameter (LVID) at diastole(d) and systole(s) in Kv 1.5\(^-\) (panel D) and WT (panel E) mice. Panel F- Fractional shortening shortening (FS) at baseline and after NE treatments. Measurements were done at mid-papillary muscle level, and volumes were calculated by Teichholz formula. Data are presented as Mean±SD, *P<0.05 WT mice compared to Kv 1.5\(^-\).
Online Figure X. MBF measured by myocardial contrast echocardiography (MCE) and by microsphere (BioPal, Inc.). MBF was measured at baseline and during Norepinephrine (0.5-5.0 µg/kg min⁻¹) injection. MBF estimated by MCE was higher compared to microspheres, but there was an excellent correlation between the two methods. \( R^2 = 0.98 \) and \( Y = 1.39X - 0.7 \).
Online Figure XI. Correlation between double and triple product as estimates of cardiac work (CW). $R^2 = 0.95$. 

$Y = 16.76X + 17849$