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Requisite Role of Kv1.5 Channels in Coronary Metabolic Dilation

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Abstract

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, 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 (MAP), myocardial blood flow (MBF), myocardial tissue pO_2, and ejection fraction (EF) before and after inducing cardiac stress with norepinephrine (NE). Cardiac work (CW) was estimated as the product of MAP and heart rate. Isolated arteries were studied to establish if genetic alterations modified vascular reactivity.

Despite higher levels of CW in the Kv1.5$^{-/-}$ (versus WT at baseline and all doses of NE), MBF was lower in Kv1.5$^{-/-}$ than in WT. At high levels of CW, tissue pO_2 dropped significantly along with EF. 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 H_2O_2 was impaired, but responses to adenosine and acetylcholine were normal compared to WT.

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DISCLOSURES

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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.

Keywords

Coronary microcirculation; coronary blood flow; voltage-gated potassium channels; vasodilation; ion channel; contrast echocardiography; mouse; cardiac function; transgenic mice; mouse blood pressure measurement/monitoring; hydrogen peroxide

INTRODUCTION

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 75-80% under baseline physiological conditions, leaving very 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 of myocardial work must be met, nearly instantaneously, by an increase of 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.

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 vascular smooth muscle cells to intravascular pressure (the vascular myogenic response) and release of vasoactive metabolites from cell types including endothelium, nerves, and cardiomyocytes.[4-7] 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 (VGCC). Membrane hyperpolarization through the opening of potassium channels in VSMCs reduces activation of VGCC, leading to reduction of Ca^{2+} entry and vasodilatation. In contrast, closure of K^+ channels leads to membrane depolarization and causes vasoconstriction.[8-10]

Four major classes of potassium channels have been identified in VSMCs: ATP sensitive (K_{ATP}), inward rectifier (Kir), large conductance Ca^{2+}-activated potassium channels (BK), and voltage-dependent potassium (Kv) channels.[8, 9, 11-16] Of these channel families, our previous results have suggested that the Kv family is involved in coronary metabolic flow regulation,[17] in a scheme where production of H_2O_2 from mitochondria produces opening of the channels, and thus dilation in feed-forward manner.[18] We also found that H_2O_2-induced redox-sensitive coronary vasodilatation[19] is mediated by 4-aminopyridine-sensitive K^+ channels.[17] However, it is important to recognize that the use of a pharmacological antagonist does not irrefutably test for a specific ion channel as drugs, such as 4-aminopyridine, can antagonize other classes of ion channels, e.g., K_{ATP}.[20] 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 vascular smooth muscle cells.[21-23] These results, when taken together with our previous observations,[17-19] provoked us to hypothesize that Kv1.5 channels play a critical role in the coupling of myocardial blood flow to cardiac work. Accordingly, we studied coronary metabolic dilation (changes in myocardial blood flow [MBF] in response to increases in cardiac work, i.e., the connection of coronary blood flow to myocardial metabolism) in wild-type 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 wild type backgrounds). We also measured tissue oxygenation to understand if the balance between oxygen supply (product of blood flow and oxygen content) and cardiac work, 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 below.

All procedures were conducted with the approval of the Institutional Animal Care and Use Committee of the Northeastern Ohio Medical University and [3]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 (NIH 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

Wild type 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.[24] $Kv1.5^{-/-}$ 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 (more than 6 generations) to obtain C57Bl/6N null for *KCNA5*.

Generation of inducible double transgenic mouse with smooth muscle specific expression of Kv1.5 Channels (Figure 1)

Transgenic mice with smooth muscle–specific expression (SM22-α promoter) of the reverse tetracycline transactivator gene (rtTA), SM22-rtTA, were purchased from Jackson Lab. Transgenic mice expressing Td-Tomato and Kv1.5 channel with tet-on expression were made by pronuclear 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 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, 7-10 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 if expression of Kv1.5 channels in smooth muscle is critical for coronary flow regulation during changes in cardiac work, we determined if 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 4-5 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 was calculated. It is also worth noting that differences in cardiac function (EF and Fractional shortening, FS) between WT and Kv1.5$^{-/-}$ were apparent at all doses of NE (Supplementary material, Figure I). During NE infusion, EF was significantly lower at all time points in Kv1.5$^{-/-}$ mice compared to WT mice (Figure 2B). In the double transgenic mice on the Kv1.5$^{-/-}$ background, with 7-10 days of doxycycline treatment to induce smooth muscle expression of Kv1.5 channels, cardiac function (EF) was increased by NE to levels comparable to WT mice (P=NS), and significantly greater than Kv1.5$^{-/-}$ ($P<0.05$). The time course of changes in systolic arterial pressure in WT (Figure 2C) and Kv1.5$^{-/-}$ (Figure 2D) show a striking difference. In WT, during the highest dose of NE blood pressure increased to a steady state level, and dropped only after the NE infusion was stopped. In contrast, Kv1.5$^{-/-}$ mice maintained arterial pressure only transiently, for about 30 sec, during infusion of NE, then pressure dropped. If the NE infusion was not stopped, mortality ensued in the null mice. Figure 2E illustrates the effects of NE 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, had a steady-state increase in arterial pressure to NE, which fell only when infusion was stopped. To obtain measurements of myocardial blood flow, we used the 30 sec period when pressure was elevated as a quasi-steady state to obtain flow and work measurements during high dose NE (5 μg/kg.min^{-1}). 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. MAP, HR and cardiac work was not significantly different between WT and Kv1.5$^{-/-}$-RC mice after doxycycline (Supplement, Figure I). Left ventricular mass in Kv1.5$^{-/-}$ mice was significantly higher compared to WT mice, but in Kv1.5$^{-/-}$-RC mice

doxycycline treatment LV mass was not significantly than that of WT mice (Supplement, 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 three groups are shown in Figure 3. MBF in Kv1.5$^{-/-}$ mice was significantly ($P<0.05$) lower at any given DP than in either WT or the transgenic (Kv1.5$^{-/-}$ -RC) mice after doxycycline treatment. In Kv 1.5$^{-/-}$ mice at baseline and any given does of NE the MAP was higher compared to WT and Kv 1.5$^{-/-}$-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 about 8 fold (Supplement, 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 two transgenes, increased expression of Kv1.5 channels shifted the relationship between DP and MBF to the left (Supplement, Figure IV). This resulted in the observation that for any work load, MBF in the WT-RC animals was greater than in WT, ($P<0.05$). We also analyzed MBF against CW (Supplement, Figure V) to ascertain if this relationship provided different insights than the plot of MBF vs 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 if 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 (Supplement, 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 in Kv1.5$^{-/-}$ mice expression of other ion channels was altered. In particular we noted that expression of Kv1.2, Kir6.1, and Kir6.2 channels was upregulated (mRNA measured by real time PCR), Kv1.3 and Kv7.1 appeared to be downregulated, and Kv2.1 was not altered (Supplement, Figure VII).

To determine if the imbalance between metabolism and flow, i.e., insufficient coronary blood flow to meet cardiac metabolic demands, resulted in tissue hypoxia, we measured myocardial tissue oxygenation. Myocardial oxygen tension was significantly ($P<0.05$) lower in Kv1.5$^{-/-}$ mice compared to WT at baseline and any time point after high dose of NE injection (Figure 4). We also used Hypoxyprobe-1 to identify hypoxic regions in the myocardium. As shown in Supplemental 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 to WT ($P<0.05$ compared to WT). In Kv1.5$^{-/-}$-RC mice after 7 days of doxycycline treatment the hypoxic areas were significantly less intense compared to Kv1.5$^{-/-}$ mice. It is important to note the re-expression of the Kv1.5 channels in smooth muscle minimized tissue hypoxia, i.e., the fluorescent signals were comparable to controls.

Figure 5 illustrates vasodilatory responses of small coronary arteries (internal diameter averaged 100-150 μm) to hydrogen peroxide, adenosine, and acetylcholine. In arterioles isolated from Kv1.5$^{-/-}$ mice, vasodilation to H_2O_2 was significantly lower than WT. Re-expression of Kv1.5 channels in smooth muscle (Kv1.5$^{-/-}$-RC after 7 days of doxycycline) increased the dilation compared to Kv1.5$^{-/-}$ ($P<0.05$) to vasodilation equivalent to WT. 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 due to a non-specific 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 species[25, 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 due to 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 myocardial blood flow to cardiac metabolism. This observation was based on impaired increases in MBF during increased cardiac work in Kv1.5$^{-/-}$ mice. This inadequate dilation during increased cardiac work 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 could be rescued by re-expressing Kv1.5 channels in vascular smooth muscle cells, supporting the concept that the expression of the potassium channels in smooth muscle is critical for metabolic flow control. Taken together these results support the conclusion that Kv1.5 channels play a key role in coronary metabolic dilation, i.e., the connection of myocardial blood flow to cardiac metabolism. We will discuss this conclusion from the perspective of the importance of the coronary microcirculation in health and disease, results in the literature that are cogent to our findings, and implications of our findings in understanding the control of coronary blood flow.

The importance of the coronary microcirculation in health and disease

The microcirculation of the heart comprises the bulk of vascular resistance,[27] 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-E. Figure 2D illustrates the outcome when flow, and thus, oxygen delivery, is uncoupled from cardiac work (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 about 30 seconds after norepinephrine induced the initial increase in arterial pressure. Note, in wild-type mice (Figure 2C) or those with smooth muscle specific expression of Kv1.5 channels on the null background (Kv1.5 RC, Figure 2E), arterial pressure was maintained during the entire period of norepinephrine infusion indicating 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 and Supplement, Figure VIII), but tissue oxygenation was sustained at basal levels in WT and Kv1.5 RC mice. The maintenance of myocardial oxygen tension indicates that oxygen consumption was matched to oxygen delivery; whereas a decrease in oxygen tension suggests that flow and oxygen delivery were inadequate to meet the needs of the working heart. We also will add that the myocardial PO_2's were higher than what we expected. Perhaps this is due to 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 very different response to metabolic stress with decreases in oxygen tension, 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 wild type animal, which could be inferred from the results in Supplemental 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 $Kv1.5^{-/-}$ compared to the wild type mice.

We believe our observations facilitate an understanding of “microvascular disease” in the heart. There have been several clinical indications; for example, the WISE trial (Women's Ischemia Syndrome Evaluation) has revealed that women, without large vessel disease, show symptoms consistent with myocardial ischemia when stressed.[31-33] Also these women show abnormal coronary vasodilator reserve to adenosine.[34] 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 wild type and $Kv1.5^{-/-}$ mice. We believe 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 GUSTO IIb (Global Use of Strategies to Open Occluded Coronary Arteries in Acute Coronary Syndromes 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 had normal coronary angiographies.[35] 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.[36] 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 non-obstructive coronary

disease); rather, we speculate that they may be. More likely, there may be several genetic polymorphisms, perhaps in different combinations, involved in non-obstructive 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.[37] Previously, polymorphisms in several ion channels and in eNOS, but not in Kv1.5 channels were associated with coronary microvascular disease.[38]

We would like to speculate about an implication of our results that may also bear upon clinical observations in patients with non-obstructive coronary disease. For example in mice null for Kv1.5 channels cardiac function as defined by measurements of ejection fraction and fractional shortening were less than wild type mice even during basal conditions (Supplement, 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 non-obstructive coronary disease the higher incidence of cardiovascular complications relates to insufficient blood flow to the myocardium.[39]

Considerations from the literature

The factors and their 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 cardiac work.[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 K_{ATP} channels and the K_{Ca} 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.[43, 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 upon 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, or nitric oxide are critically involved in coronary metabolic dilation.

Previously, we proposed that that mitochondrial production of H_2O_2 is a feed-forward link between metabolism and flow in the heart.[18] We further established that the vasodilatory actions of H_2O_2 were redox-dependent[19] and mediated by 4-aminopyridine sensitive ion channels.[17] Although it is not unreasonable to speculate that 4-AP 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, e.g., tetraethylammonium

(TEA) to block MaxiK channels,[46] glibenclamide to block K_{ATP} channels,[45] and 4-aminopyridine to block all Kv channels.[51, 52] In these experiments, the antagonists were used to attenuate coronary metabolic dilation to exercise, administration of inotropes and/or pacing in anesthetized dogs. Definitive conclusions are limited, however, due to the relative non-specificity of the ion channel antagonists. For example, TEA blocks virtually any K channel,[20, 53, 54] and glibenclamide antagonizes both the sarcolemmal K_{ATP} channel and the mitochondrial K_{ATP} channel,[55-57] as well as Kv channels.[54, 58] In addition to blocking Kv channels, 4-aminopyridine also can antagonize K_{ATP} channels.[20] Accordingly, we opine that conclusions about a specific ion channel transducing metabolic signals into changes in coronary blood flow are 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,[18, 52] 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[21-23] 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 myocardial blood flow and cardiac metabolism. Before we discuss evidence supporting this conclusion, we would like to emphasize that we do not believe this ion channel is the only effector modulated by metabolites that connects flow to metabolism. If it were, then we would expect the knockout to be lethal, but this is not the observation. Although there were modest changes in myocardial blood flow at rest between the $Kv1.5^{-/-}$ mice and wild type, 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 myocardial blood flow (metabolic dilation) was insufficient to sustain cardiac function as indicated by acute decreases in cardiac function and the development of profound tissue hypoxia (Figures 2D, 3 and 4, respectively). In contrast, wild type 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 as cardiac function cannot be sustained by anaerobic metabolism. Second, we observed a connection, albeit blunted, between cardiac work 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_2O_2, were not completely abolished in the Kv1.5 null mice. This implies other mechanisms, perhaps other redox sensitive ion channels, e.g., Kv1.3[59], or ion channels modulated directly by oxygen, e.g., Kv1.2[60], and/or other redox-dependent signaling processes, e.g., dimerization of protein kinase G[61] 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 (Supplement Figure VII), which both may compensate for the loss of this membrane ion channel through their control of smooth muscle membrane potential and vascular tone.[60, 62]

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, e.g., cardiac myocytes,[63, 64] endothelial cells,[65, 66] and neurons,[67] 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, i.e., 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 upon our previous results suggesting the H_2O_2 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 to wild type mice) and doxycyclineinducible 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 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. [25, 26] However, we do not think this explanation is plausible given that re-expression of the Kv1.5 channels only in smooth muscle restored myocardial blood flow during the norepinephrine stress test.

The application of contrast echocardiography to measure myocardial blood flow was described over two decades ago,[68] and since this observation the technique has been used to measure blood flow in a variety of animal models[69, 70] and in the clinical setting.[71-73] 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 myocardial blood flow in the mouse heart were confined to buffer-perfused isolated heart models,[74-77] 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 myocardial blood flow in anesthetized mice, and these values were about 40% higher compared to the flows measured with microspheres. Raher *et al*[78] compared microsphere measurements of flow to those obtained with contrast echocardiography and similar to our results found a very 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 MCE measurement in units of db/sec. Therefore it is

difficult to relate our comparison of blood flows derived from MCE and microspheres to theirs. Magnetic resonance imaging (MRI) was used to measure myocardial perfusion in a murine model, and these investigators reported baseline flows in the range of 7 ml/min per g.[79] This range was less than our MCE measurements of about 12 ml/min per g 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 (Supplement 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 measurements—the impairment in metabolic dilation in Kv1.5$^{-/-}$ mice and restoration of flows to values comparable to wild type mice in the Kv1.5$^{-/-}$-RC mice in that values for all mice would be decreased by a certain factor and the differences would remain.

Conclusions

In the heart, vascular Kv1.5 channels play a critical role in coupling myocardial blood flow 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 cardiac work.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

ATP	adenosine triphosphate
CW	cardiac work
DP	double product
EF	ejection fraction
EPR	electron paramagnetic resonance
FS	fractional shortening
H_2O_2	Hydrogen peroxide
HR	heart rate
KCNA-5	Potassium voltage-gated channel subfamily A member 5 (Voltage-gated potassium channel subunit Kv1.5)

Kir	inward rectifier
Kv	channel voltage gated potassium channel
LVEDV	left ventricular volume at end diastole
LVESV	left ventricular volume at end systole
LVID,d-	left ventricular internal diameter at end diastole
LVID,s	left ventricular internal diameter at end systole
LV	left ventricle
MAP	mean arterial pressure
MBF	myocardial blood flow
MCE	myocardial contrast echocardiography
MI	mechanical Index
NIH	National Institute of Health pO2 oxygen tension
RBV	relative blood volume
RC	reconstituted
ROI	region of interest
rtTA	reverse tetracycline transactivator gene
SBP	systolic blood pressure
SV	stroke volume
TEA	tetraethylammonium
TP	triple product
VGCC	voltage-gated calcium channels
VSMCs	vascular smooth muscle cells
WT	wild type

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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_2O_2 from mitochondria during aerobic metabolism.
- H_2O_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.
- 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 non-obstructive coronary disease show impairments in flow regulation that can lead to myocardial ischemia.

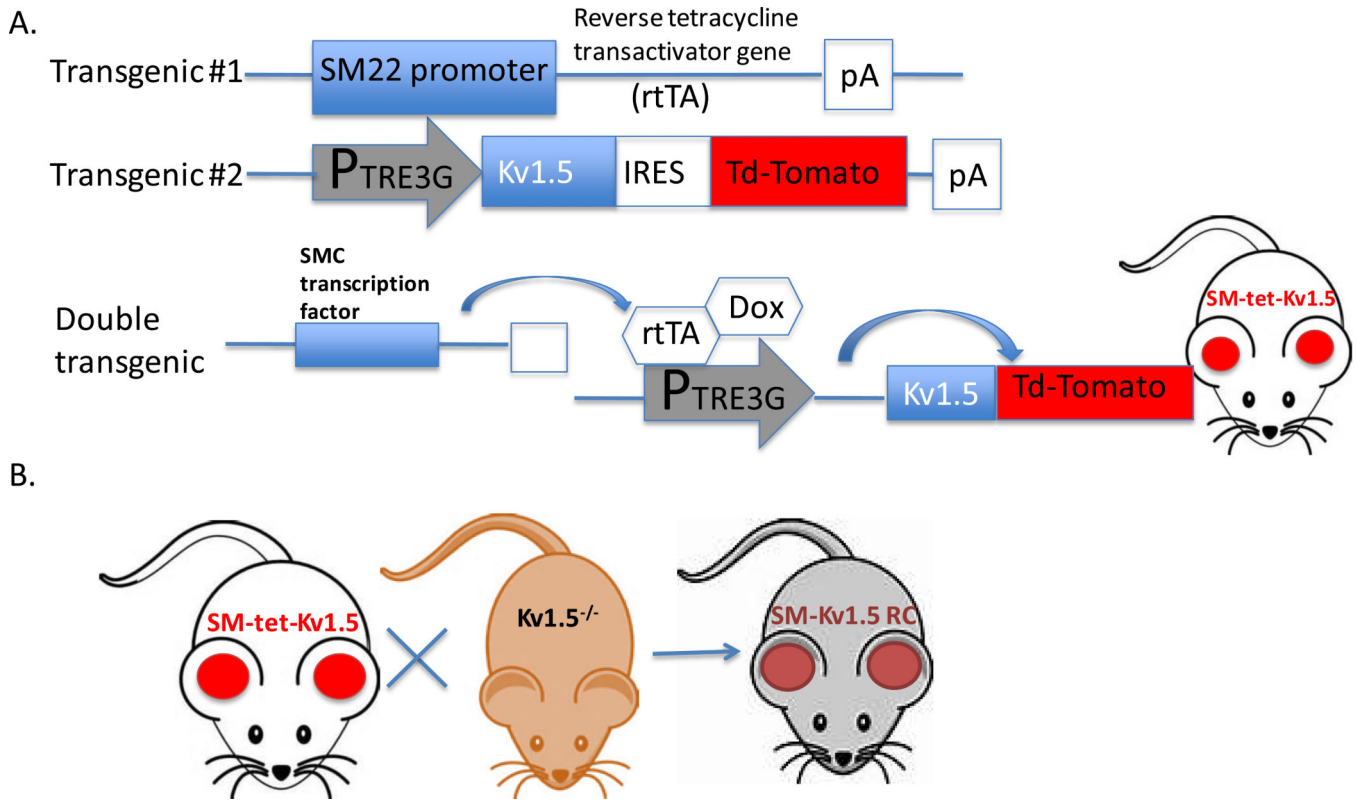

Figure 1.

A) Generation of the SM22-tet-KV1.5-Td-Tomato transgenic mouse. The generation of this mouse results in doxycycline-inducible, smooth muscle-specific expression of Kv1.5 channels. B) Smooth Muscle-Specific Expression of Kv1.5 channels in smooth muscle in Kv1.5 null mice. Crossing the SM-tet-Kv1.5 with $Kv1.5^{-/-}$ followed by subsequent back crossing enables the development of $Kv1.5^{-/-}$ mice with reconstituted doxycycline-inducible expression of Kv1.5 smooth muscle (SM-Kv1.5 RC).

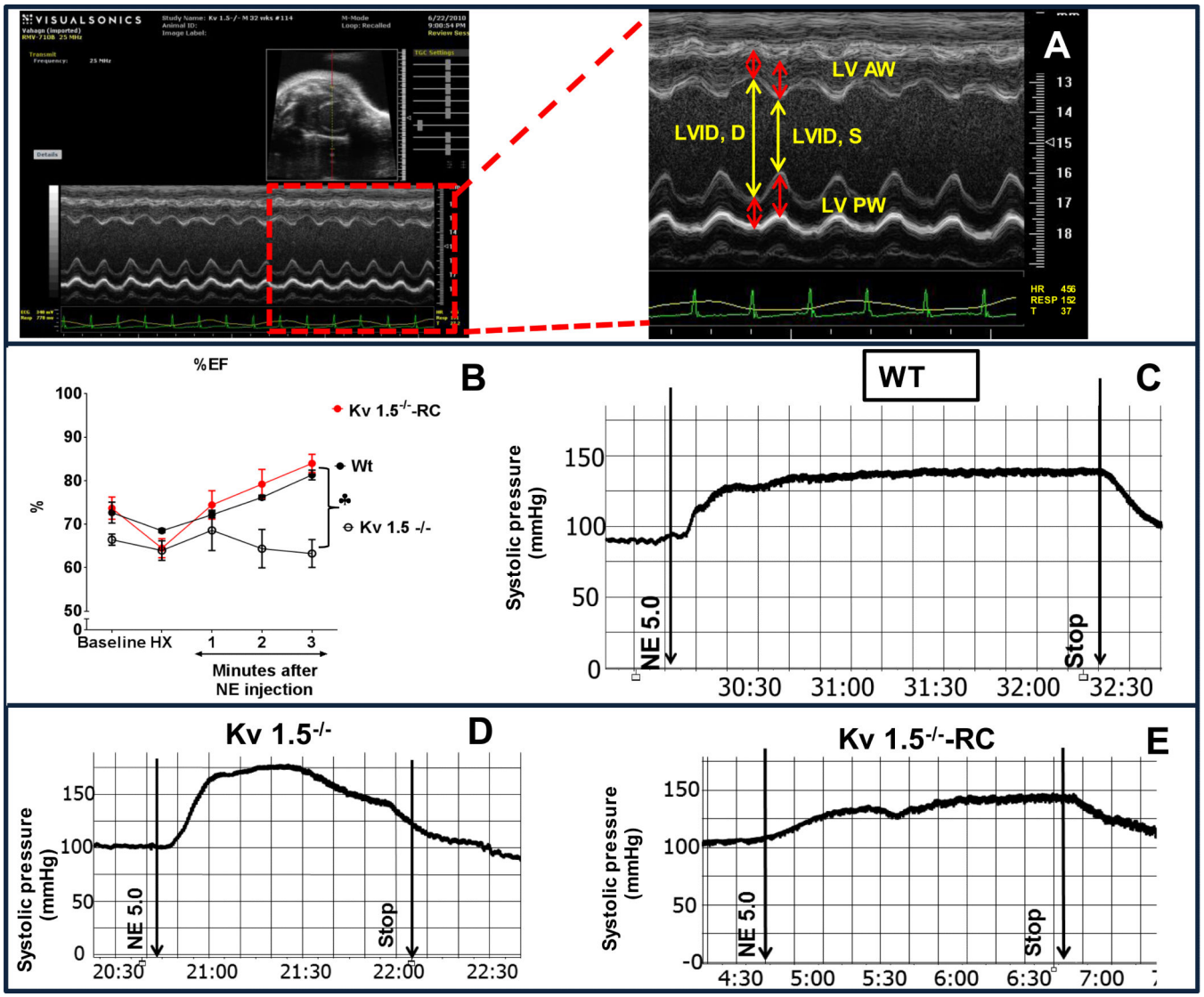

Figure 2. Cardiac Function and Hemodynamics

A) Transthoracic echocardiographic M-mode images at the mid-papillary muscle level. LVID- left ventricular internal diameter at diastole and at systole. LVAW- left ventricular anterior wall, LVPW-left ventricular posterior wall. B) Time course of changes in ejection fraction during norepinephrine infusion (5 μg/kg min^{-1}) in WT, $Kv1.5^{-/-}$, and Double Transgenic on the $Kv1.5^{-/-}$ background ($Kv1.5^{-/-}$-RC) with 7 days' doxycycline treatment. Note in the $Kv1.5^{-/-}$ EF decreased after one minute of NE infusion, whereas in the other groups EF rose during the duration of infusion. C) Systolic blood pressure (SBP) in Wild Type (WT) and D) in Kv $1.5^{-/-}$ mice under basal conditions and during infusion of norepinephrine (NE 5 μg/kg/min). Note, in $Kv1.5^{-/-}$ mice SBP started to decrease one minute after injection (prior to stopping the infusion). In contrast, in WT mice SBP increased during the infusion, and decreased only after NE infusion was stopped. E) Systolic blood pressure in double transgenic (Kv $1.5^{-/-}$-RC) after high dose of NE injection. In Kv

$1.5^{-/-}$-RC mice SBP was similar to WT mice and dropped only after NE infusion was stopped. Data are presented as Mean±SD, ♣-P<0.05 Kv $1.5^{-/-}$ mice compared to WT.

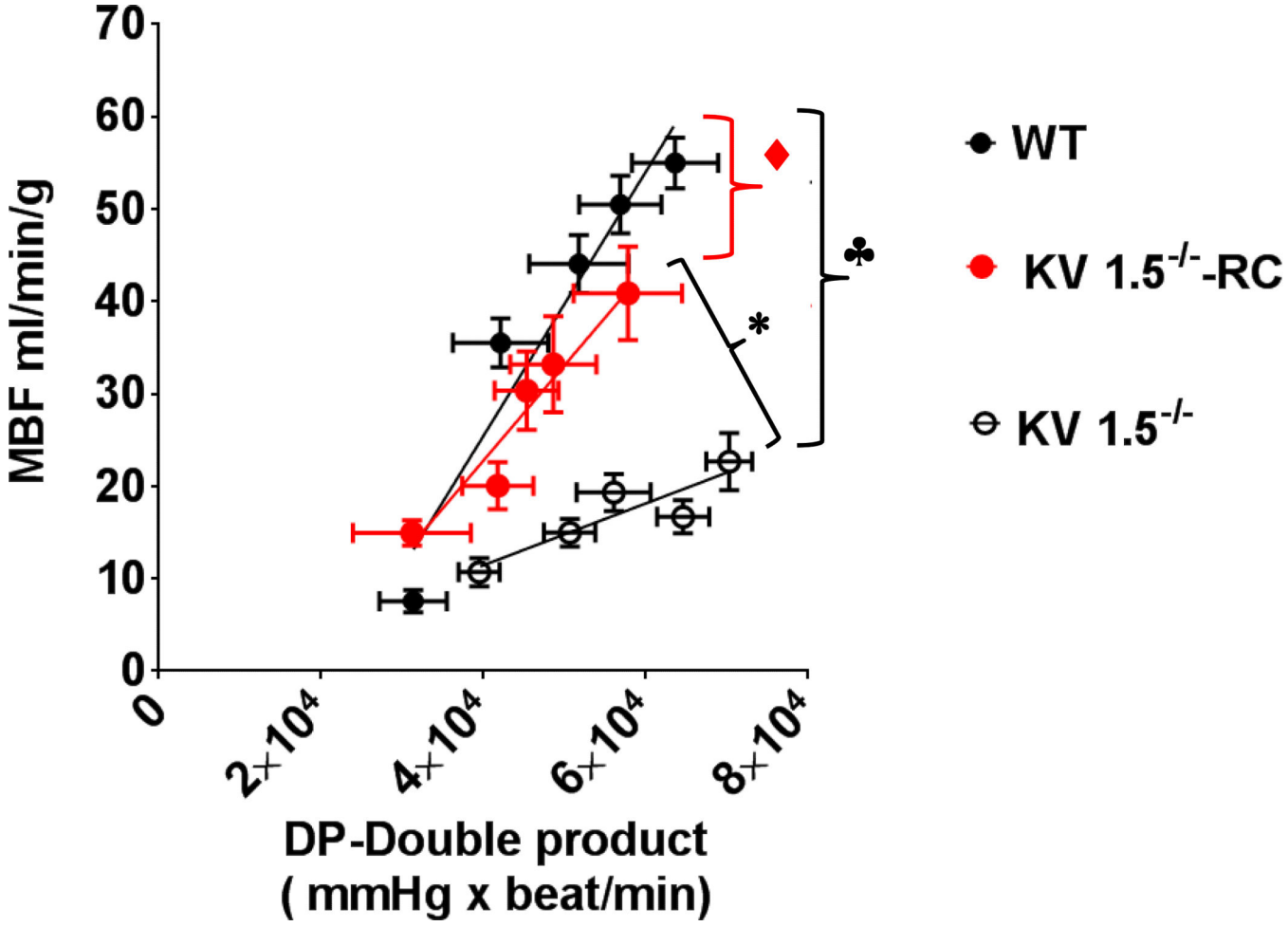

Figure 3. Relationship between Double Product (DP, mean arterial pressure X heart rate) and myocardial blood flow (MBF)

MBF and DP were measured at baseline (5-10 minutes after Hexamethonium) and during norepinephrine administration (0.5 -5.0 μg/kg/min iv,). In Kv $1.5^{-/-}$ mice MBF was significantly lower compared to WT mice at any given dose of NE (♣-P< 0.05). In Kv$1.5^{-/-}$ double transgenic mice (Kv$1.5^{-/-}$-RC) treated with doxycycline, MBF was comparable to WT at all levels of DP(♦- P=NS, Kv $1.5^{-/-}$-RC vs WT). MBF in Kv $1.5^{-/-}$-RC mice was significantly higher compared to Kv $1.5^{-/-}$ mice (⁎- P<0.05).

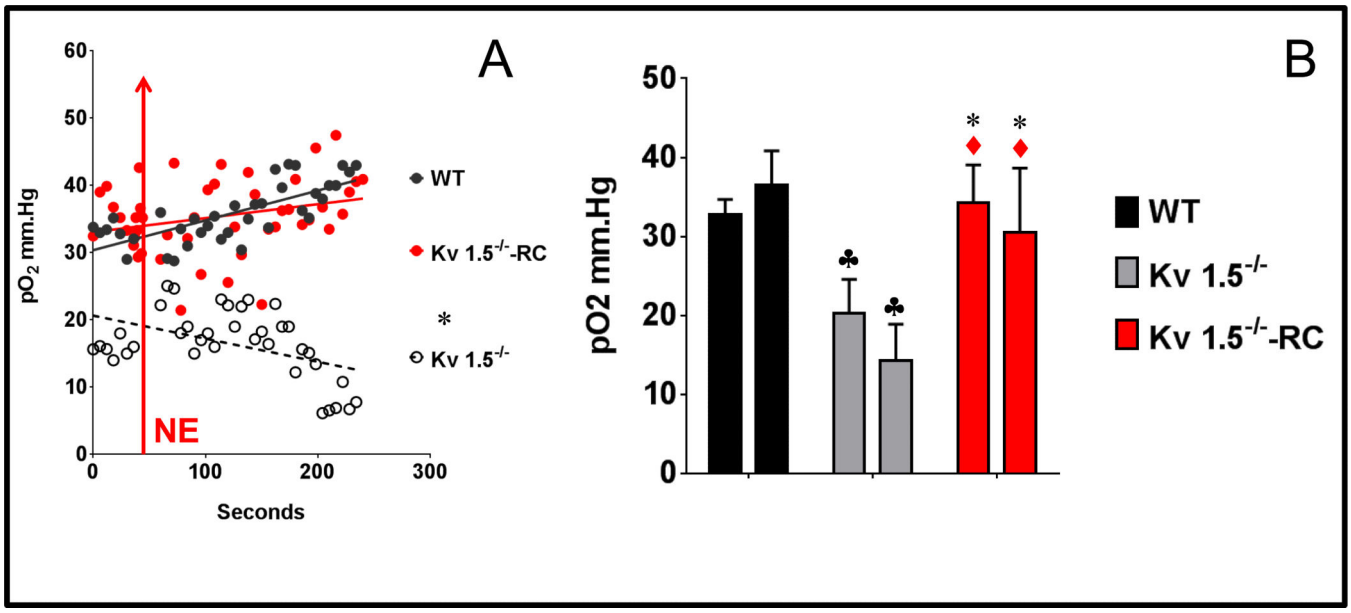

Figure 4. Myocardial oxygen tension (pO_2). A) Time course of pO_2 values in WT and Kv1.5$^{-/-}$ mice during the high dose of norepinephrine (NE, 5 μg/kg min^{-1}) (up to 4 minutes after the start of infusion)

Oxygen tension was significantly lower in Kv1.5$^{-/-}$ mice compared to WT mice and declined in Kv 1.5$^{-/-}$ during NE infusion indicating that myocardial oxygen demand increased beyond the capacity of supply, resulting in tissue hypoxia. **B**) Mean values of pO_2 at baseline and during NE; pO_2 was significantly lower at baseline and during NE injection in Kv1.5$^{-/-}$ vs WT (♣-P<0.05). in Kv 1.5$^{-/-}$-RC pO_2 was significantly higher at baseline and NE infusion (♦-P<0.05) compared to Kv 1.5$^{-/-}$ mice and was comparable to WT mice (∗-P=NS).

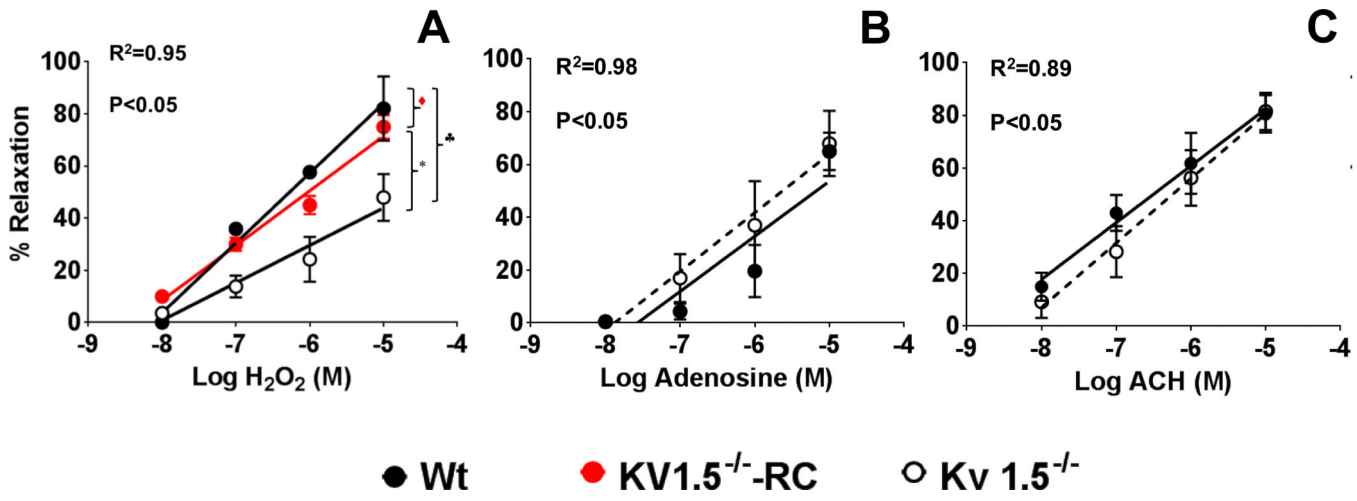

Figure 5. Isolated coronary microvessel responses to H_2O_2, adenosine and acetylcholine (ACH)

A) Vasodilatation response to hydrogen peroxide in coronary vessels isolated from Kv $1.5^{-/-}$ mice was significantly lower (♣-P<0.05) compared to vessels from WT mice or doxycycline-treated transgenic Kv $1.5^{-/-}$ mice (Kv $1.5^{-/-}$-RC, ∗-P<0.05). Vasodilation to H_2O_2 was not different between Kv $1.5^{-/-}$RC and WT mice (♦-P=NS). There were no significant differences between WT and Kv $1.5^{-/-}$ dose-response curves to adenosine (B) or acetylcholine (C).