ATP-sensitive potassium (K\textsubscript{ATP}) channels are widely expressed in the cardiovascular system, where they regulate a range of biological activities by linking cellular metabolism with membrane excitability. K\textsubscript{ATP} channels in vascular smooth muscle have a well-defined role in regulating vascular tone. K\textsubscript{ATP} channels are also thought to be expressed in vascular endothelial cells, but their presence and function in this context are less clear. As a result, we aimed to investigate the molecular composition and physiological role of endothelial K\textsubscript{ATP} channels. We first generated mice with an endothelial-specific deletion of the channel subunit Kir6.1 (eKO) using cre-loxP technology. Data from qRT-PCR, patch clamp, \textit{ex vivo} coronary perfusion Langerdorff heart experiments, and endothelial cell Ca\textsuperscript{2+} imaging comparing eKO and wild-type mice show that Kir6.1-containing K\textsubscript{ATP} channels are indeed present in vascular endothelium. An increase in intracellular [Ca\textsuperscript{2+}], which is central to changes in endothelial function such as mediator release, at least partly contributes to the endothelium-dependent vasorelaxation induced by the K\textsubscript{ATP} channel opener pinacidil. The absence of Kir6.1 did not elevate basal coronary perfusion pressure in eKO mice. However, vasorelaxation was impaired during hypoxia in the coronary circulation, and this resulted in greater cardiac injury during ischemia–reperfusion. The response to adenosine receptor stimulation was impaired in eKO mice in single cells in patch clamp recordings and in the intact coronary circulation. Our data support the existence of an endothelial K\textsubscript{ATP} channel that contains Kir6.1, is involved in vascular reactivity in the coronary circulation, and has a protective role in ischemia reperfusion.

ATP-sensitive potassium channels (K\textsubscript{ATP}) are expressed throughout the body including in the brain, pancreas, and cardiovascular system. Their ability to open in response to declining ATP and/or increasing ADP allows them to couple cellular metabolism with membrane excitability (1). As a result, they play an important role in the regulation of a range of biological activities such as insulin release, vascular tone, and adaptation to stresses such as exercise and ischemia (1). Structurally, K\textsubscript{ATP} channels form as hetero-octomeric complexes consisting of four pore-forming inward rectifier Kir6.x subunits (Kir6.1 or Kir6.2) and four large regulatory sulfonylurea receptor subunits (SUR1, SUR2A, or SUR2B) (1). These subunits associate in various tissue-specific combinations to form functional K\textsubscript{ATP} channels, and these currents can have distinct pharmacological and electrophysiological properties. K\textsubscript{ATP} channels in the vasculature have been implicated in the regulation of tone and blood flow (2–6). The function and molecular identity of the vascular smooth muscle cell K\textsubscript{ATP} channel has been the subject of intensive research over the past 25 years and has been aided by the development of a number of genetically modified murine models (1, 7). Global deletion of Kir6.1 and SUR2 were initially used to explore the role of K\textsubscript{ATP} channels and ascertain the contribution of these subunits to the K\textsubscript{ATP} current in smooth muscle cells. Both KO mice had a similar hypertensive phenotype consistent with previous \textit{in vitro} electrophysiological data, suggesting a role for vascular smooth muscle K\textsubscript{ATP} channels in the regulation of resting membrane potential and vascular tone (2, 3). Although informative, a global genetic deletion of Kir6.1 or SUR2 is not selective for the smooth muscle channel, and potentially channels in the endothelium, nervous system, and heart might all be affected and contribute to the observed phenotype. Recently, a tissue-specific targeting strategy has substantially clarified the role of K\textsubscript{ATP} channels in smooth muscle (1, 4). Mice expressing a smooth muscle-specific gain of function mutant K\textsubscript{ATP} channel have low blood pressure (4). In addition, we developed a mouse model in which it was possible to conditionally delete Kir6.1 in smooth muscle cells. Mice with a conditional vascular smooth muscle K\textsubscript{ATP} deletion lacked a functional K\textsubscript{ATP} current in smooth muscle cells and were moderately hypertensive consistent with Kir6.1 being the pore-forming subunit of these channels and Kir6.1-containing channels playing a role in the regulation of blood pressure (5). However, the specific deletion of Kir6.1 did not fully recapitulate the global Kir6.1 KO phenotype, suggesting that Kir6.1-containing channels in other tissues are also important in the regulation of vascular tone (5, 6). Potentially, K\textsubscript{ATP} channels in many cell types could be involved in the control of vascular function, but given its control over vasoactive substances, the endothelium is the most likely candidate. Indeed, based mainly on pharmacological evidence, K\textsubscript{ATP} channels in the endothelium have been shown to exist and contribute to the regulation of blood flow in the coronary vasculature (8–13). The vasodilatory actions of adenosine may be in part due to its effect on

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endothelial $K_{ATP}$ channels (14). Pharmacological approaches, however, suffer from a lack of tissue selectivity, especially given the prominent expression of $K_{ATP}$ channels in striated and smooth muscle. The expression of a dominant-negative Kir6.1 in endothelium leads to the increased release of endothelin-1 and increased basal coronary perfusion pressure (15). However, such dominant-negative approaches are not subunit-specific, because Kir6.1 and Kir6.2 heteromultimerize and the high expression of such constructs can have off-target effects (16). Kir6.1 is thought to be the significant but not the only pore-forming subunit of the endothelial $K_{ATP}$ channel, with Kir6.2 thought to also be involved (17).

Thus in this report, we investigate the role of Kir6.1 containing $K_{ATP}$ channels in the endothelium on vascular function, particularly in the coronary circulation, by using cre-loxP technology to generate an endothelial-specific Kir6.1/Kir6.2 KO mouse model. We show that Kir6.1 is the likely pore-forming subunit of the vascular endothelial $K_{ATP}$ channel. The channel is involved in vascular function and has an important protective role in metabolically challenging conditions.

Results

Kir6.1 expression is reduced in the endothelium from the aorta and mesenteric arteries of Kir6.1 knock-out mice

In our previous study, we used endothelium denuded aorta and other blood vessels for expression and functional studies (5). We used cre-loxP technology to specifically delete Kir6.1 ($Kcnj8$) in endothelium (Ref. 5 and Fig. 1, A and B). To confirm the reduced expression of Kir6.1 ($Kcnj8$) in the endothelium of tie2cre+ Kir6.1(flx,flx) (eKO) mice, quantitative real-time PCR was performed on RNA isolated from the aorta and mesenteric arteries with and without endothelium (Fig. 1C and D). Kir6.1 expression was substantially reduced in endothelium-intact aorta (40%) and mesenteric arteries (40%) from eKO mice compared with littermate control animals (WT) ($n = 6$, $p < 0.01$ and 0.001, respectively). In both vessel types where the endothelium had been denuded, Kir6.1 expression was reduced to a similar level in both the WT and eKO mice and was comparable with the amount of deletion observed in endothelium-intact vessels from the eKO mice ($p > 0.05$). The expression of Kir6.2 ($Kcnj11$) was not affected in either the intact aorta or mesenteric arteries in either mouse line ($n = 6$, $p > 0.05$). Interestingly, expression of the Kir6.2 transcript was also unchanged in the denuded vessels ($n = 6$, $p > 0.05$). SUR2 (Abcc9) expression was significantly reduced in the denuded aorta and mesenteric arteries of endothelium-specific Kir6.1 knock-out mice, suggesting that SUR2 was the major endothelial SUR subunit (Fig. 1C and D). However, the effect of endothelial removal was more pronounced in the eKO mice, suggesting perhaps some compensatory up-regulation. SUR1 subunit was not expressed in either aorta or mesenteric arteries (data not shown).

$K_{ATP}$ current is attenuated in acutely isolated aortic endothelial cells from endothelium-specific Kir6.1 KO mice

To investigate the functional relevance of Kir6.1 deletion on the $K_{ATP}$ current in the endothelium, whole-cell patch clamp recordings were performed on acutely isolated aortic endothelial cells from WT and eKO mice (Fig. 2). Endothelial cells from WT mice show a K+ selective current that was activated by the $K_{ATP}$ opener pinacidil ($p < 0.05$) and inhibited by the $K_{ATP}$ blocker glibenclamide (Fig. 2, A, C, and D). In contrast, currents from eKO endothelial cells lacked a response to pinacidil ($p > 0.05$) or glibenclamide, suggesting that the $K_{ATP}$ current is absent or present at very low levels (Fig. 2, B–D). It is possible that deletion of Kir6.1 from endothelium may lead to changes in expression in smooth muscle cell $K_{ATP}$ currents (SMCs). To see whether this is the case, we subjected acutely isolated aortic smooth muscle cells from eKO mice to whole-cell patch clamp (Fig. 2, E–H). Both WT and eKO vascular SMCs showed a similar amplitude glibenclamide-sensitive current ($p > 0.05$).

Pinacidil-induced elevation of cytosolic [Ca$^{2+}$] is absent in endothelium-specific Kir6.1 knock-out mice

Ca$^{2+}$ is an important mediator of endothelial cell function including release of vasoactive species. To further characterize the specific deletion of Kir6.1 in the endothelium, we studied mouse aortic valve (MAV) leaflets using an imaging-based approach to measure Ca$^{2+}$ dynamics in the cytosol of endothelial cells (18). It is technically highly challenging to selectively image Ca$^{2+}$ signals in endothelial cells in blood vessels because endothelial cells are flat, and there will be contamination of the endothelial fluorescent signal from overlaying smooth muscle cells, which will also take up Fluo-4 even using confocal microscopy. This methodology excludes the possibility of vascular smooth muscle cells contaminating the Ca$^{2+}$ signal (Fig. 3). In both WT and eKO MAV endothelial cells, Fluo-4 fluorescence was elevated to a similar extent ($F/F_0 \sim 3.0$) by the SERCA inhibitor, cyclopiazonic acid (CPA) (100 μM), which initiates Ca$^{2+}$ entry through Ca$^{2+}$ store depletion ($p < 0.001$). Pinacidil (10 and 100 μM) also significantly increased cytosolic Ca$^{2+}$ in a concentration-dependent manner in WT MAV endothelial cells ($p < 0.05$ and $p < 0.001$ compared with baseline), although it was smaller ($F/F_0 \leq 1.5$) than those induced by CPA. The pinacidil-induced increase in cytosolic Ca$^{2+}$ was completely inhibited by prior application of 10 μM glibenclamide. In contrast, in eKO MAV endothelial cells, pinacidil failed to significantly increase cytosolic Ca$^{2+}$ from baseline levels ($p > 0.05$). These data support the presence of Kir6.1 and its involvement in Ca$^{2+}$ signaling in the endothelial cells of MAVs.

Endothelial $K_{ATP}$ channels in the coronary vasculature

To investigate the presence and role of Kir6.1-containing $K_{ATP}$ channels in the endothelium of the coronary circulation, we measured coronary perfusion pressure (CPP) at a constant flow rate. Basal CPP was higher in hearts from eKO mice compared with WT mice, but this increase was not statistically significant (93.1 ± 4.4 mmHg versus 103.5 ± 6 mmHg, $n = 23$, $p > 0.05$). Pinacidil (10 μM) significantly reduced CPP in both WT and eKO mice (Fig. 4), although the reduction of CPP in eKO (17%) mice was significantly less compared with WT (35%, $p < 0.05$). The addition of glibenclamide (10 μM) reversed the pinacidil-induced...
fall in CPP back to baseline (Fig. 4, A–C). We also compared this with mice in which Kir6.1 is deleted from smooth muscle (see “Experimental procedures” and Ref. 5), and in this case the response to pinacidil was also reduced but not completely abolished (Fig. 4). The basal CPP was not significantly different.

Function of endothelial K\textsubscript{ATP} channels in the coronary circulation

To examine the role of the endothelial K\textsubscript{ATP} at times of metabolic stress, we exposed the coronary circulation to local hypoxia with a perfusate saturated with N\textsubscript{2} and measured the change in CPP during that period. Both WT and eKO hearts responded with a significant drop in pressure; however, the effect was much larger in WT (37%) hearts compared with eKO (10%) hearts (Fig. 5A, \(p < 0.01\)). In the smKO mice, there was a trend to reduction in the hypoxic response (WT, 37%; eKO, 20%), although this was not statistically significant (Fig. 5A).

Additionally, hearts from all groups of mice were subjected to global ischemia (no flow) followed by reperfusion. Staining of sections from these hearts with 2,3,5-triphenyltetrazolium...
chloride (TTC) revealed a greater infarcted area in Kir6.1 eKO (77%) hearts than in WT (56%) hearts (Fig. 5B). Taken together, these data suggest that endothelial Kir6.1-containing KATP channels in the coronary circulation may have a protective role in the heart during ischemia–reperfusion injury. Hearts from smKO mice (Fig. 5C) also had an increase in infarcted area (p < 0.05) compared with littermate controls, suggesting that both the smooth muscle and endothelial KATP channel are important in limiting the ischemic damage. In our hands there is no deletion of Kir6.1 in cardiac tissues using the sm22 cre, and thus these effects likely derive from the deletion in smooth muscle (5).

Expression and role of Kir6.2 in vascular endothelium

The endothelium of blood vessels is postulated to express KATP channels containing Kir6.2, as well as Kir6.1. To test for this, we used a mouse model in which Kir6.2 was selectively deleted from endothelium using a cre driven by the Tie2 promoter (see “Experimental procedures”). Whole-cell patch clamp recordings from endothelial cells isolated from the aortas of WT and Kir6.2 eKO mice show that the amplitude of the pinacidil and glibenclamide-sensitive current is not affected by the ablation of Kir6.2 (Fig. 6A–C). Next, we investigated whether deletion of Kir6.2 in endothelium affected the function of the coronary circulation. The data from the Langendorf experiments show that hearts from WT and Kir6.2 eKO mice have similar basal CPP values, suggesting that Kir6.2-containing KATP channels are not involved in regulating basal vascular tone in the coronary circulation (Fig. 6D). In addition, the pinacidil and hypoxia response in Kir6.2 eKO mouse hearts was not significantly different from WT hearts (Fig. 6E, p > 0.05). Furthermore, there was no difference in infarct size between Kir6.2 eKO and WT hearts following ischemia–reperfusion (Fig. 6F, p > 0.05).
which in turn activates adenosine receptors in vascular smooth muscle and endothelial cells. Thus we investigated whether this was the case and isolated K\textsubscript{ATP} currents in endothelial cells as described above. In WT endothelial cells, NECA, an adenosine receptor agonist not metabolized by adenosine deaminase, was able to activate K\textsuperscript{+} currents (p < 0.01). In contrast, in endothelial cells from eKO mice, no such increase of current was seen (Fig. 7, A–C). We also investigated changes in perfusion pressure in the coronary circulation in response to NECA. NECA induced a substantial fall in CPP (−42%) comparable with that of hypoxia. This response was significantly reduced in the eKO mice (−30%, p < 0.05) (Fig. 7, D and E).

**Discussion**

The major findings in this study are that an endothelial K\textsubscript{ATP} channel exists composed of Kir6.1 and likely SUR2B. Activation of the channel can promote Ca\textsuperscript{2+} entry into endothelial cells. In the coronary circulation, the channel contributes significantly to the vasodilation in response to hypoxia and loss of Kir6.1 in the endothelium increases cardiac injury after ischemia reperfusion. The endothelial K\textsubscript{ATP} channel responds to adenosine receptor activation, and this regulation is impaired in eKO cells and blood vessels. In earlier studies, we and others have shown the important contribution of vascular smooth muscle K\textsubscript{ATP} channels comprising the Kir6.1 subunit to blood vessel tone and the regulation of blood pressure (2–5). The location of the endothelium as a first point of contact for sensing circulatory changes, its ability to synthesize vasoactive mediators (19), and direct electrical coupling to vascular smooth muscle cells allow the endothelium to govern vascular smooth cell tone (20). To further understand the role of K\textsubscript{ATP} channels in the vasculature, we have used the cre-loxP system to create an endothelium-specific Kir6.1 knock-out mouse model. In this study, we particularly focused on establishing the presence of this channel and its potential role in vascular physiology. Our data show the significant contribution of Kir6.1-containing K\textsubscript{ATP} channels to the current in the endothelium. Although endothelial K\textsubscript{ATP} currents have been shown in a number of vessels and species (8, 9, 11), the subunit composition of these channels was not systematically investigated except in one study where a heteromultimer of Kir6.1 and Kir6.2 was proposed using a human coronary endothelial cell line (17). We used a different approach by selectively deleting Kir6.1 in murine endothelial cells. Our data show that Kir6.1 expression in the endothelium

**Figure 3. Pinacidil-induced elevation of cytosolic [Ca\textsuperscript{2+}] is attenuated in eKO mouse aortic valves.** A, confocal images showing a pinacidil-evoked concentration-dependent elevation in cytosolic [Ca\textsuperscript{2+}] in WT mice. 100 μM CPA (sarcoplasmic reticulum uptake inhibitor) was used as a control to induce an increase in cytosolic [Ca\textsuperscript{2+}] at baseline. B, confocal images showing the effect of CPA and pinacidil on cytosolic [Ca\textsuperscript{2+}] in valves from eKO mice. Scale bar represents 25 μm. C, summary time-course traces (S.E. in the dashed lines) illustrating the effects of 100 μM CPA, 10 and 100 μM pinacidil (Pin), and 10 μM glibenclamide (Glib) on cytosolic [Ca\textsuperscript{2+}] in aortic valve cells from WT (black) and eKO (gray) mice. Changes in cytosolic [Ca\textsuperscript{2+}] are expressed as a relative fluorescence ratio, F/F\textsubscript{0}. D, bar graph showing the mean data from traces in C. The data are shown as means ± S.E. (n = 6 mice). *, p < 0.05; ***, p < 0.001 compared with WT; ++, p < 0.001 compared with WT 10 μM pinacidil.
and transcriptomic analysis confirmed deletion of Kir6.1 in the eKO murine line. Functionally, whole-cell patch clamp recordings using an intracellular solution favorable for activity of both Kir6.1 and Kir6.2 subunit containing K_{ATP} currents (0.1 mM ATP and 1 mM ADP) from acutely isolated aortic endothelial cells confirmed the presence of a pinacidil and glibenclamide-sensitive current. This current was not detectable in endothelial cells from Kir6.1 eKO mice, suggesting the presence of Kir6.1-containing K_{ATP} channels in vascular smooth muscle cells (18). We found that deletion of Kir6.1 from endothelium prevented the pinacidil-induced increase in cytoplasmic Ca^{2+}. The increase in [Ca^{2+}]_{i} is likely because of K_{ATP} opening leading to membrane hyperpolarization and as a result initiating a change in the electrochemical gradient favorable to Ca^{2+} influx from the extracellular space. In contrast to vascular smooth muscle cells, endothelial cells do not prominently express voltage-gated Ca^{2+} channels, and Ca^{2+} entry occurs through transient receptor potential channels and store-operated entry (21). Changes in the response to Ca^{2+} store-depletion seem an unlikely mechanism because there was no difference in the effects of the SERCA inhibitor CPA between the lines. Finally, the effect of pinacidil on CPP was also reduced in eKO mice, suggesting the presence of Kir6.1-containing K_{ATP} channels in the mouse coronary circulation. The basal CPP was not significantly elevated in eKO hearts, although there was a trend to an increase. This is in contrast to the finding in an endothelial-specific dominant-negative mouse model, where the CPP was substantially elevated, and this discrepancy might reflect differences in the experimental strategy (15). In an analogous vein, knock-out of Kir6.1 in vascular smooth muscle cells also did not affect the basal CPP. It is known that glibenclamide can inhibit basal coronary flow, and it may be that combined inhibition of both vascular smooth muscle and endothelial K_{ATP} channels is necessary for this to occur (22, 23). Deletion of Kir6.1 does not rule out the possibility of Kir6.1-Kir6.2 heteromultimers. To investigate this, we used an endothelial-specific Kir6.2 KO mouse and looked at whole-cell K^+ current in aortic endothelial cells and CPP in isolated hearts. We found that the K_{ATP} current was largely unaffected in Kir6.2 eKO mice, and the CPP was not significantly different at rest or during challenge with pinacidil and hypoxia. The previous study showing a Kir6.1/Kir6.2 heteromultimer used a human cell line, and it is possible there are species differences. For example, SUR1 is preferentially expressed in the atria of mice, but this is not prominent in man (24, 25).

Figure 4. Kir6.1 containing K_{ATP} channels are present in both endothelium and smooth muscle of the coronary circulation. A, mean basal CPP in eKO and smKO mice and their littermate controls (n = 9–23). CPP was measured using the Langendorff set-up under constant flow. B, representative CPP traces from Langendorff hearts from eKO, smKO mice, and their littermate controls (WT, top panels; KO, lower panels) in response to 10 μM pinacidil (Pin) and 10 μM glibenclamide (Glib). C, summary of the mean change in CPP (relative to baseline) of WT and eKO mouse hearts in response to 10 μM pinacidil and 10 μM glibenclamide. The data are shown as means ± S.E. (n = 5–10 mice). *, p < 0.05; **, p < 0.01 compared with WT.

Figure 5. Endothelial K_{ATP} channels containing Kir6.1 may be protective during metabolic challenge. A, mean CPP of eKO and smKO mouse hearts and hearts from their respective littermate controls in response to hypoxia (Krebs solution gassed with 95% N2/5% CO2) (n = 6–10). CPP was measured using the Langendorff set-up under constant flow. B and C, representative sections (left panel) and mean infarct size (right panel) following 30 min of global ischemia and 60 min of reperfusion of eKO (B) and smKO (C) mouse hearts stained with 1% TTC; pale tissue signifies infarction. The data are shown as means ± S.E. (n = 5–16 mice). *, p < 0.05; **, p < 0.01 compared with WT.
One of the major gains of our tissue-selective strategy is that it allows us to explore the contribution of the endothelial channel to whole animal cardiovascular physiology. One potentially important role was revealed when we studied the coronary circulation. In the coronary arteries of eKO mice, hypoxia-induced vasodilation was reduced by more than 65%. Although there was a trend for a reduction in the hypoxic response in coronary smooth muscle cells with Kir6.1 deletion, it was not as pronounced as that for endothelial deletion. This suggests that the endothelial channel may be of preeminent importance as a hypoxic sensor in the coronary circulation. The pronounced changes in coronary perfusion pressure and loss in eKO mice may mean it is particularly important in resistance vessels.

Figure 6. Ablation of Kir6.2 does not attenuate K_ATP current in aortic ECs and is not protective in IR injury. A, representative time-course traces at +40 mV taken from ECs isolated from WT (left panel) and Kir6.2 eKO (right panel) mice showing the effects of pinacidil (Pin) and glibenclamide (Glib). Currents were elicited using a 1-s ramp protocol (−150 mV to +50 mV) from a holding potential of −80 mV. B, summary of the mean current densities at +40 mV from ECs isolated from WT (left panel) and Kir6.2 eKO (right panel) mice. C, glibenclamide-sensitive current in ECs from WT and Kir6.2 eKO mice (n = 9–14 cells from three mice). D, mean basal CPP of WT and Kir6.2 eKO mouse hearts (n = 10–13). CPP was measured using the Langendorff set-up under constant flow. E, mean change in CPP (relative to baseline) in the presence of pinacidil, glibenclamide, and hypoxia of WT and Kir6.2 eKO hearts (n = 5–7). F, representative sections (left panel) and mean infarct size (right panel) following 30 min of global ischemia and 60 min of reperfusion of WT and Kir6.2 eKO mouse hearts stained with 1% TTC, pale tissue signifies infarction (n = 11). The data are shown as means ± S.E. *** p < 0.001 compared with control.

We also challenged the hearts to a period of ischemia reperfusion injury. In eKO hearts, this led to a ~20% increase in cardiac tissue death compared with littermate controls. These data support the idea that K_ATP channels in endothelium may have a protective role in times of metabolic stress. Specifically they may contribute together with the smooth muscle channel in matching blood flow to tissue metabolic demand and thus act to preserve tissue. The cooperation of the smooth muscle channel in this response is supported by the increased tissue injury also seen in the smKO mouse. It is also possible that the endothelial channel might have a more general protective function on surrounding tissues through the release of endothelial mediators (26). Even more broadly some protective functions attrib
uted to potassium channel openers on the heart may occur because of the activation of endothelial KATP channels (13). The endothelial channel is responsible because endothelial deletion of Kir6.1 does affect smooth muscle KATP currents.

One of the major potential mediators in the hypoxic response is the local release of adenosine from metabolically challenged tissue. It has been suggested for some time that channel activation occurs as much through hormonal regulation via adenosine receptors as direct metabolic sensitivity of the KATP channel complex (27). It is known that the vascular KATP channel can be activated by signaling coupled to the stimulatory G-protein and downstream activation of adenylate cyclase and protein kinase A (28–32). Subsequent studies in heterologous expression systems identified likely serine threonine residues on Kir6.1 and SUR2B subunits responsible for direct protein kinase A phosphorylation (33, 34). There is some disagreement as to the exact adenosine receptor responsible for the signaling, but it is known that A2A and A2B both couple to the stimulatory G-protein and activate protein kinase A signaling (30, 35). However, less is known about KATP in endothelial cells (14).

Our data show that NECA can activate KATP currents in endothelial cells and this is abrogated in eKO endothelial cells. Furthermore, the response of coronary artery blood vessels to NECA is also impaired, consistent with impaired regulation of the endothelial KATP channel.

A key question remains: how do endothelial cells couple to smooth muscle cells and what is the role of the KATP channels in that coupling? We have shown that activation of the channel leads to Ca2+ entry, which is a prerequisite for endothelial mediator release (36). Given the extensive work of others, we do not think that these channels necessarily contribute to the release of K+ acting as an endothelial hyperpolarizing factor (37, 38). The other issue is that the particular set of mediators is likely to vary with vascular bed and the nature of the vessel, whether it be conduit or resistance. The exact delineation of this is best addressed by separate studies.

In conclusion, we have established the identity of an endothelial KATP channel and shown that it can influence Ca2+ entry into the cells. The endothelial KATP channel in the coronary circulation plays an important role in vasodilatation to hypoxia and limiting ischemia reperfusion injury in the heart.

Experimental procedures

Animal husbandry

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the British Home Office regulations (covered by Project Licenses PPL/6732 and PPL/7665) and by the National Institutes of Health (Publication No. 85-23, revised 1996).

Generation of the endothelium-specific Kir6.1 mouse strains

Kir6.1(+/flx) mice were previously generated in our laboratory in collaboration with Genoway (Lyon, France; project number genOway/EV/TIN1-Kcnj8 070206), the detailed method has been described previously (5). Endothelium-specific Kir6.1 KO mice were generated by crossing endothelium

Figure 7. Effect of the adenosine agonist NECA is reduced in aortic ECs and the coronary circulation of eKO mice. A, representative time-course traces at +40 mV taken from ECs isolated from WT (left panel) and eKO (right panel) mice showing the effects of NECA. B, representative current-voltage traces from a WT (left panel) and eKO (right panel) cell in the presence and absence of NECA. Currents were elicited using a 1-s ramp protocol (−150 to +50 mV) from a holding potential of −80 mV. C, summary of the mean current-densities at +40 mV from ECs isolated from WT (left panel) and eKO (right panel) mice (n = 8–9 cells from three mice). D, representative CPP traces from Langendorff hearts from WT and eKO mice challenged with NECA and glibenclamide. E, mean change in CPP (relative to baseline) of WT and eKO mouse hearts in the presence of NECA (n = 6–10). CPP was measured using the Langendorff set-up under constant flow. The data are shown as means ± S.E. *, p < 0.05; **, p < 0.01 compared with control/WT.
Tie-2 promoter driven cre-transgenic mice (tie2 cre; The Jackson Laboratory) with Kir6.1 homozygous floxed (Kir6.1(flx/flox)) mice. A further cross of the offspring resulted in genotypes of tie2cre+ Kir6.1(flx/flox) (eKO) and littermate controls. Similarly, smooth muscle-specific Kir6.1 KOs (smKO) were generated by crossing Kir6.1(flx/flox) mice with mice expressing a sm22 promoter driven cre to give sm22 cre+ Kir6.1(flx/flox) mice. Littermate controls are referred to as wild-type mice below.

**Generation of the endothelium-specific Kir6.2 mouse strains**

Kir6.2 (+/flx) mice were generated by the IMPC (Harwell). Detailed strategy including targeting constructs can be found on the International Mouse Phenotyping Consortium website. The conditional floxed allele (tm1c) was generated from the targeted KO first allele (tm1a, with a reporter-tagged insertion with conditional potential) by flp-mediated excision of the reporter tag and neomycin selection cassette. Kir6.2 floxed mice were crossed with endothelium Tie-2 promoter driven cre-transgenic mice to generate tie2cre+ Kir6.2 (flx/flx) (Kir6.2 eKO) and littermate controls.

**Genotyping**

Genomic DNA was prepared from ear biopsies for genotyping by PCR using standard cycling parameters. The Kir6.1 floxed allele was identified using the following primers: sense 5′-ACTAGCACCTCTATCCCCCGCTCTACC-3′ and antisense 5′-CCGCCCCTCCTCTGAACTATAC-3′ yielding WT bands of 458 bp and floxed allele bands of 600 bp. The presence of the cre recombinase gene in the Kir6.1 endothelium-specific KO line was determined using the following primers: sense 5′-CCCTCTTGCATGACAGAAATGAG-3′ and antisense 5′-CGATAACGATGAAACGATGC-3′, yielding a band of 512 bp in cre positive mice and no band in cre negative mice. The presence of the cre recombinase gene in the Kir6.1 smooth muscle-specific knock-out line was determined using the following primer set: sense 5′-CCATTTCTGACGCTACCC-3′ and antisense 5′-GTTCATATCCAGGTGTAC-3′, yielding a band of 900 bp in cre positive mice and no band in cre negative mice. Both the floxed and cre PCRs used Taq polymerase (New England Biolabs). The presence of the WT and Kir6.2 floxed alleles was detected using the following primer sets: WT (110-bp band), sense 5′-CTGTCCGAAAAGGCCATT-3′ and antisense 5′-AGTGTGGGCCATTGG-3′; and for the floxed allele (190-bp band), sense 5′-CTGTCCGAAAGGCCATT-3′ and antisense 5′-GAACCTCCTGGAGATGGACCTCG-3′.

**qRT-PCR**

The RNeasy kit (Qiagen) was used to extract the total RNA from mouse tissues, treated with DNase I, and reverse-transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems). cDNA (50 ng) was used for qRT-PCR, which was performed using customized TaqMan gene expression assays (Applied Biosystems). Commercially available probes (Life Technologies) were used for all K\textsubscript{ATP} channel subunit genes as listed below: Mm00434620_m1 for *Kcnj8* (Kir6.1), Mm00441638_m1 for *Abcc9* (SUR2). Each gene was assayed in triplicate, and relative expression was calculated by using the comparative CT method normalized to GAPDH. Aortas were denuded with forceps and mesenteric arteries with coarse horse hair. The data are presented as a relative change compared with WT + endothelium.

**Ca\textsuperscript{2+} imaging of aortic valve leaflets**

To assess the intracellular calcium concentration in the cytosol of endothelial cells ((Ca\textsuperscript{2+})), aortic valve leaflets from the mouse heart were dissected out, mounted into a custom-made glass-bottomed 35-mm Petri dish (MatTek), and placed in HEPES buffer of the following composition: 10 mm HEPES, 140 mm NaCl, 5 mm KCl, 10 mm glucose, 1 mm CaCl\textsubscript{2}, pH 7.4 (18). The valves were then incubated with the Ca\textsuperscript{2+} indicator Fluo-4 (2 μm) in HEPES buffer at room temperature for 2 h followed by a wash with indicator-free buffer for 30 min. The valve leaflets were imaged on a Zeiss LSM510 confocal microscope. Changes in Fluo-4 fluorescence following experimental interventions were normalized to the value at the beginning of each experiment (F0).

**Measurement of coronary perfusion pressure**

The Langendorff isolated perfused heart system was used to measure basal CPP and the effects of the K\textsubscript{ATP} channel opener pinacidil and blocker glibenclamide and in hypoxic and ischemic conditions. Briefly, the mice were injected with heparin sodium (250 IU) and anesthetized with a combination of ketamine/xylazine. The hearts were rapidly excised and placed in ice-cold Krebs-Henseleit solution (containing 118 mm NaCl, 4.75 mm KCl, 1.19 mm KH\textsubscript{2}PO\textsubscript{4}, 25 mm NaHCO\textsubscript{3}, 1.19 mm MgSO\textsubscript{4}, 7H\textsubscript{2}O, 1.4 mm CaCl\textsubscript{2}, 2 mm sodium pyruvate, and 10 mm glucose) equilibrated with 95% O\textsubscript{2}/5% CO\textsubscript{2}, pH 7.4. The aorta was located and cannulated with a 20-gauge aortic cannula. Hearts were placed on the Langendorff system and retrogradely perfused with Krebs solution under constant flow at 2 ml/min at 37 °C. Spontaneously beating hearts were allowed to stabilize for 30 min with CPP measured continuously via a pressure transducer connected to a data acquisition system (Powerlab 5; AD Instruments) and visualized on LabChart 8 (Powerlab 5; AD Instruments). Following stabilization, the hearts were subjected to drug challenge (constant perfusion), hypoxia (Krebs bubbled with 95% N\textsubscript{2}/5% CO\textsubscript{2}), or global ischemia and reperfusion (no flow for 30 min followed by 60 min of reperfusion) (39, 40). In these experiments, the investigators were blinded to genotype until the analysis was complete.

**Quantification of infarct size with TTC staining**

Hearts were frozen for 10 min prior to being sliced into 1-mm-thick transverse sections using a heart cradle and razor blades. Sections were placed in 1% (in PBS) TTC and incubated at 37 °C for 15–20 min. The sections were then placed in a Perspex envelope and scanned. The infarct size of stained hearts was quantified using ImageJ software. In these experiments, the investigators were blinded to genotype until the analysis was complete.
**K\textsubscript{ATP} in vascular endothelium**

**Isolation of aortic endothelial and smooth muscle cells and patch clamp electrophysiology**

To isolate endothelial cells, the mice were killed by cervical dislocation, and the aortas removed and placed in ice-cold Ca\textsuperscript{2+}-free physiological saline solution (PSS) containing 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1.1 mM EDTA, 1.7 mM MgCl\textsubscript{2}, 1 mM EGTA, 10 mM HEPES, and 11 mM glucose, pH 7.4. The vessels were cleaned of fat and connective tissue, cut into rings, and treated with 2 mg/ml collagenase type II (Worthington) in low-Ca\textsuperscript{2+} PSS (containing 136 mM NaCl, 5.6 mM KCl, 4.17 mM NaHCO\textsubscript{3}, 0.44 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.42 mM Na\textsubscript{2}HPO\textsubscript{4}, 10.47 mM MgCl\textsubscript{2}, 0.1 mM CaCl\textsubscript{2}, 10 mM HEPES, pH 7.4) for 10 min at room temperature followed by 20 min at 37 °C. The rings were washed with Ca\textsuperscript{2+}-free PSS and slit open, and single endothelial cells were obtained by gentle trituration. Smooth muscle cells were isolated from endothelium-de-nuded aorta by treating with 0.7 mg/ml papain, 0.25 mg/ml BSA, 0.5 mM DTT in low calcium dissociation medium (containing 125 mM NaCl, 5 mM KCl, 0.1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM NaHCO\textsubscript{3}, 0.5 mM KH\textsubscript{2}PO\textsubscript{4}, 0.5 mM NaH\textsubscript{2}PO\textsubscript{4}, 10 mM glucose, 10 mM HEPES, pH 7.2, with NaOH) with shaking at 37 °C for 15 min followed by 15 min of incubation in dissociation solution containing 0.5 mg/ml collagenase and 0.25 mg/ml BSA. Tissue was subsequently washed in enzyme-free dissociation solution and gently tritutated. The cells were kept on ice and placed directly into the recording chamber 5 min prior to use. Whole-cell patch clamp recordings were performed as described previously (41). Whole-cell currents were amplified by an Axoclamp 700, low-pass filtered at 1 KHz (4 pole Bessel), and sampled at 5 kHz using a Digidata 1440 (Axon Instruments). The currents were acquired and analyzed using pClamp10 (Axon Instruments). Whole-cell currents were recorded using a ramp protocol (−150 to +50 mV for 1 s from holding potential of −80 mV). Mean current-density values were taken at +40 mV. The pipette solution contained 107 mM KCl, 1.2 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 10 mM EGTA, and 5 mM HEPES with 0.1 mM MgATP and 1 mM NaADP, pH 7.2, using KOH. The bath solution contained 110 mM NaCl, 5 mM KCl, 1.2 mM MgCl\textsubscript{2}, 1.8 mM CaCl\textsubscript{2}, 10 mM glucose, and 10 mM HEPES, pH 7.2.

**Reagents**

Pinacidil and glibenclamide were purchased from Sigma–Aldrich. NECA was obtained from Tocris. All the reagents for the extracellular and intracellular solutions were from Sigma–Aldrich.

**Statistical analysis**

The data are presented as the means ± S.E. The data were analyzed using Microsoft Excel (Microsoft) and GraphPad Prism. Student’s t test and analysis of variance were used to compare means where appropriate. p ≤ 0.05 was taken to be significant.

**Author contributions**—Q. A. and A. T. devised the research and wrote the paper. A. T. raised the funding for the research, Q. A., Y. L., N. A., L. O., and E. T. performed the research. All authors discussed and approved the paper.

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