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Molecular and functional characterization of the endothelial ATP-sensitive potassium channel

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Running Title: K_{ATP} in vascular endothelium

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ABSTRACT

ATP-sensitive potassium (K_{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_{ATP} channels in vascular smooth muscle have a well-defined role in regulating vascular tone. K_{ATP} channels are also thought to be expressed in vascular endothelial cells (ECs), but their presence and function in this context is less clear. As a result, we aimed to investigate the molecular composition and physiological role of endothelial K_{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, ex-vivo coronary perfusion Langendorff heart experiments and endothelial cell Ca^{2+} imaging comparing eKO and wild type mice show that Kir6.1-containing K_{ATP} channels are indeed present in vascular endothelium. An increase in intracellular [Ca^{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_{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 ischaemia-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_{ATP} channel that contains Kir6.1, is involved in vascular reactivity in the coronary circulation and has a protective role in ischaemia reperfusion.

INTRODUCTION

ATP-sensitive potassium channels (K_{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 ischaemia (1). Structurally, K_{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 sulphonylurea receptor subunits (SUR1, SUR2A or SUR2B) (1). These subunits associate in various tissue-specific combinations to form functional K_{ATP} channels and these currents can have distinct pharmacological and electrophysiological properties.

K_{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_{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_{ATP} channels and ascertain the contribution of these subunits to the K_{ATP} current in smooth muscle cells. Both knockout (KO) mice had a similar hypertensive phenotype consistent with previous in vitro electrophysiological data suggesting a role for vascular smooth muscle K_{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_{ATP} channels in smooth muscle (1,4). Mice expressing a smooth muscle-specific gain of function mutant K_{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_{ATP} deletion lacked a functional K_{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_{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_{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 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 as Kir6.1 and Kir6.2 heteromultimerise 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 knockout 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 ((5) and Figure 1A 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 to 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 knockout mice suggesting SUR2 was the major endothelial SUR subunit (Figure 1C and D). However the effect of endothelial removal was more pronounced in the eKO mice suggesting perhaps some compensatory upregulation. 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 (Figure 2). Endothelial cells from WT mice show a K^+ selective current which was activated by the K_{ATP} opener pinacidil ($P<0.05$) and inhibited by the K_{ATP} blocker glibenclamide (Figure 2A, 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. 2B, C and 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 if this is the case, we subjected acutely isolated aortic smooth muscle cells from eKO mice to whole-cell patch-clamp (Figure 2E-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 knockout mice - Ca^{2+} is an important mediator of endothelial cell function including release of vasoactive species. To further characterise 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 bloods vessels as endothelial cells are flat and there will be contamination of the endothelial fluorescent signal from overlying 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 (Figure 3). In both WT and eKO MAV endothelial cells, Fluo-4 fluorescence was elevated to a similar extent ($F/F_0 \approx 3.0$) by the SERCA inhibitor, 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 to 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 μmol/L 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+} signalling 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 vs 103.5±6 mmHg, n=23, $P>0.05$). Pinacidil (10 μM) significantly reduced CPP in both WT and eKO mice (Figure 4), although the reduction of CPP in eKO (17%) mice was significantly less compared to WT (35%, $P<0.05$). Addition of glibenclamide (10 μM) reversed the pinacidil-induced fall in CPP back to baseline Figure 4A-C). We also compared this with mice in which Kir6.1 is deleted from smooth muscle (see Experimental Procedures and (5)) and in this case the response to pinacidil was also reduced but not completely abolished (Figure 4). The basal CPP was not significantly different.

Function of endothelial K_{ATP} channels in the coronary circulation - To examine the role of the endothelial K_{ATP} at times of metabolic stress, we exposed the coronary circulation to local hypoxia with a perfusate saturated with N_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 to eKO (10%) hearts (Figure 5A, $P<0.01$). In the smKO mice there was a trend to reduction in the hypoxic response (WT- 37%, eKO- 20%) though this was not statistically significant (Figure 5A).

Additionally, hearts from all groups of mice were subjected to global ischaemia (no flow) followed by reperfusion. Staining of sections from these hearts with TTC revealed a greater infarcted area in Kir6.1 eKO (77%) hearts than in WT (56%) hearts (Figure 5B). Taken together, these data suggest that endothelial Kir6.1-containing K_{ATP} channels in the coronary circulation may have a protective role in the heart during ischaemia-reperfusion injury. Hearts from smKO mice (Figure 5C) also had an increase in infarcted area ($P<0.05$) compared to littermate controls suggesting that

both the smooth muscle and endothelial K_{ATP} channel are important in limiting the ischaemic 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 smooth muscle (5).

Expression and role of Kir6.2 in vascular endothelium - The endothelium of blood vessels is postulated to express K_{ATP} 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. Data from the Langendorf experiments show that hearts from WT and Kir6.2 eKO mice have similar basal CPP values suggesting Kir6.2-containing K_{ATP} channels are not involved in regulating basal vascular tone in the coronary circulation (Figure 6D). In addition, the pinacidil and hypoxia response in Kir6.2 eKO mouse hearts was not significantly different from WT hearts (Figure 6E, $P>0.05$). Furthermore, there was no difference in infarct size between Kir6.2 eKO and WT hearts following ischaemia-reperfusion (Figure 6F, $P>0.05$).

The effect of the adenosine receptor agonist, NECA, is diminished in endothelial cells and the coronary circulation in eKO mice. - A major mediator of the response to cellular ischaemia is thought to be adenosine release from compromised tissue which in turn activates adenosine receptors in vascular smooth muscle and endothelial cells. Thus we investigated if this was the case and isolated K_{ATP} currents in endothelial cells as described above. In WT endothelial cells, NECA, an adenosine receptor agonist not metabolised by adenosine deaminase, was able to activate K^+ currents ($P<0.01$). In contrast, in endothelial cells from eKO mice no such increase of current was seen (Figure 7A-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 to that of hypoxia. This response was significantly reduced in the eKO mice (~30%, $P<0.05$) (Figure 7D-E).

DISCUSSION

The major findings in this study are that an endothelial K_{ATP} channel exists composed of Kir6.1 and likely SUR2B. Activation of the channel can promote Ca^{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 ischaemia reperfusion. The endothelial K_{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_{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_{ATP} channels in the vasculature we have used the cre-loxP system to create an endothelium-specific Kir6.1 knockout 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_{ATP} channels to the current in the endothelium.

Though endothelial K_{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, Kir6.1 expression in the endothelium and transcriptomic analysis confirmed deletion of Kir6.1 in the eKO murine line. Functionally, whole-cell patch-clamp recordings using an intracellular solution favourable for activity of both Kir6.1 and Kir6.2 subunit containing K_{ATP} currents (0.1 mmol/L ATP/1 mmol/L 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 Kir6.1 as the main pore-forming subunit in endothelial cells in the aorta. Furthermore Ca^{2+} imaging studies of the aortic valve leaflets add functional evidence for

Kir6.1-containing K_{ATP} channels in vascular endothelium. Using mouse aortic valve leaflets allowed us to look at pure endothelial Ca^{2+} signalling without contamination from 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 hyperpolarisation and as a result initiating a change in the electrochemical gradient favourable to Ca^{2+} ion 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 as there was no difference in the effects of the SERCA inhibitor CPA between the lines. Finally, the effect of pinacidil on coronary perfusion pressure (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 though 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 knockout 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 doesn't 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).

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%. Though 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. There was a trend for a reduction in coronary perfusion pressure with hypoxia in the smooth muscle knockout though this was not statistically significant.

We also challenged the hearts to a period of ischaemia reperfusion injury. In eKO hearts this led to a ~20 % increase in cardiac tissue death compared to 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 acting to preserve tissue. The co-operation 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 attributed to potassium channel openers on the heart may occur because of the activation of endothelial K_{ATP} channels (13). The endothelial channel is responsible as endothelial deletion of Kir6.1 does affect smooth muscle K_{ATP} 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 K_{ATP} channel complex (27). It is known that the vascular K_{ATP} channel can be activated by signalling 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 signalling but it is known that A2A and A2B both couple to the stimulatory G-protein and activate protein kinase A signalling (30,35). However less is known about K_{ATP} in endothelial cells (14). Our data show that a non-specific adenosine agonist (NECA) can activate K^+ 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 K_{ATP} channel.

A key question remains: how do endothelial cells couple to smooth muscle cells and what is the role of the K_{ATP} channels in that coupling? We have shown that activation of the channel leads to Ca^{2+} 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 hyperpolarising 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 K_{ATP} channel and shown that it can influence Ca^{2+} entry into the cells. The endothelial K_{ATP} channel in the coronary circulation plays an important role in vasodilatation to hypoxia and limiting ischaemia 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 licences PPL/6732 and PPL/7665) and by the US National Institutes of Health (NIH 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 Tie-2 promoter driven cre-transgenic mice (tie2 cre, http://jaxmice.jax.org/strain/004128.html) with Kir6.1 homozygous floxed (Kir6.1(flx/flx)) mice. A further cross of the offspring resulted in genotypes of tie2cre+ Kir6.1(flx/flx) (eKO) and littermate controls. Similarly, smooth muscle-specific Kir6.1 KOs (smKO) were generated by crossing Kir6.1(flx/flx) mice with mice expressing a sm22 promoter driven cre to give sm22 cre+ Kir6.1 (flx/flx) mice. Littermate controls are referred to as wildtype 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 at: https://www.mousephenotype.org/data/alleles/MGI:107501/tm1c(EUCOMM)Wtsi.

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'-ACTAGCACCTCTATCCCCAGCTCCTACC-3' and antisense 5'-CCGCCCCTCCCTCTGAACCTATATC-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'-CCCTGTGCTCAGACAGAAATGAG-3' and antisense 5'-CGCATAACCAGTGAAACAGCATTGC -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'-CCAATTTACTGACCGTACACC-3' and antisense 5'-GTTTCACTATCCAGGTTACGG-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 (NEB, UK).The presence of the WT and Kir6.2 floxed alleles was detected using the following primer sets: WT (110 bp band); sense 5'-CTGTCCCGAAAGGGCATTAT-3' and antisense 5' AGTGTGTGGCCATTTGAGGT 3'

and for the floxed allele (190 bp band); sense 5'-CTGTCCCGAAAGGGCATTAT-3' and antisense 5'-GAACTTCGGAATAGGAACTTCG-3'.

Quantitative real-time PCR (qRT-PCR) - The RNAeasy kit (Qiagen, UK) 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, UK). cDNA (50 ng) was used for qRT-PCR, which was performed using customised Taqman gene expression assays (Applied Biosystems, UK). Commercially available probes (Life Technologies Ltd, UK) were used for all K_{ATP} channel subunit genes as listed below: Mm00434620_m1 for *Kcnj8* (Kir6.1), Mm00440050_s1 for *Kcnj11* (Kir6.2), Mm01701349_m1 for *Abcc8* (SUR1), and Mm00441638_m1 for *Abcc9* (SUR2). Each gene was assayed in triplicate and relative expression was calculated by using the comparative CT method normalised to GAPDH. Aortas were denuded with forceps and mesenteric arteries with course horse hair. Data are presented as a relative change compared to WT + endothelium.

Ca^{2+} imaging of aortic valve leaflets - To assess the intracellular calcium concentration in the cytosol of endothelial cells ($[Ca^{2+}]_i$), aortic valve leaflets from the mouse heart were dissected out, mounted into a custom-made glass bottomed 35 mm petri dish (MatTek, USA) and placed in HEPES buffer of the following composition (mmol/L): HEPES 10, NaCI 140, KCI 5, Glucose 10, $CaCI_2$ 1, pH 7.4 (18). The valves were then incubated with the Ca^{2+} indicator Fluo-4 (2 μmol/L) in HEPES buffer at room temperature for 2 hours followed by a wash with indicator-free buffer for 30 minutes. 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 (F_0).

Measurement of coronary perfusion pressure - The Langendorff isolated perfused heart system was used to measure basal coronary perfusion pressure (CPP) and the effects of the K_{ATP} channel opener pinacidil and blocker glibenclamide and in hypoxic and ischaemic conditions. Briefly, mice were injected with heparin sodium (250 IU) and anaesthetised with a combination of ketamine/xylazine. The hearts were rapidly excised and placed in ice-cold Krebs-Henseleit solution (containing in mmol/L: NaCl 118, KCl 4.75, KH_2PO_4 1.19, $NaHCO_3$ 25, $MgSO_4.7H_2O$ 1.19, $CaCI_2$ 1.4, Na Pyruvate 2 and glucose 10) equilibrated with 95% O2/5% CO2, 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 37ºC. Spontaneously beating hearts were allowed to stabilise for 30 mins with CPP measured continuously via a pressure transducer connected to a data acquisition system (Powerlab 5, AD instruments) and visualised on LabChart 8 (Powerlab 5, AD instruments). Following stabilisation, hearts were subjected to drug challenge (constant perfusion), hypoxia (Krebs bubbled with 95% N_2/5% CO_2) or global ischaemia and reperfusion (no flow for 30 minutes followed by 60 minutes reperfusion) (39,40). In these experiments the investigators were blinded to genotype until the analysis was complete.

Quantification of infarct size with 2, 3, 5-triphenyltetrazolium chloride (TTC) staining - Hearts were frozen for 10 minutes 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 37ºC for 15-20 minutes. 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.

Isolation of aortic endothelial and smooth muscle cells and patch-clamp electrophysiology - To isolate endothelial cells, mice were killed by cervical dislocation, aortas removed and placed in ice-cold Ca^{2+}-free physiological saline solution (PSS) containing (mmol/L); NaCl 125, KCl 4.8, KH_2PO_4 1.2, EDTA 1.1, $MgCI_2$ 1.7, EGTA 1, HEPES 10, and glucose 11, pH 7.4. Vessels were cleaned of fat and connective tissue and cut into rings and treated with 2mg/mL collagenase type II (Worthington) in low-Ca^{2+} PSS (containing in mmol/L); NaCl 136, KCl 5.6, $NaHCO_3$ 4.17, NaH_2PO_4 0.44, Na_2HPO_4 0.42, $MgCI_2$ 10.47, $CaCl_2$ 0.1, HEPES 10, pH 7.4) for 10 minutes at room temperature followed by 20 minutes at 37ºC. Rings were washed with Ca^{2+}-free PSS, slit open and single endothelial cells were obtained by gentle trituration. Smooth muscle cells were isolated from endothelium-denuded aorta by treating with 0.7 mg/mL papain, 0.25 mg/mL BSA, 0.5 mM DTT in low calcium dissociation medium (containing (mM); 125 NaCl, 5 KCl, 0.1 $CaCl_2$, 1 $MgCl_2$, 10 $NaHCO_3$, 0.5 KH_2PO_4, 0.5

NaH_2PO_4, 10 Glucose, 10 HEPES, pH 7.2 with NaOH) with shaking at 37 °C for 15 minutes followed by 15 minutes 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 triturated. Cells were kept on ice and placed directly into the recording chamber 5 minutes 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). Currents were acquired and analysed 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 (mmol/L); KCl 107, $MgCl_2$ 1.2, $CaCl_2$ 1, EGTA 10 and HEPES 5 with MgATP 0.1 and NaADP 1, pH 7.2 using KOH. The bath solution contained (mM); 110 NaCl, 5 KCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 10 Glucose, 10 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 - D*ata are presented as mean ± S.E.M. Data were analysed using Microsoft Excel (Microsoft) and GraphPad Prism. Student's *t* test and ANOVA were used to compare means where appropriate. $P \leq 0.05$ was taken to be significant.

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Disclosures: None and the authors have no conflicts of interest.

Author Contribution: QA and AT devised the research and wrote the paper. AT raised the funding for the research. QA, YL, NA, LO and ET performed the research. All authors discussed and approved the paper.

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Figure Legends

Figure 1. Generation and characterisation of the endothelium-specific knockout mouse (eKO). **A**, Cre-LoxP targeting strategy for the deletion of exon 2 of the *Kcnj8* gene. A targeting vector construct for *Kcnj8* with a 5' LoxP site together with a FRT-flanked neomycin (Neo) selection cassette within intron 1 upstream of exon 2 and the second loxP site in intron 2 and diptheria toxin A (DTA) negative selection marker downstream. Mice with the recombined *Kcnj8* locus were crossed with global flp-deleter mice to allow Flp-mediated excision of the neomycin selection cassette and generate Kir6.1 (+/flx) offspring. Kir6.1 (flx/flx) mice were then crossed with mice in which the Cre recombinase expression is driven by an endothelium-specific promoter associated with the Tie2 receptor to produce tie2cre+ Kir6.1(flx/flx) (eKO) mice. **B**, Genotyping of DNA isolated from tail for the floxed alleles (top) and Cre recombinase (bottom). Relative levels of expression of Kir6.1, Kir6.2 and SUR2 in the aorta (**C**) and mesenteric arteries (**D**) with intact endothelium (+E) and with endothelium denuded (-E) from WT and eKO mice. No CT values were detectable for SUR1. The gene expression level in each case was normalized to the WT + endothelium (WT+E). Data is shown as mean ± S.E.M, n=6 mice, **P<0.01, ***P<0.001 compared to WT+E.

Figure 2. Endothelial-specific Kir6.1 deletion results in an attenuated K_{ATP} current in aortic endothelial cells. Representative time-course traces at +40 mV (right panel) and whole-cell current density-voltage traces (left panel) taken from ECs isolated from WT (**A**) and eKO (**B**) mice showing the effects of pinacidil (Pin) and glibenclamide (Glib). The control (Con) trace is shown in grey. Current-voltage relationships were recorded using a 1s ramp protocol (-150 mV 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) and eKO (right) mice. **D**, Mean glibenclamide-sensitive current in ECs from WT and eKO mice. n=8-20 cells from 4-9 mice**.** Representative (**E**) and mean data (**G** and **H**) of whole-cell currents recorded from SMCs isolated from WT and eKO mice. n=10-20 cells from 4-9 mice. Data is shown as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 compared to control/WT.

Figure 3. Pinacidil-induced elevation of cytosolic [Ca^{2+}] is attenuated in eKO mouse aortic valves. **A**, Confocal images showing a pinacidil-evoked concentration-dependent elevation in cytosolic [Ca^{2+}] in WT mice. 100 μM CPA (SR uptake inhibitor) was used as a control to induce an increase in cytosolic at baseline. **B**, Confocal images showing the effect of CPA and pinacidil on cytosolic [Ca^{2+}] in valves from eKO mice. Scale bar represents 25 μm. **C**, Summary time-course traces (S.E.M in the dashed lines) illustrating the effects of 100 μM CPA, 10 and 100 μM pinacidil and 10 μM glibenclamide on cytosolic [Ca^{2+}] aortic valve cells from WT (black) and eKO (grey) mice. Changes in cytosolic [Ca^{2+}] are expressed as relative fluorescence ratio, F/F_0. **D**, Bar graph showing the mean data from traces in panel C. Data is shown as mean ± S.E.M, n=6 mice, *P<0.05, ***P<0.001 compared to WT. $^{+++}$P<0.001 compared to WT 10 μM pinacidil.

Figure 4. Kir6.1 containing K_{ATP} channels are present in both endothelium and smooth muscle of the coronary circulation. **A**, Mean basal coronary perfusion pressure (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 panel and KO-lower panel) in response to 10 μM pinacidil and 10 μM glibenclamide. **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. Data is shown as mean ± S.E.M, n=5-10 mice, *P<0.05, **P<0.01 compared to WT.

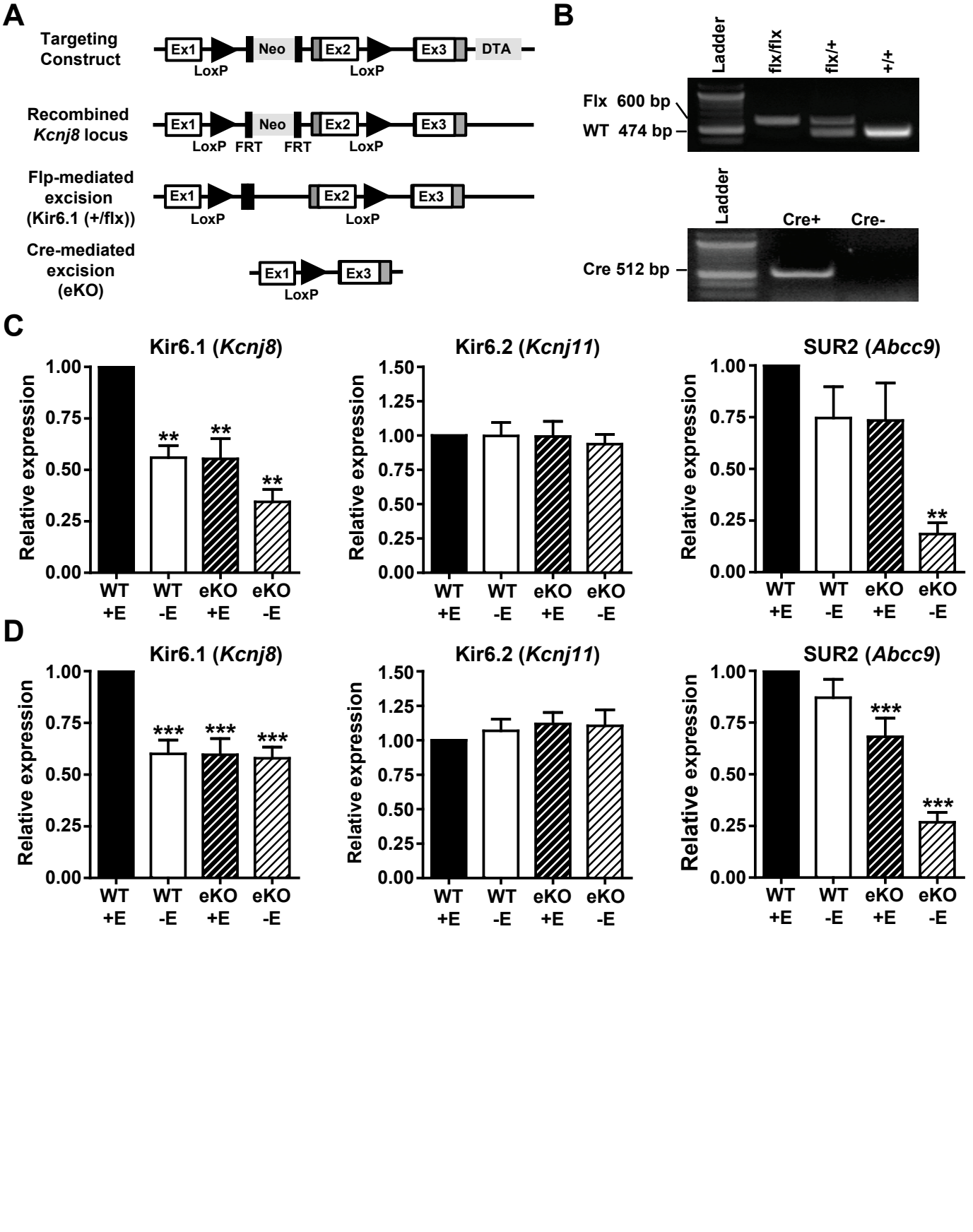
Figure 5. Endothelial K_{ATP} channels containing Kir6.1 may be protective during metabolic challenge. **A**, Mean CPP of eKO and smKO mouse hearts and hearts from their respective littermate controls in response to hypoxia (Krebs solution gassed with 95% N_2/5% CO_2) (n=6-10). CPP was measured using the Langendorff set-up under constant flow. Representative sections (left) and mean infarct size (right) following 30 minutes of global ischemia and 60 minutes of reperfusion of eKO (**B**)

and smKO (**C**) mouse hearts stained with 1% TTC, pale tissue signifies infarction. Data is shown as mean ± S.E.M, n=5-16 mice, *P<0.05, **P<0.01 compared to WT.

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) and Kir6.2 eKO (right) mice showing the effects of pinacidil (Pin) and glibenclamide (Glib). Currents were elicited using a 1s 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) and Kir6.2 eKO (right) mice. **C**, Mean glibenclamide-sensitive current in ECs from WT and Kir6.2 eKO mice. n=9-14 cells from 3 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) and mean infarct size (right) following 30 minutes of global ischemia and 60 minutes of reperfusion of WT and Kir6.2 eKO mouse hearts stained with 1% TTC, pale tissue signifies infarction (n=11)**.** Data is shown as mean ± S.E.M. ***P<0.001 compared to control.

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) and eKO (right) mice showing the effects of NECA. **B**, Representative current-voltage traces from a WT (left) and eKO (right) cell in the presence and absence of NECA. Currents were elicited using a 1s ramp protocol (-150 mV 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) and eKO (right) mice. n=8-9 cells from 3 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**.** Data is shown as mean ± S.E.M. *P<0.05, **P<0.01 compared to control/WT.

Figure 1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Cre-mediated excision (eKO)
B
Ladder
flx/flx
flx/+
+/+
Flx 600 bp
WT 474 bp
Cre+
Cre-
Cre 512 bp
C
Kir6.1 (Kcnj8)
Kir6.2 (Kcnj11)
SUR2 (Abcc9)
Relative expression
WT +E
WT -E
eKO +E
eKO -E
D
Kir6.1 (Kcnj8)
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Relative expression
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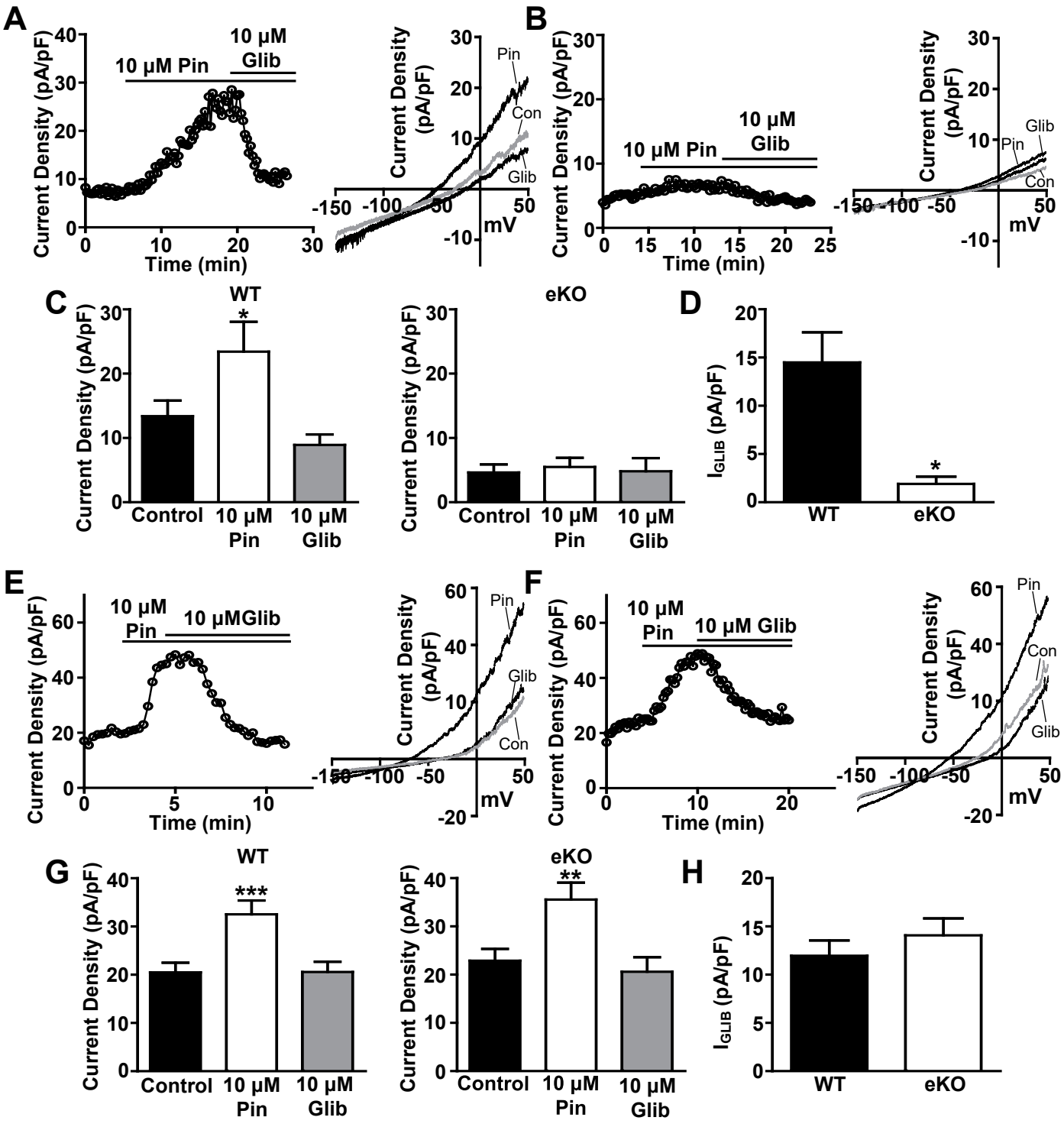
Figure 2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Time (min)
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mV
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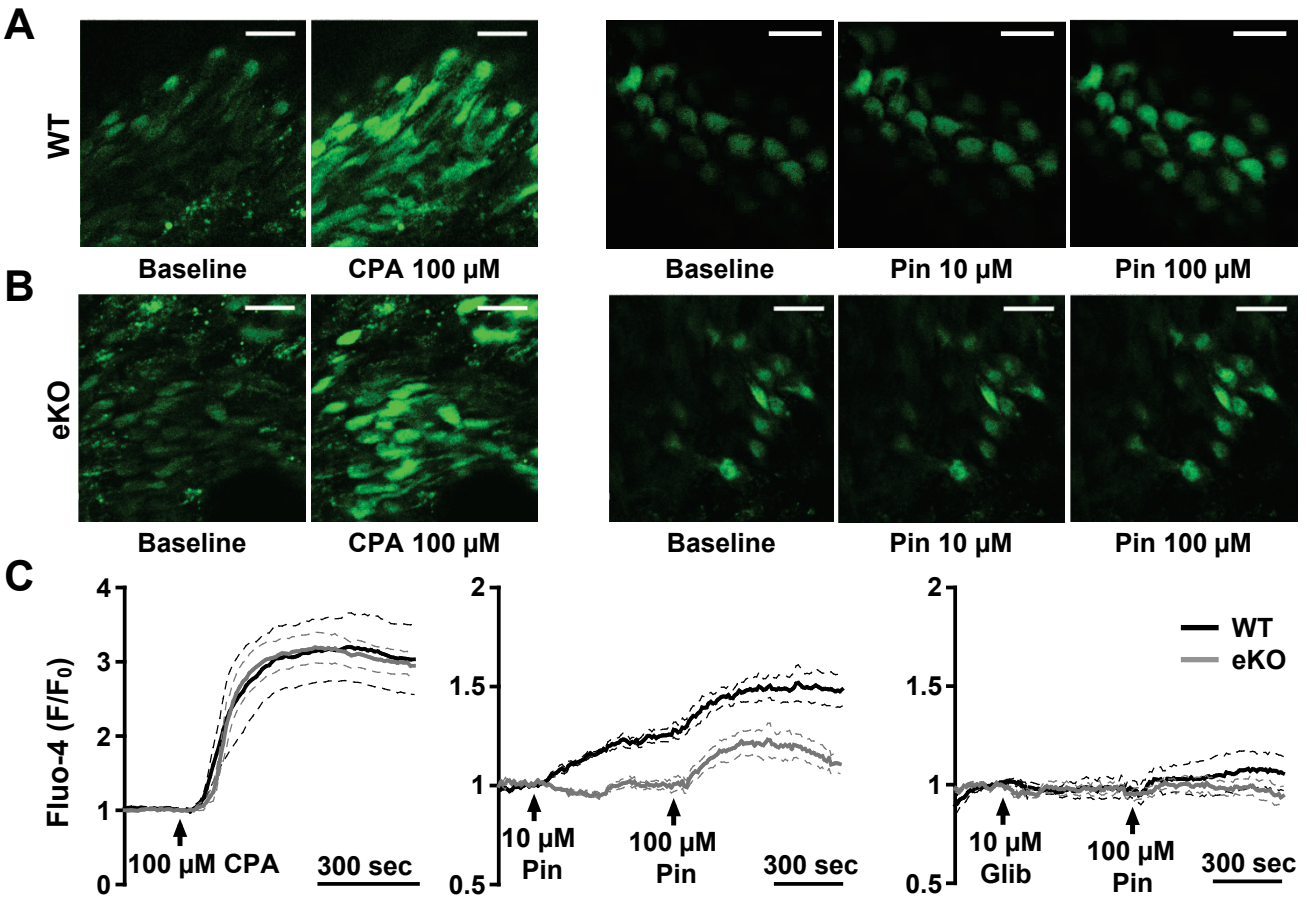
Figure 3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100 µM CPA
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10 µM Pin
100 µM Pin
300 sec
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D
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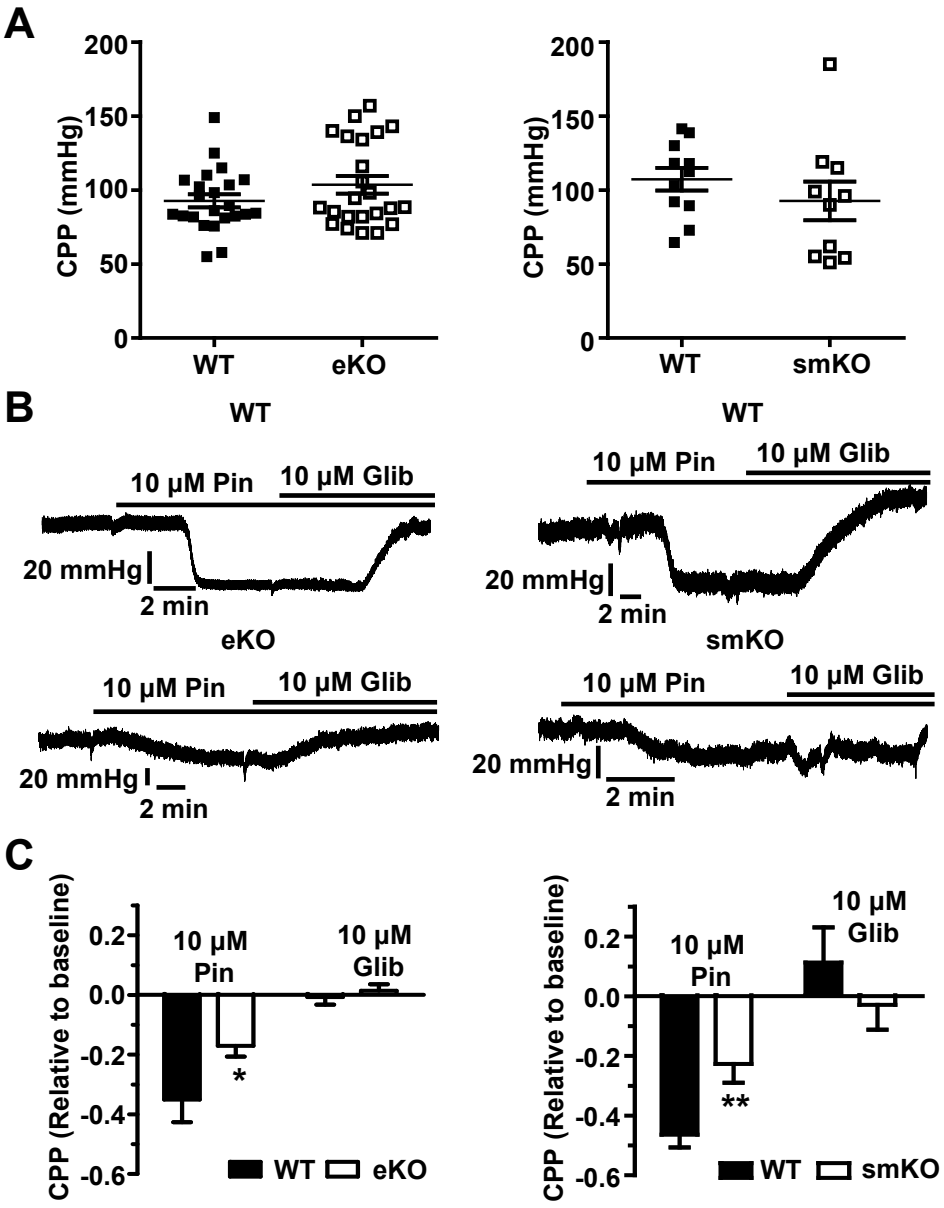
100 µM CPA

Figure 4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smKO
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0.2
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-0.6
CPP (Relative to baseline)
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10 μM Glib
*
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WT
eKO
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Figure 5

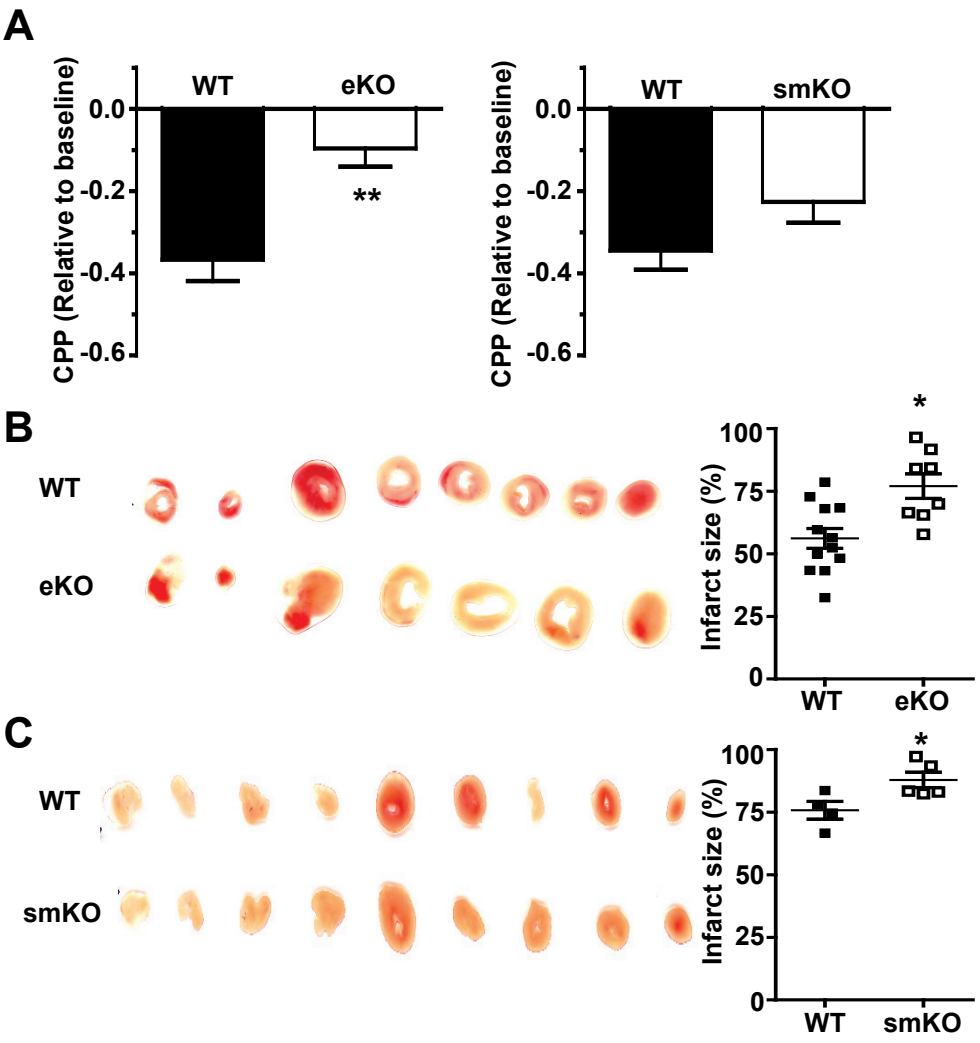
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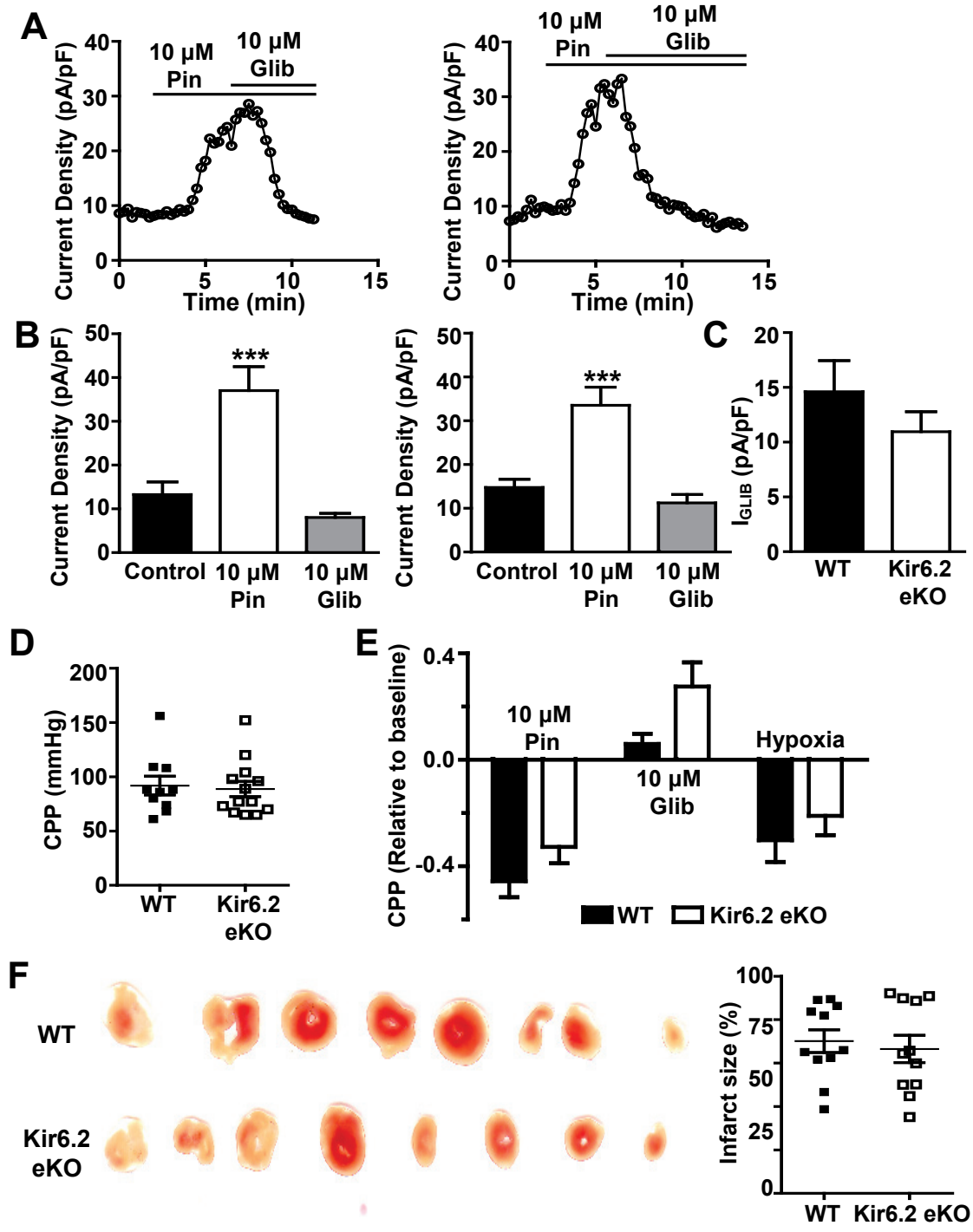
Figure 6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WT
Kir6.2 eKO
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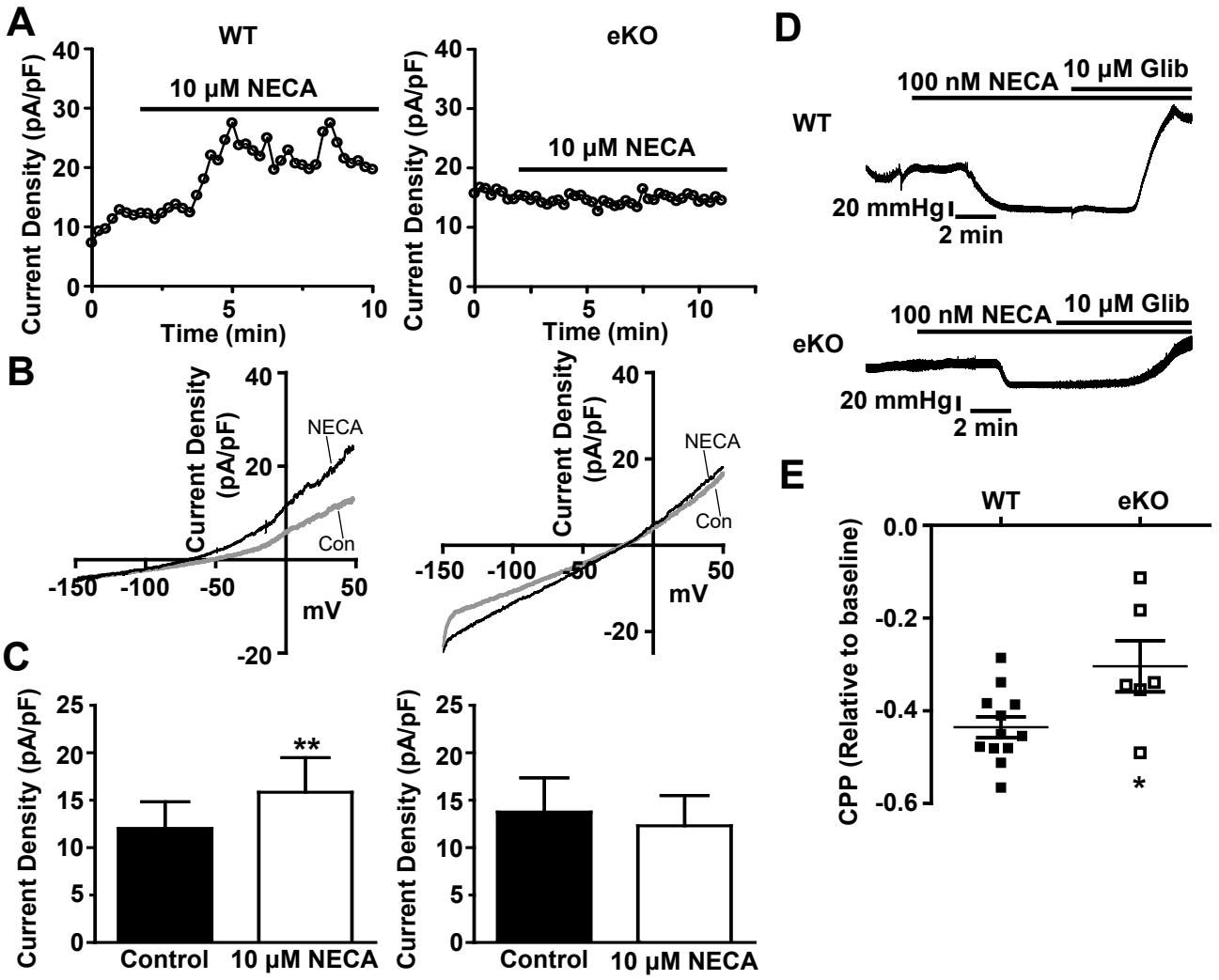
CPP (mmHg)
E
CPP (Relative to baseline)
Hypoxia
F
WT
Kir6.2 eKO
Infarct size (%)

Figure 7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D
100 nM NECA
10 μM Glib
WT
eKO
20 mmHg
2 min
E
CPP (Relative to baseline)
*

Molecular and functional characterization of the endothelial ATP-sensitive potassium channel

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