ORIGINAL ARTICLE

Mechanism of succinate efflux upon reperfusion of the ischemic heart

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Abstract

Aims Succinate accumulates several-fold in the ischemic heart and is then rapidly oxidised upon reperfusion, contributing to reactive oxygen species (ROS) production by mitochondria. In addition, a significant amount of the accumulated succinate is released from the heart into the circulation at reperfusion, potentially activating the G-protein coupled succinate receptor (SUCNR1). However, the factors that determine the proportion of succinate oxidation or release, and the mechanism of this release, are not known.

Methods and results To address these questions, we assessed the fate of accumulated succinate upon reperfusion of anoxic cardiomyocytes, and of the ischemic heart both *ex vivo* and *in vivo*. The release of accumulated succinate was selective and was enhanced by acidification of the intracellular milieu. Furthermore, pharmacological inhibition, or haploinsufficiency of the monocarboxylate transporter 1 (MCT1) significantly decreased succinate efflux from the reperfused heart.

Conclusion Succinate release upon reperfusion of the ischemic heart is mediated by MCT1 and is facilitated by the acidification of the myocardium during ischemia. These findings will allow the signalling interaction between succinate released from reperfused ischemic myocardium and SUCNR1 to be explored.

Translational Perspectives In this study we demonstrate that succinate efflux upon reperfusion of the ischemic myocardium is mediated by the monocarboxylate transporter 1 (MCT1) and is enhanced by the ischemic acidification of the heart. These findings are an important advance in understanding how succinate is released upon reperfusion of ischemic organs. While this pathway is therapeutically tractable, greater understanding of the effects of succinate release is required before exploring this possibility.

Keywords Ischemia/reperfusion injury • Succinate • MCT1 transporter • Mitochondria • SUCNR1

1. Introduction

Succinate accumulates several-fold in a range of ischemic tissues, including the heart.[1–5] Upon reperfusion, the succinate levels very rapidly (<1.5 – 2 min) return to baseline values.[3,4] A proportion of this accumulated succinate is oxidised by the mitochondrial respiratory chain, contributing to the formation of the reactive oxygen species (ROS), superoxide (*Figure 1*).[4] This ROS production initiates a cascade of damage that culminates in ischemia/reperfusion (I/R) injury.[4] In addition to its oxidation, upon reperfusion a significant amount of the accumulated succinate is released from the heart into the circulation.[2,6] The succinate accumulated during ischemia is thought to move from the mitochondria to the cytosol, catalysed by the dicarboxylate carrier (DIC) in exchange for malate.[7] Therefore, upon reperfusion of the ischemic heart, the cytosolic succinate has two fates – it either re-enters mitochondria as a respiratory substrate to drive ROS production, or effluxes from the cell (*Figure 1*). Efflux of succinate from the ischemic heart upon reperfusion has been demonstrated *ex vivo* in the mouse heart and in human hearts *in vivo* during primary percutaneous coronary intervention (PPCI) on ST-elevated myocardial infarction (STEMI) patients.[2,6] However, the mechanism of succinate release is unknown.

Succinate accumulation is a conserved signature of ischemia in different organs and species,[1–5] suggesting that its release upon reperfusion may be a signal of tissue ischemia and/or damage. Furthermore, there is a G-protein coupled succinate receptor (SUCNR1) that can respond to the succinate released into the circulation (*Figure 7*).[8–10] SUCNR1 is highly expressed on the surface of immune cells and its ligation has been associated with a range of both pro- and anti-inflammatory phenotypes, depending on context.[8,11–13] In addition, succinate release into the circulation may have other effects in addition to signalling from ischemic tissue, for example succinate was shown to activate thermogenesis by brown adipose tissue.[14] Together these findings suggest that succinate released from ischemic tissues into the circulation may promote a range of responses, such as the infiltration of immune cells and thereby contribute to the pathology and/or resolution of I/R injury (*Figure 7*).[6,9,15]

To address the mechanism of succinate release into the circulation during reperfusion, we assessed the efflux of succinate from ischemic cardiomyocytes, mouse hearts *ex vivo* and *in vivo*, and in a pig model of myocardial infarction (MI). We show that succinate was one of only a few metabolites released upon reperfusion of the ischemic heart. Furthermore, succinate efflux was mediated by the monocarboxylate transporter 1 (MCT1) and

acidification of the myocardium during ischemia enhanced release upon reperfusion. This understanding of how succinate is released upon reperfusion of ischemic organs has translational implications for targeting succinate signalling following MI.

2. Methods

All experiments were performed under UK Home Office Licences and conducted according to the Animals Scientific Procedures Act 1986 (UK) and directive 2010/63/EU of the European Parliament guidelines on the protection of animals used for scientific purposes. All experiments were approved by the Institutional Animal Welfare and Ethical Review Body. C57BL/6J male mice (~25 g, 8-12 weeks old, n = 102) were from Charles River, UK. The *MCT1*$^{+/-}$ mice (8-12 weeks old) were initially generated by homologous recombination[16] and bred to produce MCT$^{+/-}$ and corresponding MCT$^{+/+}$ littermate controls. Female Wistar rats (~250 g, 10-12 weeks old) were from Charles River, UK. All mice and rats were kept in individually ventilated cages with a 12 h light-dark cycle, controlled humidity and temperature (20–22 °C), fed standard chow and water *ad libitum*. Experiments in pig were carried out under Home Office Project Licence No 7008975 at the University of Bristol Translational Biomedical Research Centre (TBRC), Bristol, UK, and advanced facility for large animal research (http://www.bristol.ac.uk/health-sciences/research/tbrc/).

For Langendorff perfusions, mice were administered terminal anaesthesia via intra-peritoneal pentobarbitone injection (~140 mg/kg body weight). For in situ ischemia/reperfusion, mice were anesthetised with isofluorane (2 x minimum alveolar concentration and O_2 at 2 L/min Abbott Laboratories, US) before performing a laparotomy and administering 100 µl heparin bolus (100 iU; Leo Pharma A/S, Denmark). Mice were culled via exsanguination by division of the abdominal inferior vena cava (IVC) and aorta. For isolation of adult cardiomyocytes, mice were culled by cervical dislocation (no anaesthetic used). For isolation of rat heart mitochondria, rats were culled by cervical dislocation (no anaesthetic used). In porcine myocardial infarction model, landrace female pigs were premedicated with intramuscular injection of ketamine (10 mg/kg) and dexmedetomidine (15 µg/kg); for general anaesthesia, IV boluses of propofol (1 mg/kg) were used followed by isoflurane in oxygen with the vaporiser set at 2% for maintenance. At the end of the experiment, pigs were terminated by administration of 2 litres cold cardioplegia solution via the aorta at a delivery pressure of 300 mmHg.

2.1 Animal ischemia/reperfusion experimental models

2.1.1. Langendorff-perfused mouse hearts

Mice were administered terminal anaesthesia via intra-peritoneal pentobarbitone injection (~140 mg/kg body weight). While anaesthetics such as pentobarbitone can affect mitochondrial function, in our experiments the effects of inhibitors and other interventions are compared with control using identical anaesthetic regimes. Beating hearts were rapidly excised, cannulated and perfused in isovolumic Langendorff mode at 80 mm Hg pressure maintained by a STH peristaltic pump controller feedback system (AD Instruments, UK), with phosphate-free Krebs-Henseleit (KH) buffer continuously gassed with 95% O_2/5% CO_2 (pH 7.4, 37 °C) containing (in mM): NaCl (116), KCl (4.7), $MgSO_4.7H_2O$ (1.2), $NaHCO_3$ (25), $CaCl_2$ (1.4), glucose (11). Cardiac function was assessed using a fluid-filled cling-film balloon inserted into the left ventricle (LV) connected via a line to a pressure transducer and a Powerlab system (AD Instruments, UK). The volume of the intraventricular balloon was adjusted using a 1.0 mL syringe to achieve an initial LV diastolic pressure (LVDP) of 4–9 mmHg. Functional parameters (systolic pressure, end diastolic pressure, heart rate, coronary flow, perfusion pressure) were recorded using LabChart software v.7 (AD Instruments, UK) throughout the experiment. LVDP was calculated from the difference between systolic (SP) and diastolic pressures (DP). After 20 min equilibration, hearts were subjected to 20 min global ischemia prior to reperfusion. Perfusate was collected in 1 min intervals for the first 6 min of reperfusion and snap frozen in liquid nitrogen. Where inhibitors were used, these were added in the reperfusion buffer, with the heart reperfused for 6 min containing the inhibitors throughout (unless specified otherwise). Hearts were immediately snap frozen using Wollenberger tongs pre-cooled in liquid nitrogen either after equilibration, ischemia or the reperfusion period (6 min) and stored at –80 °C until further analysis.

2.1.2. In situ mouse heart ischemia/reperfusion

Mice were anesthetised with isofluorane (2 x minimum alveolar concentration and O_2 at 2 L/min Abbott Laboratories, US) before performing a laparotomy and administering 100 µl heparin bolus (100 iU; Leo Pharma A/S, Denmark). Mice were exsanguinated by division of the abdominal inferior vena cava (IVC) and aorta. Global ischemia was maintained within the body for 20 min, with physiological temperature (37 °C) maintained using a core-temperature controlled heating-mat. During ischemia, fine borosilicate tubing was inserted into the root of the aorta and a collection tube inserted into the IVC lumen. Immediately before reperfusion, the superior vena cava, pulmonary artery and hemiazygos vein were clamped to prevent back flow from the right atrium on flush. The heart was retrograde perfused with phosphate-free

KH buffer (37 °C) at a flow rate of 1 ml/min. Perfusate was collected at 1 min intervals for 6 min and the heart clamp frozen.

2.1.3. Porcine myocardial infarction model

Landrace female pigs (n=3; 5-6 months, median weight 62.5 kg) were premedicated with intramuscular injection of ketamine (10 mg/kg) and dexmedetomidine (15 µg/kg); for general anesthesia, IV boluses of propofol (1 mg/kg) were used followed by isoflurane in oxygen with the vaporiser set at 2% for maintenance. Mechanical ventilation targets of tidal volume of 10-20 ml/kg and a respiratory rate of 10-20 breaths per minute were used, aiming for end tidal carbon dioxide between 35 and 45 mmHg. Full monitoring included electrocardiogram, invasive arterial blood pressure, temperature, saturation and central venous pressure via a line in the central jugular vein (JV). Following median sternotomy and heparinization (150 IU/kg) a 5-0 prolene suture was passed around the proximal left anterior descending artery (LAD) just distal to the first diagonal branch; a catheter was inserted into the coronary sinus (CS) under direct vision and a sampling line was inserted into the aortic root (AR). Under stable conditions the LAD was gently snared to start a period of 60 min of ischemia, after which the snare was released to allow reperfusion for another period of 60 min before termination and myocardial sampling. Blood was drawn serially from JV, CS, and AR at the following time points: baseline, before intervention and at 5, 15, 30, and 60 min during ischemia and at 1, 3, 5, 15, 30, and 60 min of reperfusion.

2.1.4. In vivo murine myocardial infarction model

The left anterior descending (LAD) coronary artery was occluded to induce MI in an acute open chest, in situ mouse model as described previously[17] to assess the effects of AR-C141990 on I/R injury. Briefly, mice were anaesthetized by administration of sodium pentobarbital (70 mg/kg intraperitoneally), endotracheally intubated, ventilated with 3 cm H_2O positive end expiratory pressure and kept at 37 °C using a rectal thermometer-controlled heatpad (TCAT-2LV, Physitemp, USA). Ventilation frequency was maintained at 110 breaths/min, with tidal volume between 125 and 150 µl. The heart was exposed and a suture was placed around the prominent branch of the LAD and passed through a small plastic tube used to initiate ischemia by pressing the tube against the heart surface to occlude the LAD. Mice were subjected to 30 min of ischemia and 120 min of reperfusion, after reperfusion, hearts were stained with Evans Blue and 2% triphenyltetrazolium chloride (TTC) and blindly analyzed by an independent researcher.

2.2 Cardiomyocyte ischemia/reperfusion model

2.2.1. Isolation of adult primary mouse cardiomyocytes

Adult primary mouse cardiomyocytes were isolated as described previously.[18] Mice were culled by cervical dislocation before rapidly excising the heart and cannulating via the aorta in ice-cold, sterile perfusion buffer (in mM: NaCl (113), KCl (4.7), KH_2PO_4 (0.6), Na_2HPO_4 (0.6), $MgSO_4.7H_2O$ (1.2), $NaHCO_3$ (12), $KHCO_3$ (10), HEPES sodium salt (0.922), taurine (30), 2,3-butanedione monoxime (10) and glucose (5.5)). Hearts were retrogradely perfused for 5 min with perfusion buffer (37 °C) to clear residual blood, then the hearts were digested by perfusing digestion buffer (30 ml perfusion buffer supplemented with 5 mg Liberase (Roche, UK) and 12.5 µM $CaCl_2$) for 20 min. After digestion, the heart was removed from the cannula and carefully broken apart with tweezers and gentle pipetting in 4 ml digestion buffer. The cell suspension was transferred to a 15 ml centrifuge tube before making up to 10 ml with stop buffer (37 °C) (10% [v/v] fetal bovine serum [FBS] in perfusion buffer) and allowed to pellet by gravity for 10 min at RT. After pelleting, the supernatant was removed and the cells were resuspended in sequentially increasing Ca^{2+} concentrations (62 µM, then 212 µM, then 1 mM) in 5 ml stop buffer. Cells were subsequently resuspended in M199 media (Gibco, Thermo Fisher Scientific) supplemented with 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 25 µM blebbistatin, 100 IU/ml penicillin and 100 IU/ml streptomycin and plated (1 x 10^5 cells/dish) in laminin-coated (0.1 mg/ml from Engelbreth-Holm-Swarm murine sarcoma basement membrane; Sigma Aldrich, UK) glass dishes.

2.2.2 Anoxic cardiomyocyte incubations

Anoxic incubations were carried out using an anaerobic chamber (0.4 ppm O_2; Belle Technologies, UK). Equipment and solutions were degassed overnight in the transfer compartment of the anoxic chamber before the experiment was performed. Experiments were performed in Tyrode's buffer (in mM: NaCl (137), KCl (5.4), $MgCl_2$ (0.4), HEPES (10), Glucose (10), $CaCl_2$ (1), pH 7.4). The MCT1 inhibitor AR-C141990 (Tocris, Biotechne) was used at a concentration of 10 µM in Tyrode's buffer. Cardiomyocytes were plated (1 x 10^5 cells/plate), washed once with Tyrode's buffer (37 °C) before 2 ml fresh Tyrode's buffer were added to each dish. Anoxia was induced for different time points by placing dishes in the anaerobic chamber on a 37 °C heat block, before the cells were lysed under anoxia, transferred to Eppendorf tubes and snap frozen on dry ice. Cells were reperfused by removing from the anaerobic chamber, replacing buffer with fresh Tyrode's buffer and incubating (37 °C, 15 min) before lysing cells for subsequent succinate analysis.

2.3 Metabolite measurement by mass spectrometry

2.3.1. Succinate extraction

For tissue samples, frozen tissues were weighed out on dry ice to achieve approximately 25 mg of each tissue sample. Tissues were extracted with 25 µl/mg MS extraction buffer (50% [v/v] methanol, 30% [v/v] acetonitrile and 20% [v/v] H_2O), supplemented with 1 nmol of [$^{13}C_4$]-succinate (Sigma Aldrich, UK) in a pre-chilled Precellys tube (Hard tissue homogenising CK28-R – 2 ml; Bertin Instruments, France). Tissues were homogenised using a Precellys 24 tissue homogeniser (6,500 rpm, 15 s; Bertin Instruments, France) and then immediately placed back on dry ice for 5 min. Samples were re-homogenised (6,500 rpm, 15 s) and again placed on dry ice to cool before agitating in a shaking heat block (1,400 rpm, 15 min, 4 °C; Thermo Fisher Scientific, UK) in a cold room (4 °C) and then incubating (-20 °C, 1 h). Samples were subsequently centrifuged (17,000 x g, 10 min, 4 °C) and the supernatant transferred to a pre-chilled microcentrifuge tube and re-centrifuged (17,000 x g, 10 min, 4 °C). The resulting supernatants were transferred to pre-cooled MS vials which were stored at -80 °C until analysis for succinate by liquid chromatography- tandem mass spectrometry (LC-MS/MS).

For perfusate from the Langendorff heart and plasma samples, the perfusate or plasma was centrifuged (17,000 x g, 10 min, 4 °C) before extracting 50 µl in 750 µl MS extraction buffer supplemented with 1 nmol [$^{13}C_4$]-succinate. The samples were agitated in the cold (1,400 rpm, 15 min, 4 °C; Thermo Fisher Scientific, UK) before incubating (-20 °C, 1 h). The samples were centrifuged (17,000 x g, 10 min, 4 °C), then the supernatant transferred to a new tube and recentrifuged. The clear supernatant was transferred to MS vials and stored at -80 °C until analysis.

For adult primary cardiomyocytes, cardiomyocytes were extracted in 500 µl MS extraction buffer supplemented with 1 nmol [$^{13}C_4$]-succinate and agitated in the cold (1,400 rpm, 15 min, 4 °C; Thermo Fisher Scientific, UK) before incubating (-20 °C, 1 h). The samples were centrifuged (17,000 x g, 10 min, 4 °C), then the supernatant transferred to a new tube and recentrifuged. The clear supernatant was transferred to MS vials and stored at -80 °C until analysis.

2.3.2. Quantification of succinate

LC-MS/MS analysis of succinate was performed using an LCMS-8060 mass spectrometer (Shimadzu, UK) with a Nexera X2 UHPLC system (Shimadzu, UK). Samples were stored in

a refrigerated autosampler (4 °C) before injection of 5 µl using a 15 µl flowthrough needle. Separation was achieved using a SeQuant® ZIC®-HILIC column (3.5 µm, 100 Å, 150 x 2.1 mm, 30 °C column temperature; MerckMillipore, UK) with a ZIC®-HILIC guard column (200 Å, 1 x 5mm). A flow rate of 200 µl/min was used with mobile phases of Buffer A: 10 mM ammonium bicarbonate and Buffer B: 100% acetonitrile. A gradient of 0-0.1 min, 80% MS buffer B; 0.1-4 min, 80%-20% B; 4-10 min, 20% B, 10-11 min, 20%-80% B; 11-15 min, 80% B was used. The mass spectrometer was operated in negative ion mode with multiple reaction monitoring (MRM) and spectra were acquired using Labsolutions software (Shimadzu, UK), with compound quantities calculated from relevant standard curves in MS extraction buffer and comparing against [$^{13}C_4$]-succinate internal standard.

2.3.3. Metabolomic analysis by LC-MS

Samples were extracted as for succinate quantification but ^{13}C-succinate omitted and 5 µM d_8-valine added instead. LC-MS analyses were performed on a Q Exactive Orbitrap (Thermo Scientific) mass spectrometer coupled to an Ultimate 3000 UHPLC system (Dionex). The liquid chromatography system was fitted with either a ZIC-HILIC column (150 mm × 4.6 mm) or a ZIC-pHILIC column (150 mm × 2.1 mm) and respective guard columns (20 mm × 2.1 mm) (all Merck, Germany). The metabolites were eluted with previously described gradients.[19] The mass spectrometer was operated in full MS and polarity switching mode. Samples were randomised in order to avoid bias due to machine drift and processed blindly. The acquired spectra were analysed using XCalibur Qual and XCalibur Quan Browser software (Thermo Fisher Scientific) by referencing to an internal library of compounds.

2.4 Isolation of rat heart mitochondria

Rat heart mitochondria (RHM) were isolated as described previously.[20] Briefly, freshly excised rat hearts were homogenised in STEB buffer (250 mM sucrose, 5 mM Tris, 1 mM EGTA, 0.1% [w/v] BSA, pH 7.4) using a dounce homogeniser. Homogenates were centrifuged (3,000 x g, 5 min, 4 °C) to pellet nuclei and unbroken cells. The resulting supernatant was then centrifuged (10,000 x g, 10 min, 4 °C) to pellet mitochondria. Pelleted mitochondria were resuspended in STEB buffer and re-centrifuged (10,000 x g, 10 min, 4 °C). The pelleted mitochondria were resuspended in STEB buffer with the BSA omitted (400 µl/heart) and the mitochondrial protein quantified by BCA assay (Thermo Fisher Scientific).

2.5 Measurement of ROS production by RET

ROS production by RET was measured by following the conversion of Amplex Red to resorufin.[20] Isolated RHM were incubated in KCl buffer (120 mM KCl, 10 mM HEPES, 1 mM EGTA, pH 7.4; 37 °C) supplemented with Amplex Red (12.5 μM; Invitrogen, Thermo Fisher Scientific), horseradish peroxidase (20 μg/ml), BSA (200 μg/ml), superoxide dismutase (40 μg/ml) and succinate (0-10 mM) in a 96-well plate. Resorufin fluorescence was detected by λ_{ex}= 570 nm and λ_{em}= 585 and calibrated against known concentrations of hydrogen peroxide (46.6 $M^{-1}cm^{-1}$ at 240 nm).

2.6 Statistics and experimental design

All data in figures are presented as mean ± S.E.M., unless stated otherwise in the figure legend. Statistical analysis was performed using either one or two-way ANOVA with the suitable post-hoc correction for multiple comparisons described in the figure legend. Where only two groups were compared, statistical significance was assessed by two-tailed Student's unpaired t-test. A *p* value of less than 0.05 was considered significant. Statistics were calculated in Prism 8.0 software (GraphPad Software Inc, USA).

3. Results

3.1 Succinate efflux upon reperfusion of an *ex vivo* ischemic heart

To quantify succinate efflux from the ischemic heart upon reperfusion, we used the Langendorff isolated heart perfusion model. Mouse hearts following 20 min functional equilibration (LVDP 94 ± 3 mmHg, heart rate 453 ± 20 beats/minute, coronary flow 3.5 ± 0.14 mean±S.E.M., n=17) were then exposed to 20 min global, no-flow ischemia, followed by reperfusion with oxygenated perfusion buffer (*Figure 2A*). Langendorff hearts exposed to ischemia led to a 14-fold increase in succinate levels (*Figure 2B*), comparable to the succinate accumulation seen previously in the ischemic heart *in vivo*.[4,5] Upon subsequent reperfusion, tissue succinate levels returned to baseline (*Figure 2B)*. Halving the ischemic time decreased succinate accumulation, but following reperfusion the levels of succinate were the same (*Figure 2B*). To see if any of the succinate that had accumulated during ischemia was released from the heart upon reperfusion, we next measured succinate in the coronary effluent immediately after reperfusion (*Figure 2A*). Succinate was released from the

myocardium into the circulation over the first two min of reperfusion, with little further release after 3 min (*Figure 2C*). Note that the quantification of succinate release is normalised to perfusate volume and is thus independent of the flow rate. Comparing the total amount of succinate in the perfusate to that in the heart at the onset of ischemia, and correcting for baseline levels, showed that about half of the succinate accumulated during ischemia was released (*Figures 2C-D*), as was shown previously by Brookes and colleagues.[6] To confirm that this succinate efflux was largely from the cardiomyocytes, we used a murine primary adult cardiomyocyte model exposed to anoxia and reperfusion. Succinate accumulated significantly in anoxic cardiomyocytes and returned to baseline levels upon reperfusion (*Figure 2E*). After reperfusion, 1.3 ± 0.25 nmol succinate/10^5 cells (mean ± SEM, n = 6) was released into the incubation medium. This succinate release is about 60% of that accumulated within the cardiomyocytes during ischemia (corrected for succinate remaining after reperfusion; *Figure 2E*), and is consistent with the amount released from the heart upon reperfusion (*Figures 2C-D*).

3.2 Dependence of ROS production by RET on succinate concentration

In our *ex vivo* system we found that after 20 min global ischemia, the level of succinate per heart increased to about 360 – 400 nmol succinate/heart, while in a previous study we found that 30 min global ischemia of the heart *in situ* leads to ~800 nmol succinate/heart.[5] As the mouse hearts used in these studies weigh ~184 ± 16 mg (n = 8, mean ± SEM), this corresponds to 2 - 4 µmol succinate/g wet weight. The water content of the rodent heart is 615 µl/g wet weight intracellular and 174 µl/g wet weight extracellular.[21] Assuming that the succinate stays within the cells during ischemia and is distributed roughly equally throughout the cell, this corresponds to an intracellular succinate concentration of 3-6 mM at the onset of reperfusion. Even if during ischemia the succinate was also equally distributed between the intracellular and extracellular water, this would still give an intracellular succinate concentration of 2.5-5 mM at the onset of reperfusion. The level of succinate in the normoxic mouse hearts here is 22 ± 3 nmol/heart (Mean ± SEM n = 5) and previously we reported a succinate level in the normoxic mouse heart of 34 nmol/heart.[5] This corresponds to normoxic succinate levels of 120 - 185 nmol/g wet weight or an intracellular succinate concentration of 195 - 300 µM. Thus, even if we assume that the efflux of ~50% of the intracellular succinate occurred immediately upon reperfusion, this would still leave mitochondria exposed to 1.5 – 3 mM succinate at the onset of reperfusion, decreasing down to ~ 200 - 300 µM after 2-3 min. To see if these levels of succinate were sufficient to drive RET at complex I, we

366 determined the dependence of complex I ROS production by RET in isolated heart
367 mitochondria on succinate concentration (*Figure 2F*). This showed that RET production
368 driven by succinate saturated at ~ 4 mM with a half maximal effective concentration (EC_{50})
369 of ~ 1 mM (*Figure 2F*). This ROS production was largely inhibited by the complex I
370 inhibitor rotenone, which blocks RET but does not affect ROS production at complex III.
371 This is consistent with the protective effect of rotenone on IR injury/infarct size and suggests
372 that the ROS production measured here is essentially all generated by RET at complex I.
373 Thus, the level of succinate present within the ischemic heart at the onset of reperfusion is
374 more than adequate to generate ROS by RET and this level of succinate must decrease by
375 ~80% before substantially affecting RET. Thus, the efflux of succinate from the tissue upon
376 reperfusion does not impact on its ability to drive RET and cause pathological I/R injury.
377
378 **3.3 Selectivity of succinate release upon reperfusion**
379 To determine whether the succinate release from the heart was selective, or part of a general
380 metabolite release due to tissue damage upon reperfusion, the metabolome of the perfusate
381 from the Langendorff hearts was assessed (*Figure 3A*). For this, we could quantify the levels
382 of 47 metabolites in the perfusate from the normoxic heart under equilibration, as well as in
383 the perfusate during the first 3 min reperfusion of the ischemic heart (*Figure 2A*). We
384 calculated the cardiac release ratio,[2] the difference in metabolite levels between reperfusion
385 and normoxia, normalised to the levels in the normoxic effluent (*Figure 3A*). This showed
386 that only a few of the 47 metabolites measured were released upon reperfusion. To assess this
387 further, we plotted the differences in metabolites in the perfusate between reperfusion and
388 normoxia (*Figure 3B*). This demonstrated that only 10 metabolites showed a statistically
389 significant release upon reperfusion. Among these were nicotinamide and the adenine
390 nucleotide breakdown products hypoxanthine, adenine and inosine which are known to
391 accumulate during ischemia.[4,5,22] These metabolites are all neutral so they may diffuse
392 passively through the cell membrane.[23] In contrast, the other metabolites released - succinate,
393 lactate and some amino acids – are charged and will require transporters to leave the cell
394 upon reperfusion. Among charged substrates the cardiac release ratio was highest for
395 succinate (*Figure 3A*), indicating that succinate is a major charged metabolite released upon
396 reperfusion of the ischemic tissue probably via a selective transport pathway.
397 As the Langendorff heart is perfused with an oxygenated, hyperglycaemic crystalloid
398 buffer, we were uncertain how this might affect succinate accumulation, efflux and
399 consumption upon reperfusion compared to the *in vivo* situation. To address this potential

concern, we assessed mouse hearts that were rendered ischemic *in situ*, prior to which they had been supplied with normal blood. To do this we induced global no-flow ischemia by exsanguination and then left the heart in the body for 20 min maintained at 37˚C. Then, the blood vessels were clamped and the heart was flushed with Krebs buffer and the perfusate collected. During global *in situ* ischemia we found significant accumulation of succinate (*Figure 4A*) and flushing the heart with oxygenated buffer led to a return to baseline levels of tissue succinate (*Figure 4A*). Measurement of succinate in the perfusate following flushing showed loss of succinate from the heart to the perfusate over the first 2-3 min reperfusion (*Figure 4B*), very similar to that for the Langendorff hearts. Comparing the total amount of succinate released with that present at the onset of ischemia and correcting for baseline levels showed that about 33% of the succinate that was present in the tissue at the end of ischemia was released upon flushing (*Figure 4C*). Thus, the succinate efflux seen in the *ex vivo* perfused heart is replicated by the more physiological *in situ* mouse heart.

To extend this analysis to a large animal model closer in size to human, we used a pig MI model of ischemia/reperfusion in which a 5-0 prolene suture was passed around the coronary artery and gently snared to block blood flow and to hold the heart ischemic for 60 min and then released to reperfuse the ischemic tissue with oxygenated blood (*Figure 4D*). We assessed the efflux of succinate from the ischemic tissue into the coronary sinus, which reflects any metabolites released by the ischemic myocardium upon reperfusion, as well as that in the aortic root and in the jugular vein (*Figure 4E;* Supplementary material online*, Figure S1*). Upon reperfusion, there was a large increase in the succinate levels in the coronary sinus during the first 5 min reperfusion (*Figure 4E*), but not in the blood from the aortic root or jugular vein (Supplementary material online*, Figure S1*). To understand whether the levels of succinate released from the pig heart during reperfusion were significant compared to other metabolites, we measured the levels of 40 metabolites in the coronary sinus upon reperfusion and in the aortic root, enabling us to generate a cardiac release ratio (*Figure 4F*). This showed that only a few metabolites were released upon reperfusion of the ischemic heart tissue of pigs undergoing myocardial infarction and that prominent among them was succinate (*Figure 4F*).

3.4 Succinate efflux can be inhibited

The charged nature of succinate at physiological pH (pK_a 4.2 and 5.6) and the selectivity of its efflux upon reperfusion of ischemic tissue suggests that its release is carrier mediated. To assess this hypothesis, we measured the effect on succinate efflux of the general succinate

transport inhibitors succimer and 2-phenylsuccinate, which are structurally similar to succinate (*Figure 5A*).[24,25] Both these inhibitors decreased succinate efflux (*Figure 5B*), with a concomitant increase in succinate retention within the heart at the end of the reperfusion period (Supplementary material online, *Figure S2*).

3.5 Succinate efflux is enhanced by the plasma membrane proton gradient

The pH of the ischemic myocardium can decrease to ~6.5, compared to a pH of 7.4 for the heart perfusate.[26,27] Hence, during reperfusion of the ischemic heart there will be a pH gradient of ~1 pH unit across the plasma membrane, acidic inside. As many metabolite transport processes are coupled to proton movement, we next assessed whether this pH gradient affects succinate efflux.[28,29] To do this, we reperfused the ischemic heart with pH 6 perfusion buffer to abolish the pH gradient (*Figure 5C*). Strikingly, this intervention greatly decreased succinate efflux (*Figure 5C*). To further assess the role of the pH gradient in succinate efflux we used a range of ionophores - gramicidin (H^+, Na^+, K^+), monensin (H^+, Na^+) and nigericin (H^+, K^+), all of which can disrupt the plasma membrane pH gradient (*Figure 5D*). As these ionophores are all large hydrophobic molecules they act by inserting into the plasma membrane and are unlikely to redistribute to intracellular membranes such as the mitochondrial inner membrane over the timescale of these experiments.[30] Thus, their effects are primarily due to disruption to the plasma membrane pH gradient. All these ionophores significantly decreased succinate efflux (*Figure 5E*). These ionophores are large hydrophobic molecules which are unlikely to migrate from the plasma membrane to disrupt mitochondrial membranes. In contrast use of the small protonophores FCCP and DNP significantly disrupted cardiac function (data not shown). We conclude that succinate efflux upon reperfusion of the ischemic heart is greatly enhanced by the pH gradient.

3.6 Succinate efflux from the reperfused ischemic heart is mediated by MCT1

The above data show that succinate efflux upon reperfusion of the ischemic heart is carrier-mediated and enhanced by a pH gradient. Monocarboxylate transporter 1 (MCT1), which usually transports lactate in symport with a proton, is a potential carrier for this process.[28,31,32] As the first succinate pKa is ~5.6,[33] at the pH within ischemic tissues (~6.5)[26,27] about 10% of the succinate in the myocardium would be in the monocarboxylate form, which may be transported by MCT1.[6,28,34] Supporting this possibility, expressing MCT1 in *Xenopus* oocytes led to succinate uptake into the cells, but only when incubated in a medium at acidic (pH ~6) pH.[28] Furthermore, MCT1 is highly expressed in heart tissue.[16]

To assess whether MCT1 could mediate succinate efflux in the reperfused ischemic heart, we included lactate in the perfusion buffer to inhibit the activity of MCT1. This decreased succinate efflux (*Figure 6A*). Next, we reperfused ischemic hearts with AR-C141990, a selective MCT1 inhibitor (MCTi),[35,36] which led to a dose-dependent decrease in succinate efflux when added to the reperfusion buffer (*Figure 6B*). A similar effect was seen when hearts were administered the MCTi before ischemia (*Figure 6B*), which did not alter the ischemic levels of succinate (Supplementary material online*, Figure S3*). MCTi also enhanced succinate retention within the reperfused tissue (Supplementary material online*, Figure S4*). Succinate efflux was also reduced by MCT1 inhibition with MCTi, when it was added upon reoxygenation of anoxic cardiomyocytes. Here it decreased succinate efflux by about 90% from 1.28 ± 0.25 to 0.14 ± 0.02 nmol succinate/10^5 cells (mean ± SEM, n = 6).

To confirm that MCT1 mediates succinate efflux from the ischemic heart upon reperfusion, we utilised the *MCT1*$^{+/-}$ mouse model.[16,37,38] Whilst the *MCT1*$^{-/-}$ mouse is embryonic lethal, *MCT1*$^{+/-}$ mice have ~40% reduction in the expression of MCT1 in the heart[16] and have no obvious phenotype in normoxic conditions when compared to *MCT*$^{+/+}$ controls.[16,37,38] While the levels of succinate in the *MCT1*$^{+/-}$ and *MCT*$^{+/+}$ hearts were the same after 20 min ischemia (Supplementary material online*, Figure S3*), succinate efflux from reperfused *MCT1*$^{+/-}$ hearts was significantly lower than that of *MCT1*$^{+/+}$ control hearts (*Figure 6C*). The levels of succinate retained in the heart after 6 min of reperfusion did not differ between the *MCT1*$^{+/+}$ and *MCT1*$^{+/-}$ hearts (Supplementary material online*, Figure S5*). This result suggests that the reduction in MCT1 level in the *MCT1*$^{+/-}$ hearts is sufficient to decrease succinate efflux.

4. Discussion

The accumulation of succinate during ischemia and its oxidation upon reperfusion is a key driver of I/R injury.[1–5] While succinate is made within the mitochondrial matrix during ischemia, most succinate will be present in the cytosol at the onset of reperfusion. In addition, it is also clear that upon reperfusion some of the succinate accumulated during ischemia is released from the tissue upon reperfusion.[2,6] Here we show that this succinate efflux from the cell is greatly enhanced by the pH gradient (acidic inside) between the tissue and the circulation upon reperfusion, and that this efflux is mediated by the monocarboxylate transporter, MCT1. The best understood role of MCT1 is as a plasma membrane lactate transporter, which leads to the electroneutral efflux of lactate along with a proton. At the low

pH (~6.5) within ischemic tissue,[26,27] about 10% of the succinate will be in the monocarboxylate form (pKa ~ 5.6),[33] which can then be transported by MCT1,[28] presumably because of its similarity in structure to lactate. In addition, because MCT1 transports a monocarboxylate in symport with a proton, the pH gradient between the tissue and the circulation present upon reperfusion of the ischemic tissue will also drive succinate efflux. This model of succinate efflux, which was first suggested by Halestrap and colleagues,[28] is shown in *Figure 7*. Importantly, this work shows that the succinate accumulated in the heart during ischemia has two fates: it is either oxidised by the mitochondrial respiratory chain, or it is released into the circulation, potentially acting as a metabolic signal. A precedent for such signalling is when circulating succinate is taken up and activates thermogenesis in brown adipose tissue[14]. elucidate the mechanism of succinate efflux from the ischemic heart during reperfusion raises the prospect of targeting MCT1-dependent succinate in heart attack. The kinetics and current understanding of succinate transport by MCT1 is poor. Whilst some initial characterisation of this process has been carried out,[28] more complete experiments are required to understand the interplay of the pH gradient and succinate transport.

There are a number of experimental points that should be considered in interpreting our findings. Perfused heart experiments have a number of limitations, such as the use of supra-physiological concentration of glucose and the lack of fatty acids or other physiological respiratory substrates. However, it should be noted that an isolated perfused heart which was supplied with fatty acids as an energy source accumulates succinate to a similar extent as hearts perfused with glucose-containing medium.[4] The second is that ischemic hearts *in vivo*, which had been perfused with blood prior to ischaemia and were thus respiring on physiological substrates, also accumulate succinate to the same extent as the isolated perfused heart.[4,5] The third point is that reperfusion of the ischaemic human heart,[2] or pig heart (this work), *in vivo* with blood leads to similar efflux of succinate. Thus, our *in vitro* heart perfusion system shows similar succinate accumulation during ischemia and efflux upon reperfusion as the heart *in vivo*.

The potential role of succinate efflux as a signal from the ischemic tissue is supported by the fact that its efflux is carrier mediated, that succinate accumulates dramatically within ischemic tissues, the pH within ischemic tissues is lowered. The accumulation of succinate during ischemia seems to be a universal phenomenon and has now been shown by us and many others for hearts from mice, rats, rabbits, pigs and humans.[1-5] Importantly, this accumulation of succinate during ischemia also occurs *in vivo* within tissues utilising

endogenous substrates, as well as in the Langendorff model, presumably because during ischemia the heart relies on glycogen as its main energy source.[1-5,39]

The succinate efflux from the ischemic mouse hearts *in vivo* and *ex vivo* was associated with a limited number of other metabolites. The metabolome of the pig heart attack model mirrored that seen in STEMI patients,[2] suggesting a conserved mechanism of release of these metabolites from the ischemic heart during reperfusion, with succinate being particularly elevated in both pigs and humans. We note that the pig is a widely used model for human cardiac metabolism, and importantly these were young, healthy animals analysed under tightly controlled conditions, compared to the human subjects[1,2] who were all suffering from cardiac disease. Hence these data indicate that the succinate efflux in the human subjects was not simply a consequence of pathology. Lactate was prominent in the perfusate from mouse, a metabolite surprisingly not significantly elevated in the human plasma.[2] This difference may be due to global ischemia in the Langendorff heart compared to regional ischemia in the heart attack models, or due to the high levels of lactate already present in the plasma masking changes.

The action of succinate ligating its cognate receptor, SUCNR1 and subsequent immune activation during I/R injury by succinate released upon reperfusion may contribute to the damage associated with I/R injury (*Figure 7*).[8,9,40,41] However, the pathological role of succinate efflux and the signalling that occurs on ligation to SUCNR1 are currently unknown. Furthermore, inhibition of succinate efflux upon reperfusion with MCT1 inhibitors might be expected to elevate tissue succinate oxidation and thus exacerbate I/R injury. To test this possibility, we carried out a preliminary experiment to assess the effect of the MCT1 inhibitor AR-C141990 on cardiac I/R injury in an *in vivo* mouse model of cardiac I/R injury (*Figure S6*). Administration of AR-C141990 upon reperfusion was protective, despite the elevated tissue levels of succinate it caused in the isolated perfused heart (*Figure S4)*. Further work is required to understand the mechanistic basis of this protection. One possibility is that the lack of lactate efflux has an impact on the cell, perhaps by its impact on glycolytic flux or cell pH. One possibility is that these factors disrupt mitochondrial succinate oxidation or ROS production during reperfusion. Future work will be required to determine fully the (patho)physiological roles of MCT1-dependent succinate efflux.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

H.A.P, M.P.M and D.A. conceived and designed the studies. D.A. carried out Langendorff perfusions. M.M.H, and T.E.B. carried out *in situ* I/R perfusions with guidance from K.S.-P. J.F.M. carried out in vivo LAD model supervised by TK. A.V.G. carried out cardiomyocyte experiments. H.A.P. carried out succinate quantification and ROS measurements. T.Y., L.T., E.N. carried out metabolomics with guidance from C.F. R.A. carried out pig I/R experiments. A.H. and L.P. developed $MCT1^{+/-}$ mouse model and genotyping. All authors interpreted data. H.A.P., M.P.M. and D.A. wrote the original manuscript and all authors reviewed the revised manuscript.

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Author notes

Hiran A. Prag and Anja V. Gruszczyk contributed equally to this study.

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FIGURE LEGENDS

Figure 1 Schematic of metabolite changes occurring during reperfusion.
Upon reperfusion, succinate is oxidised producing ROS fed by succinate re-entry into mitochondria. We favour RET at complex I as the mechanism of this ROS production. Some of the succinate is also released from the cell. SDH, succinate dehydrogenase; RET, reverse electron transport; FH, fumarate hydratase; DIC, dicarboxylate carrier.

Figure 2 (*A-F*) Succinate accumulation and efflux from the ischemic Langendorff heart and primary cardiomyocytes. (*A*) Langendorff model experimental design of ischemia and reperfusion. (*B*) Time dependent succinate accumulation in a Langendorff perfused mouse heart exposed to 10 or 20 min global no-flow ischemia ± 6 min reperfusion (mean ± S.E.M., n=3-8). Statistical significance was assessed by one-way ANOVA with Tukey's post hoc test (**p <0.01, **** p <0.0001 *relative to equilibration, $^{\#\#\#\#}p$ <0.0001 $^{\#}$relative to 10 min ischemia). (*C-D*) Succinate efflux from the Langendorff heart exposed to 10 or 20 min ischemia over the first 6 min of reperfusion (*C*) and compared to the succinate levels achieved in the heart exposed to 20 min ischemia (*D*) (mean ± S.E.M., n=5-8). (*E*) Succinate accumulation and efflux in primary cardiomyocytes exposed to anoxia (1 h) ± reperfusion (15 min) (mean ± S.E.M., n=4-6). Statistical significance was assessed by one-way ANOVA with Dunnett's post hoc test **** p <0.0001, relative to control (Ctl) values). (*F*) ROS production by RET in isolated heart mitochondria. Isolated rat heart mitochondria were incubated with varying succinate concentrations and where indicated rotenone (0.5 μM), and the production of H_2O_2 measured by the conversion of Amplex Red to resorufin (mean ± S.E.M., n=3). The EC_{50} for dependence of ROS production by RET on succinate concentration is shown.

Figure 3 (*A-B*) Succinate is selectively effluxed from the ischemic heart upon reperfusion (*A*) Release of metabolites in the reperfusion coronary effluent (1-3 min) from the Langendorff heart, compared to equilibration coronary effluent expressed as cardiac release ratio ([reperfusion-equilibration]/equilibration). (*B*) Plot showing -$\log_{10}$ of the adjusted p value plotted against the difference in normalised ion counts between equilibration and 1-3 min reperfusion coronary effluent (n=5). Plot generated in Prism 8.0 using multiple t-tests corrected for multiple comparisons using the Holm-Sidak method.

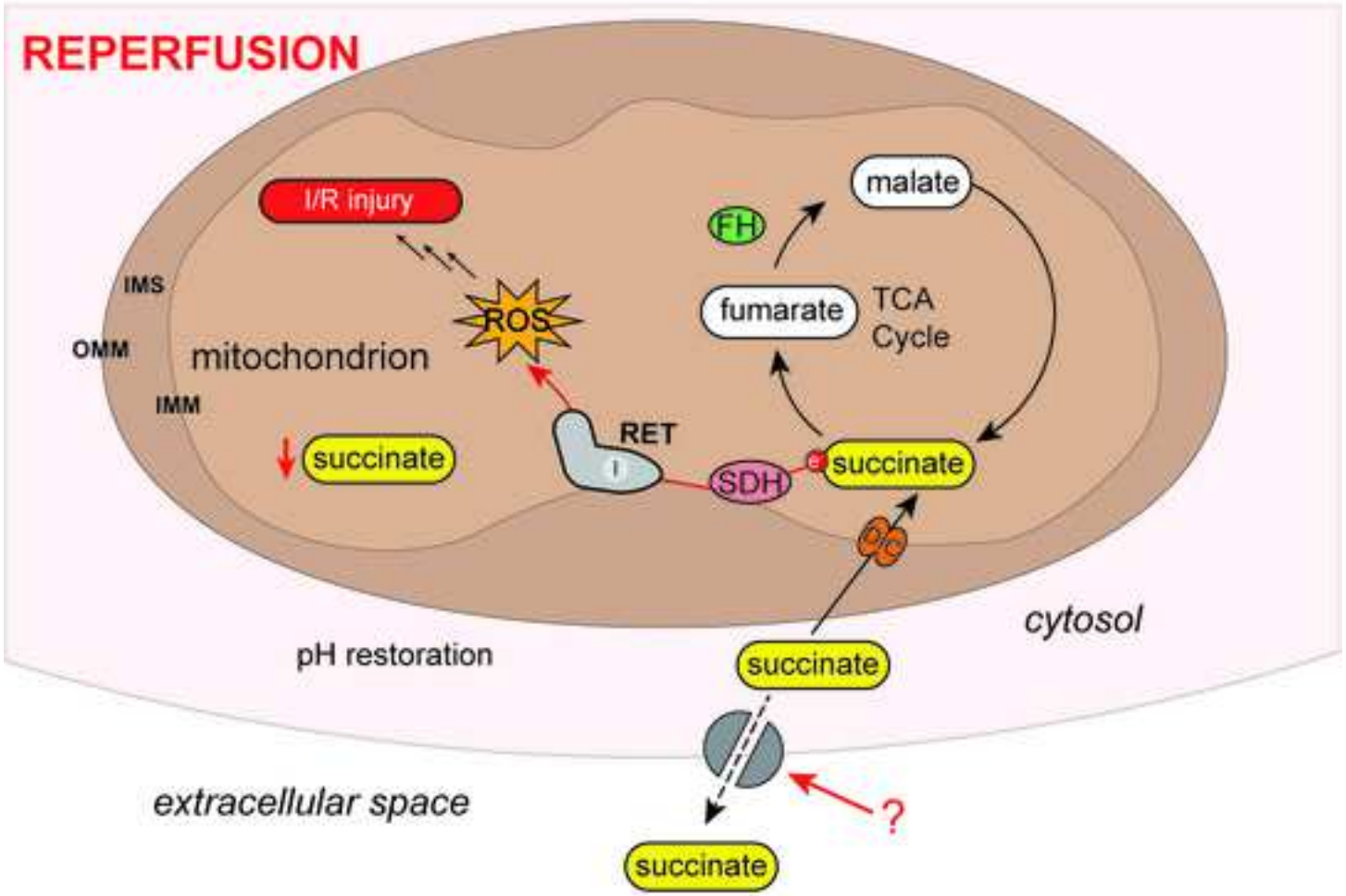
Figure 4 (*A-F*) Succinate accumulation and efflux from the ischemic heart *in situ* and *in vivo*. (*A*) Succinate accumulates during 20 min ischemia and rapidly returns to baseline values after 6 min reperfusion in a murine *in situ* perfusion model (mean ± S.E.M., n=3). Statistical significance was assessed by one-way ANOVA with Dunnett's post hoc test (*** p <0.001, relative to control (Normoxia) values). (*B-C*) Succinate efflux from the *in situ* perfused heart exposed to 20 min ischemia over the first 6 min of reperfusion (*B*) and compared to the succinate levels achieved in the heart exposed to 20 min ischemia (*C*) (mean ± S.E.M., n=3). (*D*) Schematic of porcine MI model and coronary sinus blood sampling (*E*). Succinate is elevated during early reperfusion in the coronary sinus in a porcine MI model. The LAD was occluded by gentle snaring for 60 min before snare released and blood sampled (mean ± S.E.M., n=3). Statistical significance was assessed by two-way ANOVA with Tukey's post hoc test (**p <0.01, **** p <0.0001). (*F*) Release of metabolites in the coronary sinus at reperfusion (1-5 min) compared to aortic root blood expressed as cardiac release ratio ([coronary sinus-aortic root]/aortic root) in porcine MI model.

Figure 5 Modulating succinate efflux during reperfusion. (*A*) Succinate is structurally similar to phenylsuccinate and succimer. (*B-C*) Langendorff mouse hearts were exposed to 20 min global no-flow ischemia, before reperfusing for 6 min and the succinate measured in the perfusate collected during each minute of reperfusion. Control reperfusion from *Figure 2B* (n=5) and non-specific transport inhibitors added at 1 mM succimer (n=3) and 1 mM phenylsuccinate (n=3) at the onset and during reperfusion. (*C*) Mouse hearts were exposed to ischemia as in *Figure 5B* but reperfused with buffer at pH 6 (n=5). (*D*) Diagram of the mechanism of action of ionophores gramicidin, monensin and nigericin. (*E*) Mouse hearts were exposed to ischemia as in *Figure 5B* but reperfused in the presence of ionophores for 6 min (all at 10 μM): gramicidin (n=5), monensin (n=4), nigericin (n=4). All data (*B,C,E*) are presented as mean ± S.E.M.) and statistical significance was assessed by two-way ANOVA with Dunnett's post hoc test (*p <0.05, **p <0.01,*** p <0.001, ****p <0.0001 relative to control reperfusion).

Figure 6 Succinate efflux during reperfusion is mediated by MCT1. (*A-B*) Langendorff hearts were treated as in Figure 5 but reperfused in the presence of either (*A*) 10 mM L-lactate (n=5) or (*B*) 1, 10 or 50 μM AR-C141990 (MCTi; n=3-5). AR-C141990 was also used as a pre-treatment by infusing it during the equilibration phase prior to inducing ischemia (MCTi-Pre at 50 μM; n=5) (all mean ± S.E.M.). Statistical significance was assessed by two-

818 way ANOVA with Dunnett's post hoc test (*p <0.05, ** p <0.01, ***p <0.001, ****p
819 <0.0001 relative to standard reperfusion). (*C*) Succinate efflux from *MCT1*$^{+/-}$ and *MCT*$^{+/+}$
820 mouse hearts exposed to 20 min ischemia upon reperfusion. (mean ± S.E.M., MCT$^{+/+}$ n=7,
821 MCT$^{+/-}$ n=5). Statistical significance was assessed by two-way ANOVA with Dunnett's post
822 hoc test (*p<0.05, **p <0.01 relative to *MCT1*$^{+/+}$ reperfusion).
823
824 **Figure 7** Model of the mechanism of succinate efflux from the ischemic heart during
825 reperfusion. In the ischemic cardiomyocyte, succinate protonation to a monocarboxylate is
826 favoured by the more acidic intracellular pH. During reperfusion, the intracellular pH is
827 restored by proton efflux due to the proton gradient across the plasma membrane. Here,
828 succinate monocarboxylate together with a proton is effluxed from the cardiomyocyte by
829 MCT1, reducing intracellular succinate levels. Succinate release upon reperfusion may be a
830 signal of tissue ischemia and/or damage. SUCNR1 is a G-protein coupled succinate receptor
831 that can respond to the succinate released into the circulation. SUCNR1 is highly expressed
832 on the surface of immune cells and its ligation has been associated with a range of both pro-
833 and anti-inflammatory phenotypes, depending on context.
834

Figure 1



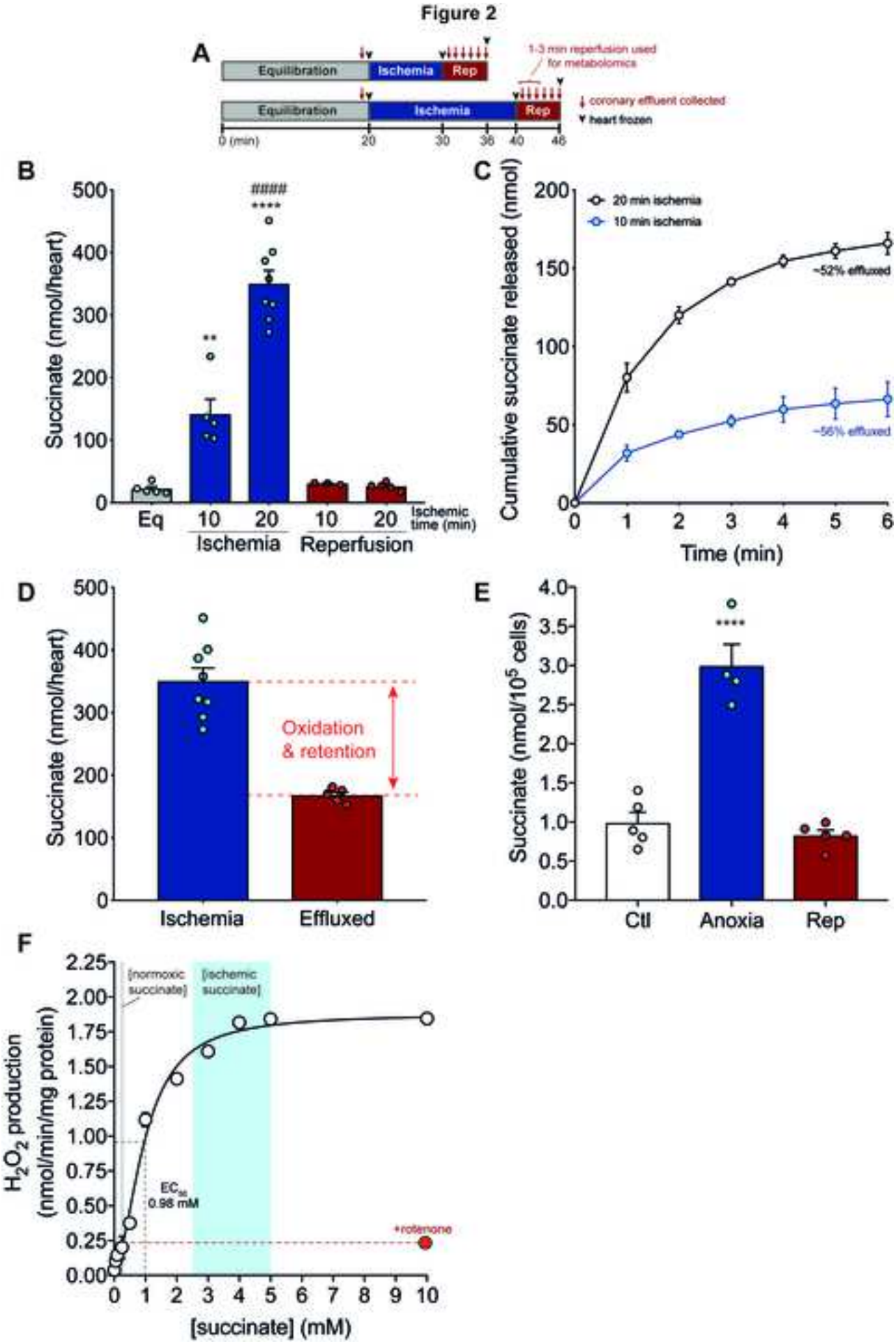


Figure 2
A
Equilibration
Ischemia
Rep
1-3 min reperfusion used for metabolomics
coronary effluent collected
heart frozen
0 (min)
20
30
36
40
46
B
Succinate (nmol/heart)
Eq
10
20
10
20
Ischemic time (min)
Ischemia
Reperfusion
C
Cumulative succinate released (nmol)
20 min ischemia
10 min ischemia
~52% effluxed
~56% effluxed
Time (min)
D
Succinate (nmol/heart)
Oxidation & retention
Ischemia
Effluxed
E
Succinate (nmol/10^5 cells)
Ctl
Anoxia
Rep
F
H2O2 production (nmol/min/mg protein)
[normoxic succinate]
[ischemic succinate]
EC50 0.98 mM
+rotenone
[succinate] (mM)

Figure 3

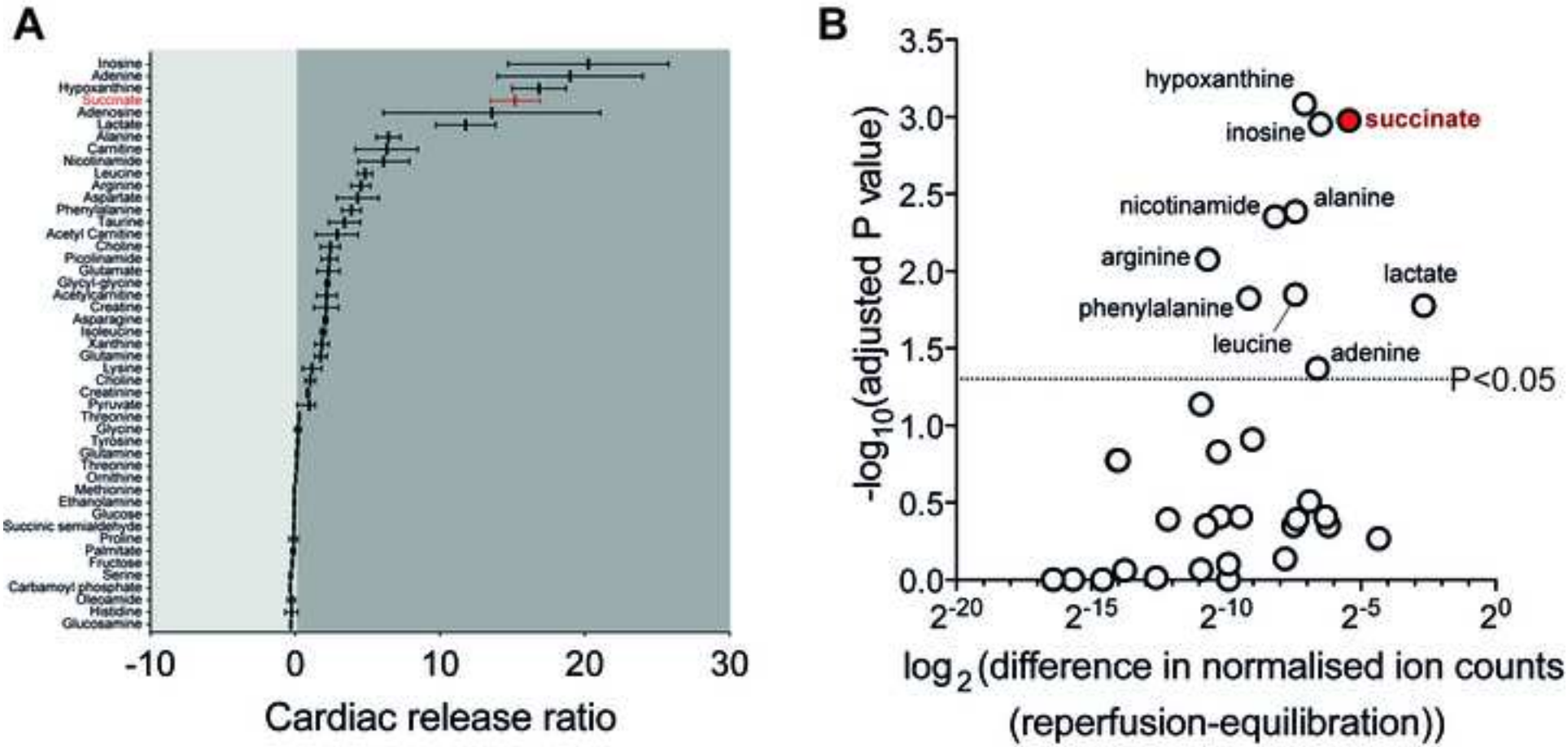


Figure 4

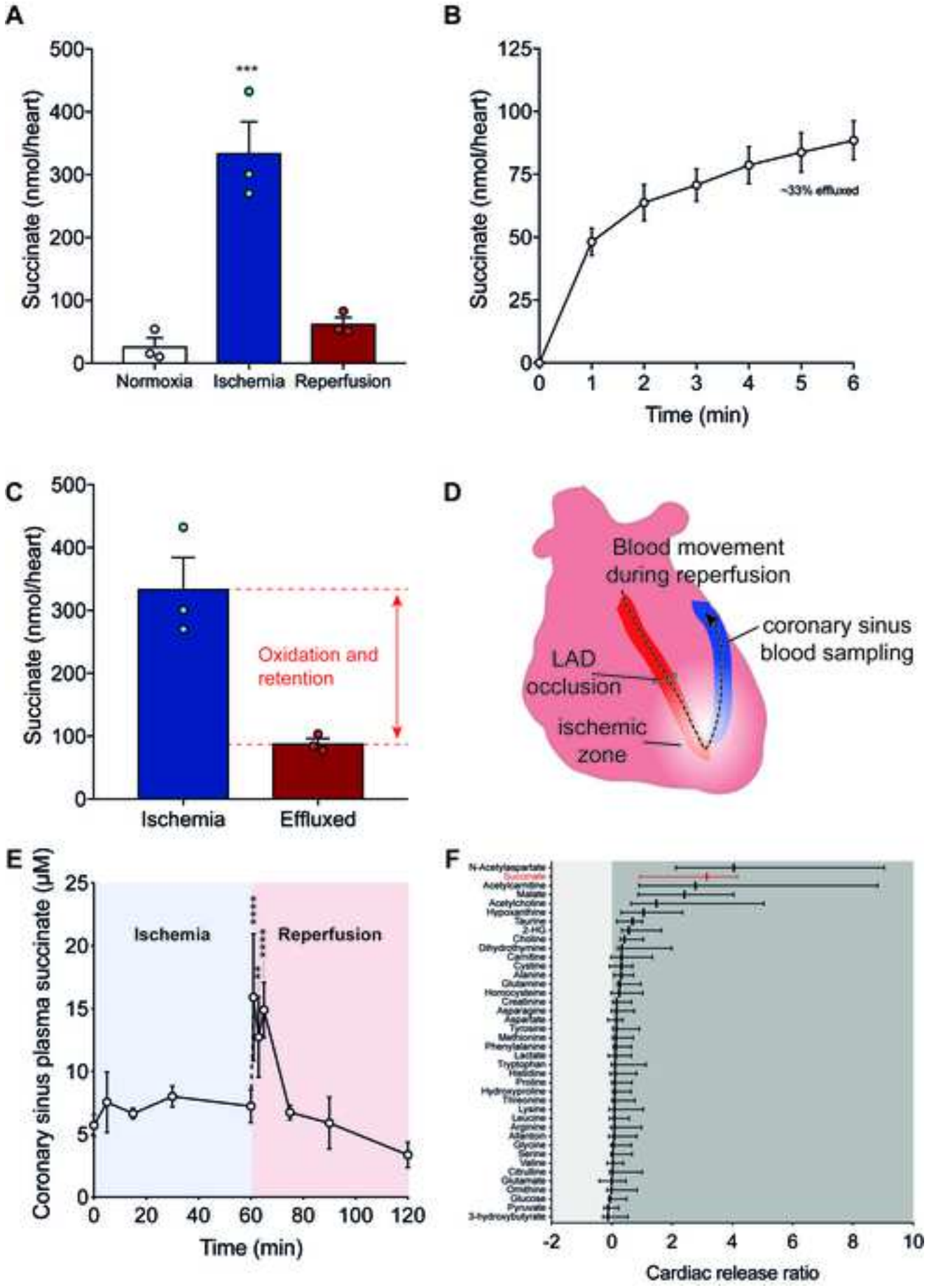


Figure 5

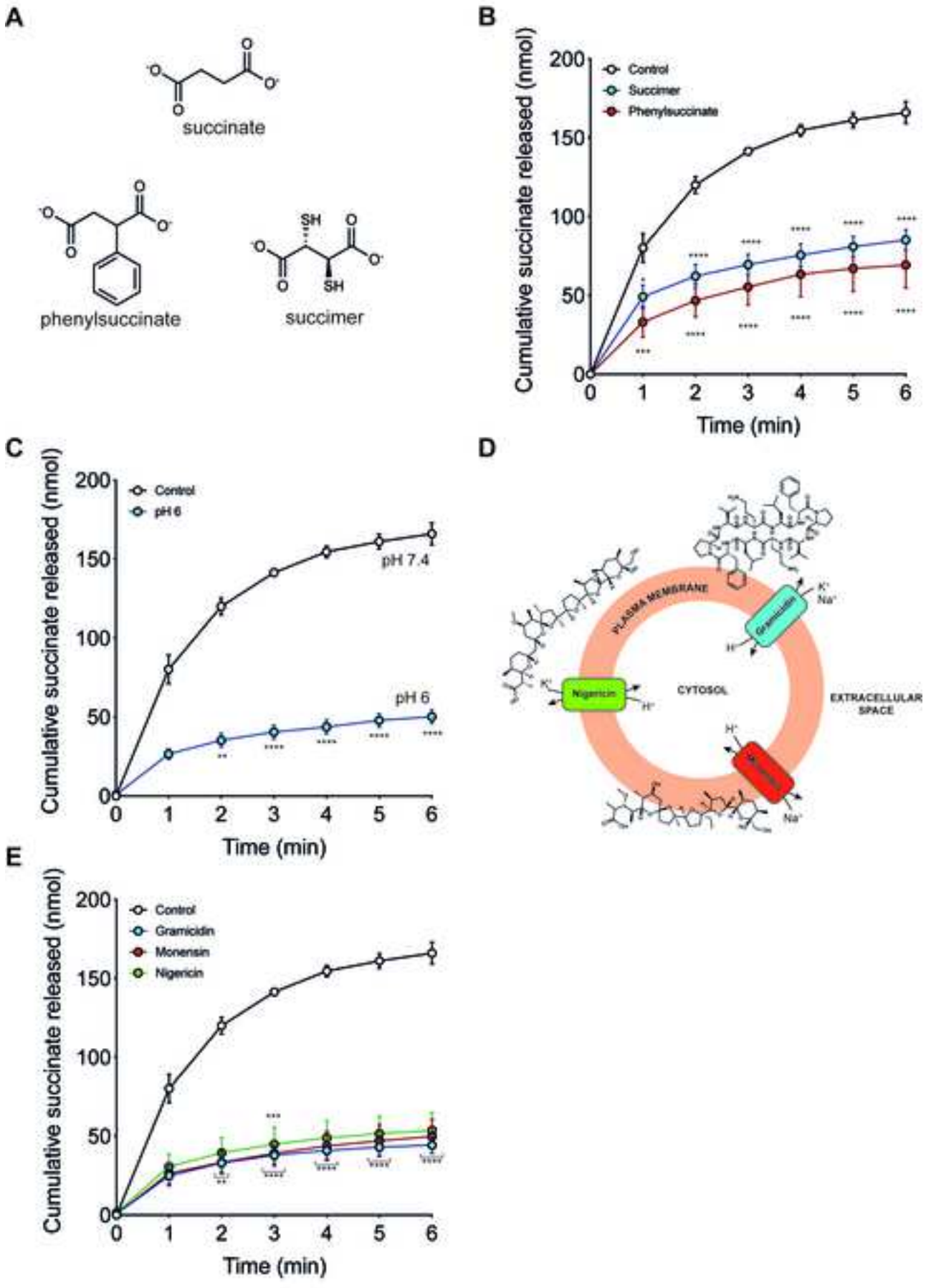


Figure 6

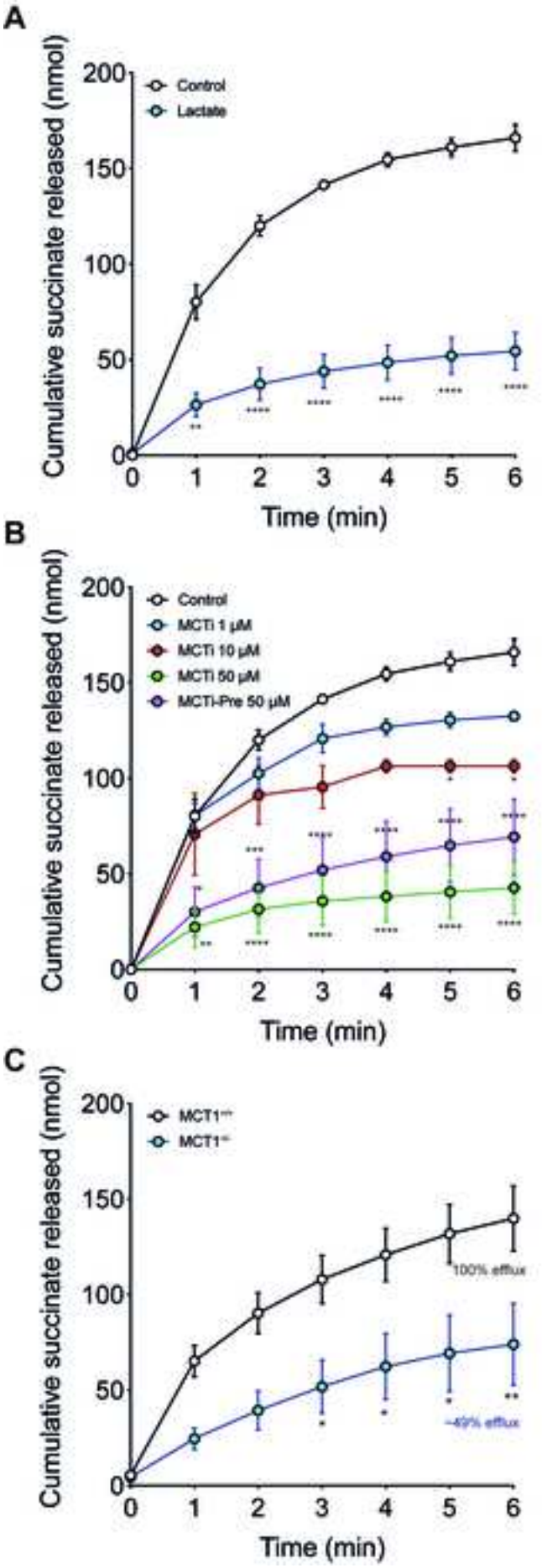
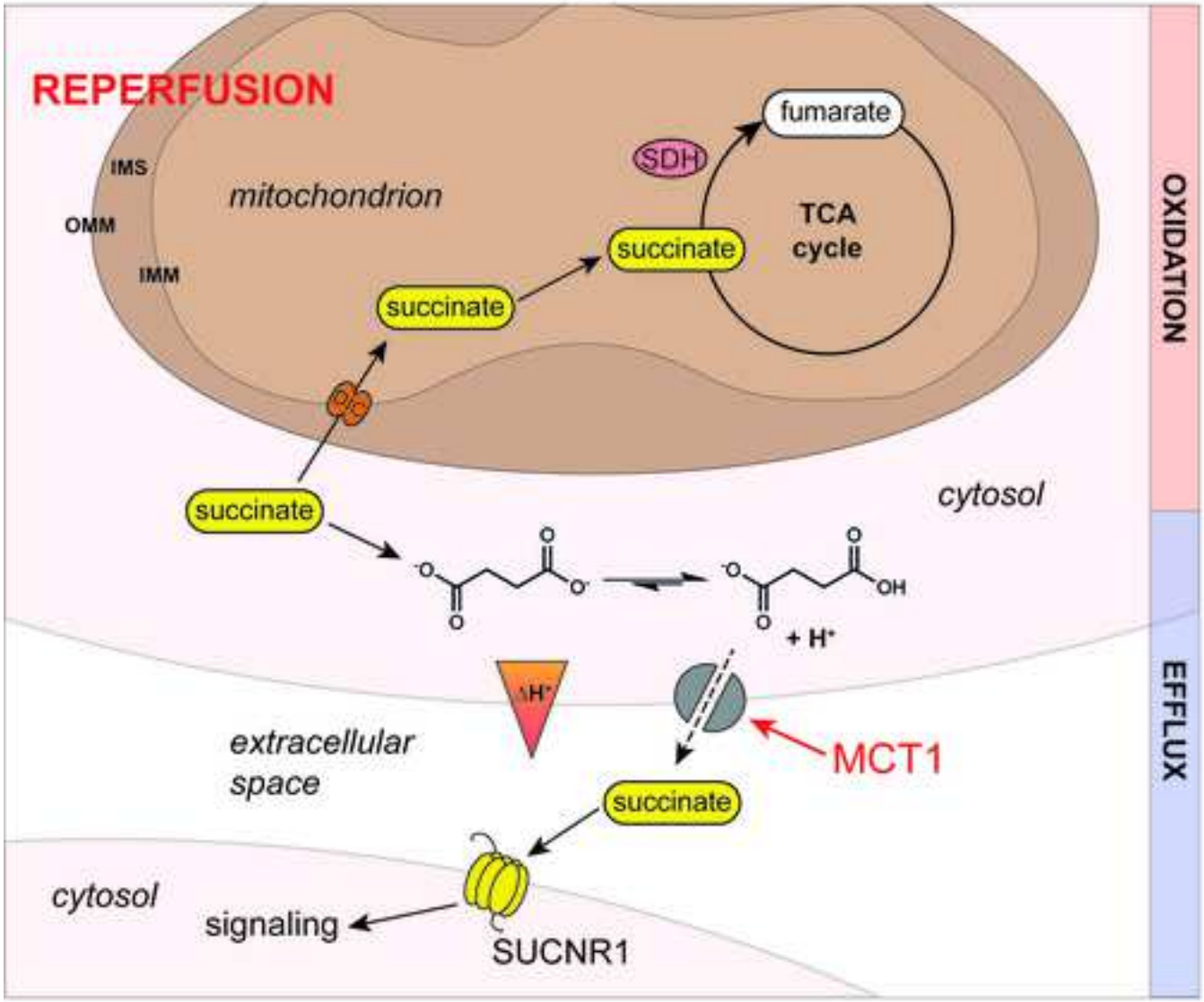


Figure 7



SUPPLEMENTARY FIGURE LEGENDS

Figure S1 Succinate efflux in a porcine MI model only occurs from the ischemic tissue. Pigs were treated as in *Figure 4E*: **s**uccinate is elevated during early reperfusion in the coronary sinus plasma but not jugular vein or aortic root in a pig heart attack model. The LAD was occluded by a balloon catheter for 60 min before removing the occlusion and blood sampled (mean ± S.E.M., n=3). Statistical significance was assessed by two-way ANOVA with Tukey's post hoc test (***p* <0.01, *****p* <0.0001).

Figure S2 Succinate is retained in the heart when reperfused with nonspecific transport inhibitors. Succinate levels in hearts after 6 min reperfusion with 1 mM succimer or 1 mM phenylsuccinate (Phsucc) from *Figure 5B* were measured (mean ± S.E.M., n=3-5). Statistical significance was assessed by two-way ANOVA with Dunnett's post hoc test (*****p* <0.0001 relative to control reperfusion).

Figure S3 MCT1 inhibition or haploinsufficiency does not affect succinate accumulation. Hearts were perfused in Langendorff mode and equilibrated with Krebs buffer alone, or for MCTi-pre with 50 μM AR-C141990 for 20 min, before 20 min global no-flow ischemia and snap freezing tissue for succinate quantification by LC-MS/MS (mean ± S.E.M or mean ± range for $MCT1^{+/-}$; WT n=8 (from *Figure 2A*), $MCT1^{+/-}$ n=2, MCTi-pre n=4).

Figure S4 Succinate is retained in the heart when reperfused with MCTi. Succinate levels in hearts after 6 min reperfusion with MCTi from *Figure 6B* were measured (mean ± S.E.M., n=3-5). Statistical significance was assessed by two-way ANOVA with Dunnett's post hoc test (*****p* <0.0001 relative to control (ctl) reperfusion).

Figure S5 Succinate retained in the heart after reperfusion in $MCT1^{+/-}$ mice is no different from $MCT1^{+/+}$ hearts. Succinate levels in $MCT1^{+/+}$ and $MCT1^{+/-}$ hearts after 6 min reperfusion from *Figure 6C* were measured (mean ± S.E.M., $MCT1^{+/+}$ n=7, $MCT^{+/-}$ n=5).

Figure S6 Inhibition of MCT1 with AR-C141990 decreases cardiac ischemia-reperfusion injury. Anesthetized mice were subjected to occlusion of the LAD for 30 min and then the occlusion was removed and the hearts were reperfused for 120 min. The mice were infused IV for 20 min from 5 min before reperfusion with either saline, or saline supplemented with the MCT1i AR-C141990, at a total delivered dose of 1.5 mg/kg body weight. Infarct sizes were determined histologically as a percentage of risk area. Data are mean ± S.E.M., n=5. Statistical significance was assessed by unpaired, two-tailed Student's t-test where *p <0.05.

Figure S1

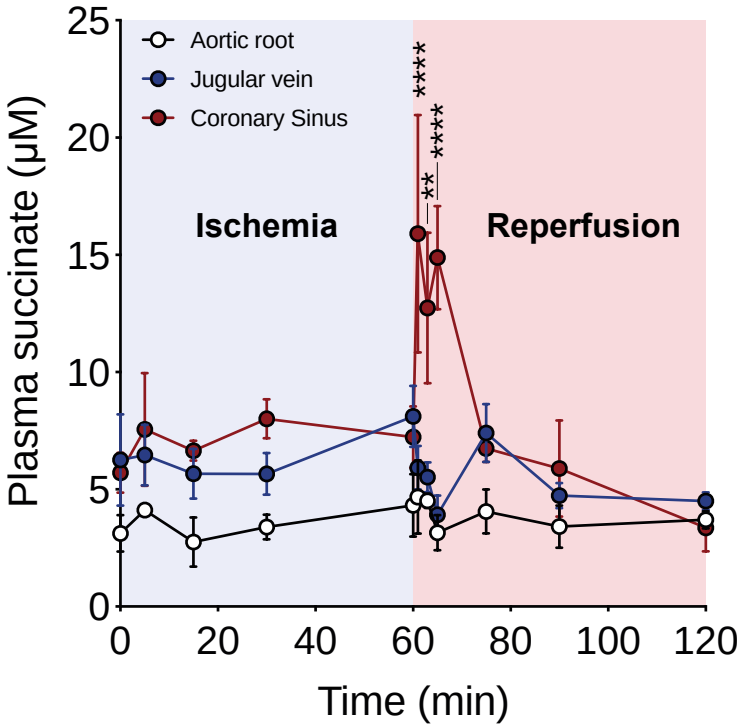

Aortic root
Jugular vein
Coronary Sinus
Ischemia
Reperfusion
****
**
****
Plasma succinate (µM)
Time (min)
0
5
10
15
20
25
0
20
40
60
80
100
120

Figure S2

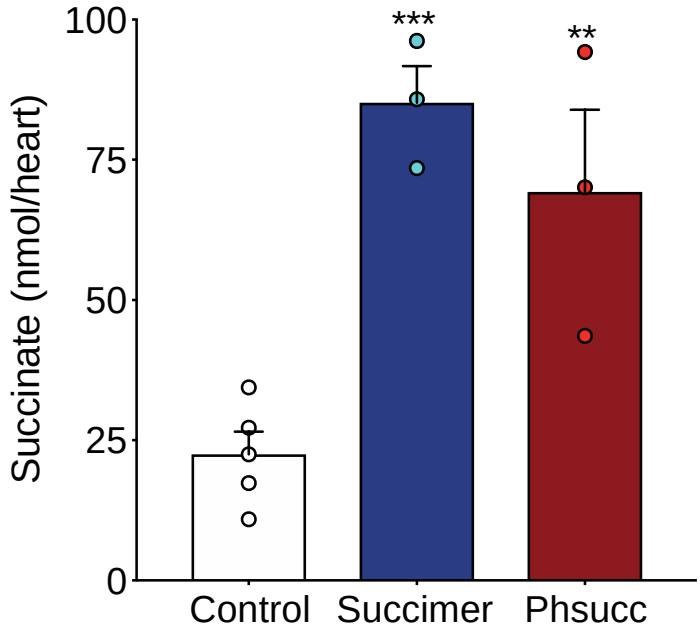


Figure S3

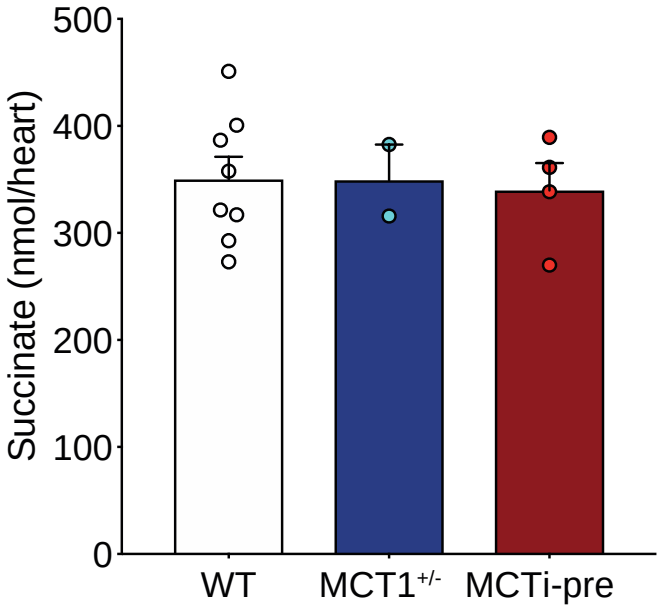


Figure S4

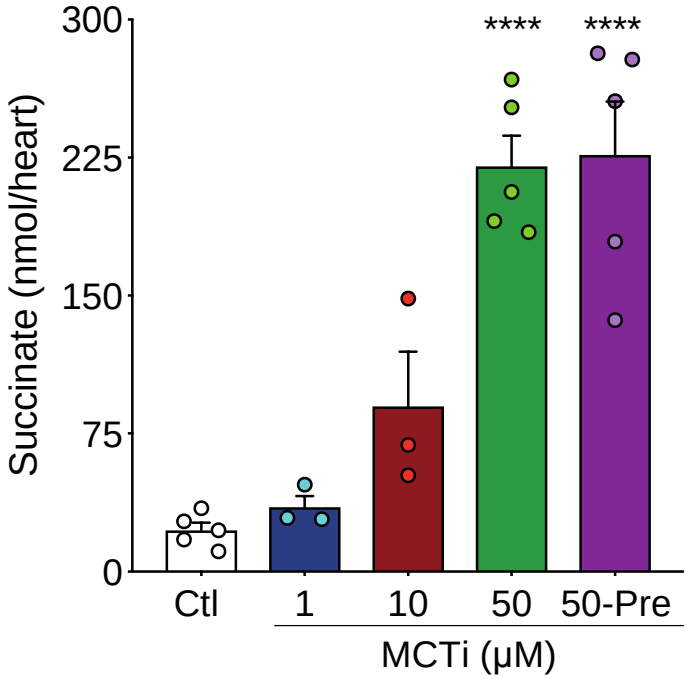


Figure S5

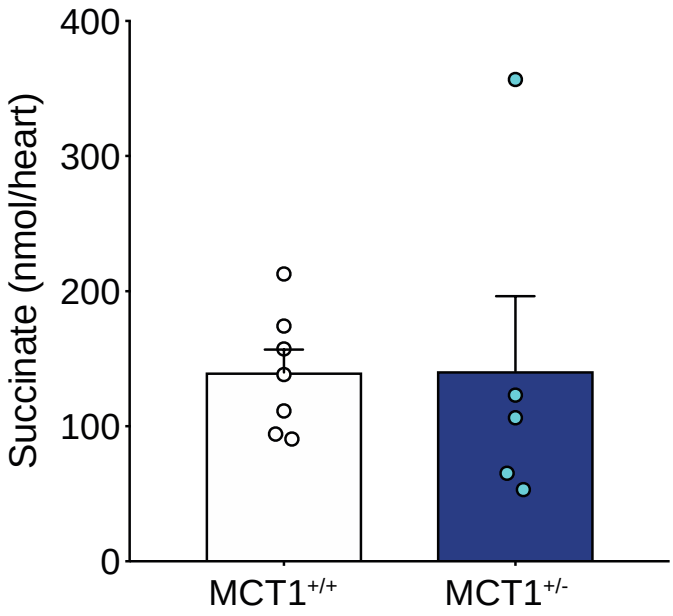


Figure S6