Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS

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LETTER

Ischaemia-reperfusion injury occurs when the blood supply to an organ is disrupted and then restored, and underlies many disorders, notably heart attack and stroke. While reperfusion of ischaemic tissue is essential for survival, it also initiates oxidative damage, cell death and aberrant immune responses through the generation of mitochondrial reactive oxygen species (ROS)1–4. Although mitochondrial ROS production in ischaemia reperfusion is established, it has generally been considered a nonspecific response to reperfusion1,3. Here we develop a comparative in vivo metabolomic analysis, and unexpectedly identify new pathway for metabolic control of ROS production in vivo, while ischaemia-reperfusion injury. Furthermore, these findings reveal a universal metabolic signature of ischaemia in a range of tissues and is responsible for mitochondrial ROS production during reperfusion. Ischaemic succinate accumulation arises from reversal of succinate dehydrogenase, which in turn is driven by fumarate overflow from purine nucleotide breakdown and partial reversal of the malate/aspartate shuttle. After reperfusion, the accumulated succinate is rapidly re-oxidized by succinate dehydrogenase, driving extensive ROS generation by reverse electron transport at mitochondrial complex I. Decreasing ischaemic succinate accumulation by pharmacological inhibition is sufficient to ameliorate in vivo ischaemia-reperfusion injury in murine models of heart attack and stroke. Thus, we have identified a conserved metabolic response of tissues to ischaemia and reperfusion that unifies many hitherto unconnected aspects of ischaemia-reperfusion injury. Furthermore, these findings reveal a new pathway for metabolic control of ROS production in vivo, while demonstrating that inhibition of ischaemic succinate accumulation and its oxidation after subsequent reperfusion is a potential therapeutic target to decrease ischaemia-reperfusion injury in a range of pathologies.

Mitochondrial ROS production is a crucial early driver of ischaemia-reperfusion (IR) injury, but has been considered a nonspecific consequence of the interaction of a dysfunctional respiratory chain with oxygen during reperfusion1–4. Here we investigated an alternative hypothesis: that mitochondrial ROS during IR are generated by a specific metabolic process. To do this, we developed a comparative metabolomics approach to identify conserved metabolic signatures in tissues during IR that might indicate the source of mitochondrial ROS (Fig. 1a). Liquid chromatography–mass spectrometry (LC–MS)–based metabolic analysis of mouse kidney, liver and heart, and rat brain, subjected to ischaemia in vivo (Fig. 1a) revealed changes in several metabolites (Supplementary Table 1). However, comparative analysis (Supplementary Tables 2 and 3) revealed that only three were increased across all tissues (Fig. 1b, c and Extended Data Fig. 1a). Two metabolites were well-characterized by-products of ischaemic purine nucleotide breakdown, xanthine and hypoxanthine6, corroborating the validity of our approach. Xanthine and hypoxanthine are metabolised by cytosolic xanthine oxidoreductase and do not contribute to mitochondrial metabolism7. The third metabolite, the mitochondrial citric acid cycle (CAC) intermediate succinate, increased 3–19-fold to concentrations of 61–72 ng mg−1 wet weight across the tested tissues (Fig. 1d, Supplementary Table 4 and Extended Data Fig. 1b, c), and was the sole mitochondrial feature of ischaemia that occurred universally in a range of metabolically diverse tissues. Therefore, we focused on the potential role of succinate in mitochondrial ROS production during IR.

Because mitochondrial ROS production occurs early in reperfusion1–4,6,9, it follows that metabolites fuelling ROS should be oxidized quickly. Notably, the succinate accumulated during ischaemia was restored to normoxic levels by 5 min reperfusion ex vivo in the heart (Fig. 1e), and this was also observed in vivo in the heart (Fig. 1f and Extended Data Fig. 2a), brain (Fig. 1g) and kidney (Fig. 1h). Of note, the accumulation of succinate by the in vivo heart was proportional to the duration of ischaemia (Extended Data Fig. 2a). These changes in succinate were localized to areas of the tissues where IR injury occurred in vivo, and took place without accumulation of other CAC metabolites (Fig. 1f–h). These data demonstrate that, uniquely, succinate accumulates markedly during ischaemia and is then rapidly metabolized on reperfusion at the same time as mitochondrial ROS production increases.

To determine the mechanisms responsible for succinate accumulation during ischaemia and explore its role in IR injury we focused on the heart, because of the many experimental and theoretical resources available. In mammalian tissues succinate is generated by the CAC, via oxidation of carbons from glucose, fatty acids, glutamate, and the GABA (γ-aminobutyric acid) shunt8,10 (Fig. 2a and Extended Data Fig. 2b). To assess the contribution of these carbon sources to the build-up of ischaemic succinate we performed an array of 13C-isotopologue labelling experiments in the ex vivo perfused heart followed by LC–MS analyses. Glucose is a major carbon source for the CAC, and therefore ischaemic CAC flux to succinate was first investigated by measuring its isotopologue distribution after infusion with [U-13C]glucose (in which U denotes uniformly labelled) (Fig. 2a). As expected, 13C–glucose was quickly oxidized via the CAC under normoxia, as indicated by the diagnostic (m + 2) and (m + 4) isotopologues of the CAC intermediates (Fig. 2b and

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However, the contribution of $^{13}$C-palmitate to succinate was notably (Fig. 2a and Extended Data Fig. 4a). The CAC was readily enriched in oxidation to the CAC activity by perfusing hearts with [U-$^{13}$C]palmitate (Extended Data Fig. 3). We then assessed the contribution of fatty acid succinate was significantly reduced in ischaemic hearts (Fig. 2b and Figure 1 | Comparative metabolomics identifies succinate as a potential lation during ischaemia, we considered earlier speculations that dur- carbon sources for the CAC under normoxia do not significantly con-

Extended Data Fig. 5c, d). Together, these data demonstrate that the major (Fig. 2a) did not decrease ischaemic succinate accumulation (Fig. 2d and mitochondrion metabolite that drives reperfusion ROS production. 

To explore other mechanisms that could lead to succinate accumulation during ischaemia, we considered earlier speculations that dur-

Because aspartate is a common carbon source for fumarate in both the PNC and the MAS pathways (Fig. 2e), we used $^{13}$C-labelled aspar-

To investigate the potential mechanisms underlying succinate-driven mitochondrial ROS production, we modelled in silico changes in ischaemic cardiac metabolism after reperfusion. The simulations predicted that SDH oxidizes the accumulated succinate and, with complex III and IV at full capacity, drives reverse electron transport (RET) through mitochondrial complex I (refs 23–26; Extended Data Fig. 8a–c). Notably, succinate drives extensive superoxide formation from complex I by RET in vitro, making it a compelling potential source of mitochondrial ROS.
**GABA and succinate levels in the ischaemic myocardium** (ND, not detected. 27 fusion, consistent with increased superoxide production27 (Fig. 3a).

**SDH inhibition by dimethyl malonate on CAC metabolite abundance in the ischaemic myocardium in vivo (n = 3). g) Relative incorporation of 13C-aspartate to the indicated CAC metabolites in the normoxic and ischaemic myocardium (n = 4). h) Effect on CAC metabolite abundance in the ischaemic myocardium in vivo of blocking aspartate entry into the CAC through aminooxyacetate-mediated inhibition of aspartate aminotransferase, or blocking PNC by inhibition of adenylosuccinate lyase with AICAR (n = 3). **P < 0.05, ***P < 0.01, ****P < 0.001 (two-tailed Student’s t-test for pairwise comparisons, one-way ANOVA for multiple comparisons). Data are mean ± s.e.m. of at least three biological replicates.

Assess the role of succinate in driving ROS production further, we used a cell-permeable derivative of succinate, dimethyl succinate, which is readily taken up by cells, where it is then hydrolysed thereby increasing succinate levels (Extended Data Fig. 7b, c). Addition of dimethyl succinate to ischaemic primary cardiomyocytes significantly amplified reperfusion DHE oxidation, suggesting that succinate levels controlled the extent of reperfusion ROS (Fig. 3b). Importantly, selective inhibition of complex I RET with rotenone (Fig. 3c and Extended Data Fig. 9a) or the mitochondria-targeted S-nitrosothiol MitoSNO49 (Fig. 3c) abolished both ischaemic succinate and dimethyl succinate-driven DHE oxidation after reperfusion, indicating that ischaemic succinate levels drove superoxide production through complex I RET. Succinate-dependent

Figure 2 | Reverse SDH activity drives ischaemic succinate accumulation by the reduction of fumarate. a, Potential inputs to succinate-directed flux by conventional cardiac metabolism and 13C-metabolite labelling strategy. b, c, 13C-isotopologue profile of succinate in the normoxic and ischaemic myocardium after infusion of 13C-glucose (b) and 13C-palmitate (c) (n = 4). ND, not detected. d, Effect of inhibition of GABA shunt with vigabatrin on GABA and succinate levels in the ischaemic myocardium (n = 4; ischaemia n = 5). e, Summary of in silico metabolic modelling of potential drivers of ischaemic succinate accumulation, and 13C-succinate metabolic labelling strategy. AOA, aminooxyacetate; AS, adenylosuccinate; IMP, inosine 5’-monophosphate; OAA, oxaloacetate; QH2, dihydrobiouniquine. f, Effect of during IR12,26. However, the role of complex I RET in IR injury has never been demonstrated. To test whether the succinate accumulated during ischaemia could drive complex I RET on reperfusion, we tracked mitochondrial ROS with the fluorescent probe dihydroethidine (DHE), and mitochondrial membrane potential from the potential-sensitive fluororosence of tetramethylrhodamine methyl ester (TMNR), in a primary cardiomyocyte model of IR injury27. DHE was rapidly oxidized after reperfusion, consistent with increased superoxide production27 (Fig. 3a). Inhibition of SDH-mediated ischaemic succinate accumulation with dimethyl malonate reduced DHE oxidation on reperfusion (Fig. 3a). To

Figure 3 | Ischaemic succinate levels control ROS production in adult primary cardiomyocytes and in the heart in vivo. a, b, DHE oxidation during late ischaemia and early reperfusion, with/without inhibition of ischaemic succinate accumulation (no additions n = 6; dimethyl malonate n = 5) (a) or addition of dimethyl succinate during ischaemia (n = 6) (b). c, Inhibition of mitochondrial complex I RET reduces DHE oxidation on reperfusion after addition of dimethyl succinate (n = 5; dimethyl succinate n = 6). d, Effect of dimethyl malonate on mitochondrial re-polarization at reperfusion as determined by the rate of TMNR quenching (n = 3). e, Effect of dimethyl succinate and oligomycin on mitochondrial ROS in aerobic C2C12 myoblasts (n = 4). AU, arbitrary units. f, g, Effect of inhibition of ischaemic succinate accumulation by dimethyl malonate on mitochondrial ROS during IR injury in vivo assessed by MitoB oxidation (n = 5; dimethyl malonate n = 6) (f). 0.05, ***P < 0.001 (two-tailed Student’s t-test for pairwise comparisons, one-way ANOVA for multiple comparisons). Data are mean ± s.e.m. of at least three biological replicates. For cell data replicates represent separate experiments on independent cell preparations.
RET was further supported by the observation that NAD(P)H oxidation at reperfusion was suppressed by increasing succinate levels with dimethyl succinate (Extended Data Fig. 9b, c). Tracking the mitochondrial membrane potential revealed that inhibition of ischaemic succinate accumulation with dimethyl malonate slowed the rate of mitochondrial repolarization after reperfusion (Fig. 3d and Extended Data Fig. 9d–f), consistent with accelerated repolarization, and RET at complex I, driven by succinate on reperfusion. Increasing succinate in C2C12 mouse myoblast cells with dimethyl succinate while hyperpolarizing mitochondria with oligomycin increased oxidation of the mitochondrial ROS indicator MitoSOX independently of IR (Fig. 3e), suggesting that combining high succinate levels with a large protonmotive force is sufficient to drive complex I ROS production by RET.

We next investigated whether succinate-driven complex I RET leads to ROS production in the heart in vivo, during IR injury. To do this we used the ratiometric mass spectrometric mitochondria-targeted ROS probe MitoP. This probe is rapidly taken up by mitochondria in the heart in vivo and then oxidized to MitoP by hydrogen peroxide and peroxynitrite. Consequently measuring the MitoP/MitoB ratio by liquid chromatography–tandem mass spectrometry (LC–MS/MS) indicates changes in mitochondrial ROS in vivo. At the onset of cardiac reperfusion there was an increase in the MitoP/MitoB ratio, and this increase was prevented by blocking the accumulation of ischaemic succinate with dimethyl malonate (Fig. 3f). Furthermore, the activity of the mitochondrial superoxide-sensitive CAC enzyme aconitase was decreased in the first few minutes of reperfusion, and this oxidative damage was also prevented by infusing dimethyl malonate during ischaemia to prevent succinate accumulation (Fig. 3g). Together, these data indicate that succinate oxidation after reperfusion drives a burst of mitochondrial ROS production from complex I by RET during cardiac IR injury in vivo, and that this ROS production is prevented by dimethyl malonate.

Our findings suggest the following model (Fig. 4a): during ischaemia, fumarate production increases, through activation of the MAS and PNC, and is then reduced to succinate by SDH reversal. After reperfusion, the accumulated succinate is rapidly oxidized to maintain the Q pool reduced, thereby sustaining a large protonmotive force by conventional electron transport through complexes III and IV to oxygen, while also driving RET at complex I to produce the mitochondrial ROS that initiate IR injury. This model provides a unifying framework for many hitherto unconnected aspects of IR injury, such as the requirement for time-dependent priming during ischaemia to induce ROS upon reperfusion, protection against IR injury by the inhibition of complexes I (ref. 8) and II (ref. 28), and by mild uncoupling.

Notably, our model also generates an unexpected, but testable, prediction. Manipulation of the pathways that increase succinate during ischaemia and oxidize it on reperfusion should determine the extent of IR injury. Because the reversible inhibition of SDH blocks both succinate accumulation during ischaemia (Fig. 2f) and its oxidation upon reperfusion, it should protect against IR injury in vivo. Intravenous infusion of dimethyl malonate, a precursor of the SDH inhibitor malonate, during an in vivo model of cardiac IR injury was protective (Fig. 4b, c). Importantly, this cardioprotection was suppressed by adding back dimethyl succinate (Fig. 4b, c and Extended Data Fig. 10a), which restored increased levels of ischaemic succinate (Fig. 4d), indicating that protection by dimethyl malonate resulted solely from blunting succinate accumulation. Finally, intravenous infusion of dimethyl malonate during rat transient middle cerebral artery occlusion (tMCAO), an in vivo model of brain IR injury during stroke, also suppressed ischaemic accumulation of succinate (Fig. 4e and Extended Data Fig. 10b) and was protective, reducing the pyknotic nuclear morphology and vacuolation of the neuropil (Extended Data Fig. 10c), decreasing the volume of infarcted brain tissue caused by IR injury (Fig. 4f, g), and preventing the decline in neurological function and sensorimotor function associated with stroke (Fig. 4h and Extended Data Fig. 10d). These findings support our model of succinate-driven IR injury, demonstrating that succinate accumulation underlies IR injury in the heart and brain and suggests decreasing myocardium in vivo (n = 4). e, Effect of intravenous infusion of dimethyl malonate on succinate accumulation in the ischaemic brain in vivo (n = 4). f–h, Protection by dimethyl malonate against brain IR injury in vivo. Quantification of brain infarct volume (f) and rostro-caudal infarct distribution (g) ± dimethyl malonate after brain IR injury by tMCAO in vivo (untreated n = 5, dimethyl malonate n = 4). h, Neurological scores for rats after tMCAO ± dimethyl malonate (untreated n = 6; dimethyl malonate n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed Student’s t-test for pairwise comparisons, and one-way ANOVA (c–e) or two-way ANOVA (f–h) for multiple comparisons). Data are mean ± s.e.m. of at least three biological replicates, except for h, for which data are median ± confidence interval.
succinate accumulation and oxidation as a new therapeutic approach for IR injury.

We have demonstrated that the accumulation of succinate, via fumarate production and reversal of SDH, is a universal metabolic signature of ischaemia in vivo. In turn, succinate is a primary driver of the mitochondrial ROS production on reperfusion that underlies IR injury in a range of tissues. Ischaemic accumulation of succinate may be of further relevance via its role in inflammatory and hypoxic signalling. Thus succinate could contribute to both the acute pathogenesis of IR injury by mitochondrial ROS, and then upon secretion also trigger inflammation and neo-vascularisation. This further suggests that mitochondrial ROS produced by RET at complex I may normally act as a redox signal from mitochondria that responds to changes in electron supply to the Q pool and ATP demand, but is grossly over-activated in IR injury. Besides determining the metabolic responses that underlie IR injury, these data demonstrate that preventing succinate accumulation during ischaemia is protective against IR injury in vivo, suggesting novel therapeutic targets for IR injury in pathologies such as heart attack and stroke.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Author Contributions** E.T.C. designed research, carried out biochemical experiments, analysed data from in vivo experiments and co-wrote the paper. T.K., V.R.P. and C.-H.H. carried out cell experiments. S.Y.S., S.M.D., E.N.J.O. and R.S. designed and carried out brain experiments. A.J.D., S.R. and K.S.-P. designed and carried out kidney experiments. M.R.D., S.M.N., E.L.R. and P.S.B. designed and carried out cell experiments. L.M.W., and M.J.S. designed and carried out in vivo experiments and co-wrote the paper. T.K., V.R.P. and C.-H.H. carried out ROS analyses. E.N.J.O. and R.S. designed and carried out brain experiments. A.L. and R.C.H. carried out ROS analyses. S.Y.S., S.M.D., E.N.J.O. and R.S. designed and carried out brain experiments. A.L. and R.C.H. carried out ROS analyses. S.E. carried out analyses. A.M.J. helped with data interpretation. A.C.S., A.J.R. and F.E. designed and performed biochemical experiments, E.T.C., T.K., C.F. and M.P.M. directed the research and co-wrote the paper, with assistance from all other authors.

**Author Information** Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to C.F. (CF366@mrc-cu.cam.ac.uk), T.K. (tk382@medschl.cam.ac.uk) or M.P.M. (mpm@mrc-mbu.cam.ac.uk).
In vivo mouse myocardial experiments. For in vivo heart IR, an open-chest, in situ heart model was used\textsuperscript{11,12}. Male mice (8–10 weeks; Charles River Laboratories) were anaesthetized with sodium pentobarbital (70 mg kg\textsuperscript{-1} intraperitoneally), intubated endotracheally and ventilated with 3 cm H\textsubscript{2}O positive-end expiratory pressure. Adequacy of anaesthesia was monitored using corneal and withdrawal reflexes. Ventilation frequency was kept at 110 breaths per minute with tidal volume between 125 and 150 \mu L. A thoracotomy was performed and the heart exposed by stripping the pericardium. A prominent branch of the left anterior descending coronary artery was not occluded. At the end of each protocol, tissue was removed from the at risk and peripheral areas of the heart, selected visually by comparing white versus red tissue, and snap-frozen in liquid nitrogen. Sham-operated tissue was removed from the presumed risk zone.

Infarct size was assessed after 30 min of ischaemia followed by 120 min reperfusion using 2% triphenyltetrazolium chloride staining, and is expressed as a percentage of the risk zone\textsuperscript{13}. Metabolic inhibitors (all from Sigma) in sterile saline were infused intravenously via a tail vein 10 min before and throughout ischaemia at the following concentrations: 1 mM L-glutamine for 10 min followed by standard normoxic perfusion for 30 min with unlabelled KH buffer (n = 4); 10 min perfusion with 1 mM [U-\textsuperscript{13}C]-glutamine, followed by 30 min global ischaemia (n = 4); 10 min perfusion of 1 mM [1-\textsuperscript{13}C]-aspartic acid, followed by normoxic perfusion for 30 min with unlabelled KH buffer; 10 min perfusion with 1 mM [1-\textsuperscript{13}C]-aspartic acid, followed by 30 min global ischaemia. At the end hearts were snap frozen in liquid nitrogen and stored at \textasciitilde 80 °C. In vivo rat brain ischaemia and reperfusion. Male spontaneously hypertensive stroke prone rats from the colony maintained at the University of Glasgow (270–310 g) were anaesthetized with 5% isoflurane in oxygen and were intubated and ventilated throughout surgery (\textasciitilde 2.5% isoflurane/oxygen). Body temperature was maintained at 37 \pm 0.5 °C. Animals underwent pre-stroke burrhole surgery\textsuperscript{14} before transient middle cerebral artery occlusion (tMCAO, 45 min). In brief, a silicone-coated monofilament (Docoll Corporation) was advanced through the common carotid artery to block the origin of the middle cerebral artery. Animals were maintained under anaesthesia during ischaemia. Immediately after removal of the filament, or after 5 min of reperfusion, the brain was removed after cervical dislocation and infarct tissue separated from surrounding tissue on the ipsilateral side and snap-frozen in liquid nitrogen for metabolomic analysis. Corresponding regions were taken from the contralateral side. A separate group was infused with dimethyl malonate (6 mg kg\textsuperscript{-1} min\textsuperscript{-1}) by intravenous infusion 10 min before and during tMCAO or carrier, allowing for recovery for 3 days, over which time they were scored for neurological function\textsuperscript{15} as modified\textsuperscript{16}, and locomotor and sensorimotor activity by the tapered beam walk test, quantifying the average number of footfalls as described previously\textsuperscript{17}. These rats were then killed by transcardiac perfusion fixation and the infarct area was assessed across seven coronal levels following haematoxylin and eosin staining\textsuperscript{18}.

Ex vivo Langendorff heart experiments for metabolic analysis. Mice were heparinized (200 U intraperitoneally) and anaesthetized with sodium pentobarbital (100 mg kg\textsuperscript{-1} intraperitoneally). The chest was then opened and the heart rapidly excised and arrested in cold Krebs–Henseleit (KH) buffer (0.5 mM EDTA, 118 mM NaCl, 4.7 mM KCl, 25 mM NaHCO\textsubscript{3}, 11 mM glucose, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, and 2 mM CaCl\textsubscript{2}) at pH 7.4. The aorta was then cannulated with a 22 G heparinized (200 U intraperitoneally) and anaesthetized with sodium pentobarbital (100 mg kg\textsuperscript{-1} intraperitoneally) intubated endotracheally and ventilated with 3 cm H\textsubscript{2}O positive-end expiratory pressure. Adequacy of anaesthesia was monitored using corneal and withdrawal reflexes. Ventilation frequency was kept at 110 breaths per minute with tidal volume between 125 and 150 \mu L. A thoracotomy was performed and the heart exposed by stripping the pericardium. A prominent branch of the left anterior descending coronary artery was not occluded. At the end of each protocol, tissue was removed from the at risk and peripheral areas of the heart, selected visually by comparing white versus red tissue, and snap-frozen in liquid nitrogen. Sham-operated tissue was removed from the presumed risk zone.

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Cells were imaged within 36 h of plating. Images using a Zeiss LSM 510 META confocal microscope with a Fluar 20x/0.75 numerical aperture ultraviolet objective, or a microscope equipped with an Orca ER cooled CCD camera (Hamamatsu), a monochromator (Cairn Research) and emission filter wheel (Prior) with a Fluar 20x/0.75 numerical aperture objective. Cells attached to coverslips, which formed the base of custom-built imaging chambers, were placed on a heated stage at 37 °C on the microscope with normoxic recording buffer (156 mM NaCl, 3 mM KCl, 2 mM MgSO4, 1.25 mM K2HPO4, 2 mM CaCl2, 10 mM HEPES, 10 mM t-glucose; pH 7.4) and by covering the heated stage with a transparent, gas-impermeant lid, forming a small chamber into which argon was forced to maintain hypoxia. PO2 was routinely measured as <2.0 mm Hg during simulated ischaemia. To simulate reperfusion, the lid was removed from the chamber, and the buffer replaced with normoxic recording buffer.

Mitochondrial membrane potential was measured using TMRM (Life Technologies) in dequench mode. In this mode, mitochondrial depolarization causes redistribution of a high concentration of quenched TMRM from mitochondria to cytosol, where the concentration gradient results in dequenching and an increase in fluorescence. Cells were loaded at room temperature with normoxic recording buffer containing 3 μM TMRM for 30 min. Before imaging, loading buffer was removed and replaced with normoxic recording buffer. TMRM fluorescence was excited at 543 nm and emission was collected using a LP 560 filter.

ROS production was estimated by oxidation of DHE and ratiometric assessment. For this cells were loaded with 5 μM DHE (Invitrogen), which remained present throughout normoxic and ischaemic conditions. DHE was excited at 514 nm and the emitted signal was acquired with a BP 435–485 nm bandpass filter. Oxidized DHE was excited at 543 nm and emission was collected with a LP 560 nm filter. NADH autofluorescence was excited at 351 nm and the emitted signal was collected using a BP 435–485 nm bandpass filter. All measured cell parameters were analysed with Fiji image processing software.

Assessment of succinate-dependent mitochondrial superoxide production in myoblasts. C2C12 myoblasts were seeded in 35 mm glass bottom culture dishes (MatTek) and incubated for at least 4 h at 37 °C, 5% CO₂.

In siRNA analysis of metabolic flux during ischaemia and reperfusion. Simulations were performed using an expanded version of the myocardial mitochondrial metabolic model iAS253 (ref. 11). The model was expanded to include additional mitochondrial reactions by using the latest version of MitoMiner, a mitochondrial proteomics database15. MitoMiner was used to identify new mitochondrial reactions for inclusion by cross-referencing these data with information from BRENDAn16. Human literature relevant to confirming new reactions was present in human, expressed in heart tissue and localized to the mitochondrial matrix. In addition, cytosolic reactions were included that could contribute to energy production, such as amino acid degradation and conversion reactions as well as the purine nucleotide cycle. Proteon states of metabolites in the model were calculated by using the Marvin suite of computational chemistry software (ChemAxon Ltd). Reactions were then charge-balanced according to the protonation state of the major microspecies found at pH 7.85 for the mitochondrial matrix17 and pH 7.30 for the cytosol. In addition, directionality constraints were imposed on the basis of general rules of irreversibility, thermodynamics and information from public resources such as BRENDA16. Human literature and capacity constraints were taken from the literature18. The final model contained 227 mitochondrial matrix reactions, 76 cytosolic reactions, 91 transport steps between the two compartments and 84 boundary conditions representing inputs and outputs into the system. The expanded model is a manually curated and highly refined model of the mitochondrial, and as with iAS253, no metabolite dead ends were present and all reactions were capable of having flux.

Metabolism of the mitochondrial network was simulated using flux balance analysis, a technique that has been described in detail19. The objective function used to optimise the reaction fluxes was maximum ATP production. All the FBA simulations were carried out using MATLAB R2012b (Math Works, Inc.) with the COBRA Toolbox20, and the linear programming solver GLPK (http://www.gnu.org/software/glpk/).

To represent ischaemia, the maximum uptake of oxygen was reduced to 20% of its level under normal conditions (4.0 versus 19.8 μmol min⁻¹ g⁻¹ dry weight). Simulations were run with boundary conditions for metabolites set to either their normoxic values, or with various metabolites in excess to determine whether they could contribute to ATP production under ischaemia. To represent reperfusion, the oxygen level was restored to its normal level and simulations were run with the availability of succinate, lactate, pyruvate and NADH increased to various levels to reflect the ischaemic accumulation of these metabolites. The flux capacity of ATP synthesis was reduced by up to 50% to represent the delay in generating ADP from AMP required for ATP synthesis to function and also to model hyperpolarization of the mitochondrial membrane, in effect by constraining the efficiency of the other proton pumping complexes of the electron transport chain.

Assessment of mitochondrial aconitase activation in mouse heart tissue. Aconitase activity was measured as described previously21. In brief, following the relevant in vivo intervention (normal respiration, or 30 min ischaemia and 15 min reperfusion ± dimethyl malonate), mouse hearts were rapidly excised and homogenised in mitochondrial isolation buffer (250 mM sucrose, 2 mM EDTA, 10 mM sodium citrate, 0.6 mM MnCl₂, 100 mM Tris-HCl, pH 7.4) followed by mitochondrial isolation by differential centrifugation. Samples (10 μl; 1–2 μg mitochondrial protein) were added to a 96-well plate and 190 μl assay buffer (50 mM Tris-HCl (pH 7.4), 0.6 mM MnCl₂, 5 mM sodium citrate, 0.2 mM NADP⁺, 0.1% (v/v) Triton X-100, 0.4 mM NaCl, 1CDH). Absorbance was measured at 340 nm for 7 min at 37 °C. To determine the background rate of NADP⁺ reduction, 100 μM fluorocitrate was added in a parallel experiment. In all cases the background NADP⁺ reduction was <10% of the observed rate. In parallel, we determined the citrate synthase activity of each sample22. To control for mitochondrial content we normalized aconitase activity to the citrate synthase activity and expressed the result as a percentage of control levels.

Statistics and experimental design. Data were expressed as mean ± s.e.m. and P values were calculated using two-tailed Student’s t-test for pairwise comparisons, and one-way analysis of variance (ANOVA) followed by Bonferroni’s test for multiple comparisons, unless otherwise stated. Experimenters analysing samples from metabolomics, histological, and neurological animal experiments were blinded to the experimental interventions. No statistical method was used to predetermine sample size.

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Prevalence of accumulation (number of tissues) | Metabolites significantly accumulated in ischaemic pro-ROS conditions (> 2 fold, p < 0.05) | Tissue conditions
---|---|---
5 | Succinate | All
5 | Xanthine | All
5 | Hypoxanthine | All
4 | Adenine | B,L,K,H
4 | Propionylcarnitine | B,L,H,HL
4 | Choline | L,K,H,HL
3 | Lactate | L,K,H
3 | Proline | L,K,H
3 | Leucine | L,K,H
3 | Tryptophan | L,K,H
3 | Phenylalanine | L,K,H
2 | Stearoylcarnitine | L,H,L
2 | Alcaptoine | B,H
2 | Adenylsuccinate | B,H
2 | Cysteine | B,L
2 | Cytosine | B,H
2 | Creatine | B,L
2 | Lysine | L,K
2 | Ornithine | L,K
2 | AMP | L,H
2 | Asparagine | L,K
2 | Arginine | L,K
2 | Palmitoylcarnitine | L,H,L
2 | Adenosine | L,H,L
2 | Guanine | K,H,L
2 | Tyrosine | L,H
2 | Alanine | L,H
2 | Methionine | L,H
1 | Urine | L
1 | N-Acetylaspartate | L
1 | Acetate | L
1 | Oleoylamine | L
1 | Threonine | L
1 | 2-hydroxyglutarate | L
1 | Glucose | L
1 | Glyceraldehyde | L
1 | 3-Hydroxybutyrate | L
1 | Acetoacetate | L
1 | Acetylcholine | L
1 | Carnitine | L
1 | Palmitoleic acid | HL
1 | Butyrylcarnitine | HL
1 | Myristic acid | HL
1 | Stearic acid | HL
1 | Valine | H
1 | Dihydrothymine | H
1 | Glutamine | H
1 | Glutamine | H
1 | Histidine | H
1 | Pyroglutamic Acid | H

Extended Data Figure 1 | Comparative analysis of metabolites significantly accumulated in ischaemic conditions. a, Various rat and mouse tissues exposed to sufficient periods of ischaemia to prime for reperfusion ROS production were subjected to targeted LC–MS metabolomics analysis and comparison of metabolites that accumulated significantly when compared to normoxic levels. After this, metabolites were scored according to the prevalence of their accumulation across five ischaemic tissue conditions. B, brain; H, whole heart ischaemia ex vivo; HL, left anterior descending coronary artery ischaemia in vivo; K, kidney; L, liver. b, Determination of linearity of the relationship between LC–MS metabolite peak intensity and concentration for CAC and related metabolites. c, Quality control determination of coefficient of variation for LC–MS quantification of CAC and related metabolites.
Extended Data Figure 2 | Time course of succinate levels in the *in vivo* heart during ischaemia and reperfusion and potential metabolic inputs for succinate. a, Time course of succinate levels during myocardial ischaemia and reperfusion for the *in vivo* heart (5 min and 15 min ischaemia n = 4; 30 min ischaemia n = 9; 5 min reperfused n = 5). b, Summary of the three potential metabolic inputs for succinate-directed ischaemic flux. To understand the metabolic pathways that could contribute to succinate production under ischaemia, an updated version of the iAS253 model of cardiac metabolism was used to simulate ischaemia using flux balance analysis. The model showed three possible mechanisms for producing succinate: from α-ketoglutarate produced by the CAC, derived from glycolysis, fatty acid oxidation, and glutaminolysis (grey box), from succinic semialdehyde produced from the GABA shunt (blue box), and from fumarate produced from the malate-aspartate shuttle and purine nucleotide cycle (red box) via the reversal of SDH. Data are mean ± s.e.m. of at least four biological replicates.
Extended Data Figure 3 | Metabolic labelling of CAC and proximal metabolites by $^{13}$C-glucose in the ischaemic and normoxic myocardium. Proportional isotopic labelling profile of CAC and proximal metabolites during normoxic and ischaemic myocardial perfusion. Mouse hearts were perfused with 11 mM [U-$^{13}$C]glucose (+6 labelled) for 10 min followed by either 30 min no flow ischaemia or 30 min normoxic perfusion followed by snap-freezing and LC–MS metabolomic analysis ($n = 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (two-tailed Student’s t-test). Data are mean ± s.e.m. of at least four biological replicates.
Extended Data Figure 4 | Metabolic labelling of CAC and proximal metabolites by $^{13}$C-palmitate in the ischaemic and normoxic myocardium.

a, Mouse hearts were perfused with 0.3 mM [U-$^{13}$C]palmitate (+16 labelled) for 10 min resulting in a significant proportion of the endogenous palmitate pool being +16 labelled. Following this, hearts were subjected to either 30 min ischaemia or continued normoxic respiration with $^{13}$C-palmitate followed by snap-freezing and metabolomic analysis.

b, Isotopic flux from palmitate to CAC and proximal metabolites following normoxic and ischaemic myocardial respiration. The isotopic profile for each metabolite is expressed as a proportion of the total pool ($n = 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (two-tailed Student’s $t$-test). Data are shown as the mean ± s.e.m. of at least four biological replicates.
Extended Data Figure 5 | Metabolic labelling of CAC and proximal metabolites by $^{13}$C-glutamine in the ischaemic and normoxic myocardium, and measurement of the effect of inhibition of GABA transaminase on succinate accumulation in the ischaemic myocardium. a, Mouse hearts were perfused with 4 mM [U-$^{13}$C]glutamine ($^{15}$ labelled) for 10 min followed by either 30 min no flow ischaemia or 30 min normoxic respiration followed by snap freezing and metabolomic analysis. The isotopic profile for each metabolite is expressed as a proportion of the total pool ($n=4$). b, Furthermore, flux to $\alpha$-ketoglutarate was determined relative to the proportion of the +5 glutamine pool in the heart ($n=4$). c, d, Perfused mouse hearts were subjected to 30 min no flow ischaemia $\pm$ continuous infusion of vigabatrin (vig; 100, 300 and 700 mM) 10 min before ischaemia. Heart tissue was snap frozen and GABA (c) and succinate (d) abundance quantified relative to normoxic levels by LC–MS ($n=4$; ischaemia $n=5$). *$P < 0.05$ (two-tailed Student’s $t$-test). Data are mean ± s.e.m. of at least four biological replicates.
Extended Data Figure 6 | Unabridged metabolic model identifying pathways that can become activated by tissue ischaemia to drive succinate accumulation. To identify the metabolic pathways that could contribute to succinate production under ischaemia, we simulated these conditions using flux balance analysis in conjunction with an expanded version of the iAS253 mitochondrial model of central cardiac metabolism. The major pathways contributing to succinate accumulation (bold red lines) were via fumarate feeding into the reverse activity of SDH. This was produced by the PNC and the MAS, which consumed glucose and aspartate, and also led to significant production of lactate and alanine. Lesser sources of succinate (thin red lines) included glycolysis and glutaminolysis but this was relatively minor as this route was constrained by the overproduction of NADH. In addition, a small amount of fumarate was generated by pyruvate carboxylase activity. The GABA shunt did not contribute (black dashed line).
Extended Data Figure 7 | Effects of dimethyl malonate and dimethyl succinate treatment of cells and in vivo on intracellular accumulation of malonate and succinate, and respiration and comparison of \(^{13}\)C-labelled ischaemic metabolite fluxes to succinate relative to isotopic donor pools.

**a.** Intravenous infusion of dimethyl malonate in vivo results in accumulation of malonate in the ischaemic myocardium (\(n = 4\)).

**b.** C2C12 cells were incubated with: no additions, glucose, 5 mM dimethyl succinate, 5 mM dimethyl malonate, or 5 mM dimethyl malonate and 5 mM dimethyl succinate. Cellular oxygen consumption rate due to ATP synthesis (b) and maximal rates (c) in the presence of p-triflouromethoxyphenylhydrazone (FCCP) were determined using a Seahorse XF96 analyser (\(n = 4\)).

**c.** Mouse hearts were perfused with \(^{13}\)C-glucose (\(16\) labelled), \(^{13}\)C-glutamine (\(15\) labelled), \(^{13}\)C-aspartate (\(11\) labelled), or \(^{13}\)C-palmitate (\(16\) labelled) for 10 min followed by 30 min no-flow ischaemia or 30 min normoxic respiration, followed by snap-freezing and metabolomic analysis. To compare the relative magnitude of metabolite flux from each carbon source, \(^{13}\)C incorporation to succinate during normoxia and ischaemia was determined relative to the proportion of the total pool of the relevant infused \(^{13}\)C donor. \(^{13}\)C incorporation into succinate was considered in terms of the proportion of the +4 isotope in the entire succinate pool for \(^{13}\)C-glucose, \(^{13}\)C-glutamine and \(^{13}\)C-palmitate infusions; and the proportion of the +1 isotope in the entire pool for the \(^{13}\)C-aspartate infusion (\(n = 4\)). \(* P < 0.05, ** P < 0.001\) (two-tailed Student's \(t\)-test for pairwise comparisons, and one-way ANOVA followed by Bonferroni's test for multiple comparisons). Data are mean ± s.e.m. of at least four biological replicates.
Extended Data Figure 8 | Predicted changes in pathways of succinate and oxidative phosphorylation metabolism during ischaemia and following reperfusion. To determine possible changes in succinate metabolism during ischaemia, reperfusion and normoxia, cardiac metabolism was simulated in these conditions using an expanded version of the iAS253 model with flux balance analysis. a, The simulations predicted that under ischaemia, SDH ran in reverse by using ubiquinol produced by complex I to reduce fumarate to succinate, thereby acting as a terminal electron acceptor instead of oxygen. Fumarate was produced from the PNC and reversal of the CAC. Flux through the rest of the respiratory chain was diminished and AMP was produced from ADP owing to insufficient ATP production. b, With oxygen restored SDH metabolised excess succinate. A delay in regenerating AMP to ADP, as typified in the first minute of reperfusion, limited the flux through ATP-synthase. This in turn prevented complex III consuming all the ubiquinol generated by SDH, as the membrane became hyperpolarized. The excess flux of ubiquinol and protons forced complex I to run in reverse, which would generate ROS by RET. c, Once the flux of succinate was reduced to normal levels, as in the transition from late reperfusion to normoxia, the fluxes through the respiratory chain and citric acid cycle returned to normal.
Extended Data Figure 9 | Tracking DHE oxidation, NAD(P)H reduction state, and mitochondrial membrane potential in primary cardiomyocytes during in situ IR. a, Inhibition of mitochondrial complex I RET reduces DHE oxidation on reperfusion (n = 6; rotenone n = 4). b, c, Effect of manipulation of ischaemic succinate levels on NAD(P)H oxidation during early reperfusion (n = 3). Primary rat cardiomyocytes were subjected to 40 min ischaemia followed by reoxygenation and NAD(P)H reduction state was tracked throughout the experiment by measurement of NAD(P)H autofluorescence. Ischaemic buffer contained no additions, 4 mM dimethyl malonate, or 4 mM dimethyl succinate. Average (b) and representative (c) traces from each condition are shown. The highlighted window in c indicates the period of the experiment expanded in detail in b. d, Effect of inhibition of ischaemic succinate accumulation on mitochondrial membrane potential following late ischaemia (left) and early reperfusion (right). e, f, Primary rat cardiomyocytes were subjected to 40 min ischaemia and reoxygenation and mitochondrial membrane potential was tracked throughout the experiment by measurement of TMRM fluorescence. Ischaemic buffer contained either no additions or 4 mM dimethyl malonate. e, TMRM signal throughout the entire experiment. f, TMRM signal during the transition from ischaemia to reoxygenation (n = 3). *P < 0.05 (two-tailed Student’s t-test and one-way ANOVA). Data are mean ± s.e.m. of at least three biological replicates. Replicates represent separate experiments on independent cell preparations.
Extended Data Figure 10 | Quantification of CAC intermediates in the heart following infusion of dimethyl succinate and in the brain after infusion of dimethyl malonate, and extended summary cytoprotection and neurological scores of rats subjected to tMCAO IR in vivo ± dimethyl malonate infusion. a, Effect of intravenous infusion of dimethyl succinate on CAC metabolite abundance in the ischaemic and non-ischaemic myocardium (normoxia and peripheral heart tissue plus dimethyl succinate n = 3; ischaemia plus dimethyl succinate n = 4; α-ketoglutarate and aconitate in peripheral heart tissue n = 2). b, Profile of mitochondrial CAC metabolite levels after tMCAO ischaemia ± dimethyl malonate (n = 4). c, Representative images of cross-sections from rat brains after undergoing tMCAO in vivo ± treatment with dimethyl malonate. Brains were treated with haematoxylin and eosin to delineate infarcted tissue. d, Locomotor and sensorimotor assessment of rats by quantification of average number of footfalls after tCMAO ± dimethyl malonate (control n = 6; dimethyl malonate n = 4). *P < 0.05, **P < 0.01 (two-tailed Student’s t-test for pairwise comparisons, and one-way (a, b) or two-way (d) ANOVA for multiple comparisons). Data are mean ± s.e.m. of at least three biological replicates, unless otherwise stated.