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Supplemental Information

Accumulation of Succinate in Cardiac Ischemia Primarily Occurs via Canonical Krebs Cycle Activity

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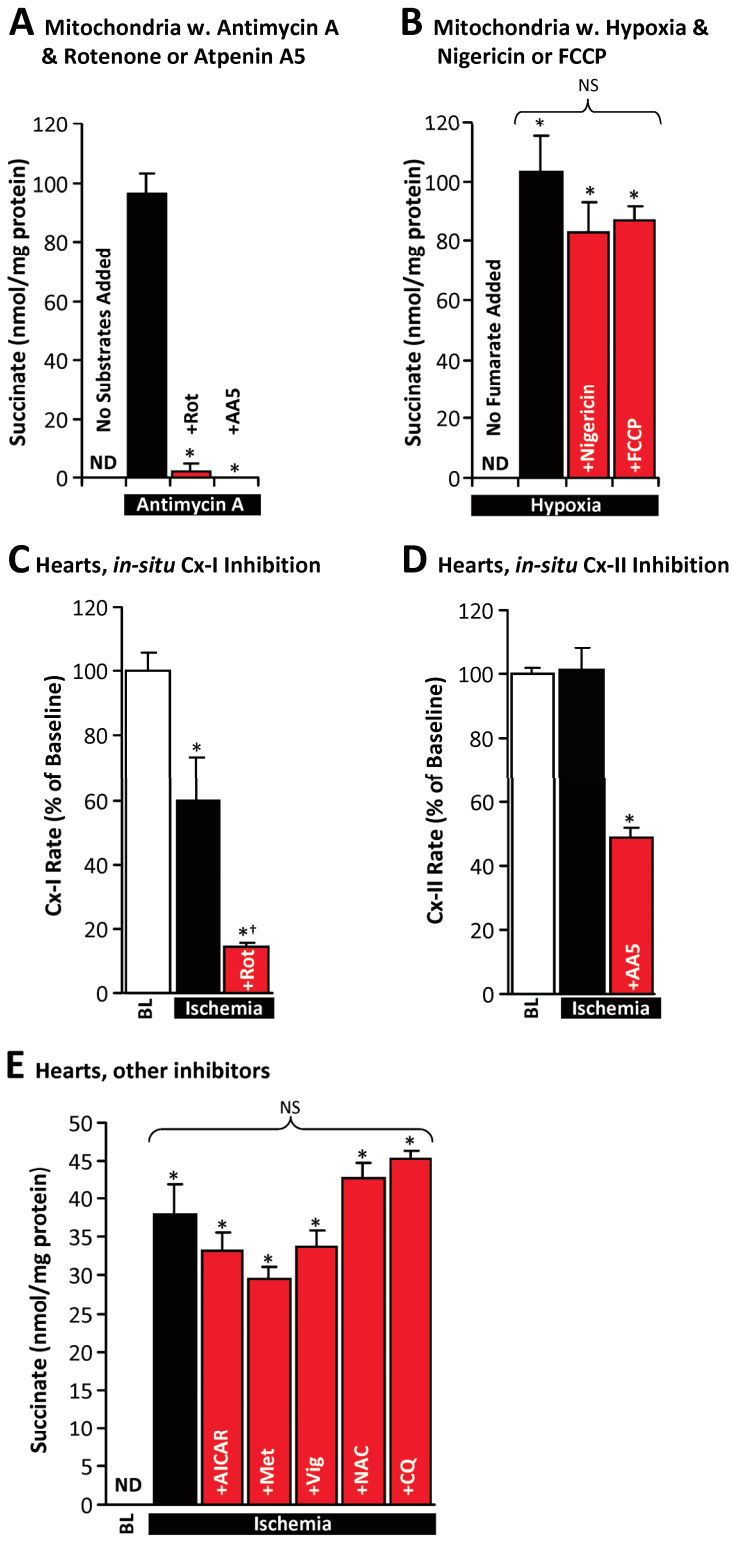

Figure S1 (legend →)

Figure S1 (related to Figure 2): Control experiments for mitochondrial or cardiac succinate generation. **(A)** Isolated mouse heart mitochondria were treated with 5 μM antimycin A under normoxic conditions. Then 1 mM fumarate was added, along with 1 μM Rot or 1 μM AA5, followed by normoxic incubation for 20 min. Succinate was measured using HPLC. *n*=3-5. *p<0.05 vs. antimycin A alone. ND: not detectable. **(B)** Isolated mouse heart mitochondria respired to hypoxia, then 1 mM fumarate was added, along with either 10 μM nigericin or 5 μM FCCP. Samples were incubated for 20 min. in hypoxia, and succinate was measured using HPLC. *n*=3-6. *p<0.05 vs. hypoxia alone. NS: no significant difference between indicated groups. **(C)** Hearts were subjected to 25 min. of normoxic perfusion (baseline, BL) or 25 min. ischemia (Isch'), with optional presence of 1 μM rotenone (Isch' +Rot), then homogenized. Cx-I activity (NADH oxidation) was assayed spectrophotometrically. *n*=3. *p<0.05 vs. baseline (BL), †p<0.05 vs. untreated ischemia (Isch'). **(D)** Hearts were subjected to 25 min. of normoxic perfusion (baseline, BL) or 25 min. ischemia (Isch'), with optional presence of 1 μM atpenin A5 (Isch' +AA5), then homogenized. Cx-II activity (succinate oxidation) was assayed spectrophotometrically. *n*=4-7. *p<0.05 vs baseline (BL). **(E)** Perfused hearts were treated with (abbreviation/concentration/duration): 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR/500 μM/5 min.), metformin (Met/10 mM/5 min.), vigabatrin (Vig/500 μM/5 min.), N-acetylcysteine (NAC/10 mM/10 min.), and chloroquine (CQ/50 μM/5 min.), then immediately subjected to 25 min. ischemia. Hearts were freeze-clamped (no reoxygenation) and succinate measured using HPLC. *n*=2-8. *p<0.05 vs baseline (BL). All data are means ± SEM.

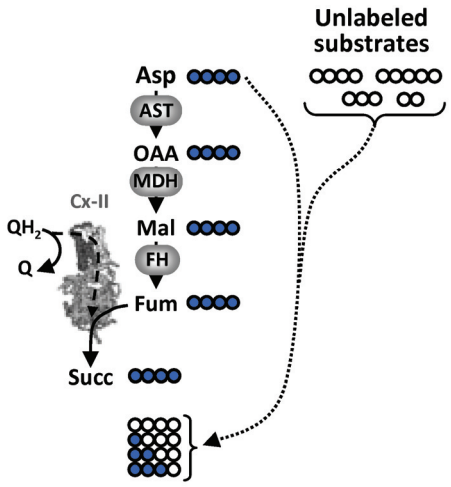

Figure S2 (related to Figure 3): Schematic of predicted isotopologue labeling from [U-^{13}C]aspartate delivery. Proposed metabolic pathways from aspartate to succinate are shown, namely via Cx-II reversal (Figure 1, model A), and via other pathways in which unlabeled carbon becomes incorporated. Fully labeled aspartate is represented by 4 blue dots. Unlabeled substrate are represented by white dots. Succinate isotopoloues are indicate by mixed white (unlabeled) and blue (labeled) dots.

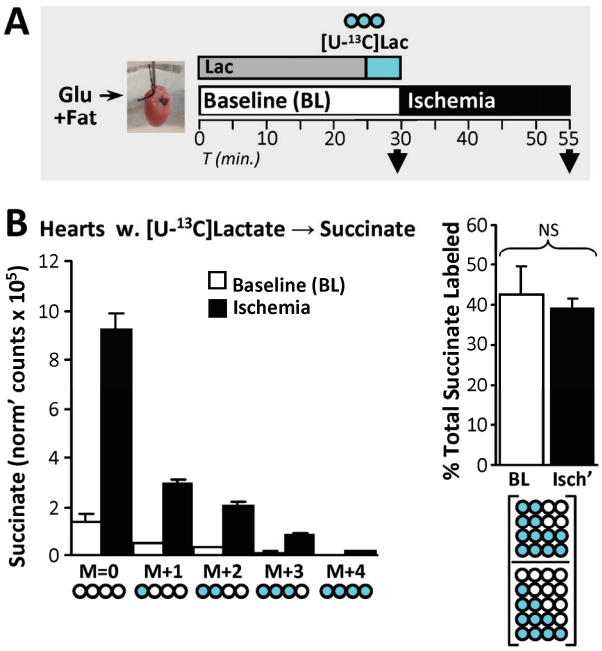

Figure S3 (Related to Figure 5E/F): Steady-state labeling with [U-^{13}C]lactate. (A) Schematic of steady-state SIRM experiments, with [U-^{13}C]lactate delivery for 5 min. achieving steady-state labeling of Krebs' cycle metabolites. Hearts were sampled immediately or after 25 min. ischemia. Succinate was measured using LC-MS/MS. **(B)** *Left.* Abundances of each ^{13}C-labelled succinate species in hearts from (A) are shown. Pictograms below represent labeling status of carbons in succinate (white = unlabeled, turquoise = labeled). *Right.* Total fractional labeling of succinate. Pictogram below represents fraction calculated. *n*=3. NS: no significant difference between indicated groups. All data are means ± SEM.

Supplemental Experimental Procedures

Animals and reagents

Animal and experimental procedures complied with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (2011 revision) and were approved by the University of Rochester Committee on Animal Resources (Protocol Number 2007-087). Male 2-4 month old mice on a C57BL/6J background obtained from The Jackson Laboratory (RRID: IMSR_JAX:000664) were housed in a pathogen-free vivarium with 12 hr. light-dark cycles, and food and water *ad libitum*. Mice were group housed, up to 5 mice per cage. Cages were changed at least every two weeks. Anesthesia was induced with intraperitoneal injection of 250mg/kg 2,2,2-tribromoethanol (Avertin). Unless otherwise stated, all biochemicals and reagents were of the highest grade possible obtained from Sigma (St. Louis MO).

Mitochondrial isolation

Mouse heart mitochondria were isolated as previously described (Zhang et al., 2016). Hearts from four mice were removed and immersed in 10 ml of ice-cold isolation medium (IM), consisting of (in mM) sucrose (300), Tris-HCl (20), EGTA (2), and 0.1% (w/v) fat-free BSA, pH 7.35 at 4°C. Tissue was chopped, washed, and homogenized in 10 ml IM using a Tekmar Tissumizer (IKA Instruments, Wilmington, NC). The homogenate was centrifuged at 800 x *g* for 5 min., the pellet was discarded, and the supernatant was centrifuged at 8000 x *g* for 5 min. The pellet was resuspended in 1.5 ml IM and centrifuged again at 8000 x *g* for 5 min. The top layer of the pellet (broken mitochondria and microsomes) was discarded, and the remaining pellet resuspended in 75 µl IM. Protein content was determined using the Lowry method (Lowry et al., 1951) against a standard curve constructed using bovine serum albumin. This protocol yielded ~4 mg of mitochondrial protein.

Hypoxic mitochondria

Respiration buffer (RB) consisted of (in mM): KCl (120), sucrose (25), $MgCl_2$ (5.0), KH_2PO_4 (5.0), EGTA (1.0), HEPES (10), 0.1% (w/v) fat-free BSA pH 7.3 at 37°C. For each experiment, 0.5 ml RB and 0.5 mg mitochondrial protein were added to a water jacketed (37°C) and magnetically stirred chamber. The chamber was sealed with a plug containing a capillary through which substrates and drugs were added using Hamilton syringes. The O_2 concentration was monitored using a Clark electrode (YSI, Yellow Springs, OH). After baseline measurements, 2 mM pyruvate, 5 mM carnitine, and 100 µM ADP, were added sequentially. This method yielded a respiratory control ratio of 3.6±0.8 (Mean±SD). Once the mitochondria consumed all of the oxygen in the chamber (<2%), 1 µM rotenone or 1 µM atpenin A5 (AA5), and 1 mM fumarate were added. After 20 min. of hypoxia, 8% perchloric acid was added to precipitate proteins, and the samples were frozen at -80°C until metabolite analysis. In a separate series of related experiments, isolated mitochondria (0.5 mg protein in 0.5 ml RB) at 37°C were provided 2 mM pyruvate, 5 mM carnitine, and 100 µM ADP, and treated with 5 mM antimycin A under normoxic conditions (open incubations). After 20 min. of incubation, 8% perchloric acid was added to precipitate proteins, and the samples were frozen at -80°C until metabolite analysis.

Hypoxic mouse cardiomyocytes

Adult mouse cardiomyocytes were isolated as previously described (Zhang et al., 2016). The heart was excised and perfused for 3 min. (flow rate 4 ml/min.) with perfusion buffer (PB), consisting of (in mM): NaCl (120), KCl (15), Na_2HPO_4 (0.6), KH_2PO_4 (0.6), $MgSO_4$ (1.2) HEPES (10), $NaHCO_3$ (4.6), taurine (30), glucose (5.5), and butadione monoxime (10), pH 7.4 at 37°C. The heart was then perfused for 10 min. with digestion buffer (DB), consisting of PB plus 12.5 µM $CaCl_2$, 0.025 % (w/v) trypsin, 6.525 U

collagenase A, and 15.375 U collagenase D (enzymes from Roche, Indianapolis IN). Ventricular tissue was teased apart, resuspended in Stop Buffer (SB), consisting of PB plus 12.5 µM $CaCl_2$ and 10% (v/v) FBS, and filtered through a 200 µm mesh. Cells were allowed to settle by gravity for 10 min., and then resuspended in 10 ml of SB. [Ca^{2+}] was increased stepwise to 1.8 mM in a shaking water bath. The final pellet was resuspended with unbuffered DMEM containing (in mM) glucose (5.5), palmitate (0.1), and pyruvate (0.1). This protocol yielded ~ $8x10^5$ rod-shaped cells per heart with > 80% viability (determined by Trypan blue exclusion assay).

$1x10^5$ cells were seeded per well onto laminin-coated 12-well plates. 10 µM rotenone and 1 µM AA5 were added, and the plate was placed into a hypoxic chamber (<0.1 % O_2, Coy Inc., Grass Lake MI) at 37°C. After 1 hr. of anoxia, 8% perchloric acid was added to lyse cells and precipitate proteins. The samples were frozen at -80°C until metabolite analysis.

Langendorff-perfused mouse hearts

Perfused hearts experiments were performed as previously described (Nadtochiy et al., 2015). The heart was excised and perfused with Krebs-Henseleit buffer (KH) consisting of (in mM): NaCl (118), KCl (4.7), $MgSO_4$ (1.2), $NaHCO_3$ (25), KH_2PO_4 (1.2), $CaCl_2$ (2.5), glucose (5), pyruvate (0.2), lactate (1.2), and palmitate (0.1), with 95% O_2 and 5% CO_2 at 37°C. A balloon connected to a pressure transducer was inserted into the left ventricle and cardiac function was recorded for the duration of the protocol. This yielded a rate x pressure product of 43,884±7,209 (Mean ± SD).

Whole heart inhibitor & metabolite labeling studies

Hearts were equilibrated for 20 min., infused with substrates or inhibitors as follows, and either immediately frozen, or subjected to 25 min. of global no-flow ischemia and then frozen. Hearts were frozen by clamping in pre-cooled Wollenberger tongs and plunged into liquid N_2, then pulverized in a mortar and pestle, and powder stored at -80°C until metabolite analysis.

Substrate and inhibitor treatments were as follows (concentration used / time infused): rotenone (1 μM/5 min.), AA5 (1 μM/5 min.), dimethylmalonate (5 mM/10 min.), malonate (5 mM/10 min.), thenoyltrifluoroacetone (TTFA, 1 mM/5 min.), 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR, 500 μM/5 min.), metformin (10 mM/5 min.), vigabatrin (500 μM/5 min.), N-acetylcysteine (NAC, 10 mM/10 min.), chloroquine (50 μM/5 min.), aminooxyacetate (AOA, 5 mM/5 min.), UK5099 (10 μM/5 min.), [U-^{13}C]aspartate (1 mM/10 min.), [U-^{13}C]glucose (5 mM/5 min.), [U-^{13}C]palmitate (100 μM/5 min.), [U-^{13}C]lactate (1.2 mM/5 min.), or [U-^{13}C,^{15}N]glutamine(2 mM/10 min.). N.B., [U-^{13}C]glucose, [U-^{13}C]palmitate and [U-^{13}C]lactate perfusions replaced their unlabeled counterparts in the KH buffer. Where indicated, dimethyl-α-ketoglutarate (5 mM/10 min.) or methyl-pyruvate (1 mM/10 min.) were infused.

Ischemia and reperfusion injury

Hearts were equilibrated for 20 min., subjected to 25 min. of global no-flow ischemia, and then 60 min. of reperfusion. AA5 (100 nM) was infused for 5 min. immediately at reperfusion. Infarct was assessed following staining with triphenyltetrazolium chloride.

Metabolite reperfusion washout studies

Hearts were equilibrated for 20 min., subjected to 25 min. of global no-flow ischemia, and then reperfused for 1, 2, or 5 min. before freeze-clamping and pulverization as described above. In addition where feasible, effluent from early reperfusion was collected and binned into 0-1 min., 1-2 min., and 2-5 min. Effluents were treated with 8% perchloric acid to precipitate proteins. Effluents and heart powder were stored at -80°C until metabolite analysis.

Metabolite Analysis by HPLC

Heart powder was serially extracted in 3 x 400 µl 8% perchloric acid, and the extracts centrifuged at 20,000 x *g* to remove insoluble materials. Perchloric acid extracts of cell or mitochondrial pellets, or cardiac reperfusion effluent (see above), were centrifuged at 20,000 x *g* to remove insoluble materials. Metabolites were resolved on HPLC (Shimadzu Prominence 20 system) using two Aminex HPX-87H columns (300 x 7.8 mm) in series with 10 mM H_2SO_4 mobile phase (flow rate: 0.7 ml/min) at 40°C (Wojtovich and Brookes, 2008). 100 µL of the sample was used for each injection. Succinate was detected using a photodiode array measuring absorbance at 210 nm. A standard curve of succinate was constructed for bracket-calibration. Data were normalized to heart protein content.

Metabolite Analysis by LC-MS/MS

Heart powder was serially extracted in 3 x 1 ml 80% methanol, evaporated under N_2, and resuspended in 200 µL of 50% methanol. LC-MS/MS analysis was essentially as previously described (Nadtochiy et al., 2015). Metabolites were resolved by HPLC (Shimadzu Prominence 20 system), with a Phenomenex Synergi Polar-RP column (150 x 2 mm) at 35 °C. Mobile phases were A: 97:3

water:methanol with 10 mM formic acid and 10 mM tributylamine, and B: 100% methanol. The flow rate was 0.2 ml/min. The solvent ramp was (% B): 0 min. 0; 5 min. 0; 10 min. 20; 20 min. 20; 35 min. 65; 38 min. 100; 42 min. 100, 43 min. 0; 50 min. 0. 10 µL of sample was used for each injection. Metabolites were identified by single reaction monitoring on a triple-quadrupole mass spectrometer (Thermo Quantum TSQ) using individually validated standards. Data were analyzed using publicly available MzRock machine learning tool kit (http://code.google.com/p/mzrock/), which automated metabolite identification based on retention time, whole molecule mass, collision energy, and fragment mass. Selected metabolites were also analyzed using XCalibur Qual Browser (Thermo Scientific). Data were normalized to heart protein content.

Glycogen Content Assay

Langendorff-perfused hearts were assayed for glycogen content using a Glycogen Assay Kit (see Key Resources Table). Briefly, glycogen is hydrolyzed into glucose, which is then reacted to form a colored product that could be detected colorimetrically at 570nm.

Mitochondrial Cx-I Assay

The reaction buffer contained (in mM): $K^+ PO_4^{3-}$ (25), $MgCl_2$ (10), fat-free bovine serum albumin (2.5mg/ml), (0.05), reduced nicotinamide adenine dinucleotide (NADH, 0.075), and KCN (1) with pH 7.2 at 37°C. 0.2 mg protein/ml of freeze-thawed heart homogenate were added per reaction. After baseline measurements, 50 µM co-enzyme Q_1 was added to start the reaction, and the disappearance of NADH absorbance was measured at 340 nm using a spectrophotometer (Beckman Coulter DU-800). After 5

min., 2 μM rotenone was added to inhibit Cx-I, and the difference between the two rates were calculated as the Cx-I rate.

Mitochondrial Cx-II Assay

The reaction buffer contained (in mM): K^+ PO_4^{3-} (50), co-enzyme Q_2 (0.05), EDTA (0.1), dichlorophenolindophenol (DCPIP, 1.2), KCN (1), and rotenone (0.01) with pH 7.4 at 37°C. 0.6 mg protein/ml of freeze-thawed heart homogenate were added per reaction. After baseline measurements, 20 mM succinate was added to start the reaction, and the disappearance of DCPIP absorbance (due to its reduction to $DCPIPH_2$) was measured at 600nm using a spectrophotometer (Beckman Coulter DU-800). After 5 min., 1 mM thenoyltrifluoroacetone (TTFA) was added to inhibit Cx-II, and the difference between the two rates were calculated as the Cx-II rate.

Quantification and Statistical Analysis

Comparisons between groups were made using ANOVA, with post-hoc Student's t-test applied where appropriate. Data are shown as means ± SEM unless otherwise noted. Numbers of replicates (*n*) are noted in Figure legends. Significance was set at α=0.05. All raw data and statistical analyses are available in the original data set on Figshare (doi: 10.6084/m9.figshare.6157631).

Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Atpenin A5	Enzo	Cat#ALX-380-313; CAS#: 119509-24-9
Rotenone	Sigma-Aldrich	Cat#R8875; CAS#: 83-79-4
Dimethylmalonate	Sigma-Aldrich	Cat#136441; CAS#: 108-59-8
Malonate	Sigma-Aldrich	Cat#M1296; CAS#: 141-82-2
Thenoyltrifluoroacetone	Sigma-Aldrich	Cat#T27006; CAS#: 326-91-0
Antimycin A	Sigma-Aldrich	Cat#A8674; CAS#: 1397-94-0
Nigericin	Sigma-Aldrich	Cat#N7143; CAS#: 28643-80-3
FCCP	Sigma-Aldrich	Cat#C2920; CAS#: 370-86-5
AICAR	Sigma-Aldrich	Cat#A9978; CAS#: 2627-69-2
Metformin	Sigma-Aldrich	Cat#PHR1084; CAS#: 1115-70-4
Vigabatrin	Sigma-Aldrich	Cat#V8261; CAS#: 68506-86-5
N-acetylcysteine	Sigma-Aldrich	Cat#A7250; CAS#: 616-91-1
Chloroquine	Sigma-Aldrich	Cat#C6628; CAS# 50-63-5
Aminooxyacetate	Sigma-Aldrich	Cat#C13408; CAS#: 2921-14-4
UK-5099	Sigma-Aldrich	Cat#PZ0160; CAS#: 56396-35-1
Dimethyl-α-ketoglutarate	Sigma-Aldrich	Cat#349631; CAS#: 12192-04-6
Methyl-pyruvate	Sigma-Aldrich	Cat# 371173; CAS# 600-22-6
[U-^{13}C]aspartate	Cambridge Isotope Laboratories	Cat#CLM-1801-H; Unlabeled CAS#: 56-84-8; Labeled CAS#: 55443-54-4
[U-^{13}C]glucose	Cambridge Isotope Laboratories	Cat#CLM-1396; Unlabeled CAS#: 50-99-7; Labeled CAS#: 110187-42-3

[U-^{13}C,^{15}N]glutamine	Cambridge Isotope Laboratories	Cat#CLM-1275-H; Unlabeled CAS#: 56-85-9; Labeled CAS#: 285978-14-5
[U-^{13}C]palmitate	Cambridge Isotope Laboratories	Cat#CLM-6059; Unlabeled CAS#: 408-35-5; Labeled CAS#: N/A
[U-^{13}C]lactate	Cambridge Isotope Laboratories	Cat# CLM-1579; Unlabeled CAS# 867-56-1; labeled CAS# 201595-71-3
Critical Commercial Assays		
Glycogen Assay Kit	Sigma-Aldrich	Cat#MAK016
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	The Jackson Laboratory	RRID: IMSR_JAX:000664

Supplement References

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265–275.

Nadtochiy, S.M., Urciuoli, W., Zhang, J., Schafer, X., Munger, J., and Brookes, P.S. (2015). Metabolomic profiling of the heart during acute ischemic preconditioning reveals a role for SIRT1 in rapid cardioprotective metabolic adaptation. J. Mol. Cell. Cardiol. *88*, 64–72.

Wojtovich, A.P., and Brookes, P.S. (2008). The endogenous mitochondrial complex II inhibitor malonate regulates mitochondrial ATP-sensitive potassium channels: Implications for ischemic preconditioning. Biochim. Biophys. Acta - Bioenerg. *1777*, 882–889.

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