Supplementary information

Surgical technique

A rapid cardiectomy was carried out while preserving the inferior and superior vena cava, and pulmonary artery (PA) and pulmonary veins up to the descending aorta vessels. The heart was emptied of blood and weighed. The vessels were surgically prepared for mounting onto the *ex situ* heart perfusion (ESHP) apparatus. The aortic arch was dissected from the PA for enabling the attachment to the cannula. The pulmonary veins were cut to create a common orifice at the left atrial (LA) interface, and a 3-0 polypropylene purse-string suture was placed around it. The LA magnetic cannula was then fitted in the orifice and secured by tightening the suture. The inferior vena cava was closed but the superior vena cava remained open to ensure decompression of the right ventricle. The aorta arch vessels were trimmed of fat and connective tissue. The hearts were mounted by aligning the LA magnetic cannula to the LA flow orifice on the silicone membrane. Subsequently, the aorta was attached to the aortic cannula on the silicone membrane and secured with a 0-silk tie. The length and positioning of the aorta were adjusted to ensure no kinking or tension of the heart. The aortic purge line was then secured to the innominate artery using a 0-silk tie. The left subclavian artery was snared and a 5F introducer sheath was placed. The aortic pump rotations per minute (RPM) were increased to reach a mean pressure of 30 mmHg, and coronary flow was established – marking the onset of reperfusion. The left atrial pressure (LAP) was maintained at 0 mmHg. A blood sample was taken before and after mounting of the heart for arterial blood gas analysis of the perfusate.

Ex-situ heart perfusion (ESHP) and administration of cardiac postconditioning

Broad-spectrum antibiotics (3 g Piperacillin/0.375 g Tazobactam) were added to the perfusate, and 2 g of anti-arrhythmic $MgSO_4$ were added to the perfusate. 20% Intralipid was added to the

circuit to a final concentration of 1% 5 min prior to DCD heart reperfusion. 2% (v/v) sevoflurane was administered to the circuit by the aid of a vaporizer connected to the membrane oxygenator prior to mounting the heart (priming the circuit), and sevoflurane administration was maintained for 30 min after the onset of reperfusion. An initial 3.3 µg bolus of remifentanil was administered to the circuit to achieve a final concentration of at least 3 nM at the onset of reperfusion, followed by a continuous maintenance infusion of 0.45 µg/min remifentanil for 30 min. The infusion delivery rate of remifentanil was calculated based on a $t_{½}$ of 3.65 min and a linear degradation of the initial bolus.[1] Epicardial electrocardiogram (ECG) electrodes were attached to the surface of the heart to measure heart rhythm. The heat exchanger was turned on after 5-10 min after the onset of reperfusion and gradually warmed up to 38.4°C. Appearance of prolonged (15 s) ventricular fibrillation was treated by defibrillation with increasing energy doses (5-20 J). Pacemaker leads were attached to the right atrial wall and paced in AAI mode. Insulin (2 U/hour) and dobutamine (4 µg/min) were infused into the circuit for metabolic support and adrenergic stimulation, respectively, after 30 minutes of reperfusion. The perfusate pH was maintained between 7.35-7.45 by titrating 3M tris(hydroxymethyl)aminomethane. Gas flow to the circuit was controlled through a gas mixer, pCO_2 was maintained between approximately 30-40 mmHg and sO_2 was maintained at ≥95%. cCa^{2+} was maintained between approximately 1.1-1.2 mM after transition into working mode. Glucose (125 mg/mL) infusion was initiated when perfusate glucose levels decline below 5 mM. An initial 500 mg bolus was given and a continuous infusion of 500 mg/hr was administered to maintain perfusate glucose levels above 5 mM. The heart was perfused in Langendorff mode for 60 min before switching to working mode. The superior vena cava was ligated and a saline-filled 5F pigtail catheter (Cordis, Milpitas, CA, USA) was inserted through the introducer sheath in the subclavian artery to monitor left ventricular pressure. The

aortic purge line was closed while the LAP was gradually increased to 6 mmHg. Aortic diastolic pressure was maintained at 40 mmHg and the heart was paced at 100 beats per minute (RA pacing). Steady state functional data were collected every hour starting at T2 (120 min) using an in-house custom-made data acquisition platform at increasing LAP values of 6, 8, 10, 12 mmHg (workload challenge). The functional parameters include: heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), LA flow (as a measure of cardiac output), PA flow (as a measure of coronary blood flow), left ventricular stroke work (LVSW), $LVdP/dt_{max}$, and $LVdP/dt_{min}$. LVSW was calculated as the product of developed pressure (DP, calculated as MAP – LAP) and stroke volume (derived from cardiac output and heart rate). Cardiac Output and LVSW were indexed for heart weight (grams) to obtain Cardiac Index (CI) and LVSW Index (LVSWI), respectively. Blood gas measurements were taken from the aortic and PA line simultaneously at the beginning of functional assessments (LAP=6 mmHg) and measured on an ABL800 FLEX blood gas analyzer (Radiometer, Mississauga, ON). Parameters include blood gas values (pH, pCO_2, pO_2, p50c), oximetry values (ctHb, sO_2, FO_2Hb, FCOHb, FHHb, FMetHb), electrolyte values (cK^+, cNa^+, cCa^{2+}, cCl^-, mOsmc), acid base status (cGlu, cLac, ctBil, cBase(Ecf)c, $cHCO_3^-$(P, st)c, $cHCO_3^-$(P)c). Myocardial oxygen consumption (MVO_2) was calculated as following:

$$MVO_2\ (mL\ O_2 \cdot min^{-1} \cdot 100\ g^{-1}) = CBF \cdot (C_aO_2 - C_vO_2)$$

where CBF, coronary blood flow; C_aO_2, arterial oxygen content; C_vO_2, venous oxygen content. Oxygen content was assessed using the following formulas:

C_aO_2 = [(1.34 mL O_2 · g Hb $^{-1}$) · Hb concentration (g · 100 mL $^{-1}$) · oxygen saturation (%)] + [(0.00289 mL O_2 · mmHg $^{-1}$ · 100 mL^{-1}) · PaO_2 (mmHg)]

C_vO_2 = [(1.34 mL O_2 · g Hb $^{-1}$) · Hb concentration (g · 100 mL $^{-1}$) · oxygen saturation (%)] + [(0.00289 mL O_2 · mmHg $^{-1}$ · 100 mL^{-1}) · PvO_2 (mmHg)]

Primers for cell-free mitochondrial DNA

The following primer pairs used for mitochondrial DNA (mtDNA) quantification. The targets are the genes encoding NADH dehydrogenase 1 (*ND1*) and (*CYTB*) as well as a non-coding region known as the displacement loop (*Dloop*).

Gene	Forward Primer Sequence (5' → 3')	Reverse Primer Sequence (5' → 3')	Product Size (bp)
DLoop	ACACCCTATAACGCCTTGCCA	AAGTGCCTGCTTTCGTAGCAC	148
ND1	GCCGTAGCATTCCTCACCCT	ATGTGCCTGGTCGTAGGGGT	151
CYTB	GCTACGTCCTGCCCTGAGGA	TGGCAGGATAAAGTGGAAGGCG	170

Metabolomics

A targeted quantitative metabolomics approach based on a custom LC-MS/MS assay (TMIC MEGA Metabolomics Profiling Assay, developed at the Metabolomics Innovation Centre of the University of Alberta; https://metabolomicscentre.ca/service/tmic-mega-assay-2/) was used to analyze metabolites extracted from left ventricular (LV) tissue samples. The assay uses a combination of direct injection mass spectrometry (MS) along with a reverse-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for the identification and absolute quantification of up 635 metabolites including amino acids and amino acid related biogenic amines, acylcarnitines, glycerophospholipids, other polar lipids (sphingomyelins, phosphatidylcholines, lysophosphatidylcholines), organic acids, nucleotide/nucleosides and neutral lipids such as diacylglycerols. The method combined the derivatization and extraction of analytes with the selective mass-spectrometric detection using multiple reaction monitoring (MRM) pairs. Internal standards, including isotope-labeled internal standards were used for metabolite quantification. LV tissue powder (100 mg) was weighed accurately and homogenized

with 3 volumes of tissue extraction buffer (85% v/v methanol in 10 mM phosphate buffer). After homogenization, the samples were centrifuged at 14,000 rpm for 20 min and the supernatant was collected for further LC-MS/MS sample preparation. Prior to loading onto the assay plate, the samples were thawed on ice, vortexed and centrifuged at 13,000g to remove debris. After loading onto the filter plate, the samples were dried in a stream of nitrogen. Subsequently, phenyl-isothiocyanate was added for derivatization. After incubation, the filter spots were dried again using an evaporator. Metabolites were extracted by adding 300 µL of extraction solvent and centrifuged into the lower 96-deep well plate, followed by a dilution step with MS running solvent. For organic acid analysis, 10 µL of sample was loaded, followed by the addition of 3-nitrophenylhydrazine (NPH) derivatization reagents and internal standard solution. After incubation for 2 hours, BHT (butylated hydroxytoluene) stabilizer and water was added before LC-MS injection. Mass spectrometric analysis was performed on an ABSciex 5500 Qtrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with an Agilent 1290 series UHPLC system (Agilent Technologies, Palo Alto, CA). MS data analysis was done using Analyst 1.6.3. Please note that the reverse-phase liquid chromatography-tandem mass spectrometry strategy used to measure metabolite concentrations allows for measurements of triglyceride content but does not allow for the complete elucidation of triglyceride molecular composition.

Potential alterations in the concentration of the 635 analyzed metabolites in treated hearts (DCD/ESHP+PoC group) as compared to untreated hearts (DCD/ESHP group) were first explored via univariate and multivariate statistical analysis, using the MetaboAnalyst 5.0 statistical analysis module. MetaboAnalyst (https://www.metaboanalyst.ca/) is a web-based tool for the analysis of metabolomics datasets that was launched in 2009 and subsequently

progressively refined and improved.[2-5] Metabolites with more than 25% of missing values were excluded from further analysis. The data were then mean-centered to generate appropriate Gaussian metabolite concentration distributions. A first analysis revealed a large number of differentially regulated metabolites belonging to the neutral lipid class of triglycerides. For this reason, lipid-related metabolites were analyzed separately. Differences in mean metabolite concentrations within the 2 experimental groups were assessed using a parametric t-test.

Lipid mediator profiling

Lipid mediators were quantified using internal standards, calibrators, and quality controls, as previously described.[6] Frozen LV tissue powder (20 mg) was mixed with methanol and internal standards prior to solid phase extraction (SPE). The mixture was vortexed for 10 sec and centrifuged at 10,000g for 5 min at 4°C. The supernatant was collected and diluted with H_2O to prevent breakthrough during SPE. 96-well reverse phase SPE plates (Strata-X, Phenomenex, Torrance, USA) were equilibrated with methanol followed by H_2O prior to sample loading. The sample-containing wells were washed with H_2O followed by a 20:80 mixture of methanol and H_2O, and then dried for 30 sec under maximum gas flow using a 96-well positive pressure SPE manifold (Biotage, Uppsala, Sweden). Lipid mediators were eluted twice with 0.9 mL methanol into a 96-well collection plate containing 6 µL glycerol/methanol (30:70). Samples were then evaporated under nitrogen and reconstituted with 50 µL methanol. The ultra-HPLC (UHPLC)-MS/MS system consisted of a Shimadzu Nexera X2 UHPLC (Shimadzu Schweiz GmbH, Reinach, Switzerland) connected to a Sciex 6500+ triple quad mass spectrometer (AB Sciex, Zug, Switzerland). Samples were separated on an Acquity UPLC CSH C18 column (Waters AG, Baden-Dättwil, Switzerland) and delivered to the mass spectrometer via a sandwich injection program.

The data acquired from the perfused heart samples (experimental groups) as well as from the healthy hearts were collectively analyzed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/)[2-5] in order to measure potential increases in lipid mediator production due to the DCD process and subsequent ex-situ perfusion. Following data scaling (mean-centering), univariate and multivariate statistical analysis was used (one-way ANOVA and post-hoc analysis, Principal Component Analysis, and hierarchical clustering using the Euclidean distance measure). Lipid mediators are listed by their abbreviated names. Abbreviations are as follows:

12-HEPE, (+-)-12-Hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid; 5S-HEPE, (+-)-5-Hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid; 17-HDHA, (+/-)-17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid; 12S-HETrE, (12S)-Hydroxy-(8Z,10E,14Z)-eicosatrienoic acid; 14,15-EET, (14R,15S)-14,15-Epoxy-5,8,11-icosatrienoic acid; 15-HEPE, (15R)-15-Hydroxyeicosa-5Z,8Z,11Z,13E,17Z-pentaenoic acid; 15S-HETE, (15S)-Hydroxy-(5Z,8Z,11Z,13E)-eicosatetraenoic acid; 5S,15S-DiHETE, (5S,15S)-Dihydroxy-(6E,8Z,11Z,13E)-eicosatetraenoic acid; 5S-HETE, (5S,6E,8Z,11Z,14Z)-5-Hydroxy-6,8,11,14-eicosatetraenoic acid; 12-HETE, (5Z,8Z,10E,12S,14Z)-12-Hydroxyeicosa-5,8,10,14-tetraenoic acid; 8S-HETrE, (8S,9Z,11E,14Z)-8-hydroxyicosa-9,11,14-trienoic acid; 15S-HETrE, (8Z,11Z,13E,15S)-15-Hydroxyicosatrienoic acid; 12,13-EpOME, (9Z)-12,13-Epoxyoctadecenoic acid; 11,12-EET, 11,12-Epoxyeicosatrienoic acid; 5,12-DiHETE , 12-Epi-Leukotriene B4; 14-HDHA, 14(S)-Hydroxy docosahexaenoic acid; 18-HEPE, 18(R)-Hydroxyeicosa-5Z,8Z,11E,14Z,16E-pentaenoic acid; 6-keto-PGF1a, 6-Keto-prostaglandin F1a; 6-trans-LTB4, 6-trans-leukotriene B4; 8-iso-PGF2a, 8-Isoprostaglandin F2a; 9,10-EpOME, 9,10-Epoxyoctadecenoic acid (coronaric acid); 9-HODE, 9-Hydroxy-10E,12Z-octadecadienoic acid; LTB4, leukotriene B4;

LTB, leukotriene B5; PGE2, prostaglandin E2; PGF2a, prostaglandin F2a (PGF2); RvD5n-3 DPA, (8Z,15E,19Z)-7,17-dihydroxydocosa-8,10,13,15,19-pentaenoic acid (n-3 docosapentaenoic acid-derived resolvin D5); TXB2, Thromboxane B2.

Transmission electron microscopy (TEM)

LV tissue was cut into cubes of approximately 1 mm^3 in a mortar chilled with liquid nitrogen. The tissue pieces were placed into a fixing solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer with 2 mM $CaCl_2$, pH 7.4) while still frozen and incubated overnight at 4°C. The tissue pieces were washed with 0.1 M sodium cacodylate buffer and post-fixed in freshly made 1.5% K_4FeCN_6 and 2% OsO_4 in 0.1 M sodium cacodylate buffer for one hour. The samples were washed 0.1 M sodium cacodylate buffer and 0.1 M sodium acetate buffer (pH 5.2) and subsequently treated with 2% uranyl acetate in 0.1 M sodium acetate buffer (pH 5.2) for one hour. The samples were washed again in 0.1 M sodium acetate buffer and deionized water, and then dehydrated in a graded series of ethanol at 30%, 50%, 70%, 80%, 90%, 95%, and two changes of 100%. Infiltration in ethanol and Spurr's resin (EMS #14300, Electron Microscopy Sciences, Hatfield, PA) mixture was done at 2:1, 1:1, 1:2 and 3 changes of 100% resin. Finally, the tissue pieces were polymerized in the fourth change of resin at 65°C for 48 hours. The tissue blocks were sectioned to 70 nm with an ultramicrotome (Leica, EM UC6) and post-stained with uranyl acetate and lead citrate. TEM images were captured with a transmission electron microscope (JEOL F21000, Gatan Orius camera with Digital micrograph) at 200kV acceleration voltage. 2 blocks were processed from each sample, and 5-8 images at 8,000x magnification were collected from each block for quantitative analysis. Mitochondrial ultrastructure scoring was done using the Flameng Score.[7] From each image, a total of 40 mitochondria were randomly selected and scored by two individuals independent of each other.

Thus, for each block, up to 8x40=320 mitochondria were scored. An individual sample score was calculated as the average of the scores from block 1 and block 2. Additional images were captured at 5,000x and 30,000x to show tissue and mitochondrial structure. All work was carried out at the Cell Imaging Core Facility of the Faculty of Medicine and Dentistry at the University of Alberta.

Metabolic enzyme activities

The activity of the mitochondrial matrix marker enzyme citrate synthase (CS) was measured according to the method by Srere.[7] Citrate synthase activity was measured in LV tissue homogenates and isolated mitochondria by monitoring the formation of thionitrobenzoate (TNB, ε=13.6 mM^{-1} cm^{-1} at 412 nm) at 25°C upon addition of 0.5 mM oxaloacetate in the presence of 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.3 mM acetyl-CoA and 0.25% Triton X-100. For preparation of total tissue homogenate samples, LV tissue was rinsed with PBS and homogenized using a glass Dounce homogenizer with 10-15 passes of the tight (B) fitting pestle. The crude homogenate was centrifuged at 1,000g for 10 min at 4°C and the supernatant was collected for the assay. Mitochondrial samples were prepared by homogenizing LV tissue in STM buffer (Sucrose [250 mM], Tris-HCl pH 7.4 [50 mM], $MgCl_2$ [5 mM], EDTA [5 mM], EGTA [5 mM]) using the Polytron® PT 1200 E (Kinematica AG, Malters, Switzerland). The resulting homogenate was subjected to differential centrifugation to obtain the mitochondrial fraction for the assay. Pyruvate dehydrogenase activity was measured in LV tissue homogenates with 10 mM NaF to preserve endogenous phosphorylation status using a commercially available colorimetric kit (#ab109902, Abcam Inc., Waltham, MA). For preparation of total tissue homogenate samples, LV tissue was rinsed and homogenized in cold PBS supplemented with 10 mM NaF using a glass Dounce homogenizer with 25-35 passes of the tight fitting pestle until

completely homogeneous. The crude homogenate was adjusted to 9.3375 mg/mL and solubilized with 20X Detergent Solution. The mixture was centrifuged at 1,000g for 10 min at 4°C and the supernatant was collected for the assay. Samples were incubated in the assay microplate overnight at 4°C and the reactions were performed at 28°C following manufacturer's instructions.

Additional references

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