Triiodothyronine Facilitates Weaning From Extracorporeal Membrane Oxygenation by Improved Mitochondrial Substrate Utilization

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Background—Extracorporeal membrane oxygenation (ECMO) provides a bridge to recovery after myocardial injury in infants and children, yet morbidity and mortality remain high. Weaning from the circuit requires adequate cardiac contractile function, which can be impaired by metabolic disturbances induced either by ischemia-reperfusion and/or by ECMO. We tested the hypothesis that although ECMO partially ameliorates metabolic abnormalities induced by ischemia-reperfusion, these abnormalities persist or recur with weaning. We also determined if thyroid hormone supplementation (triiodothyronine) during ECMO improves oxidative metabolism and cardiac function.

Methods and Results—Neonatal piglets underwent transient coronary ischemia to induce cardiac injury then were separated into 4 groups based on loading status. Piglets without coronary ischemia served as controls. We infused into the left coronary artery $[2\text{-}^{13}C]$pyruvate and $[^{13}C_6, {}^{15}N]_L$-leucine to evaluate oxidative metabolism by gas chromatography-mass spectroscopy and nuclear magnetic resonance methods. ECMO improved survival, increased oxidative substrate contribution through pyruvate dehydrogenase, reduced succinate and fumarate accumulation, and ameliorated ATP depletion induced by ischemia. The functional and metabolic benefit of ECMO was lost with weaning, yet triiodothyronine supplementation during ECMO restored function, increased relative pyruvate dehydrogenase flux, reduced succinate and fumarate, and preserved ATP stores.

Conclusions—Although ECMO provides metabolic rest by decreasing energy demand, metabolic impairments persist, and are exacerbated with weaning. Treating ECMO-induced thyroid depression with triiodothyronine improves substrate flux, myocardial oxidative capacity and cardiac contractile function. This translational model suggests that metabolic targeting can improve weaning. (*J Am Heart Assoc*. 2014;3:e000680 doi: 10.1161/JAHA.113.000680)

Key Words: cardiac metabolism • congenital heart defects • extracorporeal circulation • thyroid hormone

Extracorporeal membrane oxygenation (ECMO) is the most commonly used form of mechanical circulatory support in infants and children.[1] The venoarterial mode provides volume unloading, thereby reducing cardiac work, wall stress, and oxygen consumption requirements while maintaining systemic circulation. Accordingly, this mode provides rescue and/or a bridge to recovery after injury induced during congenital heart surgery or after other forms of cardiac insult. Ventricular unloading theoretically allows the heart to rest and recover, eventually weaning from mechanical circulatory support in some, but not all, cases. Despite technical improvements, mortality rates while on ECMO or after removal from the circuit remain near 50%.[1,2] Furthermore, both the adverse event and mortality rates directly relate to the duration of mechanical support.[3] Thus, early weaning is an important goal for practitioners caring for patients on ECMO.

Initiation of mechanical circulatory support in the form of ECMO imposes deleterious systemic reactions, which can paradoxically and adversely affect cardiac function and delay weaning.[4–6] In particular, a massive surge in proinflammatory cytokines occurs after the initiation of circuit perfusion, which then impairs hormonal and metabolic responses to stress. Elevations in specific cytokines are directly proportional to marked depression in circulating triiodothyronine (T_3) levels

induced by mechanical circulation.[7] Some studies in infants[4] and immature animal models[8] show that T₃ supplementation improves cardiac function after ischemia-reperfusion (IR) injury occurring with procedures performed under cardiopulmonary bypass. While the mechanical circulation circuit for cardiopulmonary bypass is similar to that performed for ECMO, the indications and duration of support are considerably different for each mode of support.

Although T₃ elicits ubiquitous nongenomic and genomic effects on the heart and vascular system, short-term improvements in ventricular function have been linked to changes in energy metabolism.[8–11] In particular, T₃ supplementation ameliorates impairments in myocardial pyruvate flux to the citric acid cycle caused by IR in multiple animal models.[8,12] ECMO promotes oxidative energy contribution by fatty acids relative to pyruvate produced through carbohydrate pathways such as glycolysis and/or lactate dehydrogenase. However, impairment in myocardial flux through pyruvate dehydrogenase becomes apparent only during weaning. The previously uninjured heart on ECMO support adapts by increasing oxidation of alternate substrates in order to successfully meet the ATP utilization required by weaning.[13] Substrate shifts in metabolism during successful weaning occur without increasing amino acid oxidation and without adversely affecting rates of protein synthesis. Considering the degree of pyruvate flux inhibition previously observed after IR injury,[12] we do not know if the injured heart similarly retains the ability to alter substrate oxidation to the degree required to reestablish cardiac function with weaning.

Accordingly, we used a translational experimental swine model, emulating infant ECMO for cardiac support, to test multiple hypotheses. First, we tested the hypothesis that ECMO partially resolves some of the metabolic abnormalities involving flux through the citric acid cycle (CAC) that are induced by IR. Second, we tested the hypothesis that ECMO does not adequately resolve these metabolic abnormalities to successfully accomplish weaning. Finally, we determined if thyroid hormone supplementation during ECMO moderated substrate flux and energy production in order to improve cardiac function during weaning.

Methods

Thirty-six male Yorkshire piglets between 25 and 48 days of age and weighing between 12.3 and 16.7 kg were prepared essentially as previously described.[13,14] All experimental procedures were approved by Seattle Children's Institutional Animal Use and Care Committee.

Sedation was achieved by intramuscular injection of ketamine (33 mg/kg, VEDCO) and xylazine (2 mg/kg, VE-

DCO), and the animals were placed on a circulating warming blanket. Monitors were placed for ECG, pulse oximetry (Radical SET, Masimo), and rectal temperature. A PowerLab 16/30 recorder (AD Instruments) was used to continuously record data throughout all protocols. A cutdown tracheostomy was performed, and animals underwent general anesthesia under inhaled isoflurane (1% to 2%, Baxter Healthcare Corporation). Venous and arterial access was then obtained for continuous blood pressure monitoring, blood sampling, and infusion of maintenance fluids. Arterial pH, P_{CO_2}, P_{O_2}, and hemoglobin were measured at regular intervals by use of a Radiometer ABL 800 device (Radiometer America). After the performance of a median sternotomy, a 5Fr high-fidelity pressure catheter (Millar Instruments) was inserted into the apex of the left ventricle. Baseline hemodynamic assessments were made at this point. The piglets were separated into 5 groups (Figure 1). The first group (CON) did not undergo coronary ischemia or ECMO cannulation and served as the control group. Piglets in IR did not undergo cannulation for ECMO but were subject to open chest and coronary
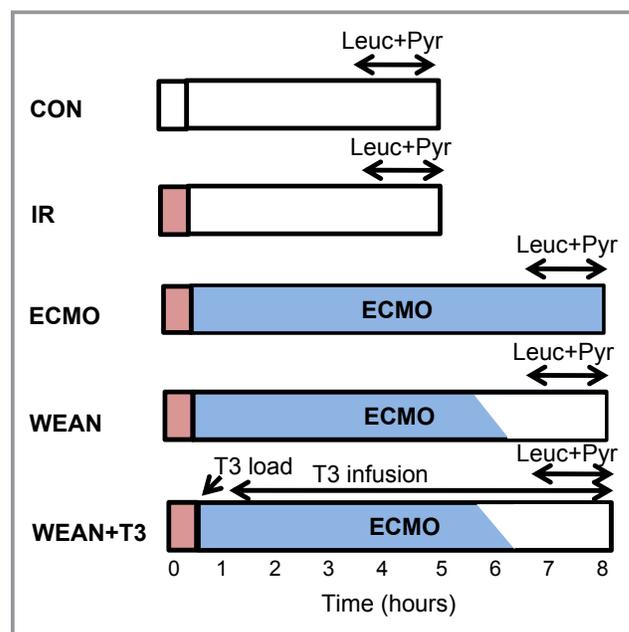

Figure 1. Schematic depicting the 5 protocol groups. Baseline hemodynamic parameters were taken at time=0. Ischemia-reperfusion (red bar) was performed for 10 minutes in the experimental groups. ECMO support was initiated after coronary release. In the groups WEAN and WEAN+T₃, ECMO flows were slowly weaned over 30 minutes to gradually reintroduce cardiac work. Isotopomers were infused into the LAD for 60 minutes. The cardiac tissue was then rapidly frozen and analyzed. T₃ was given as a bolus at initiation of ECMO (0.6 μg/kg) followed by a continuous infusion (0.2 μg/kg per hour). n=6 in CON, IR and ECMO, and n=5 in WEAN and WEAN+T₃. ECMO indicates extracorporeal membrane oxygenation; IR, ischemia-reperfusion; LAD, left anterior descending coronary arteries.

occlusion. For the animals undergoing ECMO cannulation (ECMO, WEAN, WEAN+T$_3$), the hemiazygous vein (which empties directly into the coronary sinus in swine) was ligated, and the aorta and the right atrium were cannulated and connected to the ECMO circuit (Stockert-Shiley roller pump and hollow-fiber oxygenator [CapioX Rx 05; Terumo]). The coronary sinus was cannulated for coronary venous sampling and flow monitoring (Transonic flow probe ME-4PXL; Transonic Systems) in order to determine myocardial oxygen consumption (MVo$_2$). Anticoagulation was achieved with 120 units/kg heparin (APP Pharmaceuticals) as a bolus. To minimize hemodilution anemia, we used the smallest volume of priming solution possible (80 mL of 10% Dextran 40–0.9% sodium chloride solution). In all animals, we inserted a 24-gauge BD Saf-T-Infusion catheter into the left anterior descending artery in retrograde fashion starting just distal to the first major branch for intracoronary infusion of labeling substrates. Myocardial injury (all animals except CON) was performed by suture ligature of the left anterior descending artery after the first major diagonal branch for 10 minutes, followed by release and reperfusion. The final group (WEAN+T$_3$) received systemic infusion of T$_3$ (Triostat; King Pharmaceuticals), which was given as a bolus of 0.6 μg/kg at the start of ECMO support, followed by a continuous infusion at 0.2 μg/kg per hour. In groups supported with ECMO, pump flow (80 to 100 mL/kg per minute) was adjusted to maintain a mean arterial pressure of 40 to 50 mm Hg, and gas sweep through the oxygenator was adjusted to maintain arterial Po$_2$ >120 mm Hg and Pco$_2$ between 35 and 45 mm Hg. In the ECMO group, the ECMO pump was continued for a total of 8 hours. In the WEAN groups, the ECMO circuit was weaned over 30 minutes after 6 hours of support. Weaning success was determined based on the ability to keep MAP >45 and avoidance of acidosis. No inotropic medications were used and no fluid boluses for hypotension were given. Defibrillation was performed if necessary. At the end of the ^{13}C-labeled substrate infusion, the apex of the left ventricle was rapidly excised, freeze-clamped, weighed, and stored in liquid nitrogen for further analyses. Euthanasia was performed by using rapid exsanguination.

Infusion of Labeled Substrates

During the last hour of all conditions, [2-^{13}C]pyruvate and [^{13}C$_6$,^{15}N]ʟ-leucine were infused into the left anterior descending coronary arteries for a target intracoronary concentration of 7.4 and 3.7 mmol/L, respectively. Labeled substrates were obtained from Sigma-Aldrich. These target intracoronary concentrations were determined in accordance with the requirement amount of nuclear magnetic resonance (NMR) spectral analyses for myocardial extracts and based on prior metabolic experiments in piglets.[8]

Metabolic Analyses by NMR

^{13}C-NMR was performed on the myocardium for determination of specific carbon glutamate labeling. Our labeling scheme has previously been reported.[13,14] Freeze-clamped hearts were pulverized under liquid nitrogen, and the left ventricular extracts were prepared using perchloric acid. NMR spectra were acquired on a Varian Direct Drive (VNMRS) 600-MHz spectrometer (Varian Inc) equipped with a Dell Precision 390 Linux workstation running VNMRJ 2.2C. The spectrometer system was outfitted with a Varian triple resonance salt-tolerant cold probe with a cold carbon preamplifier. Protons were decoupled with a Waltz decoupling scheme. Final spectra were obtained using 45° excitation pulse (7.05 μs at 58 dB), with an acquisition time of 1.3 seconds, a recycle delay of 3 seconds, and a spectral width of 224.1 ppm. The labeled carbon resonances (C3–C5) of glutamate were integrated using commercial software (NUTS; Acorn NMR). tcaCALC determines the relative contribution of our labeled substrates to glutamate labeling (tcaCALC kindly provided by the Advanced Imaging Research Center at the University of Texas Southwestern Medical Center).[15] [2-^{13}C]Pyruvate does not label the C4 of glutamate, whereas uniformly labeled leucine results in labeling at the C4 position. This allowed determination of relative fractional contribution to glutamate labeling via acetyl-CoA between leucine and pyruvate. Representative NMR signals are reported previously.[13]

Metabolites from tissue samples were also analyzed using ^{1}H-NMR. One-dimensional proton nuclear Overhauser effect spectroscopy (NOESY) with presaturation was collected on each sample, using the Chenomx standard data collection protocol,[16] which consisted of a nonselective 90° excitation pulse (≈7 μs at 53 dB), a mixing time of 100 ms, an acquisition time of 4 seconds, a recycle delay of 1 second, a sweep width of 12 ppm, and a temperature control set to 25°C. Obtained spectra were analyzed using Chenomx software version 7.1 (Chenomx Inc), with quantifications based on spectral intensities relative to 0.5 mmol/L 2,2-dimethyl-2-silapentane-5-sulfonate, which was added as a spike to each sample. Amino acid concentrations and ATP were normalized to total creatine in each sample.

Metabolic Analysis by Gas Chromatography–Mass Spectrometry

An Agilent 6890N gas chromatograph equipped with an HP-5 column coupled to a 5973N mass spectrometer (Agilent Technologies) was used to measure the ^{13}C-enrichment and concentrations of CAC intermediates (citrate, alpha–ketoglutarate, succinate, fumarate, and malate), lactate, and pyruvate as described previously.[12,13]

Molar Percent Enrichment Calculation

Gas chromatography–mass spectrometry (GC-MS) data are reported as the total metabolite (labeled and unlabeled) as well as ^{13}C-molar percent enrichment (MPE). Mass isotopomers of metabolites containing 1 to n ^{13}C-labeled atoms were identified as Mi, with i=1, 2, . . . n. The total and absolute MPE of individual ^{13}C-labeled mass isotopomers (Mi) of a given metabolite was calculated as follows:

$$\text{total MPE} = \% \sum A_{Mi}/(A_M + \sum A_{Mi})$$

$$\text{absolute MPE (Mi)} = \% A_{Mi}/(A_M + \sum A_{Mi}),$$

where A_M and A_{Mi} represent the peak areas from ion chromatograms corrected for natural abundance, corresponding to the unlabeled (M) and ^{13}C-labeled (Mi) mass isotopomers, respectively. A detailed description for the calculations of flux ratios has also been published previously.[12,14] CAC intermediate enrichment was evaluated via the total MPE of several intermediates in the CAC from the ground tissue samples.

Flux Ratios

Mass isotopomers of metabolites containing 1 to n ^{13}C-labeled atoms were identified as Mi, with i=1, 2, . . . n. Pyruvate decarboxylation (PDC) is the pathway from pyruvate entering into the CAC via acetyl-CoA. PDC flux rate relative to citrate synthase (CS) was determined using the following formula:

$$\text{PDC/CS} = (\text{total MPE of citrate} - \text{total MPE of malate})/ \text{M1 of intracellular pyruvate}$$

where M1 is the ^{13}C-MPE in M1 isotopomers.

Triiodothyronine

Serum levels of porcine T$_3$ were analyzed with an ELISA (ERK S8013; Endocrine Technologies) according to the manufacturer's protocol.

Statistical Analysis

Values are reported as mean±SE in the text, figures, and tables. Data were analyzed for significant variance with the Mann–Whitney test for comparisons between groups. Additionally, ANOVA for repeated measures was used where appropriate (eg, time-related changes of myocardial oxygen consumption and serum T$_3$ concentration). Significance was defined as $P<0.05$ for all comparisons. Error bars in graphs indicate SE.

Results

Cardiac Function and Thyroid Hormone During ECMO

We performed preliminary experiments to validate our model of cardiac injury. Coronary occlusion for 30 and 60 minutes produced severe and unrecoverable hemodynamic instability that could not be rescued by ECMO. However, ischemia for 10 minutes caused moderate systolic and severe diastolic dysfunction, resulting in the death of 4 of 10 piglets during or shortly after reperfusion. The 6 survivors (Table 1) showed mild to moderate systolic dysfunction with most parameters reduced to 70% to 80% of baseline. Injury also produced a dramatic increase in left ventricular end-diastolic pressure but did not affect systemic vascular resistance.

Table 1. Hemodynamic Variables

	IR	WEAN	WEAN+T$_3$
SBP	86±12	86±3	97±7
LVSP	86±11	88±5	109±4 *
LVEDP	298±84	129±16	133±21
Dp/Dt max	85±11	118±11	121±11
Dp/Dt min	64±11	73±5	91±14
Developed pressure	75±11	85±6	107±4 *
CO	85±8	76±7	88±3
Power	82±14	60±4	81±6 *
SVR	98±16	101±17	100±14
MV$_{0_2}$	98±13	101±13	92±20

Each value is represented as percent change at final measurement compared with baseline±SE. Statistic comparison between IR or WEAN and WEAN+T$_3$ was not performed as there is clear survivor bias in groups WEAN and WEAN+T$_3$. n=6 in IR and 5 in WEAN and WEAN+T$_3$. IR indicates ischemia-reperfusion; SBP, systolic blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; Dp/Dt max/min, maximum and minimum first derivative; CO, cardiac output; SVR, systemic vascular resistance; MV$_{0_2}$, myocardial oxygen consumption.
*$P<0.05$ between WEAN and WEAN+T$_3$ and is graphically represented in Figure 2.

Weaning after ECMO support (WEAN) was successful in 6 of 7 piglets. However, both systolic and diastolic functions remained depressed compared with baseline. ECMO produced a significant decrease in T$_3$ levels at 1, 4, and 8 hours (35% baseline at 8 hours), and our dosing regimen maintained levels near baseline (75% baseline) as demonstrated in Figure 2. ECMO with thyroid supplementation (WEAN+T$_3$) also allowed weaning in 6 of 7 piglets. Furthermore, piglets in the T$_3$ group achieved superior left ventricular systolic parameters compared with WEAN (Figure 3). Specifically, T$_3$ significantly increased left ventricular systolic and diastolic pressure and power, while other parameters showed a trend upward. No arrhythmias were seen with T$_3$ infusion.

Consistent with prior studies, ECMO reduced MVo$_2$ in both groups by ≈40%. Weaning from ECMO abruptly increased MVo$_2$ in both groups. After weaning, MVo$_2$ levels were equal to baseline, even though systolic performance was superior in the WEAN+T$_3$ group.

Mitochondrial Oxidative Capacity

Ischemia reduced ATP in all groups compared with CON (Figure 4A) as determined by ^{1}H-NMR. Additionally, the ratio of phosphocreatine to adenosine triphosphate (PCr/ATP) was analyzed as a surrogate for mitochondrial oxidative capacity (Figure 4B). IR significantly decreased the PCr/ATP by >50% compared with CON. ECMO after injury returned the PCr/ATP to near CON levels, yet this effect was lost during weaning.
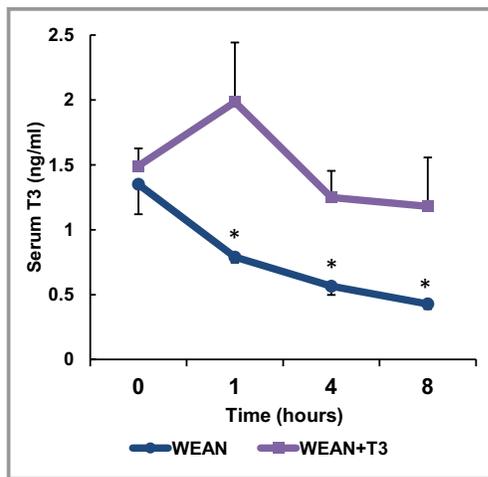

Figure 2. Serum T$_3$ following initiation of ECMO. ECMO significantly depressed T$_3$ levels within 1 hour of ECMO initiation and continued to fall over the course of support. Our supplementation protocol (WEAN+T$_3$) was able to maintain T$_3$ levels similar to baseline (all points nonsignificant vs baseline). n=5 for both groups. *P<0.05 vs baseline.

However, compared with WEAN, WEAN+T$_3$ significantly increased the PCr/ATP to levels similar to CON and ECMO.

Substrate Oxidation

Absolute concentrations of lactate and pyruvate were determined by using GC-MS (Figure 5A and 5B). All models of injury produced substantial pyruvate and lactate accumulation compared with CON. Support with ECMO after injury decreased accumulation of pyruvate and lactate. However WEAN caused reaccumulation of these metabolites. T$_3$ supplementation (WEAN+T$_3$) resulted in significantly decreased accumulation of pyruvate and lactate compared with WEAN.

Figure 6A shows the fractional substrate contribution to acetyl-CoA by ^{13}C-NMR from left ventricular tissues at the end of the protocol. Due to technical issues, NMR data was available from only 24 of 30 samples. Cardiac injury (IR) significantly decreased ^{13}C-labeled pyruvate contribution to acetyl-CoA compared with CON. ECMO significantly increased the pyruvate fractional contribution and decreased contribution for unlabeled substrates compared with IR. WEAN followed a similar pattern as IR, whereas WEAN+T$_3$ increased the fractional contribution of pyruvate compared with WEAN. NMR also provided estimate for oxidation of the branch chain amino acid leucine, which did not differ among the protocols.

GC-MS MPE data allowed estimation of total PDC relative to CS flux (Figure 6B) and are complementary to NMR data, which only evaluate contribution according to label. Ischemia substantially lowered the PDC/CS ratio compared with CON, while support with ECMO returned the ratio to near CON levels. WEAN also markedly reduced this ratio. Importantly, T$_3$ supplementation returned the PDC/CS ratio to control levels.

CAC Intermediates and MPE

Absolute CAC intermediate concentrations are shown in Figure 7A. IR did not alter concentrations for the first CAC span intermediates, citrate and alpha-ketoglutarate (αKG), but did substantially increase succinate, fumarate, and malate, reflecting the distal span. ECMO did substantially increase citrate and αKG, while reversing the injury-promoted increase for the distal span intermediates. For the most part, these changes were reversed in the opposite direction with weaning. However, T$_3$ supplementation ameliorated the increases for the distal span intermediates caused by the wean.

Figure 7B represents the MPE of substrates. We observed significant difference in the MPE of 6-carbon and 5-carbon intermediates (citrate and α-KG), whereas the 4-carbon intermediates (succinate, fumarate, and malate) did not show significant difference between protocols. IR produced slightly
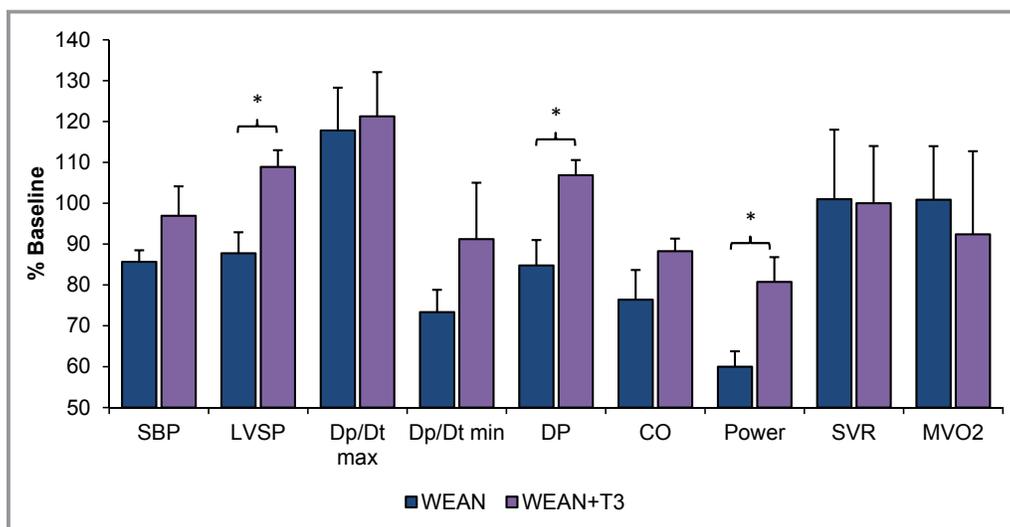
Figure 3. Hemodynamic performance following ECMO weaning. Each variable is presented as a percentage of baseline function before ischemia. Values are means±SE. n=5 in each group. *$P<0.05$ WEAN+T$_3$ vs WEAN. CO indicates cardiac output; DP, developed pressure; Dp/Dt max/min, maximum and minimum first derivative; ECMO, extracorporeal membrane oxygenation; LVSP, left ventricular systolic pressure; MVo$_2$, myocardial oxygen consumption; SBP, systolic blood pressure; SVR, systemic vascular resistance.

higher MPE in the distal span 4-carbon intermediates fumarate and malate compared with citrate and αKG, a pattern also found in WEAN but not seen in ECMO or WEAN+T$_3$.

Amino Acid Concentrations

Table 2 shows ^{1}H-NMR–derived estimates for amino acid concentrations relative to total creatine. All models of ischemic injury produced substantial decreases in aspartate and glutamate compared with CON. No other significant changes occurred in amino acid concentrations in these protocols.

Discussion

We explored the links between myocardial metabolism and contractile function after IR and subsequent support by ECMO. The pathogenesis for acute cardiac failure requiring rescue by mechanical circulatory support in infants and children varies substantially among the various clinical scenarios. IR and reoxygenation injury presumably play important roles in development of contractile dysfunction after cardiac surgery, the most common indication for ECMO in this age group. However, other factors such as inadequate myocardial protection, residual hemodynamic defects, and/or trauma caused by ventriculotomy also likely contribute.[17,18] Accordingly, we used a classic form of IR insult created by coronary occlusion and release, while recognizing the potential limitations of this model's applicability to all

pediatric patients requiring ECMO for cardiac failure. Nevertheless, the model's unique characteristics yielded substantial divergence from other experimental swine preparations, which were intended to examine pathophysiological conditions prevalent in human adults with coronary artery disease.[19–21] We observed IR-mediated metabolic abnormalities, which were partially ameliorated by ECMO but then exacerbated during reloading. Furthermore, T$_3$ supplementation partially reversed this exacerbation and improved contractile function.

Ischemia-Reperfusion

This injury mode induced myocardial stunning in all animals, exemplified by high mortality if ECMO support was not initiated, and moderate contractile dysfunction in all survivors. IR modified key metabolic parameters, when compared with previously published data from control piglet hearts treated with similar protocols but without preceding insult.[13,22–24] Importantly, IR reduced ATP and PCr/ATP ratio (≈50%). This ratio provides a surrogate for cytosolic phosphorylation potential and changes inversely in relation to cytosolic (ADP). In myocardium in vivo, the PCr/ATP falls when ATP demand nears or exceeds the maximal ATP production rate such as occurs with limitations in oxygen or substrate supply at a relatively high workload. We previously showed that rapid PCr depletion and repletion occur respectively during IR in immature swine heart in vivo.[22,25] The creatine kinase equilibrium shifts, favoring net flux toward ATP, and therefore
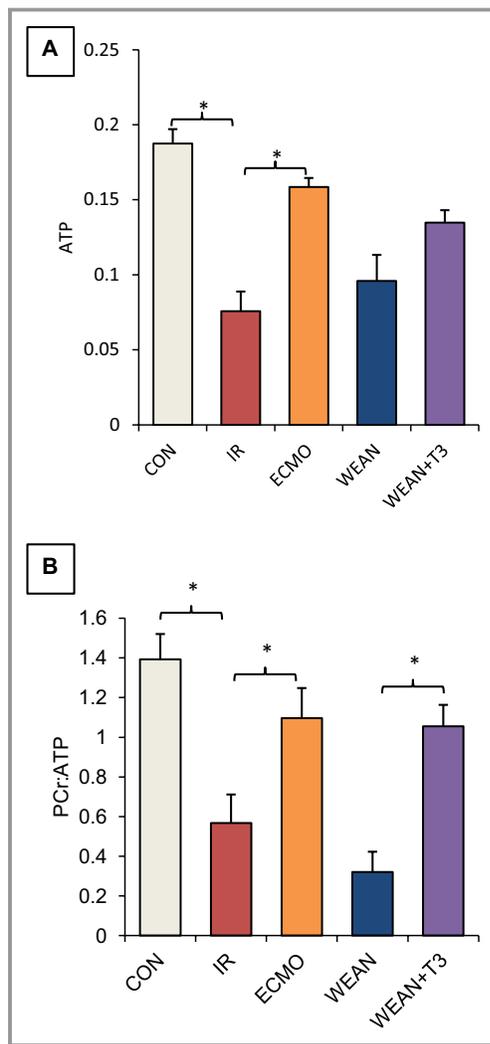
Figure 4. A, ATP concentration (normalized to total creatine) is reduced in all models of ischemia. B, Phosphocreatine to ATP ratio by ^{1}H-NMR. The ratio of PCr to ATP provides an assessment of cytosolic phosphorylation potential and mitochondrial oxidative capacity. n=6 in CON, IR, and ECMO and n=5 in WEAN and WEAN+T₃. *P<0.05. ECMO indicates extracorporeal membrane oxygenation; IR, ischemia-reperfusion; NMR, nuclear magnetic resonance; PCr, phosphocreatine.

Figure 5. Absolute quantity of lactate (A) and pyruvate (B) by GC-MS. Compared with CON, ischemia increased absolute concentrations of both lactate and pyruvate in all protocols. Following ischemia, concentrations of lactate and pyruvate were lowest in protocols ECMO and WEAN+T₃. n=6 in CON, IR and ECMO, and n=5 in WEAN and WEAN+T₃. *P<0.05. ECMO indicates extracorporeal membrane oxygenation; GC-MS, gas chromatography–mass spectroscopy; IR, ischemia-reperfusion.

favors preservation of the ATP pool over PCr during these conditions. Thus, caution should be used when interpreting studies, which depend only on adenine nucleotides to determine the high-energy phosphate state. Several investigators have used the PCr repletion rate as an index of mitochondrial respiratory function.[26,27] Although we did not dynamically monitor PCr through our protocol, the incomplete ATP and PCr replenishment, following several hours of reperfusion and ECMO, indicates impaired mitochondrial oxidative capacity. This may reflect reduced efficiency for oxidative phosphorylation, increased uncoupling, or both.
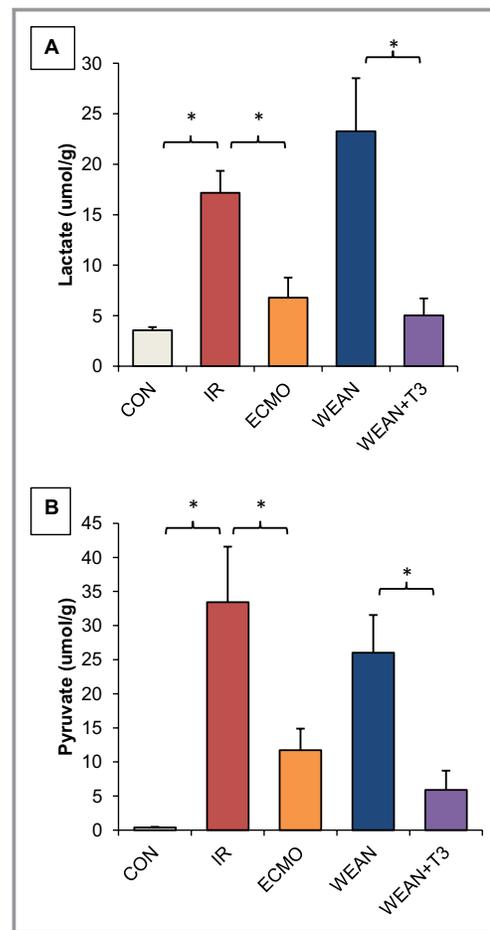
Along with the impaired mitochondrial respiration, we observed that IR promoted perturbations in pyruvate flux. IR expanded the intracellular pyruvate and lactate pools without altering MPE for these metabolites, indicating that this expansion occurs from equivalently proportional increases in ^{13}C-labeled pyruvate uptake and production from other nonlabeled glycolytic sources. Despite the marked increases in pyruvate and lactate production with IR, our GC-MS and NMR data show that IR reduced PDC relative to CAC flux, in agreement with prior studies.[8] Thus, IR under these experimental conditions appears to shift pyruvate from decarboxylation and toward anaerobic glycolysis. Our previous study, performed in immature pig hearts subjected to
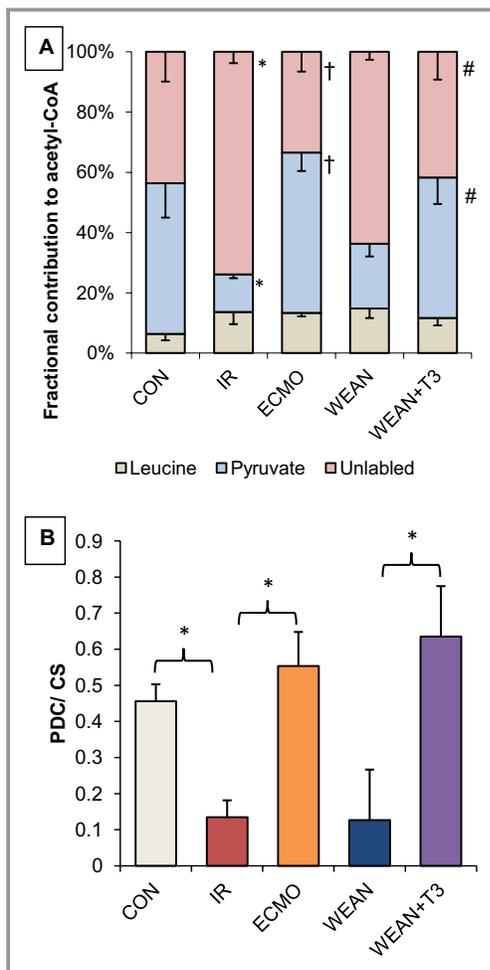
Figure 6. Pyruvate decarboxylation. A, Fractional substrate contribution to acetyl-CoA determined by ^{13}C-NMR. Both ECMO and WEAN+T$_3$ demonstrate pyruvate contribution similar to CON, while IR and WEAN show significantly decreased pyruvate contribution. Leucine contribution to the CAC under our experimental protocols did not change. n=4 in IR and WEAN, n=5 in CON and WEAN+T$_3$, and n=6 in ECMO *$P<0.05$ CON compared to IR. $^†P<0.05$ ECMO compared to IR. $^\#P<0.05$ WEAN+T$_3$ compared to WEAN. B, Pyruvate decarboxylation relative to citrate synthetase (PDC/CS) flux as determined by GC-MS. Both ECMO and WEAN+T$_3$ are similar to CON, while IR and WEAN show significantly reduced PDC/CS flux. n=6 in CON, IR and ECMO, and n=5 in WEAN and WEAN+T$_3$. *$P<0.05$. CAC indicates citric acid cycle; ECMO, extracorporeal membrane oxygenation; GC-MS, gas chromatography–mass spectroscopy; IR, ischemia-reperfusion; NMR, nuclear magnetic resonance.

ischemia under hypothermic conditions and then reperfused, showed that IR maintained absolute CAC intermediate concentrations but reduced their ^{13}C-MPE.[28] Those data corresponded to the measured reduction in ^{13}C labeling of the

intermediates through PDC and to a lesser but proportional decrease in pyruvate carboxylation. In the current investigation, the fairly severe IR injury similarly affected citrate and αKG but, in contrast to the prior study, expanded the pools for the distal CAC intermediates succinate, fumarate, and malate. Preservation for the succinate MPE suggests that IR accumulation for this metabolite occurs primarily through forward flux from αKG with partial inhibition of the succinate dehydrogenase within mitochondrial complex II. Succinate at high concentration might also readjust kinetic control of the dehydrogenase, partially releasing inhibition, and produce fumarate and malate at a higher concentration. Alternatively, IR under these altered conditions could promote anaplerotic pyruvate carboxylation through its carboxylase to oxaloacetate or via malic enzyme to malate.[19,29,30] These intermediates would then supply fumarate and even malate through reverse flux mechanisms along the CAC, which have been previously documented in heart.[31,32] Our isotopomer strategy, involving uniformly ^{13}C-labeled leucine for estimation of amino acid oxidation, precluded more specific analyses of flux through pyruvate carboxylation. However, our data show that the fumarate and malate MPE increase in comparison to citrate and αKG along with expansion in the pools for these distal span metabolites. This pattern, which does not occur with ECMO or T$_3$, suggests that this expansion occurs at least in part through anaplerotic entry of pyruvate. Although we could not directly measure CAC flux in these studies, this labeling pattern corresponds to that seen with reductions in forward flux through succinate dehydrogenase caused by either ischemia or hypoxia. Thus, these CAC absolute concentration and MPE data, along with inability to maintain high-energy phosphate stores, suggest that citric acid cycle flux is impaired after ischemia and during reloading and is partially ameliorated by T$_3$ supplementation.

The labeling strategy did affectively show that branch chain amino acid oxidation does not change in response to inhibition of PDC. However, we detected IR-induced reductions in concentrations for some amino acids, which participate in shuttling of NADH and NADPH reducing equivalents into the mitochondria. In particular, we noted decreases in concentrations for aspartate and glutamate, important components of the malate–aspartate shuttle. Studies in isolated perfused heart identified altered metabolite exchange between the cytosolic and mitochondrial compartments after IR.[33] The αKG–malate transporter, which operates either independently or in tandem with the unidirectional glutamate–aspartate shuttle, competes with αKG dehydrogenase for exchange of carbon units between the compartments and is particularly sensitive to cellular redox state after reperfusion.[33] The pyruvate/lactate ratio, a surrogate for cellular redox state, increases substantially with IR in our experiments. Thus, our data imply that shifts in pyruvate flux modulate redox state
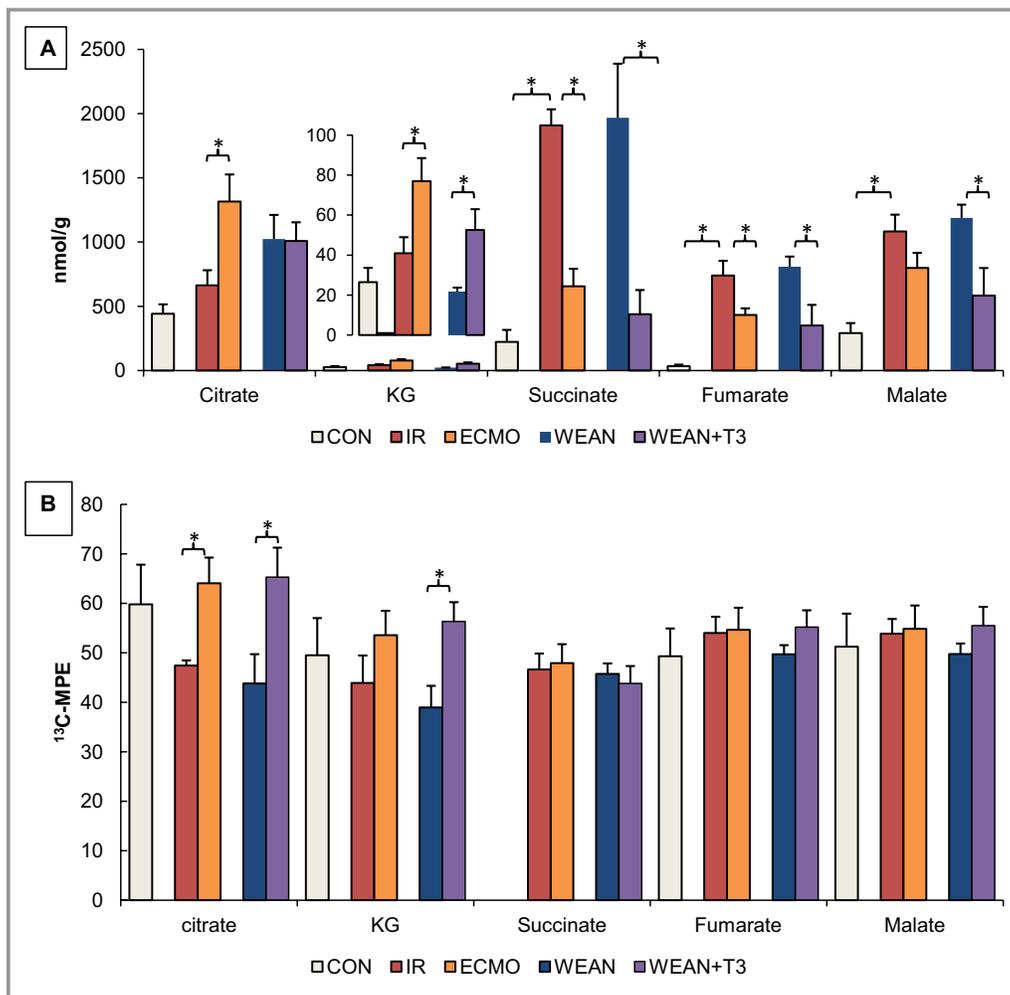
Figure 7. Concentrations (A) and their ^{13}C-molar percent enrichment (B) of CAC intermediates by GC-MS. Succinate MPE was not obtained for CON. n=6 in CON, IR and ECMO, and n=5 in WEAN and WEAN+T$_3$. *$P<0.05$ CON compared with IR. CAC indicates citric acid cycle; ECMO, extracorporeal membrane oxygenation; GC-MS, gas chromatography–mass spectroscopy; IR, ischemia-reperfusion; KG, alpha-ketogluterate; MPE, molar percent enrichment.

and influence concentrations of these amino acids. Potentially, reductions in NADH shuttling could limit substrate supply to the CAC and explain some of the mitochondrial respiratory impairments caused by IR. As these studies were performed in vivo, limitation in NADH provision to the CAC would occur only if fatty acid oxidation also incurred impairments. We previously showed that successful weaning from ECMO requires enhanced rates of fatty acid oxidation.[23] Although the noninjured heart maintains the capacity to increase fatty acid oxidation during ECMO, we have not specifically studied this issue after IR. However, rates of fatty acid oxidation are reduced in other heart models that use IR.[21]

ECMO and Reloading

Importantly, we found that the various conditions within our protocols affected, and in some cases reversed the IR-initiated metabolic perturbations. The operative mechanisms affording recovery of contractile function by ECMO have not been well defined. We show for the first time that ECMO restores the high-energy phosphate state toward preischemic values. Thus, the reduction in cardiac work lowers energy requirements, thereby raising the oxygen supply/demand ratio and permitting PCr pool restoration through oxidative phosphorylation.[27] These findings imply that the mitochondrial ATP synthesis operates near maximal capacity, as weaning with the associated elevated energy requirement disrupts the PCr/ATP. The metabolic impairments, including the reduction in relative PDC rate and the succinate accumulation, recur with reloading. Thus, these data suggest that abnormalities in acetyl-CoA supply to the mitochondria and cycling of the intermediates limit oxidative phosphorylation and impair weaning, evidenced by poor contractile function in these piglets. Although the mechanical

Table 2. Amino Acid Concentrations (Normalized to Total Creatine Concentration) From ^{1}H-NMR

	CON	IR	ECMO	WEAN	WEAN+T$_3$
Alanine	0.212	0.278	0.177	0.249	0.213
Aspartate	0.073	0.011*	0.015*	0.006*	0.013*
Glutamate	0.287	0.126*	0.138*	0.075*	0.157*
Glutamine	0.520	0.543	0.542	0.543	0.580
Glycine	0.139	0.101	0.123	0.113	0.169
Isoleucine	0.008	0.009	0.005	0.006	0.010
Leucine	0.014	0.017	0.011	0.013	0.017
Phenylalanine	0.005	0.005	0.006	0.006	0.009
Tyrosine	0.005	0.005	0.006	0.006	0.009
Valine	0.014	0.016	0.011	0.013	0.019

NMR indicates nuclear magnetic resonance; CON, control; IR, ischemia-reperfusion; ECMO, extracorporeal membrane oxygenation.
*$P<0.05$ compared with CON. n=6 in each group.

circuit lowers cardiac energy requirements, ECMO simultaneously initiates an inflammatory response, which in prior studies reduced metabolic flexibility by increasing reliance on fatty acid oxidation.[13] The mechanism for this shift requires further elucidation, but results from several studies implicate disturbances in endocrine regulation of substrate metabolism.

Triiodothyronine Supplementation

In the current study, we showed that mechanical circulation markedly depresses circulating thyroid hormone levels. This finding conforms to data from multiple previous studies in both animals and humans investigating cardiopulmonary bypass. These disturbances in thyroid hormone homeostasis have been closely related to the cytokine surge induced by the extracorporeal circuit.[7] In our current study, maintenance of thyroid hormone levels through supplementation reversed many of the metabolic impairments, which recurred during weaning. Of note, our supplementation strategy did not promote a hyperthyroid state but maintained T$_3$ levels near basal state. Thyroid hormone ubiquitously regulates the cardiovascular system through both transcriptional and posttranscriptional mechanisms.[9] Therefore, difficulty ensues when trying to link a specific metabolic action to contractile response. Furthermore, T$_3$ action on substrate metabolism varies according to baseline thyroid state.[9,34] However, the time course of our experiments suggests that T$_3$ rapidly acts predominantly through direct or nongenomic actions. Several prior studies showed that stimulation of flux through pyruvate dehydrogenase via either thyroid hormone action or other means apparently improves cardiac function after IR.[12,35] In general, those studies evaluated contractile function immediately after IR. Similarly, we previously showed that T$_3$ supplementation improved overall pyruvate flux into the CAC and contractile function immediately after reperfusion

following cardiopulmonary bypass and hypothermic aortic cross-clamping.[8] In these ECMO experiments, we showed that T$_3$ supplementation reverses many of the metabolic abnormalities leading to and within the CAC caused by reloading over a longer time course. Consistent with prior studies performed in vivo during a hypothyroid state, T$_3$ also directly increased PCr/ATP ratio without increasing MVo$_2$.[10] Our data suggest that this improvement in high-energy phosphate kinetics relates to substrate switching, or at least enhancement of more energy efficient carbohydrate oxidation following IR impairment of mitochondrial function.

Limitations

Supraphysiological concentrations of ^{13}C-labeled pyruvate and leucine were used in the coronary artery in these experiments. Similar concentrations have been used previously to delineate PDC in vivo by several investigators including ourselves.[8,12] Use of these high concentrations may be perceived as an experimental limitation but is required to achieve adequate ^{13}C metabolite enrichment for detection by NMR and GC-MS in these studies performed in pigs in vivo. We also acknowledge that the reported metabolic shifts may or may not be evident at physiological concentrations of either infused agent. The metabolic rearrangement caused by these protocols suggests but does not prove that CAC activity is defective. Indeed, the data could reflect alterations in equilibrium state with normal CAC flux.

Conclusions

Our study highlights metabolic abnormalities or shifts caused by IR in a translational model emulating ECMO after IR injury in the immature heart. We noted disruption of flux into and

through the citric acid cycle in conjunction with reduction in PCr/ATP ratio. The metabolic parameters closely related to contractile function. Although myocardial rest by ECMO restored these values towards normal, the left ventricle could not sustain their recovery or return contractile function to normal when challenged with weaning from mechanical support. However, thyroid hormone supplementation allowed superior functional recovery with near full restoration of the metabolic parameters. A large placebo controlled trial in infants and children undergoing cardiopulmonary bypass showed safety for similar thyroid hormone supplementation, while post-hoc analyses suggested efficacy in improvement of clinical outcome parameters for some age groups.[4] The data in this study reinforce the importance of thyroid hormone supplementation and its potential in enhancing weaning from ECMO. Clinical trials to determine efficacy for T$_3$ supplementation during ECMO for cardiac support in infants and children may be indicated based on the overall data from human and animal studies.

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Disclosures

None.

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