Cardiac hypertrophy in the newborn delays the maturation of fatty acid β-oxidation and compromises posts ischemic functional recovery


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Oka T, Lam VH, Zhang L, Keung W, Cadete VJ, Samokhvalov V, Tanner BA, Beker DL, Ussher JR, Huqi A, Jaswal JS, Rebeyska IM, Lopaschuk GD. Cardiac hypertrophy in the newborn delays the maturation of fatty acid β-oxidation and compromises posts ischemic functional recovery. Am J Physiol Heart Circ Physiol 302: H1784–H1794, 2012.—During the neonatal period, cardiac energy metabolism progresses from a fetal glycolytic profile towards one more dependent on mitochondrial oxidative metabolism. In this study, we identified the effects of cardiac hypertrophy on neonatal cardiac metabolic maturation and its impact on neonatal posts ischemic functional recovery. Seven-day-old rabbits were subjected to either a sham or a surgical procedure to induce a left-to-right shunt via an aortocaval fistula to cause RV volume-overload. At 3 wk of age, hearts were isolated from both groups and perfused as isolated, biventricular preparations to assess cardiac energy metabolism. Volume-overload resulted in cardiac hypertrophy (16% increase in cardiac mass, P < 0.05) without evidence of cardiac dysfunction in vivo or in vitro. Fatty acid oxidation rates were 60% lower (P < 0.05) in hypertrophied hearts than controls, whereas glycolysis increased 246% (P < 0.05). In contrast, glucose and lactate oxidation rates were unchanged. Overall ATP production rates were significantly lower in hypertrophied hearts, resulting in increased AMP-to-ATP ratios in both aerobic hearts and ischemia-reperfused hearts. The lowered energy generation of hypertrophied hearts depressed functional recovery from ischemia. Decreased fatty acid oxidation rates were accompanied by increased malonyl-CoA levels due to decreased malonyl-CoA decarboxylase activity/expression. Increased glycolysis in hypertrophied hearts was accompanied by a significant increase in hypoxia-inducible factor-1α expression, a key transcriptional regulator of glycolysis. Cardiac hypertrophy in the neonatal heart results in a reemergence of the fetal metabolic profile, which compromises ATP production in the rapidly maturing heart and impairs recovery of function following ischemia.

fatty acid oxidation; glucose oxidation; glycolysis; neonatal heart; hypertrophy

ONE OF EVERY 100 CHILDREN is born with a congenital heart defect. Cardiac hypertrophy can occur secondary to volume/pressure overload due to defects such as ventricular septal defects, atrial septal defects, or a patent ductus arteriosus representing 29, 21, and 10%, respectively, of all congenital heart diseases (31). These defects often undergo surgical repair that requires a motionless and bloodless field achieved by arresting the heart, which subjects the heart to a period of ischemia. Maturation of energy metabolism in the newborn heart is not complete until after birth. Therefore the response of the neonatal heart to ischemia may be different from the adult heart. Adding to that, hypertrophy may alter the normal course of metabolic maturation in the neonatal heart via changes in gene and protein expression. Thus, cardiac hypertrophy-related changes in cardiac energy metabolism in the neonatal heart may also impact the heart’s tolerance to ischemia.

Shortly after birth, the heart rapidly develops the ability to metabolize fatty acids, while dramatically decreasing glycolytic rates (14, 24, 25). This metabolic transition occurs in many mammals, including rabbits and pigs. In 1-day-old rabbit hearts, glycolysis contributes to nearly one-half of the total cardiac ATP production. By 7 days old, glycolysis contributes <10% of the heart’s energy requirements (19, 26). In contrast, in 1-day-old rabbit hearts, fatty acid oxidation provides <10% of total ATP production; by 7 days of age, it provides >50% (17, 30). Decreased glycolysis following birth partly occurs by allosteric inhibition of upstream regulators due to increased fatty acid oxidation (5, 25, 26, 43). Transcriptionally, decreased hypoxia-inducible factor-1α (HIF-1α) expression may also contribute to decreased glycolysis. HIF-1α regulates expression of genes that favor anaerobic glycolysis. During normal development, HIF-1α mRNA/protein levels decrease (33), resulting in decreased glycolytic enzyme activities (5, 25, 26). Simultaneously, increased acetyl-CoA from fatty acid oxidation inhibits pyruvate dehydrogenase (PDH), the first committed enzymatic step in glucose oxidation. Cardiac glucose oxidation rates remain low following birth due to low glycolysis and PDH activity (18, 30, 34).

In the adult heart, cardiac hypertrophy is associated with a reversion to a fetal metabolic profile (9, 35) causing decreased fatty acid oxidation (1, 12) and increased glycolytic rates (1, 40, 46). These metabolic changes correlate to altered expression/activity of key regulatory enzymes of energy substrate metabolism (2, 13, 21, 44, 45). While adult cardiac hypertrophy results in a fetal-like metabolic phenotype (38), the effect(s) of hypertrophy on the neonatal heart, in the midst of a fetal-to-adult transition, is unclear. We previously showed that newborn cardiac hypertrophy in pig hearts delays the maturation of key fatty acid oxidation enzymes (20). However, the effects of hypertrophy on energy metabolic rates in the neonatal heart have not been directly determined. Because energy substrate preference can have profound impact on ischemic tolerance (7, 42), it is important to understand the effect(s) of cardiac hypertrophy on energy substrate metabolism in the neonatal heart.

To examine energy metabolism in hypertrophied neonatal hearts, we developed a volume-overload hypertrophy model in

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neonatal rabbits. The model produces biventricular cardiac hypertrophy in 21-day-old rabbits, which is relevant to ventricular septal defects, atrial septal defects, and patent ductus arteriosus pathologies. Because systolic pressure in the right ventricle (RV) remains high in the perinatal period and decreases immediately after birth (13), our biventricular model is particularly valid in correctly capturing the metabolic and functional changes in both ventricles that may occur during the development of hypertrophy. Using a biventricular working heart preparation to measure cardiac function and metabolism, we show that hypertrophied neonatal heart metabolism retains a fetal profile causing a severe decrease in overall cardiac ATP production that compromises postischemic functional recovery.

**MATERIALS AND METHODS**

**Animals**

All animal procedures were approved by the University of Alberta Health Sciences Animal Welfare and Policy Committee, which adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and complies with Canadian Council of Animal Care guidelines.

**Induction of Volume-Overload Via Production of an Aortocaval Fistula in Newborn Rabbits**

Seven-day-old New Zealand White rabbits of either sex (90–200 g) were anesthetized with isoflurane (2%) via inhalation. Animals were placed on their right lateral, and a left flank retroperitoneal incision was made to expose the descending aorta and the inferior vena cava. The descending aorta was clamped with a microclamp 5 mm inferior to the renal blood vessels to cease downstream blood flow. The aorta was punctured with a 20-gauge needle tip below the microclamp, and a through-and-through fistula was generated across the aorta to reach the renal blood vessels to cease downstream blood flow. The aorta was then removed, and a drop of cyanoacrylic glue was placed on the puncture site and allowed to dry, following which the microclamp was fixed. The descending aorta was clamped with a microclamp 5 mm inferior to the renal blood vessels to cease downstream blood flow. The aorta was punctured with a 20-gauge needle tip below the microclamp, and a through-and-through fistula was generated across the aorta to reach the renal blood vessels to cease downstream blood flow. The aorta was then removed, and a drop of cyanoacrylic glue was placed on the puncture site and allowed to dry, following which the microclamp was fixed. The descending aorta was clamped with a microclamp 5 mm inferior to the renal blood vessels to cease downstream blood flow. The aorta was punctured with a 20-gauge needle tip below the microclamp, and a through-and-through fistula was generated across the aorta to reach the renal blood vessels to cease downstream blood flow. The aorta was then removed, and a drop of cyanoacrylic glue was placed on the puncture site and allowed to dry, following which the microclamp was fixed. The descending aorta was clamped with a microclamp 5 mm inferior to the renal blood vessels to cease downstream blood flow. The aorta was punctured with a 20-gauge needle tip below the microclamp, and a through-and-through fistula was generated across the aorta to reach the renal blood vessels to cease downstream blood flow. The aorta was then removed, and a drop of cyanoacrylic glue was placed on the puncture site and allowed to dry, following which the microclamp was fixed.

**Echocardiography**

Transthoracic echocardiographic assessment was performed using a two-dimensional/Doppler echocardiographic system (Acuson Sequoia 512; Siemens Medical Solutions, Mountain View, CA) using a 15-MHz probe on the day of and 7 and 13 days after surgery. The left ventricular (LV) end-diastolic and systolic dimension, the RV end-diastolic and systolic area, heart rate, and shunt flow between the ventricular (LV) end-diastolic and systolic dimension, the RV end-diastolic and systolic area, heart rate, and shunt flow between the ventricular (LV) end-diastolic and systolic dimension, the RV end-diastolic and systolic area, heart rate, and shunt flow between the ventricular (LV) end-diastolic and systolic dimension, the RV end-diastolic and systolic area, heart rate, and shunt flow between the ventricular (LV) end-diastolic and systolic dimension, the RV end-diastolic and systolic area, heart rate, and shunt flow between the ventricular (LV) end-diastolic and systolic dimension, the RV end-diastolic and systolic area, heart rate, and shunt flow between the ventricular (LV) end-diastolic and systolic dimension.

**Isolated Biventricular Heart Perfusion Model**

At 14 days postsurgery, 21-day-old rabbits were anesthetized with pentobarbital sodium (60 mg/kg body wt), and hearts were excised and cannulated and retrogradely (Langendorff) perfused with Krebs-Henseleit buffer (37°C, pH=7.4, gassed with 95% O2-5% CO2 mixture). During 15 min of retrograde perfusion, the superior vena cava (SVC), the left atria, and pulmonary artery were cannulated, and the inferior vena cava was ligated. LV work was initiated at the end of the 15-min retrograde perfusion by opening flow into the left atria and aortic afterload line and terminating retrograde perfusion. RV flow was added by opening SVC flow, thereby producing a biventricular working heart preparation. Hearts were subjected to 30 min LV perfusion (clamped SVC cannula) followed by 30 min biventricular perfusion (open SVC cannula). Hearts were perfused with modified Krebs-Henseleit solution containing 2.5 mM Ca2+, 5.5 mM glucose, 0.8 mM palmitate prebound to 3% BSA, 0.5 mM lactate, and 100 μM insulin. The preload for the left and right atria was set at 12.5 and 7.5 mmHg, respectively. LV afterload was set at 35 mmHg and RV afterload at 4.5 mmHg. In some studies, hearts were subjected to an initial 15-min LV working heart perfusion followed by 20 min of biventricular perfusion and a 25-min period of isothermal, no-flow global ischemia followed by 30 min of biventricular aerobic reperfusion. The Krebs-Henseleit solution used to perfuse hearts subjected to ischemia-reperfusion was similar to the solution described above; however, it contained 1.2 mM palmitate. At the end of the perfusion protocols, LV, RV, and septal tissues were separated and rapidly frozen in liquid N2 and stored at −80°C for further biochemical analysis. Glycolysis, glucose oxidation, lactate oxidation, and palmitate oxidation were measured as described (3, 27). ATP production rates from glycolysis, glucose oxidation, lactate oxidation, and palmitate oxidation were calculated using the values of 2 mol ATP/mol of glucose passing through glycolysis, 31 mol ATP/mol of glucose oxidized, 15 mol ATP/mol of lactate oxidized, and 105 mol ATP/mol of palmitate oxidized, respectively. These numbers are derived based on using P-to-O ratios of 2.5 for NADH and 1.5 for FADH2 (15, 37).

**Mechanical Measurements in Isolated Biventricular Working Hearts**

Heart rate and systemic and diastolic pressures were measured using a Gould P21 pressure transducer attached to the aortic and pulmonary outflow line. Left and right cardiac output and aortic and pulmonary flow were measured by using ultrasonic flow probes (Transonic T206) placed in the left atrial and SVC inflow lines and aortic and pulmonary outflow lines, respectively. LV and RV work were calculated as cardiac output × peak systolic pressure × 10−2 and served as a continuous index of left and RV mechanical function.

**Table 1. Physical and cardiac parameters in 20-day-old control and hypertrophy rabbits**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Hypertrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>360 ± 18</td>
<td>338 ± 17</td>
</tr>
<tr>
<td>Heart wt, g</td>
<td>2.06 ± 0.12</td>
<td>2.39 ± 0.11</td>
</tr>
<tr>
<td>Heart wt/body wt, mg/g</td>
<td>5.59 ± 0.37</td>
<td>6.64 ± 0.52*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>316 ± 6</td>
<td>305 ± 5*</td>
</tr>
<tr>
<td>Left ventricle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>8.6 ± 0.1</td>
<td>9.7 ± 0.2*</td>
</tr>
<tr>
<td>LV vol/d, mm3</td>
<td>406 ± 17</td>
<td>58 ± 20*</td>
</tr>
<tr>
<td>Stroke volume, mm3</td>
<td>234 ± 11</td>
<td>323 ± 10*</td>
</tr>
<tr>
<td>EF, %</td>
<td>57.6 ± 1.1</td>
<td>603.4 ± 1.3</td>
</tr>
<tr>
<td>LV vol/body wt</td>
<td>1.17 ± 0.05</td>
<td>1.62 ± 0.11*</td>
</tr>
<tr>
<td>Right ventricle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV area/diameter, mm3</td>
<td>34.4 ± 6.8</td>
<td>46.9 ± 2.9*</td>
</tr>
<tr>
<td>RV, %FAC</td>
<td>39.2 ± 4.5</td>
<td>39.3 ± 4.5</td>
</tr>
<tr>
<td>RV area/d/body wt</td>
<td>0.096 ± 0.005</td>
<td>0.129 ± 0.008*</td>
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</table>

Values are means ± SE; LVIDd, diastolic left ventricular internal diameter; LV, left ventricular; d, diameter; LV vol/d, left ventricular volume; EF, ejection fraction; RV, right ventricular. RV area/diameter, right ventricular volume; FAC, fractional area change. Rabbits from the control group underwent the sham surgical procedure at 7 days of age. Rabbits from the hypertrophy group underwent the aortocaval fistula procedure at 7 days of age. *Significant difference (P < 0.05) vs. control.
Tissue Sample Preparation for Immunoblot Analysis

Each sample of frozen ventricular tissue (~20 mg) was combined with homogenization buffer containing 50 mM Tris-HCl (pH 8 at 4°C), 1 mM EDTA, 10% (wt/vol) glycerol, 0.02% (wt/vol) Brij-35, 1 mM dithiothreitol, and protease and phosphatase inhibitors (Sigma) and homogenized for 30 s using a Polytron homogenizer. Samples were centrifuged at 800 g for 10 min. Protein concentration of homogenates was determined using the Bradford protein assay kit (Bio-Rad) and normalized to 1 μg/μl. The supernatant was boiled in protein sample buffer solution (62.5 mM Tris-HCl, 6 M urea, 10% glycerol, 2% SDS, 0.003% bromphenol blue, and 5% 2-β-mercaptoethanol) for 5 min, loaded onto SDS-PAGE gels, and resolved as described previously by Dyck et al. (11).

Immunoblot Analysis

Denatured samples of heart tissue homogenates were subjected to SDS-PAGE and transferred to nitrocellulose membranes as previously described (11). Membranes were blocked in 10% fat-free milk for 1 h and probed with anti-AMP-activated protein kinase (AMPK) (Cell Signaling Technologies), anti-phospho-threonine-172 AMPK (Cell Signaling Technologies), anti-α-tubulin (Cell Signaling Technologies) (using nuclear fraction of cell homogenates), or anti-actin (Santa Cruz Biotechnology) antibodies in 5% fatty acid-free BSA/PBS. After further washing, antibodies were visualized using the enhanced chemiluminescence Western blot detection kit (Perkin-Elmer) and quantified using Quantity One (4.4.0) software (Bio-Rad Laboratories).

PDH Activity Assay

PDH activity was assayed as described previously by Constantin-Teodosiu et al. (8). For active PDH activity, frozen heart tissue (~20 mg) was homogenized in 1:10 buffer containing 200 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 50 mM Tris-HCl, 50 mM NaF, 5 mM dichloroacetate, and 0.1% Triton X-100 (pH 7.8). For total PDH activity, homogenization was done with 1:10 tissue-to-buffer ratio, which contained 200 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 50 mM Tris-HCl, 10 mM glucose, and 4.5 U/ml hexokinase.

Active PDH activity. Warm (37°C) assay buffer (150 mM Tris-HCl, 0.75 mM EDTA, 1.5 mM MgCl₂, 0.75 mM NAD⁺, 0.75 mM CoA grade 1, and 1.5 mM thiamine pyrophosphate) was added to samples. The reaction was initiated by the addition of pyruvate to a final concentration of 1 mM and incubated at room temperature (RT) for 10 min. The reaction was stopped with perchorlic acid (80 mM). The acid was neutralized with KHCO₃. The samples were then centrifuged at 10,000 g for 3 min.

Total PDH activity. Samples were incubated with a preincubation reagent (200 mM sucrose, 50 mM KCl, 5 mM EGTA, 50 mM Tris-HCl, 24 mM CaCl₂, 600 mM MgCl₂, 125 mM dichloroacetate acid, 10 mM glucose, and 18 μM hexokinase) added at a 1:20 dilution for 15 min at 37°C. Warm (37°C) assay buffer was added. The reaction was initiated with the addition of pyruvate/Triton to a final concentration of 1 mM/0.003% and incubated for 10 min at RT. Reactions were terminated with perchorlic acid and centrifuged as above. Resulting supernatants from active and total samples were used to determine acetyl-CoA content as acetyl-CoA was converted to [1⁴C]citrate. [1⁴C]oxaloacetate was formed by reacting 50 mM (pH 7.8) HEPES, 2 mM EDTA, 2 mM α-ketoglutarate, 1.25 mM aspartate, and a total of 0.045 μCi of [1⁴C]aspartate catalyzed by glutamic-oxaloacetic transaminase (16.8–42 KU) for 20 min at RT. To a 200-μl aliquot of each sample was added dithiothreitol (0.83 mM) and CuSO₄-potassium acetate mixture (0.03 and 12 mM) and incubated for 30 min at RT. EDTA (4.6 mM, pH 7.4) was then added, and the mixture was incubated for 5 min at RT. Twenty micromolars of the [1⁴C]oxaloacetate reaction mixture were also added along with a total of 0.5 units of citrate synthase. The mixture was allowed to react for 20 min at RT, terminated with perchloric acid (60 mM), and cooled on ice for 10 min. The reaction was then neutralized with KOH (67 mM). EDTA (5.3 mM) was also added. The unreacted [1⁴C]oxaloacetate was removed by the reverse reaction, whereby the oxaloacetate is reconverted to aspartate with the addition of a glutamate/glutamicoxalacetic transaminase solution (6.6 mM glutamate; 0.8–2.0 units glutamic-oxaloacetic transaminase) incubated for 20 min at RT. One milliliter of Dowex slurry (1:2 wt/vol) was added to each reaction mixture, and the mixture was vortexed every 10 min for 40 min. The mixture is then centrifuged at 3,500 rpm for 5 min to separate out the Dowex beads. The supernatant was retrieved and added to a scintillation fluid, and radioactivity was quantified via a scintillation counter. The amount of acetyl-CoA was determined by comparison of acetyl-CoA standard curves run in parallel in each experiment.

Citrate Synthase Activity Assay

According to the method from Srere (41), tissue homogenates were prepared as for immunoblot assays. The sample was added to the reaction mixture containing 92 mM Tris-HCl (pH 8.2), 0.91 mM EDTA, 0.91 mM MgCl₂, 0.3 mM acetyl-CoA, and 0.1 mM dithionitrobenzoic acid (DNTB) to a final volume of 0.2 ml. Activity was spectrophotometrically quantified as the reaction occurred. The reaction was initiated with oxaloacetic acid (0.5 mM). Absorbance was detected at 412 nm (A₄₁₂) for 2 min. Citrate synthase activity was calculated as follows:

\[
\text{Citrate synthase activity} = \frac{(\Delta A_{412}/\text{min}) \times 0.2\text{ml}}{13.6_{\text{ECOD}}} \times \frac{0.552}{\text{path length of 96-well plate used for measurement} \times \text{tissue wt}}
\]

β-Hydroxyacyl-CoA Dehydrogenase Activity Assay

Tissue homogenates were prepared as described in Tissue Sample Preparation for Immunoblot Analysis. The sample was added to a reaction mixture containing: 50 mM imidazole (pH 7.4) and 0.15 mM NADH. Acetoacetyl-CoA (0.1 mM) was added to initiate the reaction. The final volume of the reaction was 0.2 ml. Activity was quantified spectrophotometrically as the reaction occurred by measuring absorbance at 340 nm (A₃₄₀) for 5 min. β-Hydroxyacyl-CoA dehydrogenase (β-HAD) activity was calculated as follows:

\[
\text{β-HAD activity} = \frac{(A_{340}/\text{min}) \times 0.2\text{ml}}{6.2_{\text{NADH}}} \times \frac{0.552}{\text{path length of 96-well plate used for measurement} \times \text{tissue wt}}
\]

Malonyl-CoA Decarboxylase Activity Assay

Ventricular tissue samples were homogenized in a buffer containing 75 mM KCl, 20 mM sucrose, 10 mM HEPES, 1 mM EGTA, 20 mM NaF, and 5 mM protease inhibitor (Sigma). According to the established method found previously (10, 39), to initiate the malonyl-CoA decarboxylase (MCD) assay, heart homogenates were incubated in a 210-μl assay buffer (0.1 M Tris, pH 8, 0.5 mM dithiothreitol, and 1 mM malonyl-CoA) for 10 min at 37°C. The presence or absence of NaF (50 mM) and NaPP (5 mM). Addition of 40 μM of perchorlic acid (0.5 mM) stopped the reaction. The acid was neutralized with 10 μl of 2.2 M KHCO₃ (pH 10) and centrifuged at 10,000 g for 5 min to remove precipitated proteins. Incubating the sample with malonyl-CoA converted malonyl-CoA to acetyl-CoA, which was then combined with [1⁴C]oxaloacetate (0.17 μCi/ml) to produce [1⁴C]citrate. N-ethylmaleimide was present to remove excess CoA remaining in all reactions during the latter stages of the reaction to prevent the conversion of nonmalonyl-CoA-derived acetyl-CoA by other en-
zymes in the nonpurified MCD assay sample. Sodium glutamate (6.8 mM) and aspartate aminotransferase (0.533 µU/µl) were added to remove unreacted [14C]xaloacetate during a 20-min incubation at RT. A negative control of water, to which all reagents were added accordingly, was performed alongside samples to account for possible inhibitory effects of N-ethylmaleimide and alkylating effects of GOT. However, any inhibitory effect on MCD itself is not expected, since N-ethylmaleimide was not added during the reaction whereby MCD converts malonyl-CoA to acetyl-CoA. The solution was then stirred in a 1:2 suspension of Dowex AG 50W-8X resin (100–200 mesh) and centrifuged at 400 g for 10 min. The supernatant fraction was counted for [14C]citrate. An acetyl-CoA concentration standard curve was run with each experiment and used to quantify sample acetyl-CoA.

Peroxisome Proliferator-Activated Receptor-α mRNA Expression

Peroxisome proliferator-activated receptor-α (PPARα) mRNA expression was determined by reverse transcription of total RNA followed by quantitative PCR (qPCR) analysis. Total RNA was extracted from 1-day-old whole hearts and LV and RV of 7- and 21-day-old heart and 21-day-old hypertrophied hearts as above using TRI reagent (Qiagen) according to the manufacturer’s kit. RNA is then quantified spectrophotometrically at 260 nm. Reverse transcription was then performed on 1 µg of total RNA using 100 IU of reverse transcriptase (Qiagen) with 100 ng of random hexanucleotide primers in a 20-µl reaction volume. PCR cycles were then followed with 1 µl of the reverse transcription product with 0.4 mM of each dNTP, 25 pmol specific primers, PCR buffer, and 1.25 units of Taq DNA polymerase. Two minutes of denaturation at 94°C were followed by amplification of 30 cycles: 30 s at 94°C, 35 s at 53°C, and 40 s at 72°C, then maintained for 7 min at 72°C. qPCR was then performed using 12.5 µl Sybr-Green Jump Start Taq Readymix (Sigma-Aldrich), 5 lM forward primer TGCACCCACTTTGCAAGAG, 7.5 µM reverse primer CCTTCACTTCTCAGAAA, start base pairs 211, stop base pairs 230, Tm 58; reverse primer CCGCCTACATCTTTCAGAAA, start base pairs 224, stop base pairs 255, Tm 59; and probe ATCTACAGACATGTACTGAT, start base pairs 232, stop base pairs 253, Tm 69 [Eukaryotic 18S rRNA Endogenous Control (FAM Dye/MGB Probe, Nonprimer Limited; Part No.: 4352930E)].

Statistical Analyses

Data are expressed as means ± SE. The significance of differences for multiple comparisons was estimated by one-way ANOVA. If ANOVA revealed differences, selected data sets were compared by Bonferroni’s multiple-comparison test. The significance of differences between two groups was estimated by unpaired, two-tailed Student’s t-test where appropriate. Differences were considered significant at P < 0.05.

RESULTS

Assessing Cardiac Hypertrophy by Echocardiography

An abdominal aortocaval fistula was produced in 7-day-old rabbits to increase volume returned to the RV. In volume-overload animals, at 20 days old, a significant increase in LV internal diameter (by 13%) (Fig. 1, A–C, and Table 1), LV volume (by 33%) (Table 1), and end-diastolic RV area (by 36%) (Table 1) was seen. Aortocaval fistula patency, thus volume-overload, was confirmed in 14-day-old and 20-day-old rabbits using color Doppler echocardiography to demonstrate turbulent blood flow (Fig. 1C), which was absent in sham-operated animals. Body weight was similar between the volume-overload and sham groups (Table 1). In contrast, the heart weight and heart weight-to-body weight ratio were significantly increased by 16 and 19%, respectively, in the volume-overload group compared with the sham group (Table 1), indicative of cardiac hypertrophy. Heart rate, LV ejection fraction, and RV percent fractional area change in 20-day-old rabbits were similar between the two groups.

Cardiac Function in Isolated Biventricular Working Hearts

Control hearts were isolated from animals at 21 days of age that had been subjected to sham surgery while hypertrophied hearts were isolated from animals in which volume-overload was produced secondary to a patent aortocaval fistula. With biventricular perfusion, cardiac hypertrophy did not alter cardiac function, consistent with in vivo echocardiographic studies. No differences in LV work (Fig. 2A) or cardiac output (Fig. 2B) were observed between hypertrophied and control hearts, regardless of LV or biventricular mode. A trend toward a decrease in LV work and cardiac output was observed in control hearts perfused in the biventricular mode although the differences did not reach significance. LV stroke volume was also similar between hypertrophied and control groups when perfused in the LV mode. However, hypertrophied hearts had significantly increased LV stroke volume when perfused in the biventricular mode (Fig. 2C). Heart rates were similar between hypertrophied and control hearts during LV perfusions but were significantly increased in control hearts during the biventricular perfusion (Fig. 2D).
Impact of Ischemia on Hypertrophied Hearts

Although cardiac hypertrophy did not affect biventricular aerobic function compared with controls when perfused in 0.8 mM palmitate (Fig. 2 and Table 2), it did affect functional recovery from ischemia (Table 2). While the hypertrophied heart adapted to the volume-overload during aerobic perfusion, the recovery following ischemia-reperfusion was only 52% of the function seen in the control hearts. Hearts exposed to 25 min of ischemia were perfused with 1.2 mM palmitate rather than 0.8 mM, since hypertrophied hearts did not recover with 0.8 mM palmitate (data not shown). Therefore, 1.2 mM palmitate increases substrate availability to resolve differences in recovery between control and hypertrophied hearts. This significant functional decrease during recovery was accompanied by a corresponding increase in the AMP-to-ATP ratio in the RV. Although an increase in the AMP-to-ATP ratio is present in the LV, the difference was not significant. However, the significantly higher AMP-to-ATP ratio in the RV compared with controls (Table 2) may underlie the impaired recovery of function during reperfusion following ischemia.

Metabolism in Aerobically Perfused Hypertrophied Neonatal Rabbit Hearts

Cardiac hypertrophy markedly decreased palmitate oxidation rates (by 57%) compared with controls during LV and biventricular perfusions (Fig. 3A). In contrast, glycolytic rates increased 246 and 172% during LV and biventricular perfusion, respectively, in hypertrophied hearts (Fig. 3B). Glucose oxidation rates were similar in the two groups during LV perfusion. Lactate oxidation rates were also similar between the two groups (Fig. 3C). Hypertrophied hearts failed to increase glucose oxidation, whereas these rates increased to 148% in control hearts during biventricular perfusion (Fig.

Table 2. Cardiac function and corresponding ATP-to-AMP ratios in 21-day-old control and hypertrophy hearts under aerobic biventricular perfusion or ischemic reperfusion

<table>
<thead>
<tr>
<th></th>
<th>21-day-old</th>
<th></th>
<th>Ischemia-Reperfusion</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hypertrophy</td>
<td>Control</td>
<td>Hypertrophy</td>
</tr>
<tr>
<td>Biventricular</td>
<td>18.42 ± 0.33</td>
<td>18.21 ± 0.55</td>
<td>20.70 ± 1.03</td>
<td>18.13 ± 1.03</td>
</tr>
<tr>
<td>LV AMP-ATP</td>
<td>0.051 ± 0.004</td>
<td>0.078 ± 0.015*</td>
<td>0.052 ± 0.009</td>
<td>0.077 ± 0.006</td>
</tr>
<tr>
<td>RV AMP-ATP</td>
<td>0.084 ± 0.019</td>
<td>0.095 ± 0.015</td>
<td>0.067 ± 0.007</td>
<td>0.149 ± 0.031*</td>
</tr>
</tbody>
</table>

Data are means ± SE. Units are mmHg · ml · min⁻¹ · 10⁻². All cardiac functional work was normalized between RV and LV and measured as HR × (aortic peak systolic pressure + pulmonary vein peak systolic pressure) × 10⁻⁴ as in Itoi and Lopaschuk (18). Aerobic hearts were perfused in 0.8 mM palmitate (n = 32 and 16 for control and hypertrophy, respectively) while hearts subjected to 25 min of ischemia were perfused in 1.2 mM palmitate (n = 5 and 5 for control and hypertrophy, respectively). *Significant difference (P < 0.05) vs. control.
Active PDH activity was not diminished in LV or RV of hypertrophied vs. controls (Table 3). In fact, total PDH activity is increased in the RV.

In control hearts, fatty acid oxidation was the predominant ATP source providing >75% of ATP production in LV and biventricular modes (Table 4). Glucose oxidation was the second most important source of ATP (11–16%). Consistent with hypertrophy-altered energy metabolism, ATP production rates decreased by 42 and 38% in hypertrophied hearts compared with controls during LV and biventricular perfusion, respectively (Fig. 3E). The contribution of glycolysis increased in hypertrophied hearts to 27.2% (Table 4). The overall decrease in ATP production, mainly due to the dramatic decrease in fatty acid oxidation, suggests that volume-overload cardiac hypertrophy compromises cardiac energetics. In support of this, acetyl-CoA content, which increased in control hearts (1–21 days of age), did not increase in hypertrophied hearts (Fig. 3F).

Control of Fatty Acid Oxidation in Hypertrophied Hearts

Decreased malonyl-CoA levels are an important determinant of increased cardiac fatty acid oxidation rates following birth (28). Consistent with our previous reports (20, 34), there was a maturational increase in MCD activity during the neonatal period (1–21 days of age) in LV and RV (Fig. 4A). This increase is attenuated in hypertrophied RVs. Interestingly, MCD activity and expression did not differ between hypertrophied and control LVs (Fig. 4B). Although MCD expression did not differ between hypertrophied and control RV (Fig. 4B), MCD activity was significantly lower in hypertrophied RVs compared with control (Fig. 4A). Whereas malonyl-CoA content decreased in the LV of control hearts, this decrease was attenuated in hypertrophied hearts (Fig. 4C). Malonyl-CoA content decreased in the RV of hypertrophied and control hearts over the neonatal period (1–21 days of age). AMPK phosphorylation levels were assayed in RV and LV (Fig. 5).
Although no significant differences between the hypertrophied and control LVs and RVs were detected, RV phospho-AMPK increased in control hearts (21-day-old) vs. 7-day-old hearts (P < 0.05).

In the postnatal period, increased mitochondrial biogenesis increases mitochondrial tricarboxylic acid cycle activity and fatty acid oxidation capacity. Increases in tricarboxylic acid cycle enzyme activity, specifically, citrate synthase, occurred between 7 and 21 days of age in control hearts (Table 3). Similarly, β-HAD, a fatty acid β-oxidative enzyme, also increased in its activity. Hypertrophy reverted citrate synthase and β-HAD activities to that in 7-day-old control hearts (Table 3).

**HIF-1α and PPARα Expression in Control and Hypertrophied Hearts**

As with the maturational decrease in glycolysis (26), HIF-1α expression decreased in neonatal RV but not in the LV between 1 and 21 days of age (Fig. 6A). In hypertrophied hearts, HIF-1α expression in RVs regressed to levels seen in the immediate newborn period (1 and 7 days). HIF-1α expression did not change in the hypertrophied LV. In contrast, PPARα, which activates transcription of enzymes involved in fatty acid oxidation, showed reciprocal expression to that of HIF-1α. LV PPARα mRNA expression remains low from 1 to 7 days old [1.6 ± 0.2 and 4.4 ± 1.2 arbitrary units (AU), respectively] (Fig. 6B). By 21 days old, LV PPARα mRNA expression increases dramatically to 26.6 ± 6.0 AU (P < 0.05 compared with 1- and 7-day-old hearts). Hypertrophy inhibited the rise in PPARα mRNA expression (1.9 ± 0.5 AU) to maintain levels seen in 1- and 7-day-old hearts. Hypertrophy has similar effects on RV PPARα mRNA expression. RV PPARα mRNA expression remains low at 1, 7, and to 2 days of age (1.6 ± 0.2, 1.4 ± 0.2, and 1.0 ± 0.1 AU, respectively). Hypertrophy significantly decreases the levels of PPARα mRNA further to 0.4 ± 0.17 AU (P < 0.05).

**DISCUSSION**

Newborn animal models of cardiac hypertrophy are limited, and the direct effects of hypertrophy on energy substrate preference in the neonatal heart are poorly understood. With the use of the biventricular working heart preparation, functional and metabolic changes in both sides of the heart are detected. We made a number of novel observations in our neonatal cardiac hypertrophy model using this approach. First, mild neonatal cardiac hypertrophy is not associated with significant alterations in aerobic cardiac function (in vivo or in vitro). However, the hypertrophy sufficiently altered neonatal cardiac energy metabolism, including a substantial decrease in cardiac fatty acid oxidation rates and increases in glycolytic rates. Meanwhile, glucose and lactate oxidation do not increase in response to the decreased fatty acid oxidation. This inflexibility in carbohydrate oxidation in response to lower rates of fatty acid metabolism results in a decrease in overall ATP production in hypertrophied neonatal hearts. Cardiac energetics are compromised, which contributes to the compromised recovery from ischemia in hypertrophied neonatal hearts.

In spite of increased metabolic efficiency in hypertrophied neonatal hearts during aerobic perfusion, these changes are not compatible with ischemia-reperfusion. When subjected to ischemia, the recovery of function in hypertrophied hearts is compatible with ischemia-reperfusion. When subjected to ischemia, the recovery of function in hypertrophied hearts is compatible with ischemia-reperfusion.
Cardiac fatty acid oxidation increases dramatically in the normal neonatal period (14, 24, 25). We found that hypertrophy markedly decreased fatty acid oxidation while glycolysis increased to recapitulate a fetal-like metabolic profile. In the adult heart, decreased fatty acid oxidation rates are reciprocated by increased glucose and lactate oxidation rates (7, 42). In the neonatal heart, carbohydrate oxidation rates remain low until weaning (14). Hence, carbohydrate oxidation did not increase with the decreased fatty acid oxidation rates in hypertrophied hearts despite increased PDH activity. This lack of "metabolic flexibility" in hypertrophied neonatal hearts may compromise postischemic cardiac energetics, during which energy demands increase for successful recovery. Indeed our previous report indicates that increasing fatty acid provision improves the recovery of postischemic function by increasing fatty acid oxidation. Of particular relevance are observations that increasing fatty acid oxidation via treatment with a PPARα activator (GW-7647, 3 mg/kg) also increases the recovery of postischemic function in hypertrophied neonatal hearts by increasing fatty acid oxidation (unpublished observations). These data lend further support to the suggestion of compromised energy metabolism and the utility of increasing fatty acid oxidation.
acid oxidation as a cardioprotective intervention in the neonatal heart.
Malonyl-CoA regulates fatty acid oxidation in newborn and mature hearts. Decreased acetyl-CoA carboxylase (ACC) and high MCD expression/activity decrease malonyl-CoA levels in the immediate newborn period to increase fatty acid oxidation (29). Although ACC does participate in the final production of malonyl-CoA, this study focused on MCD, and ACC was not examined. MCD was chosen since prior studies in regard to maturational changes to ACC showed that ACC expression is not affected with age (28). To add, ACC is highly controlled by AMPK in normal cardiac maturation (29) as well as neonatal cardiac hypertrophy (20). Between 1 and 7 days of age, AMPK activity rises in the rabbit heart to phosphorylate and inhibit ACC and decrease ACC activity (29). In turn, the contribution of ACC to the malonyl-CoA pool would decrease, allowing for the increase in fatty acid oxidation. Although ACC activity was not further examined in these experiments, the analysis per-

Fig. 5. AMP-activated protein kinase (AMPK) expression and phosphorylation do not change in hypertrophied hearts compared with controls in LV or RV ventricles. Relative ratios of phosphorylated AMPK to total AMPK and their corresponding representative Western immunobLOTS above. Values represent means ± SE. Differences were determined using a 1-way ANOVA with Bonferroni post hoc test. *P < 0.05, significantly different from 21-day-old controls.

Fig. 6. Hypoxia-inducible factor (HIF)-1α expression and peroxisome proliferator-activated receptor (PPAR)-α activity change with age and hypertrophy. A: HIF-1α expression. B: PPARα mRNA expression measured relative to 18S rRNA in arbitrary units (AU) in RV and LV. Values represent means ± SE. Differences were determined using a 1-way ANOVA with Bonferroni post hoc test. *P < 0.05 compared with 21-day-old controls. †P < 0.05 compared with 1-day-old controls. ‡P < 0.05, significant difference from 7 day olds.
formed on AMPK is an indicator of ACC activity. AMPK expression did not change between control and hypertrophied LV and RV and reflects on a likely lack of change to ACC activity. Therefore, focus was placed on MCD expression and activity as most responsible for malonyl-CoA level changes in the neonatal hearts.

PPARα regulates MCD transcription (22, 23, 36). We demonstrate that, with PPARα level changes, LV MCD expression increases with age. However, although hypertrophy decreases PPARα expression, MCD expression did not fall. Transcriptional responses to cardiac hypertrophy precede protein level changes, since existing MCD must be degraded to decrease its protein levels. However, RV MCD activity decreases with hypertrophy. Despite the decreased activity, RV malonyl-CoA levels do not change from controls. Meanwhile, hypertrophied LV MCD activity, unchanged, allows malonyl-CoA levels to rise significantly. This is further supported by the lower PPARα mRNA levels in RV vs. LV; thus, RV fatty acid oxidation pathway expression is less. Carnitine palmitoyltransferase-1 expression and activity were not assayed in this study. However, β-HAD, although not a rate-limiting step in fatty acid oxidation, is a surrogate marker of fatty acid oxidative capacity. When expressed at low levels, as indicated by a relatively lower activity in the hypertrophied LV, β-HAD may also limit the rate of fatty acid oxidation. Because of a lag between transcription and protein degradation, high malonyl-CoA levels may be needed to regulate the continually high fatty acid oxidation pathway in the LV. LV palmitate oxidation was low during aerobic perfusion and may be the primary contributor to total palmitate oxidation rates, since rates did not change with the addition of RV work in the biventricular mode perfusion in both groups. Thus, RV palmitate oxidation occurs at a low, undetectable rate that requires little inhibitory signaling to decrease β-HAD activity, but low hypertrophied LV palmitate oxidation rates primarily correlate to elevated malonyl-CoA levels.

As the heart matures, HIF-1α expression declines, and this is accompanied by a decreased dependence on glycolysis (33). Normal RVs showed this maturational decrease in HIF-1α expression. This decline is not replicated in the LV, since glycolytic enzymes may still be expressed early in life. The normally low glycolytic rates in the neonatal heart result from allosteric inhibition rather than lowered enzyme expression in the LV. In hypertrophy, the elevated glycolytic rates reflect its release from inhibition from fatty acid oxidation rather than upregulated glycolytic pathway expression. As with pressure-overload hypertrophy (7), the hypertrophied RV HIF-1α expression profile demonstrates the hypertrophy-recapitulated fetal profile.

MCD expression is under PPARα regulation. When PPARα activity/expression decreases, such as that found in PPARα knockout mice, so does MCD expression (6). In contrast, when PPARα activity is increased (e.g., stimulation with a PPARα agonist), MCD mRNA expression is also increased (22). The PPARα gene itself contains a consensus motif that is under HIF-1α transcriptional regulation (32). Upon hypoxia-induced HIF-1α nuclear accumulation, HIF-1α binding to the consensus sequence on the PPARα gene is increased, and PPARα expression and binding activity are decreased. Additionally, HIF-1α may also interrupt the interaction between PPARα and the retinoid X receptor to prevent PPARα-induced transcription (4). This increases lipid accumulation in cardiomyocytes and limits fatty acid oxidation (4), an effect that may be attributed to elevated malonyl-CoA content and decreased expression of MCD. Therefore, through its reciprocal interaction with PPARα, HIF-1α may influence MCD expression. In the neonatal heart, cardiac hypertrophy downregulates PPARα mRNA expression in both the RV and LV. Although this finding is associated with an increased HIF-1α expression relative to control 21-day-old hearts, whether HIF-1α has a direct influence on PPARα expression/activity and MCD expression/activity was not assessed in this study.

In conclusion, we demonstrate that neonatal hearts subjected to volume-overloaded hypertrophy have lower fatty acid oxidation and higher glycolysis rates than controls. Increased energy demands associated with modest elevations in cardiac work are met by a substantial increase in glucose oxidation rates only in control hearts. The hypertrophy-induced delayed metabolic maturation is detrimental to neonatal hearts subjected to ischemia-reperfusion, as evidenced by an impaired recovery of cardiac function. A diminished ability for ATP production may be detrimental to the stressed neonatal heart such as in surgical ischemia.

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DISCLOSURES

GDL is a major shareholder of Metabolic Modulators Research Ltd and is a consultant for Eli Lilly. Other authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS


REFERENCES


27. Lopaschuk GD, Wambolt RB, Barr RL. An imbalance between glycolysis and glucose oxidation is a possible explanation for the detrimental effects of high levels of fatty acids during aerobic repertusion of ischemic hearts. J Pharmacol Exp Ther 264: 135–144, 1993.


