Isoproterenol stimulates 5′-AMP-activated protein kinase and fatty acid oxidation in neonatal hearts

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Am J Physiol Heart Circ Physiol 299: H1135–H1145, 2010. First published July 23, 2010; doi:10.1152/ajpheart.00186.2010—Isoproterenol stimulates 5′-AMP-activated protein kinase and fatty acid oxidation in neonatal hearts. Am J Physiol Heart Circ Physiol 299: H1135–H1145, 2010. First published July 23, 2010; doi:10.1152/ajpheart.00186.2010.—Isoproterenol increases phosphorylation of LKB, 5′-AMP-activated protein kinase (AMPK), and acetyl-CoA carboxylase (ACC), enzymes involved in regulating fatty acid oxidation. However, inotropic stimulation selectively increases glucose oxidation in adult hearts. In the neonatal heart, fatty acid oxidation becomes a major energy source, while glucose oxidation remains low. This study tested the hypothesis that increased energy demand imposed by isoproterenol originates from fatty acid oxidation, secondary to increased LKB, AMPK, and ACC phosphorylation. Isolated working hearts from 7-day-old rabbits were perfused with Krebs solution (0.4 mM palmitate, 11 mM glucose, 0.5 mM lactate, and 100 μU/l insulin) with or without isoproterenol (300 nM). Isoproterenol increased myocardial O$_2$ consumption (in J g dry wt$^{-1}$ min$^{-1}$; 11.0 ± 1.4, n = 8 vs. 7.5 ± 0.8, n = 6, P < 0.05), and the phosphorylation of LKB (in arbitrary density units; 0.87 ± 0.09, n = 6 vs. 0.59 ± 0.08, n = 6, P < 0.05), AMPK (0.82 ± 0.08, n = 6 vs. 0.51 ± 0.06, n = 6, P < 0.05), and ACC-β (1.47 ± 0.14, n = 6 vs. 0.97 ± 0.07, n = 6, P < 0.05), with a concomitant decrease in malonyl-CoA levels (in nmol/g dry wt; 0.9 ± 0.9, n = 8 vs. 7.5 ± 1.3, n = 8, P < 0.05) and increase in palmitate oxidation (in nmol/g dry wt$^{-1}$ min$^{-1}$; 272 ± 45, n = 8 vs. 114 ± 9, n = 6, P < 0.05). Glucose and lactate oxidation were increased (in nmol g dry wt$^{-1}$ min$^{-1}$; 253 ± 75, n = 8 vs. 63 ± 15, n = 9, P < 0.05 and 246 ± 43, n = 8 vs. 82 ± 11, n = 6, P < 0.05, respectively), independent of alterations in pyruvate dehydrogenase phosphorylation, but occurred secondary to a decrease in acetyl-CoA content and acetyl-CoA-to-free CoA ratio. As acetyl-CoA levels decrease in response to isoproterenol, despite an acceleration of the rates of palmitate and carbohydrate oxidation, these data suggest net rates of acetyl-CoA utilization exceed the net rates of acetyl-CoA generation.

inotropes; neonatal heart; adenosine 5′-monophosphate-activated protein kinase; malonyl-coenzyme A; palmitate oxidation; carbohydrate oxidation

THE NEWBORN (1-DAY OLD) RABBIT HEART derives 44–60% of its ATP requirements from glycolysis, while lactate oxidation meets the remainder of ATP demand (35). There is a maturational increase in the contribution of fatty acid β-oxidation to cardiac ATP generation (26). The maturational increase in fatty acid β-oxidation is accompanied by the increased expression and activity of cardiac 5′-AMP-activated protein kinase (AMPK) (39) and a decrease in the activity of acetyl-CoA carboxylase (ACC) (37), the enzyme that synthesizes malonyl-CoA. During this time period (between 1 and 7 days), there is also an increase in the activity of malonyl-CoA decarboxylase (MCD), the enzyme that degrades malonyl-CoA (43). These alterations in AMPK, ACC, and MCD relieve the malonyl-CoA-induced inhibition of carnitine palmitoyl transferase I, the rate-limiting enzyme of mitochondrial fatty acid uptake, and thus allow for fatty acid β-oxidation to become a more prominent contributor to cardiac energetics. Although alterations in AMPK, ACC, and MCD activity and/or expression, and alterations in malonyl-CoA content contribute to the maturational changes in cardiac energy substrate metabolism in the neonatal period, their contribution(s) to changes in energy substrate metabolism in response to an acute increase in cardiac energy demand in the neonatal heart remains to be addressed.

Unlike the rapid/early maturational increase in fatty acid β-oxidation, the rates of glucose oxidation remain low in the early neonatal period. During the postnatal period, there is a progressive increase in the activity of pyruvate dehydrogenase (PDH), which catalyzes the committed step of carbohydrate oxidation, which coincides with increased rates of cardiac glucose oxidation occurring later in the maturation process (43). However, in the presence of physiological concentrations of palmitate (0.4 mM), the rates of cardiac glucose oxidation are markedly inhibited in the early neonatal period, due to low-PDH activity (43). Despite possessing the capacity to readily oxidize glucose, the contribution of glucose oxidation to cardiac ATP generation only increases marginally in the fatty acid-perfused neonatal heart and does not fully mature until weaning (35).

Pharmacological support with inotropic agents is an important, clinically utilized intervention for the management of impaired cardiac contractile function (60, 73). A hallmark response of the myocardium to inotropic/chronotropic stimulation is an increase in oxygen consumption (MVO$_2$); however, the effects of catecholamines on AMPK and ACC are complex. Interestingly, AMPK has been demonstrated to be activated in response to the stimulation of β-adrenoceptors (which couple primarily to G proteins and thus increase intracellular cAMP levels) in a variety of tissues (for review see Ref. 24). However, the activation of myocardial AMPK in response to β-adrenoceptor/inotropic stimulation may be dependent on the specific agent utilized. Previous reports have demonstrated that increased MVO$_2$ elicited in response to catecholamine infusion (dobutamine) does not alter the activities of myocardial AMPK or ACC (20), but does decrease malonyl-CoA content (20, 27). The mechanisms governing the catecholamine-induced decrease in myocardial malonyl-CoA content are also not completely understood, as previous reports both implicate (17), and
conversely dissociate (49) alterations in MCD as mediating this effect. Adding to this confusion is that fatty acid β-oxidation does not increase in the adult heart in response to increased MV\O2 elicited by epinephrine treatment (5, 12). Rather, the increased MV\O2 is accompanied by a preferential increase in the rates of glucose oxidation and an increase in PDH activity (6, 18).

Isoproterenol is used to support cardiovascular function in neonatal patients with congenital heart defects and signs of circulatory congestion. This is due in part to its positive chronotropic effects, as in the neonate the ability to increase cardiac output (CO) is primarily determined by heart rate (70), whereas changes in preload (29, 50), afterload (32), and contractility (4, 65) contribute to a lesser extent (12). Furthermore, inotropic stimulation with isoproterenol increases the phosphorylation and activation of AMPK and the subsequent phosphorylation and inhibition of ACC (2). Interestingly, in 3T3-L1 adipocytes, isoproterenol-induced AMPK activation occurs secondary to the phosphorylation of serine/threonine kinase 11 (LKB) (15), an effect required for the LKB-dependent phosphorylation/activation of AMPK (11, 75, 76). The associated alterations in LKB, AMPK, ACC, MCD, and malonyl-CoA content, as well as alterations in energy substrate metabolism that accompany isoproterenol-induced increases in MV\O2 in the neonatal heart, are not well characterized. The present study utilized hearts from neonatal (7-day-old) rabbits to test the hypothesis that, in neonatal hearts, isoproterenol increases the phosphorylation of LKB, AMPK, and ACC, and lowers malonyl-CoA content. Furthermore, this study tested the hypothesis that these alterations have permissive effects allowing fatty acid β-oxidation to meet the increase in energy demand required to support the increase in contractile function. Due to the low PDH activity (in the presence of fatty acids) in the neonatal heart (43), we also hypothesize that, unlike the mature heart, carbohydrate oxidation is not the major source of energy that facilitates the increase in MV\O2.

**METHODS**

**Animals.** All animals received humane care according to the guidelines of the Canadian Council on Animal Care. All studies were approved by the University of Alberta Health Sciences Animal Welfare Committee.

**Heart perfusions.** Hearts from pentobarbital sodium-anesthetized (0.24 g/kg) New Zealand White rabbits of either sex (7-day-old, 90–200 g) were excised, the aorta cannulated, and a perfusion using Krebs-Henseleit solution (37°C, pH = 7.4, gassed with 95% O2/5% CO2 mixture) was initiated. Hearts were initially perfused in the Langendorff mode for 10 min, following which they were switched to the working/ejecting mode. The perfusate (recirculating volume of 100 ml) consisted of a modified Krebs-Henseleit solution containing 2.5 mM Ca2+, 11 mM glucose, 0.5 mM lactate, 100 mM 1l, and 0.4 mM palmitate bound to 3% bovine serum albumin. Spontaneously beating hearts were aerobically perfused at a constant workload (preload, 7.5 mmHg; afterload, 30 mmHg) for a period of 40 min. In either the absence or presence of isoproterenol (dissolved in ascorbic acid). When present, isoproterenol (300 nM) was added to the perfusate at 5 min. Heart rate (beats/min) and systolic and diastolic arterial pressures were measured using a Harvard Apparatus 60–3002 pressure transducer attached to the aortic outflow line. CO (ml/min) and aortic flow (ml/min) were measured by using ultrasonic flow probes (Transonic T206) placed in the left atrial inflow line and the aortic outflow line, respectively. Left ventricular (LV) work was calculated in hearts perfused with either [5–3H]glucose and [U-14C]glucose (control: n = 9, isoproterenol: n = 8) or [9,10-3H]palmitate and [U-14C]lactate (control: n = 6, isoproterenol: n = 8) for the assessment of the rates of energy substrate metabolism (see below). The product of CO (expressed in terms of m3/s) and peak systolic pressure (expressed in terms of Pa) yields cardiac power [expressed in terms of W (i.e., J/s)]. LV work was derived from cardiac power and expressed as Joules per minute and subsequently normalized the dry weight of each heart and served as a continuous index of LV mechanical function in control (n = 15) and isoproterenol-treated hearts (n = 16). The oxygen content of the perfusate was measured using YSI micro-oxygen electrodes placed in the preload line and in a line originating from the cannulated pulmonary artery. MV\O2 was calculated according to the Fick principle using coronary flow rates and the arteriovenous difference in perfusate oxygen content in hearts perfused with [9,10-3H]palmitate and [U-14C]lactate (control: n = 6, isoproterenol: n = 8). MV\O2 was calculated as follows:

\[
\text{MV}_{\text{O}_2} = \left( P_{\text{O}_2} - P_{\text{V}_2} \right) \cdot (\text{CF}) \cdot \left( \text{arteriovenous difference} \right) \cdot \text{dry wt} \cdot g \cdot \text{min}^{-1} \cdot \text{g}^{-1}
\]

where \( P_{\text{O}_2} \) is the partial pressure of oxygen in the preload line, \( P_{\text{V}_2} \) is the partial pressure of oxygen in the line originating from the cannulated pulmonary artery, CF is coronary flow (in ml/min), \( \text{arteriovenous difference} \) is the solubility of oxygen (0.0212 ml/ml plasma), \( \text{dry wt} \cdot g \) is the atmospheric partial pressure of water (47.1 mmHg), and \( \text{arteriovenous difference} \) is the volume occupied by 1 mol O2 (25.2 l/mol). MV\O2 values were subsequently converted and expressed in terms of Joules per gram dry weight per minute. As such, cardiac efficiency (%) could be calculated as the ratio between LV work and MV\O2 (i.e., energy output/energy input). LV energy expenditure was calculated by expressing MV\O2 in terms of millijoules per beat normalized to the dry weight of the heart and provided an index of oxygen use/energy expended for cardiac contraction on a beat-to-beat basis. All hearts were frozen at the end of the perfusion protocol using Wollenberger clamps cooled to the temperature of liquid N2 for further biochemical analyses.

**Immunoblotting.** Frozen ventricular tissue was homogenized in a solution containing 20 mM Tris-Cl (pH 7.4 at 4°C), 50 mM NaCl, 50 mM NaF, 5 mM sodium-pyrophosphate, 0.25 mM sucrose, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and 1 mM dithiothreitol. After homogenization for 30 s, protein contents of the homogenates were determined using the Bradford protein assay. Samples were diluted and then immunoblotted with rabbit anti-phospho-LKB [Ser428 (human)/Ser431 (murine)] (Cell Signaling Technology, Beverly, MA), rabbit anti-AMPK (Cell Signaling Technology), anti-phospho-ACC (Ser79) (Upstate Cell Signaling Solutions, Charlottesville, VA), rabbit anti-PDH (Cell Signaling Technology), anti-phospho-ACC (Ser79) (Upstate Cell Signaling Solutions, Charlottesville, VA), anti-PDH (Cell Signaling Technology), and anti-phospho-AMPK (Thr172) (Cell Signaling Technology, Beverly, MA). After extensive washing, the membranes were incubated with a peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma-Aldrich, St. Louis, MO), rabbit anti-PDH (Cell Signaling Technology), and anti-phospho-ACC (Ser79) (Upstate Cell Signaling Solutions, Charlottesville, VA), anti-PDH (Cell Signaling Technology), and anti-phospho-AMPK (Thr172) (Cell Signaling Technology, Beverly, MA), respectively. The antibodies were visualized using a enhanced chemiluminescence Western blotting and detection system (Perkin Elmer, Waltham, MA). Densitometric analyses of immunoblots (n = 5–6 per experimental group) were performed using Quantity One (4.4.0) Software (BioRad Laboratories). Densitometric values of the phosphorylated proteins were normalized to the total amount of the protein detected and expressed as arbitrary density units.

**Measurement of AMPK activity.** The activity of AMPK (pmol·mg protein−1·min−1) was measured in 6% polyethylene glycol fractions.
extracted from 200 mg of frozen LV tissue. Activity of AMPK in the presence of 5'-AMP (200 μM) was assayed in the 6% polyethylene glycol fraction by following the incorporation of [γ-32P]ATP into a Ser79 phosphorylation site-specific AMARA peptide (AMARAASAAALARRR), as described previously (29).

**Determination of free-CoA and CoA-ester content.** CoA-esters were obtained from frozen ventricular tissue, as described previously (51). The 6% perchloric acid extract was maintained at pH 2–3. CoA-esters were measured using a modified HPLC procedure described previously (28). Separation was performed using a Beckman System Gold with UV detector 167. Each sample (100 μl each) was run through a precolumn cartridge (C18, size 4.6 × 15 mm, particle size 7 μm) and a Beckman Ultrasphere column (type C18, size 4.6 × 75 mm, particle size 3 μm). Absorbance was set at 254 nm, and flow rate at 1 ml/min. A gradient was initiated using two buffers: buffer A consisted of 0.2 M NaH2PO4 (pH = 5), and buffer B consisted of 0.25 M NaH2PO4 (pH = 5) and acetonitrile in a ratio of 80:20 (vol/vol). Buffers were filtered using a Nylon-66 filter membrane from Pierce (Rockford, IL). Initial conditions (97% buffer A and 3% buffer B) were maintained for 2.5 min and were thereafter changed to 18% buffer B over 5 min using Beckman’s curve 3. At 15 min, the gradient was changed in a linear manner to 37% buffer B over 3 min and subsequently to 90% buffer B over 4 min. At 29.5 min, the composition was returned in a linear manner to 3% buffer B over 0.5 min, and at 5 min column equilibration was complete. Peaks were integrated by a Beckman System Gold software package.

**Measurement of MCD activity.** The activity of MCD was measured as described previously (13). Ventricle homogenates were incubated in a 210 μl reaction mixture containing 0.1 M Tris, pH 8, 0.5 mM dithiothreitol, 50 mM NaF, 5 mM sodium pyrophosphate, and 1 mM hexokinase (2.25 U/ml) were added to 480 μl of assay buffer containing 150 mM KCl, 5 mM MgCl2, 5 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 50 mM Tris HCl, 50 mM NaF, 5 mM dichloroacetate (DCA), and 0.1% Triton X-100 (pH 7.8). To measure total PDH complex activity, NaF and DCA were omitted from the homogenization buffer, and 10 mM glucose and hexokinase (2.25 U/ml) were added to the buffer. The homogenate was then incubated for 15 min at 37°C in the presence of 200 mM sucrose, 4.8 mM CaCl2, 12 mM MgCl2, 2.5 mM DCA, 50 mM KCl, 0.5 mM EGTA, 100 mM glucose, 2.25 units hexokinase, and 50 mM Tris HCl. Following this, 100 μl of homogenate were added to 480 μl of assay buffer containing 150 mM Tris HCl (pH 7.8), 0.75 mM NAD+, 0.75 mM NaF, and 1.5 mM thiamine pyrophosphate. The reaction was initiated by the addition of pyruvate (30 μl of a 26 mM stock solution) to a final concentration of 1 mM. After 10 min, the reaction was terminated by the addition of 40 μl of 0.5 M perchloric acid. The solution was then neutralized and centrifuged, and the content of acetyl-CoA in the supernatant measured. Activity was based on the measurement of acetyl-CoA, where acetyl-CoA formed from the assay of PDH complex activity (active PDH complex activity or total) was converted to [14C]citrate in the presence of citrate synthase and [14C]oxaloacetate. Sodium glutamate and aspartate aminotransferase were used to remove excess [14C]oxaloacetate after the citrate synthase reaction by transamination of unreacted oxaloacetate to [14C]aspartate. AG 50W-X8 resin (100–200 mesh) was then used to separate [14C]aspartate from [14C]citrate. Acetyl-CoA content of supernatant samples was quantified by comparison with acetyl-CoA standard curves run in each experiment.

**Calculation of tricarboxylic acid cycle activity and ATP production rates from the rates of energy substrate metabolism.** Tricarboxylic acid (TCA) cycle activity was calculated from the rate of acetyl-CoA production attributable to the oxidation of palmitate, glucose, and lactate. Values of 8, 2, and 1 mol acetyl-CoA per mole of palmitate, glucose, or lactate oxidized, respectively, were used. The rates of ATP production from glycolysis, glucose oxidation, lactate oxidation, and palmitate oxidation were calculated, using the values of 2 mol ATP per mole of glucose passing through glycolysis, 31 mol ATP per mole of glucose oxidized, 15 mol ATP per mole lactate oxidized, and 105 mol ATP per mole palmitate oxidized based on fractional phosphorous-to-oxygen (P/O) ratios (21).

**Statistical analysis.** All values are presented as means ± SE. The significance of differences between two groups was estimated by two-tailed, unpaired Student’s t-test. Differences were considered significant when P < 0.05.

**RESULTS**

Effects of isoproterenol on LV function and oxygen consumption in neonatal rabbit hearts. LV work during the 40-min perfusion protocol was increased 70% (P < 0.05) in isoproterenol-treated neonatal rabbit hearts compared with vehicle-treated control hearts (Fig. 1A). Predictably, rate pressure
product was also increased 45% \((P < 0.05)\) (Fig. 1B), an effect attributed to a 44% \((P < 0.05)\) increase in heart rate in isoproterenol-treated hearts compared with vehicle-treated control hearts (Table 1). Other indexes of cardiac function, including CO and aortic flow, were also increased in isoproterenol-treated hearts compared with vehicle-treated hearts, whereas peak systolic pressure and coronary flow were not altered in response to isoproterenol (Table 1). As total dry weight \((g)\) was similar between vehicle-treated control hearts and isoproterenol-treated hearts \((0.148 \pm 0.01 \text{ g}, \text{n} = 15 \text{ vs. } 0.144 \pm 0.01 \text{ g}, \text{n} = 16)\), none of the parameters presented in Table 1 were affected by this variable. \(MV_\text{O}_2\) was elevated, as was oxygen extraction \((\mu \text{mol} \text{ O}_2/\text{g dry wt} \cdot \text{ml CF})\), by 46 and 61% \((P < 0.05)\), respectively, in isoproterenol-treated hearts; however, neither LV energy expenditure \((\text{mJ} \cdot \text{beat}^{-1} \cdot \text{g dry wt}^{-1})\) nor cardiac efficiency \((\%)\) was altered compared with vehicle-treated control hearts (Table 2).

Effects of isoproterenol on the phosphorylation of LKB, AMPK, and ACC. The increased energy demand (LV work and rate pressure product) in isoproterenol-treated hearts was accompanied by a 48% \((P < 0.05)\) increase in the phosphorylation/activation of LKB and a 60% \((P < 0.05)\) increase in the phosphorylation of AMPK (Fig. 2A), as well as an increase in AMPK activity \((\text{in pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1} )\); 914 \(\pm 61, \text{n} = 15 \text{ vs. } 676 \pm 58, \text{n} = 16, P < 0.05\) compared with vehicle-treated control hearts at the end of the 40-min perfusion. Consistent with the increases in AMPK phosphorylation and activity, the phosphorylation of an AMPK substrate, ACC-\(\beta\) (the major ACC isoform present in cardiac muscle), was also increased by 52% \((P < 0.05)\) in isoproterenol-treated hearts compared with vehicle-treated control hearts (Fig. 2B), an effect indicative of an inhibition of ACC activity.

Effects of isoproterenol on the ventricular content of malonyl-CoA, MCD activity, and the rates of palmitate oxidation. The ventricular content of malonyl-CoA at the end of the perfusion protocol, as well as the rates of palmitate oxidation during the perfusion protocol, were assessed in response to isoproterenol. There was an 88% decrease \((P < 0.05)\) in the content of malonyl-CoA in isoproterenol-treated hearts compared with vehicle-treated control hearts (Fig. 3A), which was associated with a 31% \((P = 0.05)\) increase in MCD activity (Fig. 3B). These alterations were accompanied by a marked 2.4-fold \((P < 0.05)\) increase in the rates of myocardial palmitate oxidation (Fig. 3C) in isoproterenol-treated hearts compared with vehicle-treated control hearts.

Effects of isoproterenol on glucose metabolism, lactate oxidation, and PDH phosphorylation and activity. Despite the marked stimulation of the rates of myocardial palmitate oxidation, which would be expected to cause a reciprocal inhibition of myocardial glucose metabolism, the rates of carbohy-

Table 1. Cardiac function in control and isoproterenol-treated hearts

<table>
<thead>
<tr>
<th>Cardiac Parameter</th>
<th>Control</th>
<th>Isoproterenol</th>
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<tbody>
<tr>
<td>HR, beats/min</td>
<td>241 ± 7</td>
<td>348 ± 89*</td>
</tr>
<tr>
<td>PSP, mmHg</td>
<td>50 ± 1</td>
<td>51 ± 1</td>
</tr>
<tr>
<td>CO, ml/min</td>
<td>25 ± 2</td>
<td>36 ± 3*</td>
</tr>
<tr>
<td>AF, ml/min</td>
<td>18 ± 1</td>
<td>29 ± 2*</td>
</tr>
<tr>
<td>CF, ml/min</td>
<td>7 ± 1</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>

Values are means ± SE for heart rate (HR), peak systolic pressure (PSP), cardiac output (CO), aortic flow (AF), and coronary flow (CF). Functional parameters were measured in control \((n = 15)\) and isoproterenol-treated \((n = 16)\) isolated working neonatal rabbit hearts. *\(P < 0.05\) compared with control.

Table 2. Oxygen extraction, LV energy expenditure, and cardiac efficiency in control and isoproterenol-treated hearts

<table>
<thead>
<tr>
<th>Oxygen Extraction (MV\textsubscript{O}_2/coronary flow), (\mu\text{mol} \cdot \text{g dry wt}^{-1} \cdot \text{ml}^{-1})</th>
<th>Control</th>
<th>Isoproterenol</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>3.3 ± 0.3</td>
<td>5.3 ± 0.5*</td>
</tr>
<tr>
<td>LV energy expenditure, mJ\cdot beat(^{-1}) \cdot g dry wt(^{-1})</td>
<td>70 ± 8</td>
<td>66 ± 13</td>
</tr>
<tr>
<td>Cardiac efficiency (LV work/MV\textsubscript{O}_2), %</td>
<td>17 ± 1</td>
<td>19 ± 3</td>
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</table>

Values are means ± SE. Oxygen extraction, left ventricular (LV) energy expenditure, and cardiac efficiency were measured in control \((n = 6)\) and isoproterenol-treated \((n = 8)\) isolated working neonatal rabbit hearts. MV\textsubscript{O}_2, myocardial O2 consumption. *\(P < 0.05\) compared with control.
Arate metabolism were also increased in response to isoproterenol. The rates of glycolysis were increased fourfold \((P < 0.05)\) (Fig. 4A) and accompanied by a similar fourfold \((P < 0.05)\) increase in the rates of glucose oxidation (Fig. 4B) in isoproterenol-treated hearts compared with vehicle-treated control hearts. Myocardial lactate oxidation was also increased threefold \((P < 0.05)\) in response to isoproterenol treatment (Fig. 4C). The increase in glucose and lactate oxidation was independent of alterations in the extent of PDH site 2 phosphorylation \((PDH-E1-Ser-271)\) (Fig. 5A), as phosphorylation of PDH was similar in isoproterenol- and vehicle-treated hearts at the end of the perfusion protocol. However, the increase in carbohydrate oxidation was consistent with the 45% decrease \((P = 0.06)\) in ventricular acetyl-CoA content, and 42% decrease \((P = 0.05)\) in the acetyl-CoA-to-free CoA ratio (Table 3). In addition, the activity of PDH was increased two- to threefold \((P < 0.05)\) in isoproterenol-treated hearts compared with vehicle-treated control hearts (Fig. 5B).

Effects of isoproterenol on TCA cycle activity and calculated rates of ATP production. TCA cycle activity attributable to fatty acid and carbohydrate metabolism was calculated from the rates of palmitate oxidation, as well as the rates of glucose and lactate oxidation, respectively. Palmitate-derived TCA cycle activity increased greater than twofold \((P < 0.05)\) (Fig. 6A), whereas glucose- and lactate-derived TCA cycle activity increased twofold \((P < 0.05)\) (Fig. 6B) and threefold \((P < 0.05)\) (Fig. 6C), respectively, in isoproterenol-treated hearts compared with control hearts.
with vehicle-treated control hearts. Interestingly, despite the increase in calculated TCA cycle activity from both palmitate and carbohydrate substrates in isoproterenol-treated hearts compared with vehicle-treated control hearts, there were strong trends toward a decrease in the content of the TCA cycle intermediate, succinyl-CoA (14.6/11006 4.1, n = 8 vs. 22.9/11006 2.7 nmol/g dry wt, P = 0.07). The rates of ATP production attributable to glycolysis and glucose oxidation were increased fourfold (P = 0.05), rates attributable to lactate oxidation were increased threefold (P < 0.05), and those attributable to palmitate oxidation were increased greater than twofold (P < 0.05) (Fig. 6D).

DISCUSSION

Inotropic stimulation increases myocardial MV˙O2 and the rates of energy substrate metabolism. This study investigated the contribution(s) of alterations in the phosphorylation of LKB, AMPK, and ACC, as well as malonyl-CoA content and MCD activity to the metabolic response to increased MV˙O2 elicited by isoproterenol in the neonatal rabbit heart. The isoproterenol-induced increase in myocardial MV˙O2 was accompanied by elevated phosphorylation of LKB, AMPK, and ACC, as well as a marked decrease in malonyl-CoA content. Isoproterenol also increased MCD activity, which likely contributed to the decrease in malonyl-CoA content. These alterations were accompanied by a dramatic increase in palmitate oxidation rates. Interestingly, the rates of both glucose oxidation and lactate oxidation also increased in response to isoproterenol. However, these increases in carbohydrate oxidation

Table 3. Acetyl-CoA and free CoA content and acetyl-CoA-to-free CoA ratios in control and isoproterenol-treated hearts

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Control</th>
<th>Isoproterenol</th>
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<tbody>
<tr>
<td>Acetyl-CoA, nmol/g dry wt</td>
<td>23.1 ± 4.1</td>
<td>12.6 ± 1.1*</td>
</tr>
<tr>
<td>Free CoA, nmol/g dry wt</td>
<td>89.0 ± 7.7</td>
<td>86.4 ± 9.2</td>
</tr>
<tr>
<td>Acetyl-CoA/CoA</td>
<td>0.26 ± 0.05</td>
<td>0.15 ± 0.02</td>
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Values are means ± SE. Acetyl-CoA and free CoA content and the acetyl-CoA-to-CoA ratio were determined in control (n = 8) and isoproterenol-treated (n = 8) isolated working rabbit hearts. *P < 0.05 compared with control.
What is the main finding of the study regarding the inotropic effect of isoproterenol in neonatal hearts?

The study utilized hearts from both male and female neonatal (7-day-old) rabbits. Interestingly, previous reports suggest there are possible differences in cardiac response to inotropic stimulation between both young vs. old and male vs. female experimental animals, as well as human patients. These differences are demonstrated in sexually mature animals; however, domesticated/laboratory rabbits do not reach sexual maturity until 4-6 mo of age (38). Furthermore, the functional and metabolic parameters reported in this study are very consistent and do not appear to have a large degree of variability in either vehicle-treated control or isoproterenol-treated hearts. This lack of variability suggests that both intrinsic cardiac function and the responses to isoproterenol may not have been affected by the sex of the neonatal animals utilized in this study.

Isoproterenol increased $\text{MV}^\circ_2$ parallel to an increase in heart rate and oxygen extraction. The increase in oxygen extraction occurred independent of alterations in coronary flow, an effect owing to coronary flow values in the crystalloid perfused heart preparation approaching near maximum and exceeding those observed in vivo by severalfold (63). Consistent with previous reports, isoproterenol did not alter LV energy expenditure (3, 19) or cardiac efficiency, indicating that inotropic stimulation with isoproterenol in the neonatal heart is not accompanied by an oxygen wasting effect per se. As coronary flow (hence oxygen delivery) was similar between isoproterenol-treated and vehicle-treated control hearts, it suggests that mechanisms intrinsic to the myocardium contributed to the increase in $\text{MV}^\circ_2$. The increase in $\text{MV}^\circ_2$ was accompanied by an increase in the phosphorylation of both AMPK and ACC. These observations differ from those in the porcine myocardium (27, 78), where dobutamine-induced inotropy is not accompanied by the elevated phosphorylation of either AMPK or ACC. This may, in part, be related to the inotropic agent utilized (isoproterenol vs. dobutamine), or may be attributable to possible species-specific differences.

The findings of this study indicate that, in the neonatal heart, the isoproterenol-induced increase in AMPK phosphorylation is at least in part mediated by LKB. This is consistent with a previous report demonstrating the ability of isoproterenol to increase the phosphorylation of LKB in 3T3-L1 adipocytes, an effect that itself was dependent on lipolysis (15). The phosphorylation of LKB, in response to isoproterenol, may have an effect that itself was dependent on lipolysis (15). The phosphorylation of both AMPK and ACC. These observations differ from those in the porcine myocardium (27, 78), where dobutamine-induced inotropy is not accompanied by the elevated phosphorylation of either AMPK or ACC. This may, in part, be related to the inotropic agent utilized (isoproterenol vs. dobutamine), or may be attributable to possible species-specific differences.

The LKB-AMPK-ACC signaling pathway has been demonstrated to be an important regulator of malonyl-CoA content in both cardiac and skeletal muscle (67); how-
ever, the impact of the LKB-AMPK-ACC signaling pathway on fatty acid oxidation in the neonatal heart has not been well characterized. The elevated levels of LKB, AMPK, and ACC phosphorylation, indicative of activation (LKB and AMPK) and inhibition (ACC), respectively, were accompanied by decreased levels of malonyl-CoA in the neonatal heart. An increase in MCD activity also likely contributed to the decrease in malonyl-CoA content observed in hearts treated with isoproterenol. Inotrope-induced alterations in MCD activity may be dependent on the specific inotropic agent employed and/or species studied, as dobutamine infusion in a swine model decreases myocardial malonyl-CoA content independently of alterations in the $V_{max}$ and $K_m$ of MCD (27), while epinephrine-induced decreases in malonyl-CoA in the isolated rat heart preparation are associated with a marked reduction in apparent $K_m$ (17). The decrease in malonyl-CoA content in isoproterenol-treated hearts was accompanied by elevated rates of palmitate oxidation. This differs from previous reports demonstrating the selective/preferential increase in carbohydrate oxidation in response to inotropic stimulation with epinephrine (6, 18). However, these differences may be related to the differing metabolic profile(s) with respect to fatty acid and glucose metabolism between the adult and neonatal heart (16, 34). Despite the stimulation of the rates of myocardial palmitate oxidation, there was not a reciprocal decrease in the rates of myocardial carbohydrate utilization, as not only glycolysis but also both glucose oxidation and lactate oxidation were increased in response to isoproterenol. These observations suggest that, similar to findings in skeletal muscle (41), the activation of AMPK and resulting alterations in ACC and malonyl-CoA can override the Randle cycle/glucose/fatty acid cycle (5) and increase both fatty acid and glucose utilization simultaneously.

The reciprocal relationship between fatty acid $\beta$-oxidation and glucose oxidation is manifest predominantly at the level of PDH. As described by mechanisms operative in the Randle cycle (23, 48), accelerated rates of fatty acid $\beta$-oxidation increase the generation of acetyl-CoA, which, via product inhibition, restricts flux through PDH (22, 59, 61). Interestingly, the accelerated rates of palmitate oxidation in response to isoproterenol were accompanied by decreased acetyl-CoA and malonyl-CoA content, as well as a decrease in the ratio of acetyl-CoA to free CoA. This differs strikingly from observations in swine myocardium, where inotropic stimulation is accompanied by an increase in the contents of acetyl-CoA (77), malonyl-CoA (78), an increase in the acetyl-CoA-to-free CoA ratio (54), and a decrease in free CoA content (54, 77). The increase in acetyl-CoA content occurs even in the presence of an inhibitor of fatty acid $\beta$-oxidation (i.e., oxfenicine) (77), thereby suggesting that increased carbohydrate oxidation is an important contributor to the increase in acetyl-CoA content in response to inotropic stimulation in the adult heart. Although isoproterenol increased carbohydrate (i.e., glucose and lactate) oxidation in the neonatal rabbit heart, the absolute rates of carbohydrate oxidation only represent $\sim$30% of the values observed in response to inotropic stimulation in the adult heart (6). These marked differences in the absolute rates of carbohydrate oxidation in the neonatal heart compared with the adult heart may contribute to the decrease in acetyl-CoA content. Although acetyl-CoA content is decreased in the neonatal heart in response to isoproterenol treatment, it is not completely exhausted. As carbohydrate oxidation at the level of the PDH complex and fatty acid $\beta$-oxidation at the level of the 3-ketoacyl-CoA thiolase reaction are sensitive to changes in acetyl-CoA content, a compensatory increase in the rates of fatty acid $\beta$-oxidation and carbohydrate oxidation prevents the complete exhaustion of acetyl-CoA itself.

In the neonatal rabbit heart, the decrease in acetyl-CoA content observed in response to isoproterenol may have contributed to decreased malonyl-CoA synthesis (as ACC was phosphorylated/inhibited) and resulted in the subsequent decrease in malonyl-CoA content, as the supply of cytosolic acetyl-CoA (from the transfer of mitochondrial acetyl groups via carnitine/acetyl-carnitine translocase) is an important regulator of malonyl-CoA synthesis (49). The total rates of carbohydrate oxidation (i.e., glucose oxidation and lactate oxidation) in response to isoproterenol in the neonatal heart are dramatically less than the rates of carbohydrate oxidation in hearts from adult animals. As a result, the generation of mitochondrial acetyl-CoA from the oxidative decarboxylation of pyruvate via PDH, and subsequent transfer to the cytosolic compartment, may have been insufficient to maintain cytosolic malonyl-CoA levels.

Although this study did not assess myocardial citrate content, citrate represents a source of cytosolic acetyl-CoA. A proportion of citrate not oxidized in the TCA cycle translocates from the mitochondrial matrix to the cytosolic compartment. Cytosolic citrate can allosterically activate ACC (42); as well, citrate via the action of ATP-citrate lyase generates cytosolic acetyl-CoA (8, 46). However, it should be noted that the activity of the cardiac mitochondrial tricarboxylate transporter is relatively low (33, 55). Cytosolic acetyl-CoA can function as a substrate for ACC and give rise to cytosolic malonyl-CoA. As isoproterenol increased the AMPK-mediated phosphorylation of ACC-$\beta$, it likely decreased malonyl-CoA synthesis. Previous reports indicate that the AMPK-mediated phosphorylation of ACC-$\beta$ increases its $K_m$ for ATP, acetyl-CoA, and bicarbonate, as well as increases its $K_f$ for citrate (74), effects expected to contribute to decreased malonyl-CoA synthesis and the decrease in malonyl-CoA content observed in isoproterenol-treated hearts. Isoproterenol also likely increased PKA-mediated phosphorylation of ACC-$\beta$; however, this was not assessed in this study. Interestingly, PKA-mediated phosphorylation of ACC-$\beta$ does not affect the above parameters, nor does prior PKA-mediated phosphorylation of ACC-$\beta$ eliminate the effects of AMPK-mediated phosphorylation (74). However, the effects of PKA-mediated phosphorylation of ACC-$\beta$ are equivocal, as PKA-mediated phosphorylation of purified ACC-$\beta$ has been reported to markedly decrease myocardial ACC activity (14). As such, it is possible that a combination of both AMPK- and PKA-mediated phosphorylation of ACC-$\beta$ contributed to the decrease in myocardial malonyl-CoA content in isoproterenol-treated hearts.

As the isoproterenol-induced increase in myocardial $MV_{O_2}$ in the neonatal heart was accompanied by increased rates of palmitate oxidation, as well as glucose oxidation and lactate oxidation, TCA cycle activity and calculated ATP production attributable to each process were also increased. However, despite the stimulation of both fatty acid and carbohydrate oxidation, the ventricular content of acetyl-CoA decreased. Furthermore, there was a strong trend toward a decrease in the ventricular content of the TCA cycle intermediate, succinyl-
CoA in isoproterenol-treated hearts. As succinyl-CoA itself inhibits both citrate synthase (57), and α-ketoglutarate dehydrogenase (56), a decrease in its content in response to isoproterenol may promote flux through both spans of the TCA cycle (i.e., acetyl-CoA to α-ketoglutarate and α-ketoglutarate to oxaloacetate). Moreover, an increase in TCA cycle flux may decrease the amount of citrate gaining access to the cytosolic space and hence decrease the contribution of citrate to cytosolic acetyl units, thereby decreasing cytosolic malonyl-CoA content and allowing fatty acid β-oxidation to increase. Taken together, these data suggest that the net rates of mitochondrial acetyl-CoA utilization via the TCA cycle were greater than the net rates of acetyl-CoA generation (from palmitate and carbohydrate oxidation). Such an effect may have important implications in the postischemic neonatal heart where energetic/metabolic reserve is limited (25). The effects of decreased energetic/metabolic reserve on LV work may not be apparent in the aerobically perfused neonatal heart. However, the implication of decreased energetic/metabolic reserve in the postischemic neonatal heart is supported by previous reports demonstrating that deficits in energetic/metabolic reserve in the face of preserved basal contractile function precede overt deficits in contractile function elicited in response to increased energetic demand (66, 68, 69). This is recapitulated in the neonatal heart during reperfusion following ischemia (25), where cardiac efficiency is decreased, at least in part due to the greater amount of ATP required to perform biochemical work vs. mechanical work (36). In the neonatal rabbit heart during reperfusion following ischemia, the generation of acetyl-CoA (from carbohydrate oxidation) appears to limit oxidative metabolism and the recovery of postischemic LV work (25) and lends support to the suggestion of decreased energetic/metabolic reserve in the postischemic neonatal heart.

A number of previous reports have demonstrated that inotropes increase myocardial glucose oxidation in part through alterations in intramitochondrial Ca²⁺ content, which can activate PDH (10, 40, 72). The isoproterenol-induced acceleration of carbohydrate oxidation was indeed accompanied by increased PDH activity; however, this was not accompanied by decreased PDH site 2 (PDHE1α-Ser-271) phosphorylation in the neonatal rabbit heart. The heart expresses the PDH kinase isoforms PDK1, PDK2, and PDK4, and each isoform can phosphorylate PDH site 1 (PDHE1α-Ser264), in addition to phosphorylating PDH site 2 (PDHE1α-Ser-271) (30, 31, 61). Therefore, the increase in PDH activity may have occurred secondary to decreased PDH site 1 (PDHE1α-Ser264) phosphorylation (the major phosphorylated/inactivation site), however, PDH site 1 phosphorylation was not assessed in this study. Interestingly, previous reports utilizing neonatal rat hearts demonstrate a maturational increase in the expression of PDK1, PDK2, and PDK4, with expression of each isoform reaching maximum between days 15 and 21 (62). If a similar profile for the maturational expression of these PDK isoforms exists in the neonatal rabbit heart, it may suggest that allosteric regulation of PDH activity predominates in the neonatal period. This is supported by the observation that a decrease in acetyl-CoA content and the acetyl-CoA-to-free CoA ratio (P = 0.06) and, therefore, decreased product inhibition of PDH were accompanied by elevated rates of carbohydrate oxidation in isoproterenol-treated hearts. However, alterations in the allosteric regulation of PDH do not account for the differences in measured PDH activity between control and isoproterenol-treated hearts, as allosteric regulation would be lost under the in vitro assay conditions utilized in this study. Thus the mechanisms underlying the increase in measured PDH activity in response to isoproterenol remain to be fully elucidated.

Pyruvate carboxylation via either pyruvate carboxylase and/or malic enzyme represents an alternative route of pyruvate oxidation. In vivo, myocardial pyruvate carboxylation accounts for 3–6% of TCA cycle flux, while pyruvate decarboxylation accounts for >40% of TCA cycle flux (44, 45). The contribution of pyruvate carboxylation to pyruvate oxidation is increased in the setting of pressure overload cardiac hypertrophy and is associated with the increased expression of malic enzyme (47, 58). Interestingly, the activation of PDH, and hence increased pyruvate decarboxylation, decreases pyruvate carboxylation (47) and may suggest a dynamic and reciprocal relationship between the two mechanisms for the oxidative disposal of pyruvate in the setting of cardiac hypertrophy. However, in the absence of cardiac hypertrophy, pyruvate carboxylation remains constant, despite inhibition of pyruvate decarboxylation (45). Whether a potential balance between pyruvate carboxylation and pyruvate decarboxylation is altered in response to isoproterenol, which increases PDH activity in the neonatal heart remains unknown and would require assessing the expression and activities of pyruvate carboxylase and malic enzyme.

In conclusion, the isoproterenol-induced increase in MV̇O₂ was accompanied by elevated levels of LKB, AMPK, and ACC phosphorylation, with the increased MV̇O₂ being attributable to an increase in both palmitate and carbohydrate (glucose and lactate) oxidation. A decrease in malonyl-CoA content, likely resulting from several factors, including 1) elevated phosphorylation/inhibition of ACC and hence decreased malonyl-CoA synthesis, and 2) elevated MCD activity and hence increased malonyl-CoA degradation, contributed to the stimulation of palmitate oxidation. Flux through PDH was increased independent of alterations in PDH site 2 (PDHE1α-Ser-271) phosphorylation, but did occur secondary to decreased acetyl-CoA content, and a decrease in the acetyl-CoA-to-free CoA ratio, which likely relieved product inhibition of PDH. As acetyl-CoA content and the acetyl-CoA-to-CoA ratio decreased, despite an increase in both fatty acid and carbohydrate oxidation, the utilization of acetyl-CoA by the TCA cycle may have exceeded the capacity for its generation. Furthermore, TCA cycle flux, in excess of acetyl-CoA generation from palmitate and carbohydrate oxidation, may potentially have contributed to the decrease in malonyl-CoA content by decreasing the amount of mitochondrial acetyl-CoA available to translocate to the cytosolic space, where it serves as a substrate for malonyl-CoA synthesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
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