Role of Pyruvate Dehydrogenase Inhibition in the Development of Hypertrophy in the Hyperthyroid Rat Heart

A Combined Magnetic Resonance Imaging and Hyperpolarized Magnetic Resonance Spectroscopy Study

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**Background**—Hyperthyroidism increases heart rate, contractility, cardiac output, and metabolic rate. It is also accompanied by alterations in the regulation of cardiac substrate use. Specifically, hyperthyroidism increases the ex vivo activity of pyruvate dehydrogenase kinase, thereby inhibiting glucose oxidation via pyruvate dehydrogenase. Cardiac hypertrophy is another effect of hyperthyroidism, with an increase in the abundance of mitochondria. Although the hypertrophy is initially beneficial, it can eventually lead to heart failure. The aim of this study was to use hyperpolarized magnetic resonance spectroscopy to investigate the rate and regulation of in vivo pyruvate dehydrogenase flux in the hyperthyroid heart and to establish whether modulation of flux through pyruvate dehydrogenase would alter cardiac hypertrophy.

**Methods and Results**—Hyperthyroidism was induced in 18 male Wistar rats with 7 daily intraperitoneal injections of freshly prepared triiodothyronine (0.2 mg · kg⁻¹ · d⁻¹). In vivo pyruvate dehydrogenase flux, assessed with hyperpolarized magnetic resonance spectroscopy, was reduced by 59% in hyperthyroid animals (0.0022 ± 0.0002 versus 0.0055 ± 0.0005 second⁻¹; P = 0.0003), and this reduction was completely reversed by both short- and long-term delivery of dichloroacetic acid, a pyruvate dehydrogenase kinase inhibitor. Hyperpolarized [2-¹³C]pyruvate was also used to evaluate Krebs cycle metabolism and demonstrated a unique marker of anaplerosis, the level of which was significantly increased in the hyperthyroid heart. Cine magnetic resonance imaging showed that long-term dichloroacetic acid treatment significantly reduced the hypertrophy observed in hyperthyroid animals (100 ± 20 versus 200 ± 30 mg; P = 0.04) despite no change in the increase observed in cardiac output.

**Conclusions**—This work has demonstrated that inhibition of glucose oxidation in the hyperthyroid heart in vivo is mediated by pyruvate dehydrogenase kinase. Relieving this inhibition can increase the metabolic flexibility of the hyperthyroid heart and reduce the level of hypertrophy that develops while maintaining the increased cardiac output required to meet the higher systemic metabolic demand. (Circulation. 2011;123:2552-2561.)

**Key Words**: hyperthyroidism ■ magnetic resonance spectroscopy ■ pyruvate dehydrogenase complex

**Clinical Perspective on p 2561**

Hyperthyroidism is accompanied by alterations in the regulation of cardiac substrate use. Specifically, hyperthyroidism increases the ex vivo activity of pyruvate dehydrogenase kinase (PDK), thereby inhibiting glucose oxidation via pyruvate dehydrogenase (PDH). Pyruvate dehydrogenase catalyzes the conversion of pyruvate to acetyl-CoA, which develop hypertrophy, tachycardia, palpitations, fatigue, and atrial arrhythmias.
can be used to generate adenosine triphosphate via the Krebs cycle.\textsuperscript{11,12} To date, it has not been possible to evaluate in vivo whether hyperthyroidism is associated with alterations in PDH flux and/or the mechanisms controlling in vivo PDH activity and regulation.

Magnetic resonance (MR) spectroscopy (MRS), in conjunction with \textsuperscript{13}C-labeled substrates, has been used to study cardiac metabolism since the early 1980s; however, the inherently low sensitivity of this technique has largely limited its application to the study of steady-state metabolism in perfused hearts. The recent development of hyperpolarized MRS has addressed this problem by improving the signal-to-noise ratio > 10,000-fold.\textsuperscript{13} The increase in the signal-to-noise ratio has made it possible to visualize the uptake of \textsuperscript{13}C-labeled metabolites in vivo and their metabolic conversion through specific enzymes in real time.\textsuperscript{13,14}

The primary aim of this study was to use hyperpolarized \textsuperscript{13}C-MRS to investigate the effects of hyperthyroidism on in vivo PDH flux after 7 days of chronically increased circulating thyroid hormone (triiodothyronine [T3]) concentrations. Metabolic perturbations were further defined with hyperpolarized [2,\textsuperscript{13}C]pyruvate to assess Krebs cycle metabolism\textsuperscript{15} and ex vivo metabolomics to evaluate differences in glycolytic substrate/product concentrations.\textsuperscript{16,17}

A secondary aim was to use hyperpolarized \textsuperscript{13}C-MRS to better characterize the mechanism of in vivo PDH regulation in the hyperthyroid heart. Two distinct mechanisms exist by which PDH flux can be regulated. The end products of fatty acid oxidation, acetyl-CoA and NADH, can directly inhibit PDH reducing PDH flux.\textsuperscript{18,19} Alternatively, acetyl-CoA and NADH can allosterically activate PDK, or in the longer term, transcriptional effectors may increase the specific activity and/or expression of PDK, thus increasing PDH phosphorylation and reducing PDH flux.\textsuperscript{20–22} To confirm whether any PDH inhibition observed in the hyperthyroid heart was PDK mediated, the PDK inhibitor dichloroacetic acid (DCA) was infused before MRS analysis. Dichloroacetic acid prevents PDK-mediated inhibition of PDH without affecting direct regulatory contributions from end-product inhibition on the PDH complex.\textsuperscript{23} Therefore, by assessing the effect of DCA on PDH flux, we could confirm the involvement of PDK in the in vivo regulation of PDH.

Finally, to investigate the functional and metabolic consequences of PDH inhibition in the hyperthyroid heart, hyperpolarized MRS and cine MR imaging (MRI) were used to assess hyperthyroid animals with and without DCA treatment throughout the 7-day T3 administration period. In this way, the effects specifically resulting from PDH inhibition could be defined and the functional and metabolic consequences of alleviating PDH inhibition in the hyperthyroid heart could be evaluated.

**Methods**

**Animal Preparation**

The study conformed to the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986 and to University of Oxford institutional guidelines. Eighteen male Wistar rats (weight, \textasciitilde 250 g) were housed in a 12/12-hour light/dark cycle (lights on at 7 AM and off at 7 PM) in facilities at the University of Oxford. All animal studies were performed between 8 AM and 1 PM. Hyperthyroidism was induced with 7 daily intraperitoneal injections of freshly prepared triiodothyronine (T3; 0.2 mg·kg\textsuperscript{-1}·d\textsuperscript{-1}) beginning on day 2 of the study.

At day 0 (before the first T3 injection to enable use as control data) and day 8, PDH flux was determined with hyperpolarized [1-\textsuperscript{13}C]pyruvate.\textsuperscript{24} Krebs cycle metabolism was assessed on day 1 (also before the first T3 injection) and day 9 with hyperpolarized [2-\textsuperscript{13}C]pyruvate.\textsuperscript{15} Hyperpolarized tracer (1 mL) was injected intravenously over 10 seconds into the anesthetized rat, and 60 individual cardiac spectra were acquired over 1 minute. Cardiac \textsuperscript{13}C MR spectra were analyzed with the AMARES algorithm as implemented in the jMRUI software package. The rate of exchange of the \textsuperscript{13}C label from the hyperpolarized pyruvate to each of its downstream metabolites was assessed with a kinetic model specifically designed to assess hyperpolarized pyruvate metabolism.\textsuperscript{24,25} The model accounts for many of the variables in the hyperpolarized experiment, including the rate of injection, the initial polarization level of the hyperpolarized pyruvate, and the rate of decay of each of the hyperpolarized compounds. The MRS acquisition and analysis protocol is described fully in the Methods section in the online-only Data Supplement.

**Short-Term Dichloroacetic Acid Administration**

One group of rats (n = 6) received a 1-mL bolus of DCA via the tail vein (30 mg/mL in phosphate-buffered saline neutralized to pH 7.2 with concentrated NaOH), followed by a 0.5-mL infusion over 15 minutes immediately before infusion of hyperpolarized [1-\textsuperscript{13}C]pyruvate on day 8.

**Dichloroacetic Acid Treatment**

One group of rats (n = 5) were treated with DCA throughout the 7-day T3 administration period. Drinking water was replaced on day 2 (immediately after the first T3 injection) with water containing DCA (0.75 g/L neutralized to pH 7.2 with NaOH). Water intake was monitored daily.

**Magnetic Resonance Imaging Measurements of Cardiac Function**

On days 1 and 8, after hyperpolarized MRS analysis, cardiac function in animals without DCA infusion/treatment (n = 7) and animals treated with DCA for 7 days (n = 5) was assessed with MRI as described in the online-only Data Supplement. Briefly, cine MRIs, consisting of 28 to 35 frames per heart cycle, were acquired in \textasciitilde 7 contiguous slices in the short-axis orientation covering the entire heart (field of view, 51.2 \times 51.2 mm; matrix size, 256 \times 256; slice thickness, 1.5 mm, giving a voxel size of 0.015 mm\textsuperscript{3}; echo time/repetition time, 1.43/4.6 milliseconds; gaussian radiofrequency excitation pulse, 0.5 milliseconds/17.5°), and averages, 4. End-diastolic (ED) and end-systolic (ES) frames were selected as those with the largest and smallest cavity volumes, respectively. Epicardial and endocardial borders were outlined with the freehand drawing function of ImageJ (National Institutes of Health, Bethesda, MD). Measurements from all slices were summed to calculate ED volume (EDV), ES volume (ESV), stroke volume (SV = EDV − ESV), ejection fraction (EF = SV/EDV), and cardiac output (CO = SV \times heart rate). Left ventricular mass was calculated as myocardial area times slice thickness times myocardial specific gravity (1.05).\textsuperscript{26}

**Plasma Metabolite Measurements**

Immediately before examination with hyperpolarized pyruvate on days 0 and 8, \textasciitilde 500 \muL of saphenous vein blood was sampled from anesthetized rats. All blood samples were immediately centrifuged (3400 rpm, 10 minutes, 4°C), and plasma was removed. A 50-\muL aliquot of plasma was separated, and 1 \muL tetraydroxiprostatin (30 \mug/mL) was added for nonesterified fatty acid analysis. All plasma samples were immediately frozen and stored at \textasciitilde 80°C. An ABX Pentra 400 (Horiba ABX Diagnostics) was used to perform plasma assays for glucose, nonesterified fatty acids, triglycerides, lactate, cholesterol, and \textbeta-hydroxybutyrate. Rats were euthanized by exsan-
guination after an overdose of isoflurane \( \approx \) 50 minutes after MR analysis on day 9. Hearts were immediately excised, frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\) for analysis of PDH activity and PDK4 protein expression.

**Metabolomic Analysis of Cardiac Metabolites**

Another group of rats were administered 7 daily T3 injections as described above \((n=6)\) or received 7 daily intraperitoneal injections of saline water \((0.2 \text{ mL})\) to serve as controls \((n=6)\). On day 7, rats were euthanized by exsanguination after an overdose of isoflurane, and hearts were immediately excised, frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\). From control hearts, a section of tissue was taken for assessment of PDH activity and PDK4 protein expression. Metabolites were extracted from remaining heart tissue with methanol/chloroform/water and analyzed with \(^1\text{H}\)-nuclear magnetic resonance spectroscopy as previously described.\(^6\) Nuclear magnetic resonance spectra were processed with the ACD SpecManager 1D nuclear magnetic resonance processor (version 8; ACD, Toronto, Canada; see the online-only Data Supplement).

**Pyruvate Dehydrogenase Activity Assay**

The activity of the active and total fractions of PDH \((\text{PDH}_\text{a} \text{ and } \text{PDH}_\text{t})\) were determined spectrophotometrically by the method of Seymour and Chatham.\(^2\) Briefly, the assay required the preparation of cardiac tissue with 1 of 2 homogenization buffers for either \(\text{PDH}_\text{a}\) or \(\text{PDH}_\text{t}\) measurement. We assessed \(\text{PDH}_\text{t}\) when PDH was extracted under conditions in which both PDH phosphatase and PDK were inhibited by \(\text{KH}_2\text{PO}_4\), potassium fluoride, DCA, and adenosine diphosphate. The rate of \(\text{PDH}_\text{a}\) was stimulated by \(\text{Mg}^{2+}\) and \(\text{PDK}\) was inhibited by DCA and adenosine diphosphate. The rate of NADH production over the first 30 seconds was used to determine activity \((\text{molecules } \text{min}^{-1} \text{ g}^{-1} \text{ wet weight})\).

**Western Blotting**

Protein levels of PDK4 were measured in total left ventricular homogenates with SDS-PAGE and Western blotting. All samples were run in duplicate on separate gels to confirm results. Protein levels were related to internal standards to ensure homogeneity between samples and gels.

**Statistical Analysis**

Values are reported as mean \(\pm\) SEM. Statistical significance between the control, T3, T3 plus short-term DCA treatment groups for the in vivo hyperpolarized data, in vitro metabolomics, PDH activity assay, plasma metabolite analysis, and Western blotting was assessed with a Kruskal-Wallis test followed by a Mann-Whitney test with a post hoc Bonferroni correction.\(^2\) For the cine MRI data, the interaction between the effects of T3 and DCA was assessed with repeated measures ANOVA. Statistical significance between parameters measured on days 0 and 8 was assessed with a paired \(t\) test. Statistical significance between the T3 and T3 plus DCA treatment groups was assessed with a 2-sample \(t\) test assuming equal variances. Statistical significance was considered at \(P<0.05\) level.

**Results**

**Cardiac Metabolism in the Hyperthyroid Heart**

**Cardiac Pyruvate Dehydrogenase Flux**

Using hyperpolarized \(^1\text{C}-\text{MRS}\), we measured \([1-\text{\textsuperscript{13}C}]\text{pyruvate conversion into the metabolic product bicarbonate in real time}\) (Figure 1A). The exchange of \(^{13}\text{C}\) label from \([1-\text{\textsuperscript{13}C}]\text{pyruvate into \textsuperscript{13}C-bicarbonate gave a direct assessment of metabolic flux through PDH.}\(^2\) All data from day 0 were combined to form a single control data set. Before any T3 administration, the rate of label exchange from \([1-\text{\textsuperscript{13}C}]\text{pyruvate to \textsuperscript{13}C-bicarbonate in these animals was calculated to be 0.0055\pm0.0005 second}^{-1}.\) After 7 days of T3 administration (T3 group), PDH flux was significantly reduced by 59\% to 0.0022\pm0.0002 second\(^{-1}\) \((P=0.0003;\) Figure 1B). The reduction in PDH\(_a\) versus control PDH\(_a\) was confirmed with an ex vivo spectrophotometric approach that detected a 46\% decrease compared with control values \((P=0.0084;\) Figure 1C).

**Other Metabolic Abnormalities in the Hyperthyroid Heart**

\([1-\text{\textsuperscript{13}C}]\text{alanine and } [1-\text{\textsuperscript{13}C}]\text{lactate appeared as a result of alanine aminotransferase– and lactate dehydrogenase–mediated } \textsuperscript{13}C-label exchange, respectively.\) The measured exchange rate of \(\text{^{13}C}\) label from \([1-\text{\textsuperscript{13}C}]\text{pyruvate to } [1-\text{\textsuperscript{13}C}]\text{alanine demonstrated a significant 62\% increase in the T3 group relative to control animals}\) \((P=0.0048;\) Figure 2A). The rate of exchange of \(\text{^{13}C}\) label into \([1-\text{\textsuperscript{13}C}]\text{lactate signal was also increased by 72\% in hyperthyroid animals}\) \((P=0.0049;\) Figure 2B). Ex vivo \(^1\text{H}\)-nuclear magnetic resonance spectroscopy analysis of the cardiac tissue from hyperthyroid hearts revealed that myocardial concentrations of glucose and glycogen were decreased to 73\%\% \((P=0.0043)\) and 24\%\% \((P=0.0087)\) of control values, respectively. The concentrations of the glycolytic products lactate and alanine were elevated to 137\%\% \((P=0.026)\) and 162\%\% \((P=0.0022)\) of the control values, respectively (Figure 2C). Hyperpolarized \([2-\text{\textsuperscript{13}C}]\text{pyruvate, in conjunction with MRS, enabled Krebs cycle metabolism to be investigated.}\) Exchange of \(\text{^{13}C}\) label into \([1-\text{\textsuperscript{13}C}]\text{citrate, } [5-\text{\textsuperscript{13}C}]\text{glutamate, and } [1-\text{\textsuperscript{13}C}]\text{acetylcarbinine was significantly reduced in the hyperthyroid hearts relative to controls}\) \((P<0.05;\) Table 1). This probably reflected a reduction in label reaching the Krebs cycle owing to the decrease in PDH flux. To try to account for this, values were further normalized against PDH flux values. After normalization, no significant difference was seen between the rates of \(\text{^{13}C}\)-label incorporation into citrate, glutamate, or acetylcarbinine between the control and hyperthyroid animals.

Further evaluation of the data revealed a previously unobserved peak at \(\approx78.1\text{ ppm}\), which was evident in the hyperthyroid heart spectra but barely quantifiable in the control spectra (Figure 3A). Further work enabled identification of this peak as \([3-\text{\textsuperscript{13}C}]\text{citrate, which would be formed through anaplerotic processing of } [2-\text{\textsuperscript{13}C}]\text{pyruvate via oxaloacetate.}\) Because of the relatively low amplitude of the \([3-\text{\textsuperscript{13}C}]\text{citrate peak, summing 10 seconds of data from } t=4\) seconds was necessary for quantification but revealed levels to be increased >4-fold in the T3 group relative to controls \((P=0.004;\) Figure 3B).

**Plasma Metabolites**

After 7 days of T3 administration, there was a \(\approx3\)-fold increase in the concentration of circulating free fatty acids with levels of \(\approx2.1\text{ mmol/L}\) \((P=0.0008).\) The concentration of the ketone body \(\beta\)-hydroxybutyrate was significantly increased >4-fold in the hyperthyroid rats \((P=0.0003).\) No significant differences in plasma concentrations of cholesterol, lactate, or glucose were detected (Table 2).

**Pyruvate Dehydrogenase Regulation in the Hyperthyroid Heart**

To study the predominant mechanism of PDH regulation in the hyperthyroid heart, hyperpolarized [1-\text{\textsuperscript{13}C}]pyruvate me-
metabolism was analyzed in hyperthyroid rats after the administration of DCA immediately before hyperpolarized MRS analysis. Infusion of DCA caused a 3.9-fold increase in PDH flux relative to flux in hyperthyroid hearts (P < 0.0012; Figure 1B) and a 1.6-fold increase in PDH flux over control values (P < 0.0084). Figure 1D shows the expression of PDK4 protein as assessed by Western blotting (control, 1.0 versus T3, 1.5; P < 0.06).

**Dichloroacetic Acid Treatment**

To establish whether relieving the PDK-mediated inhibition of PDH would have any effect on the hyperthyroid heart, the functional and metabolic effects of DCA treatment throughout the 7-day T3 administration period were assessed. A combined hyperpolarized MRS and cine MRI approach was used. The results of this study are summarized in Figures 1 through 4 and Tables 1 through 3. Dichloroacetic acid treatment increased PDH flux by 4.9-fold relative to levels in hyperthyroid animals (P < 0.0025) and by 2.0-fold relative to controls (P = 0.0017), which is similar in magnitude with the 1.6-fold increase observed in animals after a short-term DCA infusion. To verify these findings, a spectrophotometric assay was used to measure PDH activity (PDHa) ex vivo. This confirmed that PDHa was increased 4.4-fold in DCA treated animals relative to T3 animals without DCA treatment (P = 0.0027; Figure 1C). Figure 1D shows the expression of PDK4 protein as assessed by Western blotting (control, 1.0 ± 0.3; T3, 1.5 ± 0.3; P = 0.06).

The rate of 13C label incorporation into [1-13C]alanine was 42% lower than levels measured in hyperthyroid animals without DCA treatment (P = 0.0056) and the same as control values (Figure 2A). Incorporation of 13C label into [1-13C]lactate was not statistically different from control levels or levels in untreated hyperthyroid animals (Figure 2B). Label incorporation into [1,13C]citrate, [5,13C]glutamate, and [1-13C]acetyl carnitine at day 7 was significantly higher than in untreated hyperthyroid animals (P < 0.01); however, when values were normalized to PDH flux to account for the increase in 13C label entering the Krebs cycle, no significant differences were seen (Table 1). The amount of [3-13C]citrate in treated animals at day 7 was >3-fold higher than control levels (P = 0.01; Figure 3B). Analysis of circulating metabolite concentrations in plasma revealed increased β-hydroxybutyrate relative to control animals, whereas lactate, glucose, and cholesterol levels were decreased relative to controls (P < 0.05). Dichloroacetic acid treatment also reduced circulating free fatty acid levels back to control levels (Table 2).
Left ventricular mass and the ratio of heart weight to body weight were significantly higher in all animals relative to day 0 (P<0.05; Table 3), indicating the development of hypertrophy in both untreated and DCA-treated animals. However, the average increase in LV mass was significantly lower in the treated hyperthyroid animals (100±20 mg) than in the untreated hyperthyroid animals (200±30 mg; P=0.04; Figure 4). Cardiac output was increased in all animals compared with controls (P<0.05), but no significant difference was detected between treated and untreated animals (Table 3 and Figure 4).

Table 1. Rate of Exchange of 13C Label From [2-13C]Pyruvate to [5-13C]Glutamate, [1-13C]Citrate, and [1-13C]Acetyl carnitine After [2-13C]Pyruvate Infusion Into Control Animals (n=12), Hyperthyroid Animals (Triiodothyronine; n=7), and Hyperthyroid Animals Receiving Dichloroacetic Acid Treatment (n=5)

<table>
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<tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.0017±0.0002</td>
<td>0.0008±0.0001</td>
<td>0.0060±0.0008</td>
</tr>
<tr>
<td>T3</td>
<td>0.0009±0.0001*</td>
<td>0.0017±0.00005*</td>
<td>0.0018±0.0004*</td>
</tr>
<tr>
<td>T3 + DCA treatment</td>
<td>0.0036±0.0003†</td>
<td>0.0021±0.0004†</td>
<td>0.0093±0.0008†</td>
</tr>
</tbody>
</table>

Normalized to PDH flux

<table>
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<tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.40±0.06</td>
<td>0.18±0.04</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>T3</td>
<td>0.42±0.03</td>
<td>0.08±0.02</td>
<td>0.79±0.08</td>
</tr>
<tr>
<td>T3 + DCA treatment</td>
<td>0.34±0.05</td>
<td>0.19±0.04</td>
<td>0.87±0.09</td>
</tr>
</tbody>
</table>

T3 indicates triiodothyronine; DCA, dichloroacetic acid; and PDH, pyruvate dehydrogenase.

*P<0.05 vs control; †P<0.05 vs T3.
Discussion

In this study, we used hyperpolarized MRS to noninvasively measure the real-time conversion of \([1-^{13}C]\)pyruvate to \([^{13}C]\)bicarbonate to assess in vivo PDH flux in the hyperthyroid rat heart. We have, for the first time, demonstrated that cardiac PDH flux was decreased by 59% in vivo after 1 week of chronically elevated T3 levels. Reduced PDH flux would severely diminish the conversion of pyruvate to acetyl-CoA, thereby reducing the contribution of glucose oxidation to energy generation. The exact mechanism by which T3 reduces PDH flux is yet to be fully elucidated. Evidence suggests that T3 directly stimulates PDK4 gene expression via two thyroid response elements in the \(PDK4\) promoter.\(^{29}\)

Conversely, other studies have shown that the reduction in PDH flux occurs after an increase in fatty acid oxidation.\(^{30,31}\) In support of the latter hypothesis, analysis of circulating plasma metabolite levels from rats administered T3 revealed an increase in the concentration of fatty acids and \(\beta\)-hydroxybutyrate relative to control levels. Our results are also consistent with the findings of Sugden et al.,\(^{32}\) who observed a decrease in the ratio of cardiac free to acylated carnitines, indicating increased myocardial fatty acid oxidation, and with many other studies that have demonstrated an increase in the circulating levels of fatty acids and their subsequent oxidation in hyperthyroidism.\(^{33–35}\)

We also found evidence of upregulation of several important energy-generating metabolic pathways. The ex vivo metabolomic experiments provided evidence of increased cardiac glucose use in rats treated with T3, despite the reduction in PDH flux. In line with this, increased \([1-^{13}C]\)pyruvate and its metabolic products are annotated (unlabeled peaks are impurities in the \([2-^{13}C]\)pyruvate preparation). \(B\), The \([3-^{13}C]\)citrate/\([2-^{13}C]\)pyruvate ratio in each group was assessed with hyperpolarized \(^{13}C\) MR spectroscopy. Control indicates combined day 0 data (\(n=12\)); T3, hyperthyroid rats (\(n=7\)); T3 + DCA Treatment, dichloroacetic acid (DCA) treatment throughout the 7-day T3 administration period (\(n=5\)). *\(P<0.05\) vs control.

Table 2. Summary of Plasma Metabolite Concentrations in Control Rats (\(n=12\)), Hyperthyroid Rats (Triiodothyronine; \(n=7\)), and Hyperthyroid Rats Treated With Dichloroacetic Acid for 7 Days (\(n=5\))

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control, mmol/L</th>
<th>T3, mmol/L</th>
<th>T3 + DCA Treatment, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fatty acids</td>
<td>0.9±0.1</td>
<td>2.1±0.3*</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0±0.4</td>
<td>8.9±0.4</td>
<td>8.2±0.2*</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.33±0.09</td>
<td>1.29±0.06</td>
<td>0.64±0.04†</td>
</tr>
<tr>
<td>(\beta)-Hydroxybutyrate</td>
<td>0.11±0.01</td>
<td>0.49±0.08*</td>
<td>1.0±0.2*</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>2.5±0.2</td>
<td>2.1±0.2</td>
<td>1.3±0.2*</td>
</tr>
<tr>
<td>Triacylglycerides</td>
<td>1.3±0.2</td>
<td>1.11±0.09</td>
<td>1.4±0.2</td>
</tr>
</tbody>
</table>

T3 indicates triiodothyronine; DCA, dichloroacetic acid. *\(P<0.05\) vs control; †\(P<0.05\) vs T3.

Figure 3. \(A\), Magnetic resonance (MR) cardiac spectrum of a triiodothyronine (T3) rat at day 8 after infusion of \([2-^{13}C]\)pyruvate. Ten individual spectra were summed to generate this spectrum (t=4 to 13 seconds). \([2-^{13}C]\)pyruvate and its metabolic products are annotated (unlabeled peaks are impurities in the \([2-^{13}C]\)pyruvate preparation). \(B\), The \([3-^{13}C]\)citrate/\([2-^{13}C]\)pyruvate ratio in each group was assessed with hyperpolarized \(^{13}C\) MR spectroscopy. Control indicates combined day 0 data (\(n=12\)); T3, hyperthyroid rats (\(n=7\)); T3 + DCA Treatment, dichloroacetic acid (DCA) treatment throughout the 7-day T3 administration period (\(n=5\)). *\(P<0.05\) vs control.

Figure 4. \(A\), Cardiac mass and \(B\), cardiac function (cardiac output) assessed with magnetic resonance imaging in the hyperthyroid rat heart with and without dichloroacetic acid (DCA) treatment at day 0 (black) and day 8 (white) (*\(P<0.05\)). LV indicates left ventricular.
Compensated for by an increase in pyruvate carboxylation via real time. Previous studies investigating substrate use in the Krebs cycle can be maintained under conditions of reduced PDH flux. We have been able to identify and quantify an interesting and potentially useful application of hyperpolarized MRS is probing mechanisms of metabolic regulation in disease. In this study, hyperthyroid rats were infused with the PDK inhibitor DCA immediately before analysis by hyperpolarized MRS. In these rats, the T3-mediated decrease in PDH flux was completely reversed. Because previous work has shown that DCA inhibits PDH in the presence of inhibitory concentrations of acetyl-CoA and NADH, this result confirms that the observed reduction in PDH flux is mediated by DPK and that there is no discernable contribution from end-product inhibition. This supports the findings of Sugden et al and Holness et al, who observed an increase in PDK4 expression in hyperthyroid hearts. Although in our study the increase in PDK4 protein levels just failed to reach significance, this could be explained by the lower daily doses of T3 administered (0.2 versus 1.0 mg kg\(^{-1}\) d\(^{-1}\)) or by the fact the hearts in our study were collected >24 hours after the final T3 injection.

Effects of Dichloroacetic Acid Treatment on the Hyperthyroid Heart

The hyperthyroid heart undergoes well-characterized functional and morphological changes in that heart rate, contractility, and cardiac output all increase and cardiac hypertrophy is frequently observed. To determine whether the inhibition of PDH, which results in the inability to use glucose efficiently as a substrate for adenosine triphosphate synthesis, is a contributing factor in the development of T3-mediated hypertrophy, we treated a group of hyperthyroid rats with DCA throughout the 7-day T3 administration period. Consistent with the role of DCA, we observed a significant increase in PDH flux, a reduction in circulating glucose levels, and a reduction in \(^{13}\)C-label exchange with alanine, suggesting that the intracellular alanine pool size was reduced. Interestingly, despite the 4.9-fold increase in PDH flux with DCA treatment compared with untreated levels, no difference in PDK4 protein level was observed, confirming that long-term DCA regulatory pathway and using hyperpolarized MRS to assess subsequent changes in metabolic flux, we can obtain novel information regarding metabolic regulation in disease. In this study, hyperthyroid rats were infused with the PDK inhibitor DCA immediately before analysis by hyperpolarized MRS. In these rats, the T3-mediated decrease in PDH flux was completely reversed. Because previous work has shown that DCA inhibits PDH in the presence of inhibitory concentrations of acetyl-CoA and NADH, this result confirms that the observed reduction in PDH flux is mediated by DPK and that there is no discernable contribution from end-product inhibition. This supports the findings of Sugden et al and Holness et al, who observed an increase in PDK4 expression in hyperthyroid hearts. Although in our study the increase in PDK4 protein levels just failed to reach significance, this could be explained by the lower daily doses of T3 administered (0.2 versus 1.0 mg kg\(^{-1}\) d\(^{-1}\)) or by the fact the hearts in our study were collected >24 hours after the final T3 injection.

Table 3. Cine Magnetic Resonance Imaging Measurements of Cardiac Morphology and Function in Hyperthyroid Rats (Triiodothyronine; n=7) and Hyperthyroid Rats Treated With Dichloroacetic Acid for 7 Days (n=5)

<table>
<thead>
<tr>
<th>Group</th>
<th>T3</th>
<th>T3+DCA Treatment</th>
<th>P for Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 8</td>
<td>Day 0</td>
</tr>
<tr>
<td></td>
<td>LV mass, mg</td>
<td></td>
<td>Average LV mass, mg</td>
</tr>
<tr>
<td></td>
<td>520 ± 30</td>
<td>720 ± 40*</td>
<td>460 ± 20</td>
</tr>
<tr>
<td></td>
<td>End-diastolic lumen volume, μL</td>
<td>350 ± 20</td>
<td>300 ± 20*</td>
</tr>
<tr>
<td></td>
<td>End-systolic lumen volume, μL</td>
<td>58 ± 6</td>
<td>46 ± 7</td>
</tr>
<tr>
<td></td>
<td>Stroke volume, μL</td>
<td>290 ± 20</td>
<td>260 ± 20*</td>
</tr>
<tr>
<td></td>
<td>Ejection fraction, %</td>
<td>84 ± 1</td>
<td>85 ± 2</td>
</tr>
<tr>
<td></td>
<td>Heart rate, bpm</td>
<td>400 ± 30</td>
<td>530 ± 20*</td>
</tr>
<tr>
<td></td>
<td>Cardiac output, mL/min</td>
<td>115 ± 4</td>
<td>137 ± 9*</td>
</tr>
<tr>
<td></td>
<td>LV/BW, x10-3</td>
<td>1.7 ± 0.1</td>
<td>2.3 ± 0.1*</td>
</tr>
</tbody>
</table>

T3 indicates triiodothyronine; DCA, dichloroacetic acid; LV, left ventricular; and HW/BW, ratio of heart weight to body weight. Day 0 control data were collected before the first injection of T3. The interaction between T3 and DCA was assessed with a repeated measures ANOVA. Statistical significance between day 0 and day 8 data was assessed with a paired Student t test. Statistical significance between T3 and T3 plus DCA treatment groups was assessed with an unpaired t test.

\(^*P<0.05\) vs day 0; †\(^P<0.05\) vs T3.

13C]lactate and [1-13C]alanine were detected in hyperthyroid hearts. In a previous study, nonoxidative glucose metabolism and glycogenolysis were observed to increase with hyperthyroidism. The authors suggest that this was caused by a perturbation in energy metabolism, leading to increased levels of inorganic phosphate, which would stimulate glyco-
administration affects only the activity, not the expression, of this enzyme.

Further analysis of circulating metabolite levels revealed a normalization of circulating fatty acid concentrations and a reduction in glucose and cholesterol levels. These findings are consistent with a reduction in fatty acid oxidation, increased glucose oxidation, and decreased lipolysis in DCA-treated animals. Plasma lactate concentrations were also reduced, consistent with an increased level of lactate oxidation and a reduction in skeletal muscle glycolysis after DCA administration. The levels of β-hydroxybutyrate, which were elevated in the T3 animals relative to controls, were further increased in the DCA-treated animals. This may be due to increased ketogenesis in the liver resulting from excess acetyl-CoA being generated from the significantly increased PDH flux, demonstrating the systemic effects that DCA treatment will have had on these animals.

Perhaps the most important alteration observed in the DCA-treated hearts was the 52% decrease in cardiac hypertrophy. In the hyperthyroid heart, increases in myocyte diameter and abundance of mitochondria are well established. Such morphological changes allow increased energy generation via enhanced fatty acid oxidation, thus enabling the heart to meet the increase in workload. By allowing the hyperthyroid heart to use both fatty acids and carbohydrates as a fuel source, DCA treatment could increase mitochondrial flexibility, thereby reducing the requirement for increased abundance of mitochondria. Consistent with this, T3-mediated cardiac hypertrophy was reduced in animals treated with DCA. However, the hyperthyroid heart must still respond to the increase in workload and increase cardiac output to ensure efficient systemic O₂ delivery. This study therefore has demonstrated that by increasing the metabolic flexibility of the hyperthyroid heart, the response to increased levels of thyroid hormone is altered and cardiac hypertrophy is reduced. The side effects associated with DCA treatment, ie, pain, numbness, and peripheral neuropathy, prevent DCA from being a viable treatment for hyperthyroidism; however, our data suggest that alternative metabolic interventions may relieve some of the more serious symptoms associated with this disease and therefore warrant further investigation.

Limitations and Future Directions
The hyperpolarized [1-13C] and [2-13C]pyruvate experiments described in this work produced spectra that were localized to the heart with a surface coil placed over the chest. It is certain that a small amount of contamination from neighboring organs, notably blood, liver, and skeletal muscle, made some contribution to the measured signals, especially in the detection of [1-13C]lactate and [1-13C]alanine. However, we are confident that the appearance of metabolites downstream of PDH was dominated specifically by cardiac metabolism on the basis of both the metabolic time course of metabolite appearance and the high metabolic turnover of the heart. The time course of metabolite production closely followed the time course of injected pyruvate in that the initial detection and accumulation of bicarbonate, citrate, and acetylcarnitine occurred within 1 to 3 seconds of pyruvate arrival in the chest. It seems unlikely that metabolites produced anywhere outside the heart would appear so rapidly after cardiac pyruvate delivery. Furthermore, the extremely high rate of PDH and Krebs cycle flux in the heart compared with liver and resting skeletal muscle provides further evidence that the bicarbonate measurements in this study reflected local cardiac metabolism.

The potential to study the metabolic effects of hyperthyroidism and other cardiovascular diseases in humans with the hyperpolarized techniques presented here is clear. The first trials in humans using hyperpolarized pyruvate as a metabolic biomarker are imminent and offer many advantages over other forms of metabolic assessment, ie, no radiation exposure and minimally invasive procedure. Metabolic studies using this technology can be integrated into existing MRI assessments of cardiac structure and function, as demonstrated here with a combined cine-MRI and hyperpolarized MRS assessment.

Summary
The visualization of real-time metabolism noninvasively has revealed for the first time that a significant PDK-mediated reduction in PDH flux in vivo contributes to the pathology of the hyperthyroid heart. This has been shown to be accompanied by increased glycolytic metabolism and anaplerotic flux into the Krebs cycle. Increasing the number of metabolic substrates available to the hyperthyroid heart significantly reduced hypertrophy while maintaining an elevated cardiac output. Future work will focus on alternative metabolic interventions that, like DCA, may alleviate the more serious effects of hyperthyroidism and slow progression of the cardiac effects of this disease.

Acknowledgments
We thank Emma Carter for her technical assistance.

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Disclosures
None.

References


CLINICAL PERSPECTIVE

The primary clinical significance of this article lies in the importance of gaining a better understanding of the underlying mechanisms resulting from hyperthyroidism. Using both noninvasive and novel techniques, we have thoroughly characterized the relationship between the metabolic and functional consequences of hyperthyroidism. By studying the metabolic perturbations associated with hyperthyroidism and using this information to devise a treatment regimen to improve metabolic flexibility in the hyperthyroid heart, we have uniquely shown that the associated cardiac hypertrophy can be reduced. We have also found that under conditions of plentiful energy supply, the heart is able to alter its response to maintain cardiac output. These findings are undoubtedly important, not only for this disease but also for other metabolic diseases affecting the heart. Furthermore, the potential to study the metabolic effects of hyperthyroidism and other cardiovascular diseases in humans with the hyperpolarized techniques presented here is clear. The first trials in humans with hyperpolarized pyruvate as a metabolic biomarker are imminent and offer many advantages over other forms of metabolic assessment such as including no radiation exposure and being a minimally invasive procedure. Metabolic studies with this technology can be integrated into existing magnetic resonance imaging assessments of cardiac structure and function, as demonstrated here with a combined cine magnetic resonance imaging and hyperpolarized magnetic resonance spectroscopy assessment.
Role of Pyruvate Dehydrogenase Inhibition in the Development of Hypertrophy in the Hyperthyroid Rat Heart: A Combined Magnetic Resonance Imaging and Hyperpolarized Magnetic Resonance Spectroscopy Study

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SUPPLEMENTAL MATERIAL

Methods

**In vivo hyperpolarized MRS protocol.** On each study day, rats were anaesthetised (~2% isofluorane in oxygen) and a tail vein catheter was inserted for i.v. administration of the hyperpolarized solution. Rats were placed in a home-built animal-handling system and positioned in a 7 T horizontal bore MR scanner interfaced to an Inova console (Varian Medical Systems, Paolo Alto, USA). ECG, respiration rate and body temperature were monitored throughout the experiment. A home-built $^1$H/$^{13}$C butterfly surface coil was placed over the chest to localise signal from the heart. An ECG-gated shim was used to reduce the proton linewidth to ~120 Hz. Anaesthesia was maintained by means of isofluorane (~1.7%) delivered to, and scavenged from, a nose cone during the experiment.

**In vivo hyperpolarized MRS protocol.** Approximately 40 mg of $[1-^{13}$C$]$ or $[2-^{13}$C$]$pyruvic acid doped with 15 mM OX063 (trityl radical, GE Healthcare, UK) and 3µL of Dotarem (Gadoteric acid (1:50 in ddH$_2$O); Guerbet, France) was prepared and polarized in a prototype hyperpolarizer system with 45 min of microwave irradiation (GE Healthcare, UK). The hyperpolarized sample was subsequently dissolved in a pressurized and heated alkaline solution, containing 40mM NaOH, 40mM TRIS base buffer, and 0.27mM EDTA. Dissolution yielded a solution of 80mM hyperpolarized sodium pyruvate with a polarization of ~30% for $[1-^{13}$C$]$pyruvate and ~20% for $[2-^{13}$C$]$pyruvate, physiological temperature of ~40°C and pH of 7.0–7.8. Immediately before injection, an ECG-gated $^{13}$C MR pulse-acquire spectroscopy sequence was
initiated. One millilitre of the hyperpolarized tracer was injected i.v. over 10 s into the anesthetized rat and 60 individual cardiac spectra were acquired over 1 min (TR= 1 s; excitation flip angle= 5°; sweep width= 80 ppm for [1-13C]pyruvate and 180 ppm for [2-13C]pyruvate; acquired points= 2048).

**MR Data Analysis.** Cardiac 13C MR spectra were analysed using the AMARES algorithm as implemented in the jMRUI software package. Spectra were DC offset-corrected based on the last half of acquired points. For [1-13C]pyruvate experiments, peaks corresponding with [1-13C]pyruvate, [1-13C]lactate, [1-13C]alanine and 13C-bicarbonate were fit with prior knowledge assuming Lorentzian lineshapes and starting values for the peak frequencies and linewidths. Similarly, for [2-13C]pyruvate experiments, the peak areas of [2-13C]pyruvate, [1-13C]acetylcarnitine, [1-13C]citrate and [5-13C]glutamate at each time point were quantified.

For both [1-13C] and [2-13C]pyruvate, the fitted peak areas were used as input data for a kinetic model developed for the analysis of hyperpolarized pyruvate MRS data, based on a model initially developed by Zierhut et al. Firstly the change in [1-13C] or [2-13C]pyruvate signal over the 60 s acquisition time was fit to the data using equation [1]:

\[
M_{pyr}(t) = \begin{cases} 
\frac{\text{rate}_{inj}}{k_{pyr}} \left(1 - e^{-k_{pyr}(t-t_{arrival})}\right) & t_{arrival} \leq t < t_{end} \\
M_{pyr}(t_{end})e^{-k_{pyr}(t-t_{end})} & t \geq t_{end}
\end{cases}
\]
In this equation, $M_{pyr}(t)$ represents the pyruvate peak area as a function of time. This equation fits the parameters $k_{pyr}$, the rate constant for pyruvate signal decay (s$^{-1}$), $rate_{inj}$, the pyruvate arrival rate (a.u. s$^{-1}$), $t_{arrival}$, the pyruvate arrival time (s) and $t_{end}$, the time correlating with the end of the injection (s). These parameters were then used in equation [2] along with the dynamic metabolite data (either [1-13C]lactate, [1-13C]alanine and 13C-bicarbonate or [1-13C]acetylcarntine, [1-13C]citrate and [5-13C]glutamate) to calculate $k_{pyr\rightarrow x}$, the rate constant for exchange of pyruvate to each of its metabolites (s$^{-1}$) and $k_x$, the rate constant for signal decay of each metabolite (s$^{-1}$) which was assumed to consist of metabolite $T_1$ decay and signal loss from the low flip angle RF pulses. In equation [2], $t' = t - t_{delay}$, where $t_{delay}$ represents the delay between pyruvate arrival and metabolite appearance caused by the traversal of the pyruvate through the cardiopulmonary circulation before arrival at the coronary arteries.

\[
M_x(t') = \begin{cases} 
\frac{k_{pyr\rightarrow x}rate_{inj}}{k_{pyr} - k_x} \left( 1 - e^{-k_x (t' - t_{arrival})} \right) \frac{1 - e^{-k_{pyr} (t' - t_{arrival})}}{k_{pyr}} & t_{arrival} \leq t' < t_{end} \\
\frac{M_{pyr}(t_{end})k_{pyr\rightarrow x}e^{-k_x (t' - t_{end})}}{k_{pyr} - k_x} \left( 1 - e^{-k_{pyr} (t' - t_{end})} \right) + M_x(t_{end})e^{-k_x (t' - t_{end})} & t' \geq t_{end}
\end{cases}
\]

MRI Measurements of Cardiac Function. On day 1 and 8, post hyperpolarized MRS analysis, cardiac function in animals without DCA infusion/treatment (n=7), and animals treated with DCA for 7 days (n=5), was assessed using MRI. The animals remained anesthetized with 1.5%-2.5% isoflurane in O$_2$ and were positioned supine in a purpose-built, temperature-regulated cradle. ECG electrodes were inserted into the forepaws and a respiration loop was taped across the chest. The cradle was lowered into a vertical-bore 500MHz, 11.7 T MR system with a Bruker console (Bruker Biospin, Germany) and a 52-
mm birdcage RF coil (Rapid Biomedical, Germany). Long and short-axis scout images were acquired so that true short-axis images could be planned using a segmented, ECG-triggered fast low-angle shot (FLASH) sequence. The RF coil was tuned and matched, followed by slice selective shimming. Cine-MR images, consisting of 28–35 frames per heart cycle, were acquired in ~7 contiguous slices in the short-axis orientation covering the entire heart. The imaging parameters were as follows: field of view = 51.2×51.2 mm, matrix size= 256×256, slice thickness= 1.5 mm giving a voxel size 0.015 mm$^3$, TE/TR= 1.43 / 4.6 ms, 0.5 ms / 17.5° Gaussian RF excitation pulse and 4 averages. The total experimental time was approximately 50 min per animal. Heart rate remained stable throughout the procedure. End-diastolic (ED) and end-systolic (ES) frames were selected as those with the largest and smallest cavity volumes, respectively. Epicardial and endocardial borders were outlined using the freehand drawing function of ImageJ (National Institutes of Health, USA). Measurements from all slices were summed to calculate ED volume (EDV), ES volume (ESV), stroke volume (SV = EDV-ESV), ejection fraction (EF= SV/EDV) and cardiac output (CO= SV×heart rate). LV mass was calculated as myocardial area × slice thickness × myocardial specific gravity (1.05)$^5$.

**Metabolomic analysis of cardiac metabolites.** Metabolites were extracted from remaining heart tissue using methanol/chloroform/water. Frozen tissue (~100 mg) was placed in methanol-chloroform (2:1, 600 µl) and homogenised. Samples were then sonicated for 5 min before chloroform-water (1:1) was added (200 µl of each). Samples were centrifuged (13,500 rpm, 20 min), and the aqueous layer was pipetted off and dried overnight in an evacuated centrifuge (Eppendorf, Hamburg, Germany). The dried extracts
were rehydrated in 600 µL of D$_2$O, buffered in 0.24 M sodium phosphate (pH 7.0), containing 1 mM sodium-3-(tri-methylsilyl)-2,2,3,3-tetradecuteriopropionate (TSP; Cambridge Isotope Laboratories, Andover, MA). Samples were analysed using a Bruker Avance II+ spectrometer operating at 500 MHz for the $^1$H frequency equipped with a 5 mm Broadband TXI Automatic Tuning and Matching (ATMA) probe. Spectra were collected using a solvent suppression pulse sequence based on a 1D- nuclear Overhauser effect spectroscopy pulse sequence to saturate the residual [$^1$H] water proton signal (relaxation delay = 2 s, $t_1=3$ µs, mixing time = 150 ms, presaturation applied during the relaxation time and mixing time). One hundred twenty-eight transients were collected into 16 K data points over a spectral width of 12 ppm at 37 °C.

**Metabolomic Data Analysis.** NMR spectra were processed using an ACD SpecManager 1D NMR processor (version 8; ACD, Toronto, Canada). Spectra were Fourier transformed after multiplication by a line broadening of 1 Hz and referenced to TSP at 0.0 ppm. Spectra were phased and baseline corrected manually. Each spectrum was integrated using 0.04 ppm integral regions between 0.5–4.5 and 4.7–9.5 ppm. Each 0.04 ppm region was treated as an independent variable during analysis. To account for any difference in concentration between samples, each spectral region was normalised to total integral area. Resonances in the NMR spectra were assigned with reference to the literature.

**Pyruvate Dehydrogenase Activity Assay.** Frozen cardiac tissue was powdered and 0.2 g was homogenized in 1 mL of the appropriate homogenization buffer. The sample was
frozen in liquid N$_2$, thawed and re-homogenized 3 times. The sample was centrifuged (3400 rpm, 8 min) and the supernatant removed for analysis. PDH activity was determined by adding an aliquot of either PDH$_a$ or PDH$_t$ extract to assay buffer (containing rotenone to inhibit the respiratory chain, and LDH and lactate to generate pyruvate; pH=7.2, 30°C). The reaction was followed at 340nm using the kinetic program on a spectrophotometer for 2 min, for both PDH$_a$ and PDH$_t$ samples. The rate of NADH production over the first 30 s was used to determine activity in units of µmol/min/g wet weight.

**Western Blotting.** Protein levels of PDK4 were measured in total left ventricular homogenates using SDS-PAGE and Western blotting. Equal concentrations of protein were separated on 12.5% SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore, UK) using semi-dry transfer apparatus (BioRad, UK). Even protein loading between samples and successful transfer were confirmed using ponceau staining (Sigma, UK). Membranes were incubated with primary antibody to PDK4 (Abgent, UK) overnight. All samples were run in duplicate on separate gels to confirm results. Protein levels were related to internal standards to ensure homogeneity between samples and gels.
References


