In Vivo Measurement of Myocardial Protein Turnover Using an Indicator Dilution Technique

James H. Revkin, Lawrence H. Young, William S. Stirewalt, Douglas M. Dahl, Robert A. Gelfand, Barry L. Zaret, and Eugene J. Barrett

We applied a nondestructive tracer technique, previously developed for measuring skeletal muscle protein turnover, to the measurement of myocardial protein turnover in vivo. During a continuous infusion of L-[ring-2,6-3H]phenylalanine to anesthetized, overnight-fasted dogs, we measured the uptake of radiolabeled phenylalanine from plasma and the release of unlabeled phenylalanine from myocardial proteolysis using arterial and coronary sinus catheterization and analytic methods previously applied to skeletal muscle. Using these measurements, together with a model of myocardial protein synthesis that assumes rapid equilibration of tracer specific activity between myocardial phenylalanyl-tRNA and circulating phenylalanine, we estimated the rates of heart protein synthesis and degradation. The rate of heart protein synthesis was also estimated directly from the incorporation of labeled phenylalanine into tissue protein. The use of [3H]phenylalanine was compared with L-[1-14C]leucine in the measurement of heart protein turnover in dogs given simultaneous infusions of both tracers. Leucine uptake and release by the myocardium exceeded that of phenylalanine by 3.1±0.4- and 1.7±0.3-fold, respectively, consistent with leucine's 2.4-fold greater abundance in heart protein and its metabolism via other pathways. Phenylalanine is the preferred tracer for use with this method because of its limited metabolic fate in muscle. One theoretical limitation to the method, slow equilibration of circulating labeled phenylalanine with myocardial phenylalanyl-tRNA, was resolved by comparison of these specific activities after a 30-minute infusion of labeled phenylalanine in the rat. A second, empirical limitation involves precision in the measurement of the small decrements in phenylalanine specific activity that occur with each pass of blood through the coronary circulation. This was addressed by improving the precision of both the measurements of phenylalanine concentration and phenylalanine specific activity using high-performance liquid chromatography. We conclude that the in vivo measurement of phenylalanine tracer exchange across the myocardium permits the nondestructive estimation of heart protein turnover in the intact animal. (Circulation Research 1990;67:902–912)

It has long been recognized that muscle protein undergoes continuous turnover.1 In the perfused heart, rates of synthesis and degradation vary as a function of oxygen and substrate availability.2–3 The concentration of insulin4 and branched chain amino acids,5,6 and ventricular tension development.7 Both in vitro and in vivo investigations have revealed marked disturbances of myocardial protein turnover during ischemia, infarction, and reperfusion.3,8 Virtually all previous studies of heart protein have required the direct measurement of the incorporation of radioabeled amino acid tracers into tissue protein.2–3,6–11 This form of analysis not only limits repetitive measurements in a single animal but also essentially precludes the study of myocardial protein metabolism in the human heart.

We recently described an isotopic technique that does not require tissue sampling to estimate protein turnover in skeletal muscle.12,13 This method employs a continuous systemic infusion of radiolabeled phenylalanine and the measurement of differences in its

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concentration and specific activity across a muscle bed. Phenylalanine was chosen as a tracer because its metabolism is limited to incorporation into or release from protein in muscle tissue. In the current study, we administered a continuous infusion of L-[ring-2,6-3H]phenylalanine and measured the dilution of its specific activity within the coronary circulation. These measurements were used to estimate the rate of heart protein degradation. From the measured rate of myocardial uptake of labeled phenylalanine and the tracer’s specific activity in coronary sinus plasma, we also estimated the rate of protein synthesis.

Inherent in this approach to measuring protein turnover, as well as the conventional biopsy method, is the assumption that plasma amino acid specific activity is similar to that of aminoacyl-tRNA, the immediate precursor for protein synthesis. We have approached this issue in the following ways. First, because leucine specific activity is known to rapidly equilibrate between plasma and its tRNA pool in the heart, we compared both the relative kinetics of phenylalanine versus leucine across the heart and the incorporation rates of these tracers into heart muscle in the dog. Second, since the equilibration between plasma phenylalanine specific activity and the tRNA pool in vivo during continuous tracer infusion is unknown, we examined this relation in additional experiments in the rat.

In comparison with skeletal muscle, heart muscle has a much higher blood flow per gram of tissue, and as seen in the results, this leads to narrower arteriovenous differences in phenylalanine specific activity across the heart. To refine the technique and improve the precision of these specific activity measurements, we performed a final group of experiments using high-performance liquid chromatography (HPLC) analysis.

Materials and Methods

Animal Preparation

After an overnight fast, 14 mongrel dogs of either sex weighing 18–26 kg were anesthetized with sodium pentobarbital and ventilated on room air. In nine dogs (group 1), after induction of anesthesia, an incision was made on the right side of the neck to expose the jugular vein and carotid artery. Under fluoroscopic guidance a 6F or 7F Sones catheter and a 7F thermodilution coronary sinus catheter (Electro-Catheter Co., Rahway, N.J.) were advanced via the jugular vein into the distal half of the coronary sinus or into the great cardiac vein for blood sampling and flow measurements. Catheter position was confirmed at the conclusion of each experiment by fluoroscopy. The distal positioning of these catheters was necessary, not only to achieve stable catheter placement but also to avoid the admixture of right atrial blood during coronary sinus sampling. The right carotid artery was cannulated for the sampling of arterial blood.

Five dogs (group 2) were used for measurements of skeletal muscle protein turnover by catheterization and for biopsy material. In these dogs, we placed an 18-gauge catheter in the femoral artery and the ipsilateral femoral vein. The arterial catheter was used for infusion of indocyanine green to measure hind limb blood flow; the venous catheter was employed for sampling blood draining the hind limb. Care was taken in positioning the arterial catheter not to obstruct downstream blood flow in the femoral distribution. Patency of all catheters was maintained with a slow infusion of normal saline interrupted only for flow measurements and blood sampling. These dogs were killed at the conclusion of the study, and biopsies were taken (approximately 1 g tissue) from left ventricular myocardium and the quadriceps muscle for measurement of tracer incorporation.

Experimental Protocols

After placement of the catheters, each dog received a 90-minute continuous (3.3 µCi/min) infusion of L-[ring-2,6-3H]phenylalanine (Amersham Corp., Arlington Heights, Ill.) via the forepaw vein. Five of the nine dogs in group 1 and all of the group 2 dogs also received a primed (10 µCi) continuous (0.2 µCi/min) infusion of L-[1-14C]leucine beginning 90 minutes before and continuing along with the phenylalanine infusion. The infusion rate of labeled phenylalanine was doubled in the dogs receiving a dual isotope infusion so as to minimize the effects of any residual spillover of 14C counts into the tritium channel, which may not have been fully corrected by the external standardization method used for counting.

Four sets of paired arterial and coronary sinus samples (group 1) or arterial and femoral venous samples (group 2) were drawn over the last 30 minutes of the infusion period. For measurement of hind limb blood flow, indocyanine green was infused at a rate of 150 µg/min into the femoral artery. Coronary sinus blood flow was measured using the thermodilution method as previously described. At least four measurements of either hind limb or coronary flow were made in each dog. In the five group 2 dogs, after the last blood samples were obtained, the chest was opened, and the heart was rapidly excised. Biopsy samples of heart and hind limb muscle were obtained. These specimens were rapidly freeze-clamped in aluminum blocks to −70°C and stored frozen for subsequent analysis of tissue radioactivity.

Analytic Methods

Plasma amino acid concentrations and radioactivity in groups 1 and 2 were measured as previously described. In brief, blood was collected in heparinized tubes and centrifuged at 2,000 rpm for 10 minutes. The plasma was then removed and combined 1:1 with 6% sulfosalicylic acid containing norleucine as a standard. The deproteinized samples were centrifuged at 8,000 rpm for 30 minutes, and 50-µl aliquots of the supernatant were removed for amino acid
analysis, which was performed using an automated ion exchange chromatographic technique (model D-500, Dionex Corp., Sunnyvale, Calif.). A second aliquot of supernatant (1 ml) was passed over a Dowex 50W ion exchange column (Biorad Laboratories, Richmond, Calif.). The amino acids were eluted with 1N NaOH, collected in scintillation vials, and counted in a liquid scintillation system (Packard Instruments, Downers Grove, Ill.), using a dual isotope counting mode and external standard. In control experiments, recovery of labeled leucine and phenylalanine from the Dowex column was greater than 95%. In addition, at the end of a 90-minute infusion of labeled phenylalanine, more than 95% of the tritium counts in plasma were found in phenylalanine, and less than 1% were found in tyrosine. By the above methods, the coefficient of variation of phenylalanine specific activity is ±9% and that of leucine is ±6%.

Samples of heart and skeletal muscle protein from biopsy material were prepared as described by Morgan et al. The freeze-clamped tissue specimens obtained from group 2 dogs were weighed and pulverized while still frozen and then suspended in 10% perchloric acid (3 ml perchloric acid/g tissue). The supernatant was removed, and the pellet was resuspended in 10% perchloric acid containing 1 g/l of phenylalanine. This mixture was placed in a boiling water bath for 15 minutes and then centrifuged for 5 minutes; the pellet was washed twice with 3 ml of 10% perchloric acid and dissolved in 3 ml of 0.3N NaOH at 37° C. After adding 0.75 ml of 6N HCl, the solution was centrifuged for 3 minutes; the pellet was washed twice with ethanol: chloroform: ether (3:2:1) and twice with diethyl ether and dried. Protein samples weighing 5–10 mg were wetted with 0.15 ml water, dissolved with 1 ml tissue solubilizer (Soluene 100, Packard Instruments), and counted in the liquid scintillation system described above.

Samples of purified heart and skeletal muscle protein from three dogs were hydrolyzed in 10N H2SO4 for 1 hour at 100° C. The phenylalanine and leucine content of the hydrolysate were measured on the amino acid analyzer to determine the molar representation of these two amino acids within bulk heart protein.

Calculations

The net balance of an amino acid across the heart is given by the expression:

$$\text{balance} = \frac{[\text{amino acid}]_{a} - [\text{amino acid}]_{c}}{\text{flow}}$$

where $[\text{amino acid}]$ is the plasma amino acid concentration in artery (a) and coronary sinus (c).

The extraction ratio of labeled amino acid (ER) is given by the expression:

$$\text{ER} = \frac{\text{DPM}_{a} - \text{DPM}_{c}}{\text{DPM}_{a}}$$

where DPM is disintegrations per minute of tracer amino acid per milliliter of plasma.

The rate of protein degradation in heart muscle (expressed as the rate of phenylalanine release from protein) is determined by measuring the dilution of the specific activity (SA) of phenylalanine (PHE) within the coronary sinus:

$$\text{degradation rate} = \frac{[\text{SA}_{a}/\text{SA}_{c}] - 1}{\text{flow}} \times \text{PHE}$$

Since phenylalanine is neither synthesized nor metabolized by muscle, its net balance (see Equation 1) must also be equal to the difference between its rate of incorporation into newly synthesized protein and its rate of release from protein breakdown:

$$\text{net phenylalanine balance} = \text{synthesis} - \text{degradation}$$

or

$$\text{synthesis} = \text{balance} + \text{degradation}$$

where rates of synthesis, degradation, and balance are expressed as nanomoles of phenylalanine per minute per gram of tissue wet weight. One can determine the rate of protein synthesis by substituting the solutions for balance (Equation 1) and degradation (Equation 3) into Equation 5, or one can substitute the terms of both expressions (Equations 1 and 3) into Equation 5 and, by algebraic rearrangement, solve for protein synthesis directly, in terms of experimentally measurable parameters:

$$\text{synthesis} = \frac{(\text{DPM}_{a} - \text{DPM}_{c})}{(\text{flow}/\text{SA}_{c})}$$

The complete derivation of these expressions can be found in the “Appendix” (see also Reference 12). These rates can be converted to rates of tissue protein turnover by dividing by the measured molar representation of phenylalanine in muscle protein (290 nmol/mg heart protein), which gives a result in terms of milligrams of protein per minute per gram of tissue. In an effort to normalize results between dogs and to compare this method with the tissue biopsy technique, the mass of myocardium sampled by the coronary sinus catheter in each sample was estimated by assuming a myocardial plasma flow of 45 ml/min/100 g and a skeletal muscle plasma flow of 1.89 ml/min/100 g. All of the rates calculated (Equations 1, 3–6) in this study were divided by this flow-dependent estimate of tissue mass. This method of normalization assumes conditions of normal tissue perfusion.

An estimate of the rate of protein synthesis can be obtained from the incorporation of labeled phenylalanine directly into biopsy tissue protein of both heart and skeletal muscle as follows:

$$\text{synthesis (nmol phenylalanine/g muscle/hr)} = \frac{\text{dpm/mg protein} \times 160 \text{ mg protein/g muscle wet wt}}{\text{hrs of infusion} \times \text{SA}_{a}}$$

This method of normalization assumes conditions of normal tissue perfusion.
Measurement of Phenylalanyl-tRNA

For measurement of phenylalanyl-tRNA labeling, six Sprague-Dawley rats were fasted overnight and anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg). Polyethylene catheters were placed in the carotid artery for blood sampling and a jugular vein for tracer infusion. L-[ring-2,6-3H]Phenylalanine was given as a continuous intravenous infusion (2 mCi/100 g body wt/hr). At 30 minutes, arterial and femoral venous blood samples were obtained, the chest was rapidly opened, and the heart was excised and immediately freeze-clamped between aluminum tongs submerged in liquid nitrogen. Tissue was stored frozen at $-70^\circ$C until analyzed. Plasma samples were deproteinized by extraction with 2 vol absolute ethanol. Tissue-free amino acids were extracted from the frozen heart powder using 2 vol perchloric acid, and the extract was neutralized with potassium hydroxide. Aminoacyl-tRNA was extracted and deacetylated as previously described.\textsuperscript{21} The specific activity of phenylalanine in plasma, heart tRNA, and acid extracts of the frozen heart powder was determined using the $^{14}$C-dansyl chloride derivatization procedure.\textsuperscript{21}

Statistical Analysis

Values for grouped data are expressed as mean±SEM. Statistical comparisons were made using a paired $t$ test for grouped data in both the dog and rat studies.\textsuperscript{22}

Results

Group 1

Myocardial net amino acid balance and tracer flux. The coronary sinus plasma flow in these dogs averaged 31±3 ml/min, and the blood flow averaged 52±3 ml/min. There was a significant net uptake of the branched chain amino acids ($p<0.02$) and serine ($p<0.05$) (Figure 1). As we have previously observed, there is a net release of alanine and glutamine by the canine heart.\textsuperscript{18} The net balance for phenylalanine and the remaining amino acids assayed was near zero.

Over the 30-minute sampling period, the concentration and specific activity of phenylalanine in arterial plasma varied by less than 5%, indicating the presence of a steady state (see Figure 2). Consequently, the mean value for the four measurements of phenylalanine or leucine concentration and specific activity is used in all calculations (Table 1).

Despite the near-zero net balance of unlabeled phenylalanine, the simultaneously measured extraction ratio for uptake of $[^{3}$H]phenylalanine averaged $14±2\%$ ($p<0.01$) (see Table 1). Furthermore, we observed that the $[^{3}$H]phenylalanine specific activity was higher ($169±40$ dpm/nmol) in the artery than in the coronary sinus ($144±32$ dpm/nmol, $p<0.025$). The dilution of the arterial $[^{3}$H]phenylalanine specific activity across the coronary bed measured $10.6±2\%$, which was significantly different from zero.

Figure 1. Bar graph showing arterial–coronary sinus (ART–CS) concentration differences for seven selected amino acids (n=9 dogs). *Amino acids for which significant net uptake was observed. Error bars indicate SEM.

Figure 2. Graphs showing arterial–coronary sinus concentration differences for phenylalanine ([Phe]), $[^{3}$H]phenylalanine ([3-H]-Phe), and specific activity during the final 30 minutes of a 90-minute infusion period in nine dogs. Error bars indicate SEM.
Table 1. Phenylalanine Exchange

<table>
<thead>
<tr>
<th>Dog</th>
<th>[PHE]a (nmol/ml)</th>
<th>[PHE]a (nmol/ml)</th>
<th>DPMa (dpm/ml)</th>
<th>DPMa (dpm/ml)</th>
<th>SAa (dpm/nmol)</th>
<th>SAa (dpm/nmol)</th>
<th>Flow (ml/min)</th>
<th>Deg (nmol PHE/g/hr)</th>
<th>Syn (nmol PHE/g/hr)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>51</td>
<td>13,543</td>
<td>11,954</td>
<td>282</td>
<td>234</td>
<td>21</td>
<td>264</td>
<td>183</td>
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<td>2</td>
<td>70</td>
<td>68</td>
<td>16,563</td>
<td>14,470</td>
<td>237</td>
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<td>14,575</td>
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<td>196</td>
<td>157</td>
<td>29</td>
<td>180</td>
<td>207</td>
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<td>5</td>
<td>71</td>
<td>71</td>
<td>12,387</td>
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<td>174</td>
<td>160</td>
<td>25</td>
<td>176</td>
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<td>6</td>
<td>52</td>
<td>46</td>
<td>1,300</td>
<td>1,052</td>
<td>25</td>
<td>23</td>
<td>29</td>
<td>131</td>
<td>293</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>52</td>
<td>3,300</td>
<td>3,181</td>
<td>66</td>
<td>61</td>
<td>29</td>
<td>107</td>
<td>53</td>
</tr>
<tr>
<td>8</td>
<td>46</td>
<td>45</td>
<td>4,951</td>
<td>4,585</td>
<td>108</td>
<td>102</td>
<td>37</td>
<td>71</td>
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<tr>
<td>9</td>
<td>57</td>
<td>51</td>
<td>2,558</td>
<td>2,056</td>
<td>45</td>
<td>40</td>
<td>38</td>
<td>174</td>
<td>336</td>
</tr>
<tr>
<td>Mean</td>
<td>51</td>
<td>50</td>
<td>8,273*</td>
<td>7,125</td>
<td>169†</td>
<td>144</td>
<td>31</td>
<td>174</td>
<td>210</td>
</tr>
<tr>
<td>SEM</td>
<td>5</td>
<td>5</td>
<td>1,969</td>
<td>1,696</td>
<td>40</td>
<td>32</td>
<td>3</td>
<td>21</td>
<td>31</td>
</tr>
</tbody>
</table>

[p<0.001] (see Table 1). This can only be attributed to the ongoing release of unlabeled phenylalanine from protein breakdown. Using Equation 3, we estimated the rate of heart protein degradation in these anesthetized dogs to be 174±21 nmol phenylalanine/g heart/hr. The estimated rate of protein synthesis obtained using Equation 5 was 210±31 nmol phenylalanine/g/hr, and this was not significantly different from the rate of phenylalanine release.

We compared the kinetics of [14C]phenylalanine flux with those of L-[1-14C]leucine in five dogs (see Table 2). In these dogs, the coronary sinus plasma flow averaged 29±5 ml/min, and blood flow averaged 49±5 ml/min. Both labeled leucine and phenylalanine were extracted by the heart: 22±4% and 15±3% for leucine and phenylalanine, respectively. In the basal state, the specific activity of both leucine (p<0.02) and phenylalanine (p<0.05) was lower in the coronary sinus than in the arterial plasma. The rate of leucine release by heart muscle averaged 362±93 nmol/g/hr (see Table 3). On a molar basis, this was 1.7±0.3 times greater than the release rate of phenylalanine, which measured 213±22 nmol/g/hr. In contrast, the molar uptake of [14C]leucine exceeded that of [3H]phenylalanine by 3.1±0.4-fold (706±156 versus 221±22 nmol/g/hr, p<0.05). The molar composition ratio of leucine to phenylalanine in biopsy acquired heart protein was 2.4±0.1 (n=3).

Group 2

Tissue incorporation of tracer as measured by biopsy.

The direct measurement of [3H]phenylalanine incorporation into muscle was performed in five dogs. We observed an incorporation of 302±55 dpm [3H]phenylalanine/mg myocardial protein and 98±8 dpm/mg skeletal muscle protein at the end of the 90-minute labeled-phenylalanine infusion. By use of the mean arterial specific activity of [3H]phenylalanine (measured over the last 30 minutes of the tracer infusion), the incorporation rate of phenylalanine was estimated.

Table 2. Phenylalanine and Leucine Flux

<table>
<thead>
<tr>
<th>Phenylalanine</th>
<th>Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid concentration (μmol/l)</td>
<td>51±9</td>
</tr>
<tr>
<td>Artery</td>
<td>50±9</td>
</tr>
<tr>
<td>p</td>
<td>NS</td>
</tr>
<tr>
<td>Radioactivity (dpm/ml)</td>
<td>12,523±1,924</td>
</tr>
<tr>
<td>Artery</td>
<td>10,703±1,740</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Extraction ratio (%)</td>
<td>15±3</td>
</tr>
<tr>
<td>Specific activity (dpm/nmol)</td>
<td>257±38</td>
</tr>
<tr>
<td>Artery</td>
<td>217±27</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are mean±SEM; n=5 dogs. Values of p denote significant differences between arterial and coronary sinus values by paired t test.
Table 3. Uptake and Release of Phenylalanine and Leucine

<table>
<thead>
<tr>
<th></th>
<th>Uptake (nmol/g heart muscle/hr)</th>
<th>Release (nmol/g heart muscle/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>221±22</td>
<td>213±22</td>
</tr>
<tr>
<td>Leucine</td>
<td>706±156</td>
<td>362±93*</td>
</tr>
<tr>
<td>Leucine/phenylalanine ratio</td>
<td>3.1±0.4</td>
<td>1.7±0.3</td>
</tr>
</tbody>
</table>

Values are mean±SEM; n=5 dogs. Amino acid uptake was calculated from the product: extraction ratio×arterial amino acid concentration×plasma flow. Amino acid release was calculated from the difference between uptake and balance. Both are normalized per gram of tissue.

*p<0.02 compared with leucine uptake by paired t test.

Leucine was calculated to be 151±39 nmol/g/hr in heart (see Equation 7) and 47±9 nmol/g/hr in skeletal muscle.

In the same dogs, [14C]leucine incorporation into heart muscle measured 52±9 dpm/mg protein over the 180-minute leucine tracer infusion. When the mean arterial plasma leucine specific activity over the last 30 minutes of the leucine infusion was used, the molar incorporation of leucine exceeded that of phenylalanine by 1.6±0.1-fold (222±42 versus 151±39 nmol/g/hr for leucine and phenylalanine, respectively). Hind limb muscle contained very little labeled leucine (~10 dpm/mg). Consequently, no reliable estimate of hind limb leucine incorporation could be obtained.

Skeletal muscle amino acid balance and tracer flux.

In the five group 2 dogs, we assessed overall amino acid balance as well as the isotope dilution of [14C]leucine and [3H]phenylalanine in skeletal muscle. The hind limb plasma flow averaged 29±2 ml/min, and blood flow averaged 49±2 ml/min. In contrast to the findings in heart muscle, there was a net release of many amino acids by the canine hind limb with alanine, glutamine, and glycine predominating (p<0.001, p<0.01, and p<0.01, for their respective arteriovenous concentration differences).

Despite the net release of leucine and phenylalanine from skeletal muscle, a significant extraction of both [14C]leucine (18±3%, p<0.001) and [3H]phenylalanine (16±5%, p<0.05) was observed. Both tracers demonstrated a significant dilution of their specific activity within the venous effluent of the hind limb (p<0.001 and p<0.05 for leucine and phenylalanine, respectively) due to the release of unlabeled amino acids from tissue protein degradation. As we had observed in the myocardium, the relative rates of molar uptake and release for leucine were greater than for phenylalanine in the hind limb. Leucine uptake in skeletal muscle exceeded that of phenylalanine by 2.6±0.6-fold (24±5 versus 11±3 nmol/g/hr for leucine and phenylalanine, respectively). Leucine release exceeded that of phenylalanine by 1.5±0.2-fold (31±5 versus 22±3 nmol/g/hr).

Additional Studies

Measurements of myocardial phenylalanyl-tRNA. In the six rats infused with tritiated phenylalanine, the specific activity of phenylalanine in the arterial plasma averaged 241±10 dpm/pmol, or 1,000-fold greater than in the experimental dogs, reflecting the much higher tracer infusion rate. The specific activity of femoral venous phenylalanine measured 157±6 dpm/pmol 30 minutes into the infusion. This was very close to that of heart phenylalanyl-tRNA (162±8 dpm/pmol) and free heart tissue phenylalanine (144±10 dpm/pmol) at 30 minutes. Since we cannot selectively sample coronary sinus blood in the intact rat to measure the venous specific activity of phenylalanine, we assume that (as in the dog) it is intermediate between that of the artery and the femoral vein. These findings suggest that phenylalanine, like leucine, equilibrates very rapidly and nearly completely between the plasma and tissue pools in the heart in vivo.

HPLC analysis. These findings suggested that the phenylalanine isotope dilution method, previously developed for the measurement of skeletal muscle protein turnover, could also be applied to the heart. An intuitive concern with this approach would be whether the heart’s high blood flow would limit the applicability of the method for assessing the effects of interventions that might alter heart protein metabolism. Indeed, the specific activity of phenylalanine was diluted only 10.6% in coursing through the coronary circulation (Table 1), which is clearly less than the 38% decrement seen across the canine hind limb or the 35% decrement across human forearm skeletal muscle. The percent dilution of phenylalanine specific activity in the heart approximates the coefficient of variation for the measurement of phenylalanine specific activity using the ion-exchange techniques (see “Materials and Methods”) previously applied to skeletal muscle. Although the use of multiple (typically quadruplicate) paired arterial and coronary sinus samples can compensate, at least in part, for the limited precision, improved analytic capability is clearly desirable.

To approach this problem, we established an HPLC method to measure phenylalanine specific activity. Two milliliters of plasma is first eluted through the Dowex column as described (see “Materials and Methods”). The ammonium hydroxide eluate is dried and reconstituted in 2% trichloroacetic acid, and one quarter of the volume (0.2 ml) is applied to a reverse-phase column (Ultrasphere ODS [C-18, 5 μm], Beckman Instruments, Inc., Fullerton, Calif.) and eluted isocratically with a mobile phase consisting of 16% methanol (vol/vol) containing a phosphoric acid buffer and heptane sulfonic acid as an ion pairing agent (Low UV-PIC B7, Waters Associates, Milford, Mass.). The column eluate was monitored for ultraviolet absorbance at 214 nm, and a sharply separated phenylalanine peak is eluted in 14–16 minutes. This fraction was collected into scintillation vials and subsequently counted for [3H]radioactivity. The mass of phenylalanine in each sample was calculated by comparing its peak area with that of prepared standards using a chromatog-
The results reported in this investigation indicate that, when either radiolabeled phenylalanine or leucine is infused into the systemic circulation of the postabsorptive dog, one can measure the extraction of tracer and dilution of its specific activity within the coronary circulation. Since phenylalanine has only one metabolic fate in heart muscle, that is, incorporation into newly synthesized protein or release from protein degradation, the extraction of \(^{3}\text{H}\)phenylalanine and dilution of its specific activity must be proportional to the rates of these respective processes at steady state. By measuring these quantities with coronary sinus catheterization, one can obtain information regarding myocardial protein turnover that cannot be derived from the measurement of amino acid balance alone.

In order for the measured kinetics of phenylalanine tracer exchange across the myocardium to accurately reflect protein turnover, several criteria must be fulfilled. First, measurements should be made at a steady state, that is, at a time when the size of the myocardial free pool of both labeled and unlabeled phenylalanine is not changing. Only then does the extraction of tracer from plasma reflect its incorporation into muscle protein. Our measurements were made when the plasma phenylalanine concentration and specific activity were constant (see Figure 2). Since the heart does not synthesize phenylalanine de novo or actively accumulate phenylalanine and because the flux of phenylalanine into the heart is rapid (see Table 3), both the mass and the radioactive content present in the heart phenylalanine pool should have been constant by the time sampling began, 60 minutes into the infusion.

The second necessary condition is that there be no recycling of tracer from the turnover of short-lived proteins. If this were to occur, a systematic underestimation of both protein synthetic and degradative rates would result. In addition, if such short-lived proteins were differentially enriched with phenylalanine relative to bulk muscle protein, there would be further inaccuracy. However, since muscle protein turnover is slow (with a half-life on the order of days to weeks) and since there is no evidence for phenylalanine enrichment in specific proteins that turn over more rapidly, we expect such effects to be very small during a 90-minute infusion period. Parenthetically, such recycling of tracer would result in essentially the same errors if conventional tissue biopsy techniques are used.

Clearly, the most substantive condition imposed by the equations describing protein turnover (Equations 3 and 5) is that the protein synthetic precursor pool should include phenylalanine, which is equally available from both arterial inflow as well as proteolysis. We emphasize that both the rate of protein synthesis derived using Equation 5 or 6 and the rate of degradation obtained from Equation 3 follow algebraically from this model (see “Appendix”); this is equivalent to assuming that at steady state the specific activity of phenylalanine in the muscle phenylalanyl-tRNA equals that in the muscle venous effluent. This assumption is supported by the finding that during \(^{3}\text{H}\)phenylalanine infusion in vivo there is a close correspondence in the heart between the specific activity of phenylalanyl-tRNA, the phenylalanine in tissue extracts, and the phenylalanine in venous plasma. These results suggest that phenylalanine in the coronary circulation equilibrates very rapidly with the intracellular pool of this amino acid and with the tRNA pool. Leucine has also been reported to rapidly equilibrate between the systemic circulation and the heart leucyl-tRNA pool.

In accord with our in vivo results, when the isolated rat heart is perfused with physiological concentrations of phenylalanine, despite a very negative nitrogen balance that would favor recycling of phenylalanine derived from proteolysis, the specific activity of the phenylalanyl-tRNA pool is rapidly

**Table 4. Phenylalanine Flux Determined by High-Performance Liquid Chromatography**

<table>
<thead>
<tr>
<th>Concentration (μmol/l)</th>
<th>Specific activity (dpm/ml)</th>
<th>Dilution of specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artery</td>
<td>49.1±3.9</td>
<td>66.8±5.1</td>
</tr>
<tr>
<td>Coronary sinus</td>
<td>50.2±3.9*</td>
<td>62.0±4.3*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. The concentrations and specific activities of phenylalanine were measured using high-performance liquid chromatography in arterial and coronary sinus plasma in 10 dogs during a continuous infusion of labeled phenylalanine. *p<0.001 compared with corresponding arterial value by paired t test.

Discussion

The study of myocardial metabolism currently lacks a technique permitting the characterization of amino acid flux and protein turnover in vivo in the intact organism. The results reported in this investigation indicate that, when either radiolabeled phenylalanine or leucine is infused into the systemic circulation of the postabsorptive dog, one can measure the extraction of tracer and dilution of its specific activity within the coronary circulation. Since phenylalanine has only one metabolic fate in heart muscle, that is, incorporation into newly synthesized protein or release from protein degradation, the extraction of \(^{3}\text{H}\)phenylalanine and dilution of its specific activity must be proportional to the rates of these respective processes at steady state. By measuring these quantities with coronary sinus catheterization, one can obtain information regarding myocardial protein turnover that cannot be derived from the measurement of amino acid balance alone.
labeled to within 80–90% of that of the perfusate and remains higher than that of the intracellular compartment. Although we consider our results regarding labeling of phenylalanyl-tRNA in the rat to support the use of phenylalanine kinetics to study heart protein turnover in vivo, we cannot exclude the possibility of species differences with regard to the extent of phenylalanyl-tRNA labeling. Unfortunately, there is currently no practical means of quantifying the specific activity of labeled tRNA in large animals.

In considering the use of leucine versus phenylalanine to quantitate protein turnover, we noted that leucine release was 1.7±0.3-fold greater than that of phenylalanine, slightly less than the molar ratio of these two amino acids within heart protein (2.4±0.3). Similarly, the uptake of leucine by the heart exceeded that of phenylalanine 3.1±0.4-fold. These small differences can be explained most readily by the observation that in the heart, leucine, unlike phenylalanine, has several metabolic fates other than incorporation into or release from protein. No attempt was made to quantify the oxidation of leucine to $^{14}$CO$_2$ or to measure the release of α-ketoisocaproic acid in the current study. The net positive balance for leucine and the other branched chain amino acids, in the absence of net uptake of the other essential amino acids, argues that these pathways were contributing to leucine disposal.

Indeed, we have recently observed in studies in humans that the myocardium removes both the branched chain amino acids and branched chain keto acids in excess of the uptake of other essential and nonessential amino acids, consistent with significant oxidation of these compounds.

From the kinetic data obtained in the dogs studied using the HPLC analytic methods and the known molar contribution of phenylalanine to bulk heart protein (290 nmol/mg), we calculated an average degradation rate for heart protein of 0.44 mg/g wet wt/hr. The rate of proteolysis was significantly greater than the estimated rate of protein synthesis in these same dogs (0.30 mg/g wet wt/hr). This indicates that heart muscle, like skeletal muscle, in the overnight-fasted dog, is in negative protein balance.

The average rate of heart protein degradation in the dogs studied using the HPLC techniques was lower than in the studies performed using ion-exchange methods. This is in part due to the greater precision of the measurement but likely also reflects biological differences among the groups of dogs studied. This can be appreciated from the range of values of protein degradation seen in both group 1 (Table 1) and in the dogs studied using HPLC methods (51–169 nmol phenylalanine/g wet wt/hr). The observed interanimal variation in basel rates of heart protein turnover would complicate efforts to assess the effects of interventions on heart protein dynamics when comparisons are made across groups receiving different treatments, as is required for techniques using tissue sampling. However, since the catheter-based technique we describe here allows measurement of protein turnover in the same dog before and after an intervention, the effects of inter-animal variation are diminished. We have taken advantage of this in recent in vivo studies demonstrating an inhibitory effect of insulin and amino acid infusion on myocardial protein degradation in the dog and a stimulatory effect of branched chain amino acid infusion on heart protein synthesis in patients with stable coronary artery disease.

The rates of myocardial protein synthesis obtained using tissue biopsies (0.53±0.14 mg/g wet wt/hr [group 2]) are similar to those previously reported for the dog by Everett et al., who used a continuous infusion of $[^{14}$C]tyrosine. In both that and the current study, the data suggest that heart protein has a half-life of 6–7 days in the dog. By use of the values for tissue proteolysis obtained in the dogs studied using the HPLC method, the tissue protein half-life averaged 7.6 days. These values are considerably slower than tissue protein half-life values observed in the growing rat (2.5 days) and comparable with values reported in the rabbit (6.3 days) and the pig (7.4 days).

It is noteworthy that in the current study the rate of heart muscle protein synthesis, as estimated from the incorporation of tracer into tissue, is several times greater than the rate of tracer incorporation by resting skeletal muscle (0.53 versus 0.17 mg/g wet wt/hr). This is consistent with previous in vivo studies suggesting a more rapid rate of protein synthesis by heart than skeletal muscle. However, some caution should be exercised in considering the absolute extent of this difference since the rate of equilibration of tracer specific activity between skeletal muscle phenylalanyl-tRNA and plasma phenylalanine is not known. Significant intracellular dilution of phenylalanine specific activity in skeletal muscle would lessen the observed difference in synthetic rate when compared with heart.

In the current study, particular consideration was given to the method of normalizing tracer exchange measurements to myocardial mass, so as to permit the comparison of our results with those of others. Rates of protein synthesis and degradation are generally normalized to tissue mass. However, depending on the position of the coronary sinus catheter and the particular venous drainage pattern into the coronary sinus, varying amounts of myocardium contribute to the measured tracer flux in each animal. We attempted to overcome this limitation by approximating the sampled myocardial mass from measurements of coronary blood flow combined with estimates of flow per gram heart weight in similar anesthetized normal dogs. Alternatively, one could obtain a measurement of left ventricular mass, for example, from echocardiographic measurements, and correct it by some estimate of the fraction of the left ventricle that the coronary sinus catheter was sampling. Although both of these approaches have some degree of imprecision associated with them, this is of
less concern in studies in which protein turnover is measured before and after an intervention in the same animal or patient while catheter position (and therefore the amount of muscle contributing to the isotopic exchange) remains constant.13

In conclusion, we have demonstrated that canine myocardial protein turnover can be assessed in vivo by employing an amino acid isotope dilution technique and cardiac catheterization. The rates of heart protein synthesis and degradation estimated with this method are reproducible and comparable with those measured in the isolated heart and in vivo studies using conventional tissue biopsy techniques. With the relatively simple method described in the current investigation, one may now perform serial in vivo studies of the determinants of protein synthesis and degradation in the mammalian heart, both in normal and pathological states.

The small amount of tracer needed to demonstrate tissue uptake of amino acid should permit the safe implementation of this method in the clinical investigation of protein turnover in humans. Through further study of phenylalanine tracer kinetics in vivo, it may also be possible to develop a metabolic tracer for the noninvasive imaging of regional myocardial protein metabolism using positron emission tomography. Prior studies31,32 using leucine and glutamate tracers for positron imaging of the heart were hampered by the multiple metabolic fates of these amino acids and the short half-lives of 14C and 15N isotopes. As the current studies suggest, the limited metabolic fate of phenylalanine makes it a more logical choice for following the protein synthetic pathway, and a positron-emitting phenylalanine tracer, such as [15F]phenylalanine,33,34 with a limited metabolic fate and a longer half-life, might permit the noninvasive assessment of myocardial protein turnover and tissue viability.

Appendix

Derivation of Protein Degradation Expression

The derivation of the expression describing the rate of protein degradation (Equation 3) begins by defining the specific activity of phenylalanine (disintegrations per minute per nanomole) within the coronary sinus. Phenylalanine within the coronary sinus (both tracer and tracee) originates from both unextracted arterial inflow and the unlabeled, unre- cycled phenylalanine that is released into the venous outflow from protein degradation. As such, the arterial contribution (Aa) of unlabeled phenylalanine in the coronary sinus (expressed as nanomoles per gram per minute) can be defined as

\[ A_{a} = [\text{PHE}]_{a} \times F \times (1-ER) \]  

(A1)

where \([\text{PHE}]_{a}\) is the arterial concentration of phenylalanine in nanomoles per milliliter, \(F\) equals plasma flow in milliliters per gram per minute, and \(ER\) is the muscle extraction ratio for labeled phenylalanine (see Equation 2). Similarly, the muscle contribution (\(M_{out}\)) of unlabeled phenylalanine (expressed as nanomoles per gram per minute) to coronary sinus effluent can be defined as

\[ M_{out} = D - (D \times ER) \]

or

\[ M_{out} = D \times (1-ER) \]  

(A2)

where \(D\) represents the rate of total myocardial protein degradation in terms of unlabeled phenylalanine release (nanomoles per gram per minute) and \(D \times ER\) represents the amount of released phenylalanine that is immediately reincorporated into protein and never reaches the coronary sinus. (This expression assumes that a quantity of the released phenylalanine, \(D \times ER\), is reincorporated into protein with the same efficiency as phenylalanine entering muscle from arterial inflow.)

The total release of unlabeled phenylalanine into the coronary sinus (Flux of \(\text{PHE}_{cs}\)) in nanomoles per gram per minute can therefore be expressed as

\[ \text{Flux of } \text{PHE}_{cs} = A_{out} + M_{out} \]

Substituting Equations A1 and A2 gives

\[ \text{Flux of } \text{PHE}_{cs} = [\text{PHE}]_{a} \times F \times (1-ER) + D(1-ER) \]  

(A3)

Equation A3 simplifies to

\[ \text{Flux of } \text{PHE}_{cs} = ([\text{PHE}]_{a} \times F + D) \times (1-ER) \]  

(A4)

The flux of radioactivity into the coronary sinus (\(\text{DPM}_{cs}\)) (in disintegrations per minute per gram per minute) is the tracer that was not extracted from the arterial inflow, or

\[ \text{Flux of } \text{DPM}_{cs} = DPM_{a} \times F \times (1-ER) \]  

(A5)

where \(DPM_{a}\) represents \([\text{H}]\text{phenylalanine in disintegrations per minute per milliliter. The basis for our assumption that there is no release of tracer into the coronary sinus from protein breakdown during the relatively short duration of our sampling is explained in the discussion above.}

The specific activity of phenylalanine in the coronary sinus (\(SA_{cs}\)) is simply the flux of tracer into the coronary sinus divided by the flux of the tracee, or Equation A5 divided by Equation A4:

\[ SA_{cs} = (DPM_{a} \times F) / (\text{DPM}_{a} \times F + D) \]

Since \(SA_{s} = DPM_{a} / [\text{PHE}]_{a}\) and \(DPM_{a} = SA_{s} \times [\text{PHE}]_{a}\), one can solve for the rate of degradation (D) by substitution and algebraic rearrangement to give Equation 3:

\[ D = [(SA_{s}/SA_{cs}) - 1] \times [\text{PHE}]_{a} \times F \]

Solution for the Protein Synthetic Rate

The rate of protein synthesis (Equation 4) can be determined by knowing that balance (B) must equal the difference between the rate of synthesis (S) and
degradation (D). Since \( S = B + D \) and \( B = ([\text{PHE}]_a - [\text{PHE}]_c) \times F \) by Equation 1 and \( D = ([\text{SA}_s - \text{SA}_c]) \times F \) by Equation 3, one can substitute for \( B \) and \( D \), so that

\[
S = ([\text{PHE}]_a - [\text{PHE}]_c) \times F + ([\text{SA}_s - \text{SA}_c]) \times [\text{PHE}]_a \times F
\]

By combining terms

\[
S = F \times ([\text{SA}_s - \text{SA}_c] \times [\text{PHE}]_a - \text{SA}_c \times [\text{PHE}]_a + \text{SA}_a \times [\text{PHE}]_a - \text{SA}_c \times [\text{PHE}]_a) / \text{SA}_c
\]

Since \( \text{SA}_a = \text{DPM}_a / [\text{PHE}]_a \) and \( \text{SA}_c = \text{DPM}_c / [\text{PHE}]_c \), one can rearrange so that

\[
\text{SA}_a \times [\text{PHE}]_a = \text{DPM}_a
\]

and

\[
\text{SA}_c \times [\text{PHE}]_c = \text{DPM}_c
\]

By substitution

\[
S = F \times (\text{DPM}_a - \text{DPM}_c) / \text{SA}_c
\]

After cancellation and rearrangement one can solve for synthesis:

\[
S = F \times (\text{DPM}_a - \text{DPM}_c) / \text{SA}_c
\]

In summary, it can be seen that the protein synthetic and degradative rates can be expressed in terms of phenylalanine flux by knowing the flow rate per unit mass, the net balance of unlabeled phenylalanine, the extraction of tracer, and the dilution of its specific activity within the coronary sinus.

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