Meal Feeding Stimulates Phosphorylation of Multiple Effector Proteins Regulating Protein Synthetic Processes in Rat Hearts

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Abstract
Feeding promotes protein synthesis in cardiac muscle through a stimulation of the mRNA translation initiation phase of protein synthesis either secondary to nutrient-induced rises in insulin or because of direct effects of nutrients themselves. The present set of experiments establishes the effects of meal feeding on the potential signal transduction pathways that may be important in accelerating mRNA translation initiation. Hearts were obtained from male Sprague Dawley rats that had been trained to consume a meal consisting of nonpurified diet prior to, during, and following the test meal. Meal feeding raised the extent of phosphorylation of eukaryotic initiation factor 4E binding protein-1 (4EBP1) in the γ-form during feeding. Phosphorylation of mammalian target of rapamycin (mTOR) on Ser2448 or Ser2481 or 70-kDa ribosomal protein S6 kinase (S6K1) on Thr389 was not affected by meal feeding or following removal of food. Likewise, the extent of phosphorylation of TSC2, a potential upstream regulator of mTOR, was not significantly altered during meal feeding. Phosphorylation of protein kinase B (PKB) (Thr308) was elevated at all time points after initiating meal feeding. Similarly, the phosphorylation of protein kinase C (PKC)-ε but not PKC-δ was elevated at all time points after initiating meal feeding. We conclude from these studies that meal feeding stimulates at least 2 signal pathways in cardiac muscle that raises phosphorylation of eIF4G and 4EBP1 during meal feeding and results in sustained increases in phosphorylation of PKB and PKC-ε.

Introduction
The synthesis of skeletal muscle proteins is rapidly stimulated after the oral intake of nutrients (1,2) through an acceleration of mRNA translation initiation. Two major steps in translation initiation can control protein synthesis. The first limiting step is the binding of met-tRNA\textsubscript{met} to the 40S ribosomal subunit to form the 43S preinitiation complex, mediated by eukaryotic initiation factor 2 (eIF2) and regulated by the activity of eIF2B. Meal feeding does not appear to increase formation of 43S preinitiation complex. The second regulatory step involves the recognition, unwinding, and binding of mRNA to the 43S preinitiation complex, catalyzed by a multisubunit complex of eukaryotic factors referred to as eIF4E. In contrast to the formation of 43S preinitiation complex, acute provision of nutrients enhances assembly of active eIF4E (1,3).

The composition of eIF4E is 1) eIF4A (a RNA helicase that unwinds secondary structure in 5′ untranslated region of mRNA), 2) eIF4F [a protein that binds directly to the seven-methyl guanine triphosphate (m\textsuperscript{7}GTP) cap structure present at the 5′-end of most eukaryotic mRNAs], and 3) eIF4G (a protein that functions as a scaffold for eIF4E, eIF4A, and the mRNA and the ribosome). Binding of eIF4G with eIF4E appears important in accelerating mRNA translation initiation. eIF4G appears to be the nucleus around which the initiation complex forms because it has binding sites not only for eIF4E, but also for eIF4A and eIF3 (4). The activity of eIF4E plays a critical role in determining global rates of mRNA translation because essentially all mammalian mRNAs contain the m\textsuperscript{7}GTP cap structure at their 5′-ends.

The eIF4Gs are phosphorylated on residues in the C-terminal region, including Ser\textsuperscript{1108}, and phosphorylation results in a fully active eIF4G (5). Increased phosphorylation of eIF4G on Ser\textsuperscript{1108} is associated with enhanced formation of active eIF4G-eIF4E complex in culture cells (5,6), which leads to an increased rate of protein synthesis. Likewise, elevated phosphorylation of eIF4G correlates with mRNA translation in skeletal muscle during the

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2 Abbreviations used: ECL, enhanced chemiluminescence; eIF, eukaryotic initiation factor; 4EBP1, eIF4E binding protein-1; m\textsuperscript{7}GTP, seven-methyl guanine triphosphate; mTOR, mammalian target of rapamycin; P13, phosphoinositide triphosphate; PKB, protein kinase B; PKC, protein kinase C; PVDF, polyvinylidene fluoride; S6K1, 70-kDa ribosomal protein S6 kinase; TSC, tumor suppressor complex.
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perfusion of rat hindlimb with a buffer containing leucine (7) or following the oral administration of a single bolus of leucine (8).

The availability of elf4E depends, in part, by the translation repressor protein, elf4E binding protein-1 (4EBP1). Hence, 4EBP1 may regulate elf4E-elf4G complex assembly. 4EBP1 is a small protein that, in the hypophosphorylated state, tightly binds elf4E and blocks the ability of elf4E to bind to elf4G, thereby limiting cap-dependent translation. Phosphorylation of 4EBP1 correlates with an increased translational stimulation after treating cells with insulin or growth factors [for a review see (9,10)]. Likewise, the 70-kDa ribosomal protein S6 kinase (S6K1) has been implicated in augmenting the translation of a subset of mRNAs that possess a 5′ tract of oligopyrimidines (5′TOP). S6K1′s multistep activation involves mammalian target of rapamycin (mTOR)- and phosphoinositide (PI3)-dependent kinase-1–dependent ser/thr phosphorylation.

The cellular pathways by which meal feeding modulates protein synthesis are beginning to be elucidated. The meal is composed of several nutrients including carbohydrates and amino acids. Besides providing energy, carbohydrates also serve to enhance insulin secretion. Meal feeding also enhances insulin secretion both through carbohydrate intake and elevation of amino acids that stimulate insulin secretion. Insulin binding to its receptor can stimulate protein kinase B (PKB) through a PI3-kinase–dependent pathway. Amino acids, on the other hand, fail to stimulate PI3-kinase or PKB, which indicates that signaling pathways that become activated by insulin may not be necessary or sufficient for the mediating the effects of amino acids on protein synthesis (3,11–14). Alternatively, amino acids, and leucine in particular, consistently activate S6K1 and 4EBP1 through enhanced phosphorylation using both in vitro (15–17) and in vivo models (1,3,13,14,18). Indeed, structure–activity relations indicate that the leucine was the most potent amino acid in augmenting phosphorylation of 4EBP1 in adipocytes (19) or H4IIE hepatocytes (20) in culture. Phosphorylating S6K1 and 4EBP1 are associated with an acceleration of mRNA translation initiation, leading to a stimulation of protein synthesis. The S6K1 (21) and 4EBP1 (13,14,22) are both phosphorylated by mTOR, suggesting a role for mTOR in mediating, in part, the effects of leucine to phosphorylate these 2 proteins (11).

The purpose of the present set of experiments was to define the temporal relation between changes in phosphorylation of multiple effector proteins regulating protein synthetic processes in cardiac muscle following meal feeding and subsequent removal of food to determine whether there are tissue specific differences in the effects of meal feeding on protein synthesis. We tested the hypothesis that there is an increased phosphorylation of elf4G and 4EBP1 following meal feeding but that it returns to baseline following subsequent removal of food in cardiac muscle.

Materials and Methods

Animals and experimental design. Male Sprague-Dawley rats were purchased from Charles River and maintained at our facility for at least 7 d prior to the start of the meal-feeding protocol. The Penn State University College of Medicine Institutional Animal Care and Use Committee approved the animal protocols used in these studies and the investigations complied with the National Institute for Health Guide for the Care and Use of Laboratory Animals. Rats were caged in pairs rather than singly to reduce anxiety-induced changes in food intake and were adapted to a reverse light cycle (the dark cycle began at 0700 and the light cycle began at 1900) beginning on the eighth day after arrival in the animal quarters. The rats were caged over a period of 12 d to consume a meal when presented. Food (Teklad Diet 8604) was provided in 2 metal food cups for 3 h beginning 30 min after the beginning of the dark cycle as described previously (23,24). Each rat was presented with 17.5 g food/d. This is based on pilot studies showing that rats consume 16.5 g food/d plus there is 1 g/d for spillage from the food containers. All rats receiving the meal were given the same amount and the entire meal was consumed within the 3-h feeding period.

Three separate meal-feeding experiments were performed. On d 12 of the feeding protocol, rats were weighed (304 ± 3.9 g) and anesthetized (Nembutal; 50 mg/kg body wt, i.p.) at 6 different times relative to the start of the meal. For baseline, a group of rats was killed 0.5 h before presentation of the meal (T−0.5h). After the meal was provided, rats were sampled at 0.5, 1, 3, 6, and 9 h after the start of the meal. The heart was excised and immediately frozen between clamps that were precooled to the temperature of liquid nitrogen. Hearts were powdered under liquid nitrogen using a mortar and pestle and the powdered tissue was stored at −85°C until immunoblot analysis of phosphorylation state of regulatory proteins.

Frozen powdered tissue was homogenized in 7 v of buffer A (20 mmol/L HEPES with pH 7.4, 100 mmol/L potassium chloride, 0.2 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L diithiothreitol, 50 mmol/L sodium fluoride, 50 mmol/L β-glycerophosphate, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, 0.5 mmol/L sodium vanadate, and 1 μmol/L microcystin LR) using a Polytron PT 10 homogenizer set at 60% of maximum power. The homogenate was centrifuged at 10,000 × g for 10 min at 4°C and the pellet was discarded. An aliquot of the 10,000 × g supernatant was mixed with an equal volume of 2× Laemmli sodium dodecyl sulfate (SDS) sample buffer (65°C) and then subjected to protein immunoblot analysis. Another aliquot was used to measure the protein concentration by the Biorad method with crystalline bovine serum albumin serving as a standard. A third aliquot was used for immunoprecipitation to determine the association of elf4E with 4EBP1.

Determination of phosphorylation state of elf4G or mTOR. To measure the relative extent of phosphorylation of elf4G and mTOR, proteins in the homogenate were separated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) (3,7,10,24). Following electrophoresis, the proteins transferred to polyvinylidene fluoride (PVDF) membranes (Biorad, PALL). The PVDF membranes were then incubated with either an antibody that recognizes the phosphorylated form of elf4G (Ser1108), mTOR (Ser1838), or mTOR (Ser2448) (Cell Signaling Technology). The blots were then developed using an enhanced chemiluminescence (ECL) Western blotting kit according to the manufacturer's instructions (Amersham Pharmacia Biotech). Films were scanned using a Microtect ScanMaker II scanner equipped with a transparent media adaptor connected to a Macintosh computer. Images obtained were quantitated using NIH Image 1.63 software. Following the development of the immunoblot, the membranes were treated with a solution containing 62.5 mmol/L Tris-HCl (pH 6.7), 100 mmol/L β-mercaptoethanol, and 2% (wt/v) SDS to remove antibodies according to the manufacturer's instructions. The membranes were then immunoblotted with antibodies that recognize elf4G or mTOR independently of their phosphorylation state (Bethyl Laboratories). The blots were developed using ECL (Amersham Pharmacia Biotech) and the autoradiographs were scanned and analyzed as described above. The phosphorylated elf4G or mTOR signal densities were normalized to the respective total elf4G or mTOR signal to reflect the relative ratio of phosphorylated elf4G or mTOR to total elf4G or mTOR, respectively.

Quantification of elf4E 4EBP1 complexes. The association of elf4E with 4EBP1 was determined in cardiac muscle using immunoblot techniques, as previously described in our laboratory (3,7,10,24). 4EBP1-elf4E complexes were immunoprecipitated from aliquots of 10,000 × g supernatants using an anti-elf4E monoclonal antibody (gift of Dr. Leonard Jefferson, Penn State University College of Medicine). The elf4E in the antibody-antigen complex was subjected to PAGE on a 15% polyacrylamide gel for quantifying 4EBP1 and elf4E. Proteins were then electrophoretically transferred to a PVDF membrane (Biorad, PALL). The PVDF membranes were incubated with a mouse anti-human elf4E antibody (gift of Dr. Leonard Jefferson) or a rabbit anti-rat 4EBP1 antibody (Bethyl Laboratories). The blots were quantitated using the NIH Image 1.63 software described above. The abundance of 4EBP1 was normalized to the amount of elf4E in the immunoprecipitate.
**Results**

**Meal feeding–induced phosphorylation of eIF4G.** Increased phosphorylation of eIF4G on Ser\(^{1108}\) is associated with enhanced translation of mRNA (5). We examined the phosphorylation state of eIF4G in cardiac muscle extracts during meal feeding and following the removal of food. The extent of phosphorylation of eIF4G was significantly increased 2-fold during meal feeding (Table 1). Following withdrawal of the meal, the phosphorylation of eIF4G returned to values not different from those prior to initiating meal feeding.

**Meal feeding increases phosphorylation of 4EBP1.** Increased translation of mRNA occurs with hyperphosphorylation of 4E BP1 (27,28). Therefore, we examined the ability of meal feeding to induce phosphorylation of 4EBP1 to determine whether the decreased assembly of 4EBP1:eIF4E resulted from an increased phosphorylation of 4EBP1. Approximately 11% of the total 4EBP1 was present in the γ-form in myocardium prior to feeding (Table 2). The extent of phosphorylation of 4EBP1 was significantly elevated following meal feeding. The percentage of 4EBP1 present in the γ-form was ~3-4-fold greater in cardiac muscle 0.5, 1, and 3h after the start of the meal. The proportion of 4EBP1 present in the γ-form did not differ between T\(_0.5\), T\(_1\), and T\(_3\). Withdrawal of the meal reduced the percentage of 4EBP1 in the γ-form 50% compared with rats receiving a meal. However, the percentage of 4EBP1 present in the γ-form remained twice that (P < 0.01) at T\(_{0.5}\).

**Meal feeding–induced dissociation of eIF4E from 4EBP1.** Changes in the phosphorylation of 4EBP1 have been associated with a reduction in the association of eIF4E with 4EBP1. The association of eIF4E with 4EBP1 was determined in cardiac muscle following meal feeding by immunoprecipitating eIF4E with a monoclonal antibody followed by immunoblot analysis for eIF4E and 4EBP1. Results were normalized to the total eIF4E in the immunoprecipitate. Meal feeding decreased the assembly of the inactive 4EBP1:eIF4E complex (Table 2). Withdrawal of the meal increased the formation of the inactive 4EBP1:eIF4E complex compared with rats receiving a meal to values not different from those at T\(_{0.5}\).

**S6K1 Phosphorylation.** The S6K1 is a threonine/serine kinase that phosphorylates ribosomal protein S6 and promotes translation of mRNA containing a TOP motif. S6K1 is activated by multisite phosphorylation that results in isoforms exhibiting retarded electrophoretic mobility when subjected to SDS-PAGE comparisons (26). Differences were considered significant when P < 0.05. ANOVA assumes that the data are sampled from groups with equal SD. If the Bartlett test indicated the SD were not equal, the data were transformed by taking the reciprocal of the data and the ANOVA was recalculated by the InStat statistical software package.

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**TABLE 1** Effect of meal feeding on phosphorylation of eIF4G in rat hearts

<table>
<thead>
<tr>
<th>Time after meal feeding, h</th>
<th>eIF4G Phosphorylation, Phospho-eIF4G (Ser(^{1108}))/total eIF4G</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.5</td>
<td>7.2 ± 1.8(^a)</td>
</tr>
<tr>
<td>+0.5</td>
<td>21.4 ± 2.8(^b)</td>
</tr>
<tr>
<td>+1</td>
<td>38.0 ± 11.8(^b)</td>
</tr>
<tr>
<td>+3</td>
<td>16.2 ± 3.0(^b)</td>
</tr>
<tr>
<td>+6</td>
<td>6.1 ± 1.6(^a)</td>
</tr>
<tr>
<td>+9</td>
<td>2.7 ± 0.9(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Values are means ± SEM, n = 8-9.

\(^b\) Means without a common letter differ, P < 0.01.

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Phosphorylation of mTOR. Next, we examined the potential role of mTOR signaling in mediating the effects of meal feeding on 4EBP1 phosphorylation because mTOR is thought to be an upstream kinase responsible for phosphorylating both 4EBP1 and S6K1 (28,32). Phosphorylation of mTOR on residues Ser2448 and Ser2481 has been used to monitor the activity of mTOR (21,33). Therefore, we examined the phosphorylation state of Ser2448 and Ser2481 following meal feeding. Meal feeding did not affect the phosphorylation of mTOR at either Ser2448 or Ser2481 (Table 3). Likewise, phosphorylation of mTOR did not change following withdrawal of the food (T+0.5–T+3h).

Phosphorylation of PKB. PKB, also known as AKT, lies upstream of mTOR (34). PKB is a serine/threonine kinase that is activated by phosphorylation of 2 critical serine/threonine residues (i.e., Thr308 and Ser473)(35). Phosphorylation of Thr308 and Ser473 were coordinately regulated during meal feeding (Table 3). Immediately after the initiation of feeding, the phosphorylation of PKB was 100% greater than at T−0.5. Unlike eIF4G and 4EBP1, the extent of phosphorylation of PKB at both Thr308 and Ser473 remained constant for the remainder of the experiment.

Phosphorylation of TSC2. Several proteins potentially modulate mTOR including the TSC. TSC consists of the gene products associated with the complex, namely, hamartin (TSC1) and tuberin (TSC2) (36,37). The tuberin-hamartin complex is hypothesized to function as an mTOR suppressor, ultimately affecting phosphorylation of S6K1 and 4EBP1. The activity of TSC is regulated through phosphorylation. In vitro studies indicate that TSC2 phosphorylation is dependent upon the PI3K/PKB signaling pathway whereby activated PKB phosphorylates tuberin at residue Thr1462 in culture cells (38–40). In the present set of studies, we examined the phosphorylation of TSC2 (Thr1462) during feeding and following withdrawal of food. The extent of phosphorylation of TSC2 did not change during meal feeding (P > 0.05). In contrast, phosphorylation of TSC2 was lower (P < 0.05) at T+0.5 [Phospho-TSC2 (Thr1462)/Total TSC2] compared with prior to feeding (T−0.5) [14 ± 2 (Phospho-TSC2 (Thr1462)/Total TSC2)].

Phosphorylation of PKC-ε and PKC-δ. Activation of PKC-ε or PKC-δ has been implicated in the stimulation of protein synthesis in cardiomycocytes (41–43) and other cell types (44,45) in culture. Thus, we examined whether meal feeding could modify the level of PKC-ε phosphorylation in vivo. Phosphorylation of PKC-ε was elevated by ~100% relative to T−0.5 within one-half an hour of initiating the meal and remained elevated throughout the remainder of the experiment (Table 4). The various PKC isoforms exhibit common domains required for catalytic activity and regulatory function. Therefore, we determined whether this effect of meal feeding was observed with other novel PKCs by examining the phosphorylation of PKC-δ during feeding and following withdrawal of food. In contrast to PKC-ε, the extent of phosphorylation of PKC-δ was not significantly altered during meal feeding or after the withdrawal of food.

Discussion

Effector molecules controlling translation initiation are important regulators of myocardial protein synthesis. The findings provide additional insight into the processes involved in the stimulation of myocardial protein synthesis by meal feeding through alterations in translation initiation effector proteins. Meal feeding enhanced the phosphorylation of eIF4G (Ser1108) and 4EBP1(γ-form) and a decreased assembly of inactive 4EBP1-eIF4E complex. In contrast, meal feeding did not increase the extent of phosphorylation of S6K1 or mTOR. Phosphorylation of PKB, an upstream kinase suggested to be responsible in part for phosphorylating mTOR, was elevated within one-half an hour of initiating the meal and remained elevated over the duration of the experiment.

### Table 2

<table>
<thead>
<tr>
<th>Time after meal feeding, h</th>
<th>4EBP1 Associated with eIF4E, 4EBP1/eIF4E</th>
<th>4EBP1 Phosphorylation, γ-form/total 4EBP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.5</td>
<td>111 ± 10a</td>
<td>12 ± 1a</td>
</tr>
<tr>
<td>+0.5</td>
<td>57 ± 7a</td>
<td>56 ± 3a</td>
</tr>
<tr>
<td>+1</td>
<td>54 ± 6b</td>
<td>56 ± 2b</td>
</tr>
<tr>
<td>+3</td>
<td>71 ± 8b</td>
<td>47 ± 2b</td>
</tr>
<tr>
<td>+6</td>
<td>125 ± 12b</td>
<td>26 ± 2b</td>
</tr>
<tr>
<td>+9</td>
<td>157 ± 24b</td>
<td>22 ± 3b</td>
</tr>
</tbody>
</table>

ANOVA

- Values are means ± SEM, n = 11–13.
- Means in a row without a common letter differ, P < 0.01.

### Table 3

<table>
<thead>
<tr>
<th>Time after meal feeding, h</th>
<th>PKB (Thr308) Phosphorylation, Phospho-PKB (Thr308)/total PKB</th>
<th>PKB (Ser473) Phosphorylation, Phospho-PKB (Ser473)/total PKB</th>
<th>mTOR (Ser2448) Phosphorylation, Phospho-mTOR (Ser2448)/total mTOR</th>
<th>mTOR(Ser2481) Phosphorylation, Phospho-mTOR (Ser2481)/total mTOR</th>
<th>S6K1(Thr385) Phosphorylation, Phospho-S6K1 (Thr385)/total S6K1</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.5</td>
<td>5.0 ± 0.4a</td>
<td>7 ± 1a</td>
<td>13 ± 1a</td>
<td>11 ± 1a</td>
<td>3.3 ± 0.6a</td>
</tr>
<tr>
<td>+0.5</td>
<td>10 ± 1a</td>
<td>14 ± 1a</td>
<td>15 ± 1a</td>
<td>15 ± 2a</td>
<td>4.1 ± 0.7a</td>
</tr>
<tr>
<td>+1</td>
<td>12 ± 1b</td>
<td>15 ± 1b</td>
<td>15 ± 2a</td>
<td>15 ± 2a</td>
<td>3.3 ± 0.4a</td>
</tr>
<tr>
<td>+3</td>
<td>11 ± 2b</td>
<td>15 ± 3b</td>
<td>15 ± 2a</td>
<td>16 ± 2a</td>
<td>2.6 ± 0.4b</td>
</tr>
<tr>
<td>+6</td>
<td>10 ± 2b</td>
<td>15 ± 3b</td>
<td>15 ± 2a</td>
<td>16 ± 2a</td>
<td>2.5 ± 0.3a</td>
</tr>
<tr>
<td>+9</td>
<td>11 ± 2b</td>
<td>15 ± 3b</td>
<td>15 ± 2a</td>
<td>16 ± 2a</td>
<td>2.9 ± 0.5a</td>
</tr>
</tbody>
</table>

ANOVA

- Values are means ± SEM, n = 10–12.
- Means in a row with superscripts without a common letter differ, P < 0.05.

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conclude from these studies that meal feeding stimulates at least 2 signal pathways in cardiac muscle that raise phosphorylation of eIF4G and 4EBP1 during meal feeding and results in sustained increases in phosphorylation of PKB and PKC-ε. These pathways may be independent of mTOR signaling because the phosphorylation state of neither mTOR nor S6K1 is altered by meal feeding.

Increases in translation initiation in response to refeeding also depend on eIF2B and eIF4E availability for assembly of eIF4F, i.e., the nonphosphorylated isoforms of 4EBPs bind to eIF4E with a high affinity and prevent the assembly of a translational complex catalyzed by eIF2B or in the level of eIF2α phosphorylation in gastrocnemius muscle (46). We also previously reported that neither eIF2B activity nor phosphorylation state of eIF2α was altered following the provision of nutrient components of a meal, yet protein synthesis was stimulated (18). Likewise, protein synthesis appears independent of eIF2B activity when the concentration of leucine is elevated in the isolated perfused hindlimb (47). However, both PKB phosphorylation and the phosphorylation state of eIF2α can promote assembly of the eIF4G-eIF4E complex, which is considered an essential complex in promoting mRNAs for translation.

Phosphorylation of eIF4G has been associated with enhanced rates of mRNA translation in tissues and cells in culture under conditions known to stimulate protein synthesis (5,7). The present study indicates that meal feeding augmented the phosphorylation of eIF4G in cardiac muscle ~100% compared with unfed rats within 30 min of initiating the meal. Furthermore, the extent of phosphorylation remained elevated during the course of the meal. The 5′TOP region has been implicated in the translational regulation of various mRNAs in mammalian cells (33). PKB phosphorylation on residues Ser2448 and Ser2481 has been used to monitor the activity of mTOR (21,33). As such, altered mTOR phospho-

<table>
<thead>
<tr>
<th>PKC-ε Phosphorylation, Phospho-PKC-ε/total PKC-ε</th>
<th>-0.5</th>
<th>+0.5</th>
<th>+1</th>
<th>+3</th>
<th>+6</th>
<th>+9</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
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<td>60 ± 12</td>
<td>120 ± 23</td>
<td>121 ± 14</td>
<td>103 ± 5</td>
<td>103 ± 5</td>
<td>101 ± 2</td>
<td>P &lt; 0.05, F = 2.51</td>
<td></td>
</tr>
</tbody>
</table>

1 Values shown are means ± SEM, n = 6–8.

2 Means in a row with superscripts without a common letter differ, P < 0.05.
of mTOR or S6K1, which is inconsistent with the proposed role of PKB in phosphorylation of mTOR and S6K1 in other tissues (34,51). Likewise, stimulation of PKB phosphorylation cannot account for the enhanced phosphorylation of eIF4G, which remained significantly elevated only during the feeding period. However, the temporal changes in PKB phosphorylation did correspond with those of 4EBP1.

Hamartin and tuberin interact to form a stable TSC. It has been hypothesized that hamartin and tuberin are responsible for the activity of the TSC and function together in the complex to inhibit mTOR-mediated signaling to 4EBP1 and S6K1 in transformed cells in culture (36). TSC2 lacks a kinase domain and, as such, regulates mTOR through indirect mechanisms. Tuberin has a GTPase-activating protein (GAP) domain toward the Ras family small GTPase called Rheb (RAS homolog enriched in brain) (36,39,52,53). Rheb stimulates the phosphorylation of mTOR on Ser1448 in response to stimuli known to enhance mTOR activity, including activation of PKB (39). Tuberin-dependent stimulation of GTP hydrolysis of Rheb blocks phosphorylation of mTOR (Ser2448), which antagonizes the mTOR signaling pathway. Phosphorylation of Thr1462 on TSC2 limits the tuberin/hamartin complex reductions of Rheb-GTPase activity, thereby relieving inhibition of mTOR.

In vitro studies indicate that TSC2 phosphorylation appears to be dependent upon the PI3K/PKB signaling pathway, whereby activated PKB phosphorylates tuberin at residue Thr1462 (38–40). In the present set of studies, we examined the potential role of changes in phosphorylation in tuberin following meal feeding in heart in vivo. Meal feeding did not significantly enhance the phosphorylation of TSC2 despite an increased level of PKB phosphorylation at each time point investigated. This result is consistent with previous reports showing that enhanced PKB phosphorylation was not associated with phosphorylation of TSC2 in cardiac muscle stimulated with IGF-I (25) or skeletal muscle following meal feeding (24).

PKCε is a phosphatidyl serine/diacylglyceride-dependent, calcium-independent PKC isoform that undergoes phosphorylation. PKCε is controlled via phosphorylation at 3 sites in the catalytic domain (Thr298 in the activation loop, Thr710 in the turn motif, and Ser729 in C-terminal hydrophobic motif). Phosphorylation of these sites is required for binding to and activation by diacylglycerol (54,55). Ser729 phosphorylation is mediated by PKC itself via autophosphorylation (56), a protein complex that includes the atypical PKC-ε (57) or a rapamycin-sensitive heterologous kinase (58), implying that PKC phosphorylation lies downstream of mTOR in an amino acid–sensing pathway (58).

The results described herein provide evidence that meal feeding leads to increased phosphorylation of PKC-ε, but not PKC-δ, in cardiac muscle, indicating that the response is the specific effect of meal feeding on the level of PKC-ε phosphorylation. Activating the mTOR signaling pathway above basal conditions does not appear to be necessary to induce phosphorylation of PKC-ε, which suggests that multiple signaling pathways become activated with meal feeding. In the present study, PKC-ε phosphorylation occurred temporally with phosphorylation of PKB following meal feeding, although it remains unclear whether this represents a cause and effect event or merely mutually independent ones.

We have previously described changes in effector proteins involved in translation initiation in skeletal muscle following meal feeding (24). There are several major differences in the response to meal feeding between skeletal and cardiac muscle. First, meal feeding does not elevate the level of S6K1 or mTOR phosphorylation in the heart. Second, the extent of phosphorylation of 4EBP1 in heart tissue remains elevated when food is withdrawn for up to 6 h. Third, the level of PKB phosphorylation on Thr308 and Ser473 remains elevated in cardiac muscle during the entire course of the experiment, including the period after food withdrawal. Fourth, the level of PKC-ε phosphorylation on Ser729 remains elevated in the myocardium during the entire course of the experiment, including the period after food withdrawal. The findings indicate there are cardiac muscle-specific changes in the regulation of translation initiation effector molecules following meal feeding.

**Acknowledgments**

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**Literature Cited**


