Regulation of Protein Kinase B and 4E-BP1 by Oxidative Stress in Cardiac Myocytes

Fong H. Pham, Peter H. Sugden, Angela Clerk

Abstract—Stimulation of phosphatidylinositol 3'-kinase (PI3K) and protein kinase B (PKB) is implicated in the regulation of protein synthesis in various cells. One mechanism involves PI3K/PKB-dependent phosphorylation of 4E-BP1, which dissociates from eIF4E, allowing initiation of translation from the 7-methylGTP cap of mRNAs. We examined the effects of insulin and H₂O₂ on this pathway in neonatal cardiac myocytes. Cardiac myocyte protein synthesis was increased by insulin, but was inhibited by H₂O₂. PI3K inhibitors attenuated basal levels of protein synthesis and inhibited the insulin-induced increase in protein synthesis. Insulin or H₂O₂ increased the phosphorylation (activation) of PKB through PI3K, but, whereas insulin induced a sustained response, the response to H₂O₂ was transient. 4E-BP1 was phosphorylated in unstimulated cells, and 4E-BP1 phosphorylation was increased by insulin. H₂O₂ stimulated dephosphorylation of 4E-BP1 by increasing protein phosphatase (PP1/PP2A) activity. This increased the association of 4E-BP1 with eIF4E, consistent with H₂O₂ inhibition of protein synthesis. The effects of H₂O₂ were sufficient to override the stimulation of protein synthesis and 4E-BP1 phosphorylation induced by insulin. These results indicate that PI3K and PKB are important regulators of protein synthesis in cardiac myocytes, but other factors, including phosphatase activity, modulate the overall response. (Circ Res. 2000;86:1252-1258.)

Key Words: protein synthesis 4E-BP1 protein kinase B oxidative stress cardiac myocytes

Cardiac myocytes are terminally differentiated, withdrawing from the cell cycle in the perinatal period. Subsequent maturational or adaptive (hypertrophic) growth in the heart results from increased cell size. A key feature of hypertrophy is an increased rate of protein synthesis. Insulin and insulin-like growth factor 1 (IGF-1) potently stimulate global cardiac protein synthesis, as do hypertrophic agonists. The phosphatidylinositol 3'-kinase (PI3K) pathway regulates protein synthesis in many cells. Insulin and IGF-1 activate PI3K, which catalyzes the formation of phosphatidylinositol-3,4,5-trisphosphate. This binds to and activates the phosphatidylinositol-3,4,5-trisphosphate-dependent protein kinase B (PKB), which phosphorylates and activates protein kinase B (PKB). PKB phosphorylates and regulates a number of proteins, including the kinase mammalian target of rapamycin (mTOR). PI3K and PKB are potently activated by oxidative stress.

The overall rate of protein synthesis is partly related to the efficiency of translation, which is regulated primarily at the level of initiation of translation. In eukaryotes, a key step is the binding of eIF4F to mRNA molecules with a 5'-terminal 7-methylGTP cap. eIF4F is a complex containing eIF4E, which directly binds to the cap. One mechanism by which PI3K/PKB regulates protein synthesis is through 4E-BP1. Dephosphorylated 4E-BP1 associates with eIF4E to prevent binding to the cap, thus inhibiting formation of the initiation complex. On phosphorylation through the PI3K→PKB→mTOR pathway, 4E-BP1 dissociates from eIF4E, allowing the initiation complex to form and translation to proceed.

The regulation of protein synthesis by PI3K/PKB in the heart is not understood. IGF-1, phenylephrine, and isoproterenol stimulate PI3K in cardiac myocytes, and PI3K inhibitors (wortmannin and LY294002) and rapamycin (which inhibits mTOR) inhibit the stimulation of protein synthesis by hypertrophic agonists. 4E-BP1 is present in the heart, but its signaling to 4E-BP1 has not yet been studied. Here, we have examined the effects of insulin (which promotes growth) and oxidative stress (H₂O₂, which induces cardiac myocyte apoptosis) on the regulation of protein synthesis in cardiac myocytes and examined their effects on PKB and 4E-BP1.

Materials and Methods

Primary Cultures of Myocytes

Myocytes were dissociated from neonatal Sprague-Dawley rat ventricles and plated at 1.4×10⁶ cells/mm². Serum was withdrawn for 24 hours before myocytes were exposed to insulin or H₂O₂ with or without pretreatment (10 minutes) with inhibitors.

Protein Synthesis

Myocytes were incubated with [4-³H]phenylalanine (1 μCi/mL) in the absence or presence of agonists and inhibitors (4 hours).

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Wortmannin was reapplied after 2 hours. Myocytes were washed (PBS, 1 mL, 4°C) and dissolved in NaOH (0.2 mol/L, 1.5 mL), and 1% (wt/vol) BSA (0.5 mL) was added. Protein was precipitated with 5% (wt/vol) trichloroacetic acid (8 mL), and samples were centrifuged. Precipitates were washed (5% [wt/vol] trichloroacetic acid [4×8 mL]) and dissolved in tissue solubilizer before scintillation counting. Experiments were performed in triplicate and the mean taken.

Immunoblotting

Total myocyte extracts were prepared and immunoblotted. Blots were probed with PKB/phospho-PKB antibodies (New England Biolabs, 1/1000), 4E-BP1 antibodies (Santa Cruz, 1/100), or eIF-4E antibodies (Becton-Dickinson, 1/1000). Bands were detected by enhanced chemiluminescence, and blots were exposed to Hyperfilm. Antibodies were probed with PKB/phospho-PKB antibodies (New England Biolabs, 1/1000), 4E-BP1 antibodies (Santa Cruz, 1/100), or eIF-4E antibodies (Becton-Dickinson, 1/1000). Total myocyte extracts were prepared and immunoblotted. 31 Blots were probed with antibodies to eIF4E. Pellets were washed, resuspended, and boiled with sample buffer. Samples were analyzed by immunoblotting with antibodies to eIF4E.

Immunoprecipitation of 4E-BP1 and eIF4E

Myocytes were extracted as for JNK1 immunoprecipitation.31 For 4E-BP1, supernatants were incubated with 0.8 µg of antibody and protein G-Sepharose. For the association of eIF4E with 7-methylGTP–protein G-Sepharose (30 µL of a 50% slurry; 2 hours, 4°C), Samples were centrifuged and supernatants boiled with 0.33 vol sample buffer. 31 Pellets were washed, resuspended, and boiled with sample buffer. Samples were analyzed by immunoblotting with antibodies to eIF4E.

Results

Regulation of Protein Synthesis

Insulin stimulates protein synthesis in the heart,3,7,8 whereas H2 O2 promotes cardiac myocyte apoptosis,27–29 inducing terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL) positivity in 8 to 12 hours. 29 This effect is concentration dependent, and virtually all myocytes are TUNEL positive within 24 hours at concentrations >0.5 mmol/L. We compared the effects of H2O2 on protein synthesis in cardiac myocytes with the response to insulin and examined whether global protein synthesis in cardiac myocytes requires PI3K. Protein synthesis was linear over 5 hours (results not shown), and subsequent experiments were performed over 4 hours. PI3K inhibitors (LY294002, 50 µmol/L; wortmannin, 1 µmol/L) or rapamycin (1 µmol/L) inhibited basal protein synthesis (36%, 20%, and 17% inhibition, respectively, versus controls [n = 3]) (Table), suggesting that tonic PI3K activity contributes to protein synthesis in unstimulated cells. Insulin (50 µmol/mL) increased protein synthesis in cardiac myocytes (23% stimulation versus controls [n = 7]). In myocytes exposed to insulin in the presence of LY294002 or wortmannin, protein synthesis was reduced below basal levels (41% and 17% inhibition, respectively, versus controls [n = 4]), indicating that insulin-stimulated protein synthesis also required PI3K activity. As expected, given the effects of H2O2 on apoptosis, protein synthesis in cardiac myocytes exposed to H2O2 (1 mmol/L) was substantially inhibited (90% inhibition versus controls [n = 3]). However, H2O2 also inhibited protein synthesis in the presence of insulin (92% inhibition versus controls [n = 3]), indicating that toxic effects of oxidative stress dominate over any protective effects of insulin.

Cardiac Myocyte Protein Synthesis Is Regulated by PI3K and Inhibited by H2O2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mean±SEM</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>22 052±1948</td>
</tr>
<tr>
<td>LY294002</td>
<td>14 005±1061*</td>
</tr>
<tr>
<td>Insulin</td>
<td>25 538±1443*</td>
</tr>
<tr>
<td>LY294002+insulin</td>
<td>12 441±1527†</td>
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</table>

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23 368±1299</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>20 186±1103*</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>20 970±546*</td>
</tr>
<tr>
<td>H2O2</td>
<td>2426±875†</td>
</tr>
<tr>
<td>Insulin</td>
<td>31 545±1294*</td>
</tr>
<tr>
<td>Wortmannin+insulin</td>
<td>21 155±1336†</td>
</tr>
<tr>
<td>Rapamycin+insulin</td>
<td>24 057±814†</td>
</tr>
<tr>
<td>H2O2+insulin</td>
<td>2088±513‡</td>
</tr>
</tbody>
</table>

Myocytes were unstimulated (Control); exposed to 50 µmol/L LY294002, 1 mmol/L wortmannin, 1 µmol/L rapamycin, or 1 mmol/L H2O2 alone; or exposed to 50 µmol/L insulin in the absence or presence of inhibitor or H2O2. Results are in dpm and are mean±SEM for experiments on 3 preparations of cardiac myocytes. Experiment sets were independent. *P < 0.05 relative to controls. †P < 0.01 relative to insulin alone. ‡P < 0.001 relative to controls.

Stimulation of PKB(Ser473) Phosphorylation

Because PKB is implicated in the regulation of protein synthesis in other cells, we examined its phosphorylation in myocytes. PKBα is activated by phosphorylation of 2 residues, Thr308 and Ser473.12,13 There was no change in total PKB in any experiments in this study. As expected, insulin stimulated a large increase in PKB(Ser473) phosphorylation (Figure 1A). This was maximal within 5 to 10 minutes and sustained over at least 60 minutes. H2O2 increased PKB phosphorylation to a similar degree as insulin (Figure 1B). This response was also maximal within 5 to 10 minutes, but declined to basal levels within 30 to 45 minutes. Results are presented for phosphorylation of Ser473, but antibodies selective for phosphorylated Thr308 indicated that this site was also phosphorylated in myocytes exposed to insulin or H2O2 (results not shown). LY294002 suppressed basal PKB phosphorylation and the stimulation of phosphorylation by insulin or H2O2 (Figure 1C). Similar results were obtained with wortmannin (results not shown). These data are consistent with a role for tonic PI3K/PKB activity in the regulation of protein synthesis in unstimulated cells and after insulin stimulation (Table), but they also indicate that both insulin and H2O2 increase PI3K activity that is necessary for PKB phosphorylation.

We determined the concentration dependence of H2O2 stimulation of PKB phosphorylation. PKB(Ser473) phosphorylation was increased slightly (relative to unstimulated controls) at 10 to 100 µmol/L H2O2 and was substantially increased at 0.5 to 3 mmol/L H2O2 (Figure 1D).

Regulation of 4E-BP1 Phosphorylation

Because 4E-BP1 regulates the initiation of translation and is a substrate of the PI3K/PKB pathway,15,20 we examined its
phosphorylation. 4E-BP1 is detected as up to 3 bands on immunoblots, commonly designated \( \alpha \), \( \beta \), and \( \gamma \).\(^{32} \) \( \alpha \) has the greatest mobility and represents the least-phosphorylated form; \( \gamma \) is least mobile and represents the most highly phosphorylated form. In unstimulated myocytes, 4E-BP1 was detected as 2 dominant bands of \( \approx 12 \) kDa (Figure 2A), although a trace of a third band of greater mobility was sometimes detected (Figure 2B). By comparison of relative mobilities, the 2 dominant bands were considered to represent the commonly ascribed \( \beta \) and \( \gamma \) 4E-BP1 bands, the minor band representing 4E-BP1\( \alpha \). Thus, 4E-BP1 exists in a pre-dominantly phosphorylated state in unstimulated cells. Insulin further increased 4E-BP1 phosphorylation with an increase in 4E-BP1\( \gamma \) (within 10 minutes) and concomitant disappearance of 4E-BP1\( \beta \) (Figures 2A and 2B). LY294002, rapamycin, or wortmannin reduced 4E-BP1\( \gamma \) and increased 4E-BP1\( \alpha \) and 4E-BP1\( \beta \) (Figures 2B and 2C). These inhibitors also inhibited the insulin-induced increase in 4E-BP1\( \gamma \), with a corresponding increase in 4E-BP1\( \alpha \) and 4E-BP1\( \beta \), indicating that this further stimulation of 4E-BP1 phosphorylation is mediated through PI3K/PKB/mTOR.

In contrast to insulin, \( \text{H}_2\text{O}_2 \) increased the proportions of 4E-BP1\( \alpha \) and 4E-BP1\( \beta \) from \( \approx 10 \) minutes (Figure 3A), suggesting that \( \text{H}_2\text{O}_2 \) increases phosphatase activity to dephosphorylate 4E-BP1 and inhibit protein synthesis. Okadaic acid (6 \( \mu \)mol/L), which inhibits Ser/Thr phosphatases PP1/PP2A, alone had no significant effect on 4E-BP1 phosphorylation, but it inhibited the increase in 4E-BP1\( \alpha \) and 4E-BP1\( \beta \) induced by \( \text{H}_2\text{O}_2 \) (Figure 3B). Similar results were obtained with an alternative PP1/PP2A inhibitor, calyculin A.
suggesting that H$_2$O$_2$ stimulates PP1/PP2A to dephosphorylate 4E-BP1. This presumably overrides the effects of stimulation of the PI3K/PKB pathway by H$_2$O$_2$, because the combination of okadaic acid plus H$_2$O$_2$ increased 4E-BP1$\gamma$ compared with unstimulated cells (Figure 3B). Supporting this, LY294002 inhibited the increase in 4E-BP1$\gamma$ in myocytes exposed to H$_2$O$_2$ in the presence of okadaic acid, illustrating that in the absence of phosphatase activity, H$_2$O$_2$ stimulation of PI3K/PKB increases 4E-BP1 phosphorylation.

We further studied the effects of H$_2$O$_2$ pretreatment on insulin-stimulated PKB and 4E-BP1 phosphorylation to determine whether activation of PP1/PP2A by H$_2$O$_2$ overrides the strong, persistent stimulation of this pathway by insulin and whether the phosphatase effect is mediated at the level of PKB. Myocytes were pretreated with H$_2$O$_2$, the medium was replaced, and the cells were subsequently exposed to insulin. H$_2$O$_2$ pretreatment partially inhibited (50%) insulin-induced PKB phosphorylation on Ser473 (Figure 4A) or Thr308 (results not shown), and PKB phosphorylation was elevated $\approx$3-fold compared with myocytes exposed to H$_2$O$_2$ alone. However, in myocytes exposed to insulin after H$_2$O$_2$ pretreatment, 4E-BP1 was dephosphorylated to the same degree as in myocytes exposed to H$_2$O$_2$ alone (Figure 4B).

**eIF4E Association With 4E-BP1 and Binding to 7-MethylGTP-Sepharose**

We determined whether insulin-induced phosphorylation of 4E-BP1 or H$_2$O$_2$-stimulated dephosphorylation affects trans-
alone had no significant effect on this association. However, insulin increased the association of eIF4E with 7-methyl-GTP-Sepharose, an effect that was abolished by pretreatment with H$_2$O$_2$.

**Discussion**

PKB is a key regulator of many cellular responses, including protein synthesis, gene transcription, and cell survival/proliferation/differentiation. However, there is minimal information on PKB activation in the heart. Here, we demonstrate that insulin (Figure 1A) and oxidative stress (H$_2$O$_2$, Figure 1B) promote PKB phosphorylation in cardiac myocytes. Although this was predictable given that they activate the PI3K/PKB pathway in other cells, the time courses were markedly different: insulin stimulated a sustained response, whereas H$_2$O$_2$ induced a transient response. Stimulation of PKB phosphorylation by either insulin or H$_2$O$_2$ was inhibited by LY294002 (Figure 1C) or wortmannin (results not shown), indicating that (consistent with other studies) PKB phosphorylation is PI3K dependent.

Stimulation of PKB phosphorylation by H$_2$O$_2$ was concentration dependent (Figure 1D) and, at 1 to 3 mmol/L, was comparable with that of insulin. However, 0.5 mmol/L H$_2$O$_2$ induces apoptosis in cardiac myocytes such that virtually all cells are TUNEL positive within 24 hours, and the 1 mmol/L concentration used in this study has similar effects. In this context, therefore, other consequences of oxidative stress (possibly including phosphatase activation, as discussed below) dominate over any cytoprotection through PI3K/PKB. Insulin stimulates PI3K activity after receptor activation, but the mechanisms involved in activation of PI3K by H$_2$O$_2$ are not defined. Because there are multiple isoforms of PI3K, insulin and H$_2$O$_2$ may activate different species. One possible mechanism by which H$_2$O$_2$ may activate PI3K is through inhibition of a tyrosine phosphatase, increasing tyrosine phosphorylation of focal adhesion kinase, which recruits and activates PI3K. Whether this occurs in cardiac myocytes remains to be investigated.

The regulation of gene expression during cardiac hypertrophy has been extensively studied, but less attention has been paid to the necessary increase in protein synthesis. The PI3K/PKB pathway regulates protein synthesis in many cells. PI3K/PKB inhibitors suppressed the basal level of protein synthesis in unstimulated myocytes (Table), indicating that tonic PI3K activity promotes protein synthesis, and suppressed the increase in protein synthesis induced by insulin. However, although H$_2$O$_2$ substantially increased PKB phosphorylation, it almost completely abolished protein synthesis in cardiac myocytes in either the absence or presence of insulin. This is consistent with its apoptotic effects. Because H$_2$O$_2$ inhibited protein synthesis ≈90% over 4 hours, the response is obviously extremely rapid and precedes polyADP-ribose polymerase and caspase-3 proteolysis (2 to 4 hours). It seems probable, therefore, that the inhibition of protein synthesis either causes myocyte apoptosis or constitutes an integral part of the response, rather than being consequent to apoptosis.

One mechanism by which the PI3K/PKB pathway regulates protein synthesis is by increasing 4E-BP1 phosphorylation.
tion, which dissociates from eIF4E, thus promoting translation initiation.\textsuperscript{15} 4E-BP1 is usually detected on immunoblots as 3 bands (\(a\), \(b\), and \(\gamma\)) representing different phosphorylation states.\textsuperscript{32} In unstimulated cardiac myocytes, the 2 dominant bands were 4E-BP1\(b\) and 4E-BP1\(\gamma\), both of which represent the more highly phosphorylated forms (Figures 2 and 3). LY294002, wortmannin, or rapamycin induced a shift from 4E-BP1\(\beta\) to the lesser phosphorylated 4E-BP1\(\alpha\)/\(\beta\) (Figures 2B and 2C), indicating that, in unstimulated myocytes, 4E-BP1 is already phosphorylated, and this phosphorylation requires PI3K activity. This is consistent with tonic PI3K activity promoting the basal level of protein synthesis seen in unstimulated cardiac myocytes (Table). Insulin increased 4E-BP1 phosphorylation (Figure 2A), but (although it might be expected to decrease association with eIF4E) there was no significant change in 4E-BP1 association with eIF4E compared with unstimulated controls (Figure 5A). Because there was minimal association of 4E-BP1 with eIF4E in the unstimulated cells, it seems probable that the degree of phosphorylation of 4E-BP1 in these cells is sufficient to dissociate it from eIF4E, and further phosphorylation of 4E-BP1 has no additional effect on this system. Thus, in this context of an “unstressed” cell, 4E-BP1 binding to eIF4E may not be a principal mechanism for the regulation of translation, and insulin presumably increases protein synthesis through other (potentially PI3K-dependent) mechanisms.

In contrast to insulin, H2O2 promoted dephosphorylation of 4E-BP1 as shown by the increase in 4E-BP1\(\alpha\)/\(\beta\) and disappearance of 4E-BP1\(\gamma\) (Figure 3). This also occurs in hepatocytes subjected to hypoxia, and in various cells (including cardiac myocytes) after heat shock.\textsuperscript{32,33} Dephosphorylation of 4E-BP1 in myocytes exposed to H2O2 correlated with an increase in the association of 4E-BP1 with eIF4E (Figure 5A), presumably inhibiting formation of the initiation complex. It is possible, therefore, that although phosphorylation of 4E-BP1 may not be a key mechanism for the regulation of translation in an “unstressed” situation, dephosphorylation of 4E-BP1 after exposure to cellular stresses may allow the system to inhibit translation rapidly. H2O2 had no significant effect on the association of eIF4E with 7-methylGTP–Sepharose (Figure 5B), but considering the small proportion of eIF4E from unstimulated cells that associates with 7-methylGTP–Sepharose, this is perhaps unsurprising. It is possible that the conditions of this particular assay were not entirely favorable for complex formation, but, alternatively, a second positive signal may be necessary to promote association with the 7-methylGTP cap.

Dephosphorylation of 4E-BP1 may result from inhibition of kinase activity or activation of a phosphatase. Okadaic acid or calyculin A, structurally unrelated inhibitors of the Ser/Thr phosphatases PP1 and PP2A, not only prevented the dephosphorylation of 4E-BP1 induced by H2O2, but increased the proportion of 4E-BP1\(\gamma\), a response that was attenuated by LY294002 (Figure 3B). This suggests that H2O2 increases PP1/PP2A activity, but if this is inhibited, activation of the PI3K/PKB pathway by H2O2 is sufficient to increase 4E-BP1 phosphorylation. Activation of PP1/PP2A by H2O2 also overrides the effects of insulin, given that pretreatment with H2O2 resulted in dephosphorylation of 4E-BP1 by insulin (Figure 4B). Consistent with this, H2O2 inhibited protein synthesis even in the presence of insulin. However, there was a less effect on insulin-induced PKB phosphorylation (Figure 4A), indicating that the effects of the phosphatase activity are not indiscriminate. Our data are not fully consistent with previous studies, in which osmotic stress induces PP1/PP2A activity to suppress PKB activation.\textsuperscript{58} These differences may reflect cell-specific localization of phosphatase activities.

Given that in the presence of okadaic acid, H2O2 induced 4E-BP1 phosphorylation, one might predict that protein synthesis should be maintained or increased under such conditions. However, okadaic acid alone substantially inhibited protein synthesis (results not shown), presumably reflecting the complex regulation of this process. Certain phosphorylations, such as the phosphorylation of 4E-BP1 and of eIF4E itself, promote protein synthesis, but phosphorylation of other translation factors, including the initiation factor eIF2B and the elongation factor eEF2, is inhibitory.\textsuperscript{19} Consequently, although inhibition of phosphatase activity may increase eIF4E binding to the mRNA cap, increased phosphorylation of eIF2B and eEF2 could inhibit protein synthesis. From the results of this study, it is clear that although PI3K and PKB are important regulators of protein synthesis in cardiac myocytes, other factors, including phosphatase activity, modulate the overall response, and it is the integration of multiple signaling pathways that determines the final outcome.

Acknowledgment

This work was funded by the British Heart Foundation.

References


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doi: 10.1161/01.RES.86.12.1252

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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