Phosphorylation of Eukaryotic Translation Initiation Factor 2Bε by Glycogen Synthase Kinase-3β Regulates β-Adrenergic Cardiac Myocyte Hypertrophy

Stefan E. Hardt, Hideharu Tomita, Hugo A. Katus, Junichi Sadoshima

Abstract—Glycogen synthase kinase 3β (GSK-3β) negatively regulates cardiac hypertrophy. A potential target mediating the antihypertrophic effect of GSK-3β is eukaryotic translation initiation factor 2Bε (eIF2Bε). Overexpression of GSK-3β increased the cellular kinase activity toward GST-eIF2Bε in neonatal rat cardiac myocytes, whereas LiCl (10 mmol/L) or isoproterenol (ISO) (10 μmol/L), a treatment known to inhibit GSK-3β, decreased it. Immunoblot analyses using anti-S535 phosphospecific eIF2Bε antibody showed that S535 phosphorylation of endogenous eIF2Bε was decreased by LiCl or ISO, suggesting that GSK-3β is the predominant kinase regulating phosphorylation of eIF2Bε-S535 in cardiac myocytes. Decreases in eIF2Bε-S535 phosphorylation were also observed in a rat model of cardiac hypertrophy in vivo. Overexpression of wild-type eIF2Bε alone moderately increased cell size (+31±11%; P<0.05 versus control), whereas treatment of eIF2Bε-transduced myocytes with LiCl (+73±22% versus eIF2Bε only; P<0.05) or ISO (+84±33% versus eIF2Bε only; P<0.05) enhanced the effect of eIF2Bε. Overexpression of eIF2Bε-S535A, which is not phosphorylated by GSK-3β, increased cell size (+107±35%) as strongly as ISO (+95±25%), and abolished antihypertrophic effects of GSK-3β, indicating that S535 phosphorylation of eIF2Bε critically mediates antihypertrophic effects of GSK-3β. Furthermore, expression of eIF2Bε-F259L, a dominant-negative mutant, inhibited ISO-induced hypertrophy, indicating that eIF2Bε is required for β-adrenergic hypertrophy. Interestingly, expression of eIF2Bε-S535A partially increased cytoskeletal reorganization, whereas it did not increase expression of atrial natriuretic factor gene. These results suggest that GSK-3β is the predominant kinase mediating phosphorylation of eIF2Bε-S535 in cardiac myocytes, which in turn plays an important role in regulating cardiac hypertrophy primarily through protein synthesis. (Circ Res. 2004;94:●●●●●●●●●.)

Key Words: hypertrophy • glycogen synthase kinase 3β • eukaryotic translation initiation factor 2Bε • protein synthesis

Although signaling mechanisms positively mediating cardiac hypertrophy have been intensively investigated, much less is known about the mechanisms negatively regulating cardiac hypertrophy. Recently, the existence of several negative regulators of cardiac hypertrophy has been reported, including SOCS3,1 MCIP1,2 ICER,3 thioredoxin,4 and glycogen synthase kinase-3β (GSK-3β).5–8 GSK-3β is unique among the serine/threonine kinase family because it is active even in unstimulated cells and inhibition of GSK-3β, predominantly caused by serine (S) 9 phosphorylation by upstream kinases, mediates downstream cellular responses, including cardiac hypertrophy5–11 (see review12). GSK-3β regulates a wide range of cellular functions, including metabolism, cell growth and death, gene expression, protein translation, and cytoskeletal integrity in many cell types.13 Thus, it is expected that GSK-3β may affect cardiac hypertrophy through multiple mechanisms. However, the underlying mechanisms by which GSK-3β negatively affects cardiac hypertrophy are not completely understood. Some of these mechanisms may be related to the inhibitory effects of GSK-3β on transcription factors, such as NF-AT and GATA4,5–8 or the transcriptional activator β-catenin.14 These molecules are phosphorylated by GSK-3β and undergo either nuclear exit or proteasome degradation. Although these mechanisms regulate hypertrophy through transcriptional processes, other downstream targets of GSK-3β may also regulate hypertrophy through distinct mechanisms, such as protein translation.

Eukaryotic translation initiation factor 2Bε (eIF2Bε) is an attractive candidate mediating antihypertrophic effects of GSK-3β. Binding of eIF2 to the activated initiator tRNA (met-tRNAMet) and subsequent formation of a complex with
the 40S ribosomal subunit is one of the critical steps controlling initiation of protein translation. eIF2βε is the largest of the five subunits of eIF2B and is required for the GDP/GTP exchange reaction of eIF2. The activity of eIF2βε is negatively regulated by phosphorylation of S540 (corresponding to S535 in rat eIF2βε) by upstream kinases, including GSK-3β in many cell types.15 It has recently been shown that overexpression of a constitutively active mutant of GSK-3β [GSK-3β (S9A)] in neonatal rat cardiac myocytes blocks increases in the rate of protein synthesis by Geq-coupled receptor stimulation.7 Increased cellular activities of GSK-3β also inhibit increases in cell size in response to pressure overload and β-adrenergic stimulation in vivo.6 By contrast, inhibition of GSK-3β by LiCl increases protein synthesis.5 Because all of these observations are consistent with the notion that GSK-3β negatively regulates protein synthesis in cardiac myocytes, we hypothesized that inhibition of protein translation initiation through phosphorylation of eIF2βε is an important mechanism mediating the antihypertrophic effects of GSK-3β. Therefore, in this study we investigated (1) whether GSK-3β is the predominant kinase phosphorylating eIF2βε in cardiac myocytes, and if so, (2) whether or not eIF2βε and its phosphorylation at S535 play an important role in regulating cardiac hypertrophy.

Materials and Methods

Construction of Vectors
The full-length rat eIF2Bε cDNA was obtained by reverse-transcription-PCR (Roche), using total RNA obtained from the rat brain, and subcloned into pcDNA3.1 (Invitrogen). Two eIF2Bε mutants were constructed using a mutagenesis kit (Stratagene). One mutant has a point mutation in the N-terminal region (eIF2Bε-F259L).16 This mutant has reduced intrinsic guanine nucleotide exchange function and acts as a dominant-negative.16 In another mutant, S535 was replaced with alanine (A) (eIF2Bε-S535A), which is the phosphorylation site of GSK-3β in rat eIF2Bε.

Adenoviral Vectors
Adenovirus harboring GSK-3β (AdGSK-3β) has been described.6 Adenovirus harboring LacZ (Ad-LacZ), eIF2Bε-wild-type (Ad-eIF2Bε-WT), eIF2Bε-F259L (Ad-eIF2Bε-F259L), and eIF2Bε-S535A (Ad-eIF2Bε-S535A) were constructed using AdenoX adenovirus construction kit (BD-Clontech).

GST-eIF2Bε Fusion Protein
cDNA encoding the amino acid residues 518 to 716 of eIF2Bε was generated by PCR and subcloned into pGEX-4T-3. Bacterially expressed fusion protein was purified using glutathione-Sepharose.

Primary Culture of Neonatal Rat Ventricular Myocytes
Primary cultures of ventricular cardiac myocytes were prepared from 1-day-old BR-Wistar rats and purified by Percoll centrifugation.3 Culture media were changed to serum-free at 24 hours. Myocytes were cultured in a serum-free condition for 48 hours before experiments. This study was approved by the Institutional Animal Care and Use Committee at New Jersey Medical School.

Phosphorylation of GST-eIF2Bε by GSK-3β
The cells were lysed with 1 mL of the lysis buffer, GSK-3β was immunoprecipitated with anti-mouse GSK-3β antibody (Transduction Labs), and the kinase reaction was performed using 1 μg of GST-eIF2Bε or GST-eIF2Bε-S535A, as described previously.6 Reaction mixtures were subjected to SDS-PAGE (12%). The gels were dried and subjected to autoradiography. Kinase reaction was also performed using whole-cell lysates or cold ATP followed by immunoblot analyses using anti- phospho S535 eIF2Bε antibody (Biosource International).

Immunoblot Analysis
For immunoblotting, cultured cells were lysed with the lysis buffer A, whereas tissue homogenates were prepared with the RIPA buffer (see the online data supplement available at http://circres.ahajournals.org).

Rat Model of Myocardial Infarction (MI)
Male Wistar rats (200 g) were used. Animals were anesthetized by intraperitoneal injection of ketamine (70 mg/kg) and xylazine (2 to 5 mg/kg). After orotracheal intubation, the thorax was opened left parasternally. The left anterior descending artery was ligated just below the left atrial appendage and the quality of the infarction was confirmed visually by the change of the color of the myocardium. The thorax was closed and the animals were extubated. At 24 hours, the animals were anesthetized, and the hearts arrested in diastole by injection of KCl. Immunoblot analyses were performed using tissue homogenates from the remote area.

Myocyte Cell Surface Area and Sarcomeric Organization
Cells were fixed in PBS containing 4% paraformaldehyde. Immunostaining was performed using Texas Red phalloidin (Molecular Probes) and anti-α-sarcomeric actin antibod (Sigma). Myocyte surface area was determined as described.3 Sarcomeric organization was classified as “nonorganized” when punctuated staining with random distribution was present, whereas “organized” indicated well-matured staining forming a clear striated pattern. Myocyte organization was determined to be “partially organized” when a less marked cytoskeletal organization was present with faint striated pattern.

Determination of Cardiac Myocyte Hypertrophy
Total protein/DNA content and [3H]phenylalanine incorporation were determined as described.5

Quantitative PCR
Quantification of transcripts for atrial natriuretic factor (ANF) and α-myosin heavy chain (α-MHC) was performed by real-time PCR using GAPDH as a control.

Statistics
Data are given as mean±SEM. Statistical analyses were performed using ANOVA and the method of Tukey. Significance was accepted at the P<0.05 levels.

An expanded Materials and Methods section is provided in the online data supplement at http://circres.ahajournals.org.

Results
Adenovirus Constructs and Anti-Phospho S535 eIF2Bε-Specific Antibody
We tested whether our adenovirus constructs cause expression of the proteins of interest in cardiac myocytes. Transduction of Ad-GSK-3β induced expression of GSK-3β (6-fold versus untransduced myocytes at a multiplicity of infection (MOI) of 30 (Figure 1A) and increased the GSK-3β kinase activity as determined by immune complex kinase assays (Figure 1B). Transduction of Ad-eIF2ε-WT, Ad-eIF2ε-F259L, or Ad-eIF2ε-S535A led to similar levels of overexpression of eIF2Bε proteins in cardiac myocytes (Figure 1C, bottom). We also tested the specificity of the anti-phospho S535 eIF2Bε antibody. Immunoblot analyses
Figure 1. A, Immunoblot analyses with anti–GSK-3β antibody. Myocytes were transduced with Ad-GSK-3β at various MOI. B, Kinase activity of GSK-3β (dark columns) was determined by the immune complex kinase assay. White columns indicate activities in control immunoprecipitates. Mean of 6 samples in 3 experiments is shown. C, Immunoblot analyses with anti–phospho-S535 eIF2Be antibody (top) and anti-eIF2Be antibody (bottom). In A and C, experiments were conducted 3 times.

indicated that overexpression of eIF2Be-WT and eIF2Be-F259L increased S535 phosphorylation of eIF2Be, whereas that of eIF2Be-S535A failed to enhance phosphorylation of S535 (Figure 1C, top), confirming the specificity of the antibody detecting S535 phosphorylation.

GSK-3β Phosphorylates eIF2Be In Vitro and In Vivo in Cardiac Myocytes

We determined whether interventions known to change the total cellular activity of GSK-3β affect the extent of phosphorylation of eIF2Be in vitro. In order to increase the total cellular activity of GSK-3β, myocytes were transduced with Ad-GSK-3β. To reduce the activity of GSK-3β, myocytes were treated with either isoproterenol (ISO, 10 μmol/L), an agonist for β-adrenergic receptors (β-ARs), for 10 minutes, or LiCl (10 mmol/L), a well-established inhibitor of GSK-3β, for 1 hour. First, kinase assays were performed using myocyte lysates and GST-eIF2Be. Cell extracts prepared from GSK-3β overexpressing myocytes exhibited an enhanced kinase activity against GST-eIF2Be by 2-fold. Transduction of Ad-LacZ did not significantly affect the basal kinase activity (data not shown). By contrast, extracts from ISO- or LiCl-treated myocytes exhibited reduced kinase activities toward GST-eIF2Be by 49% and 51%, respectively (Figure 2A). Coomassie blue staining the SDS page gel with the duplicate samples indicated that protein at comparable levels were loaded (Figure 2A, bottom). These results suggest that cardiac myocyte extracts contain kinase activity toward GST-eIF2Be and that GSK-3β is a predominant kinase phosphorylating GST-eIF2Be in cardiac myocytes at baseline.

In order to further confirm that GSK-3β in the myocyte extract has kinase activities toward GST-eIF2Be, experiments were repeated using immune complex kinase assays. GSK-3β immunoprecipitated from control myocytes phosphorylated GST-eIF2Be, and its activity was enhanced in Ad-GSK-3β-transduced myocytes but reduced in ISO- or LiCl-treated myocytes (Figure 2B). The activity of GSK-3β was not affected by transduction of Ad-LacZ (not shown). Longer treatment with ISO also significantly reduced the GST-eIF2Be kinase activity (Figure 2C). Immunoprecipitates with control antibody [indicated as Ab(–)] did not show kinase activity toward GST-eIF2Be (Figure 2C), confirming the specificity of the immune complex kinase assays.

In order to test if serine 535 is subjected to phosphorylation, additional immune complex GSK-3β kinase assays were conducted using the same cell extracts and GST-eIF2Be-S535A. Only weak background phosphorylation was observed when GST-eIF2Be-S535A was used as a substrate compared with when GST-eIF2Be wild-type (WT) was used, confirming that GSK-3β predominantly phosphorylates S535 of GST-eIF2Be (Figure 2D). Kinase reaction was also performed using cold ATP, and immunoblotting was performed using the anti–phospho-S535 eIF2Be antibody. S535 phosphorylation of eIF2Be was reduced in ISO-treated myocytes, whereas it was enhanced in GSK-3β-transduced myocytes (Figure 2E). These results suggest that GSK-3β phosphorylates S535 of GST-eIF2Be.

In order to further test whether S535 of endogenous eIF2Be is phosphorylated and whether S535 phosphorylation of eIF2Be correlates with cellular activities of GSK3β, immunoblot analyses were performed using anti–phospho-S535 eIF2Be antibody. Although phosphorylation of eIF2Be at S535 was significantly stimulated by overexpression of GSK-3β in cardiac myocytes (Figure 3A), it was significantly decreased by LiCl or ISO treatment, as well as by a specific GSK-3β inhibitor [GSK-3β inhibitor 1 (20 μmol/L); Figure 3B]. Decreases in phosphorylated S535 eIF2Be were observed with both 1 and 10 μmol/L of ISO at 10 minutes and 1 hour (Figure 3C). These interventions did not significantly affect the total amount of eIF2Be, as determined using nonphosphospecific anti-eIF2Be antibody. ISO-induced decreases in S535 phosphorylation of eIF2Be was inhibited in the presence of propranolol, an antagonist for β-ARs, but not
by prazosin, an antagonist for α1-adrenergic receptors (Figure 3D), suggesting that decreases in S535 eIF2B phosphorylation induced by ISO at doses of up to 10 μmol/L are predominantly mediated by β-ARs. Endothelin-1 (100 nmol/L), a Gq-agonist, also transiently reduced phosphorylation of S535 eIF2B (Figure 3E).

We examined whether phosphorylation of S535 eIF2Be is also reduced in an in vivo model of cardiac hypertrophy. To this end, we used the rat model of MI, where significant levels of hypertrophy in the remote area can be observed within 24 to 48 hours.17,18 Tissues obtained from the remote area at 24 hours of MI exhibited significantly lower levels of S535 eIF2B phosphorylation than those from sham animals (Figure 4).

**eIF2Be Plays an Essential Role in Mediating Cardiac Hypertrophy, Although Its Ability to Mediate Cardiac Hypertrophy Negatively Correlates With the Level of S535 Phosphorylation**

To elucidate the role of eIF2Be in cardiac myocyte hypertrophy, we examined the effect of procedures that either increase or decrease S535 phosphorylation of eIF2Be on changes in myocyte size. Treatment of myocytes with ISO or LiCl, which reduces S535 phosphorylation of eIF2Be, increased myocyte size, whereas overexpression of GSK-3β, which increases S535 phosphorylation of eIF2Be, reduced myocyte size at baseline and partially prevented ISO-induced increases in myocyte size (Figure 5A). Treatment with GSK-3β-inhibitor I increased cell size to a similar extent as LiCl, whereas addition of LiCl to ISO did not further increase myocyte size (Figure 5A). Propranolol completely abolished ISO-induced myocyte size, whereas prazosin did not prevent it, confirming that the hypertrophic effects of ISO are mediated primarily by β-ARs.

In order to further dissect the role of eIF2Be and its phosphorylation, we examined the effect of wild-type or mutant eIF2Be overexpression on cardiac hypertrophy at baseline and in response to GSK-3β inhibition. Overexpression of eIF2Be-WT alone modestly increased myocyte size (+31±11%), whereas inhibition of GSK-3β in eIF2Be-overexpressing myocytes by ISO (+84±33% versus eIF2Be-WT only; P<0.05) or LiCl (+73±22% versus eIF2Be-WT only; P<0.05) versus control; Figure 5A).
Figure 3. Immunoblot analyses with anti-phospho-S535 eIF2Bε-specific antibody (top) or anti-total (nonphosphospecific) eIF2Bε antibody (bottom). A, Overexpression of GSK-3β increased, whereas addition of LiCl (10 mmol/L, 1 hour) or ISO (10 μmol/L, 10 minutes) reduced phosphorylation of eIF2Bε. B, Cardiac myocytes were treated with GSK-3β inhibitor I (20 μmol/L) for 1 hour. C, Myocytes were stimulated with ISO (1 or 10 μmol/L) for either 10 minutes or 1 hour. D, Myocytes were stimulated with ISO (10 μmol/L) for 10 minutes in the presence of propranolol (10 μmol/L) or prazosin (10 μmol/L). E, Myocytes were stimulated with endothelin-1 (100 nmol/L) for indicated durations. In C and E, the result shown is representative of 3 experiments. In A, B, and D, the graphs show the mean of 7, 3, and 3 experiments, respectively. In all graphs, *P<0.05 vs Control.
**Figure 4.** Adult male rats were subjected to coronary artery ligation (n=8) or sham operation (n=3). Left ventricular (LV) tissue homogenates from the remote region harvested at 24 hours after MI and those from comparable LV area in sham-operated rats were subjected to immunoblot analyses with anti-phospho-S535 eIF2B and total eIF2B antibody. P<0.01 vs Control.

**Result:**

The phosphorylation of eIF2B at S535 was significantly inhibited both basal and ISO-induced increases in the rate of protein synthesis (Figure 5B), which increases the levels of S535-unphosphorylated eIF2B. We also examined whether inhibition of endogenous eIF2B function affects these cardiac phenotypes in response to β-AR stimulation. Expression of eIF2Bε-F259L significantly reduces the myocyte size at baseline and the ability of eIF2Bε-S535A to mediate cardiac hypertrophy (Figure 6A), whereas increases in the rate of protein synthesis were significantly attenuated in the presence of eIF2Bε-F259L (Figure 7B), consistent with the results of the cell size analyses.

**Discussion**

GSK-3β has recently been identified as an important negative regulator of cardiac hypertrophy, 7,8 but the question remains as to which signaling mechanisms are involved in this process. Considering the versatile functions of GSK-3β, GSK-3β may affect a variety of cellular processes implicated in the development of cardiac hypertrophy, such as transcription, protein translation, and cytoskeletal organization. Thus
far, most studies have focused on the effects of GSK-3β on inhibition of transcription factors or cofactors, such as NFAT, GATA4, and β-catenin, as the major mechanism of inhibition of hypertrophy. In this study, we identify S535 phosphorylation of eIF2β as an important signaling step mediating the antihypertrophic actions of GSK-3β through inhibition of protein translation.

### elf2β Is a Regulator of Protein Translation Initiation in Cardiac Myocytes

Protein synthesis is a complex process involving three essential steps: initiation, elongation, and termination. One of the critical steps controlling initiation of protein translation is the binding of eukaryotic translation initiation factor 2 (eIF2) to the activated initiator tRNA (met-tRNAmet) and subsequent formation of a ternary complex that binds to the 40S ribosomal subunit. This process requires activities of eIF2β in order to stimulate the GDP/GTP exchange reaction of eIF2. GSK-3β phosphorylates human eIF2β at S540 (S535 in the rat sequence) and inactivates it. Thus, eIF2β is an attractive candidate molecule mediating regulation of cardiac hypertrophy by GSK-3β. Our results indicate that GSK-3β is the predominant kinase regulating the phosphorylation status of S535 eIF2β in cardiac myocytes, keeping S535 eIF2β phosphorylated at baseline, and that overexpression of eIF2β S535A is sufficient to stimulate cardiac hypertrophy. Furthermore, expression of eIF2β F259L significantly inhibited β-adrenergic hypertrophy, suggesting that regulation of eIF2β is an essential step for β-adrenergic cardiac hypertrophy. Taken together, our results strongly suggest that inhibition of protein translation initiation through phosphorylation of S535 eIF2β plays an important role in mediating anti hypertrophic effects of GSK-3β, and that its reversal by β-AR stimulation in part mediates increases in protein synthesis in cardiac myocytes. It should be noted that ISO caused a greater increase in cell size than LiCl or GSK-3β inhibitor-I. Thus, additional mechanisms are also involved in β-adrenergic hypertrophy. Although the fact that ISO failed to significantly enhance the effect of eIF2β S535A on hypertrophy at the first view contradicts from this notion, it may be explained by the possibility that the capacity of myocyte to undergo hypertrophy is almost saturated when eIF2β S535A is overexpressed in our experimental conditions.
GSK-3β Regulates S535 Phosphorylation of eIF2Be in Cardiac Myocytes
eIF2Be is phosphorylated by various kinases, including GSK-3β and casein kinases 1 and 2 (CK1 and CK2). Whereas GSK-3β has been shown to be a predominant kinase regulating phosphorylation of S535 of eIF2Be, which renders eIF2Be inactive, CK1 and CK2 stimulate the activity of eIF2Be through phosphorylation at sites distinct from S535. Our results using anti-phospho-S535-specific antibody indicate that phosphorylation of S535 eIF2Be is significantly reduced by GSK-3β inhibitors, including LiCl and GSK-3β-inhibitor I. This result clearly indicates that GSK-3β is a major kinase regulating S535 phosphorylation of endogenous eIF2Be in cardiac myocytes. It has been shown that insulin stimulation, which presumably inhibits the kinase activity of GSK-3β, fails to change eIF2Be phosphorylation in the heart. Although this result seems contradictory to our observation, changes in phosphorylation of S535 were not specifically determined in that study. It is possible that changes in S535 phosphorylation were masked by phosphorylation of other sites by CK1 and CK2 in this previous report.

A recent study showed that reduced phosphorylation of eIF2Be by a GSK-3β inhibitor was not sufficient to increase activity of eIF2Be in CHO cells. In this case, concomitant stimulation of eIF2Be by other kinases may be required to increase the activity of eIF2Be. At present, we do not exclude the role of other eIF2Be kinases, which presumably phosphorylate sites other than S535 and activate eIF2Be, in cardiac myocyte protein synthesis. It should be noted, however, that overexpression of eIF2Be-WT only modestly increased myocyte size and protein synthesis probably because some of the overexpressed eIF2Be is rendered inactive by phosphorylation through GSK-3β (Figure 1C). In fact, our results indicate that phosphorylation of S535 is increased in myocytes overexpressing eIF2Be. This result suggests that basal phosphorylation of eIF2Be by other kinases alone, even if it is present, may not overcome the effect of S535 phosphorylation by GSK-3β. This notion is consistent with

Figure 6. A, Representative immunostaining of myocytes with Texas Red phalloidin. Myocytes with the following treatment are shown: Control (Cont), Ad-eIF2Be-S535A transduction, ISO (10 μmol/L, 48 hours), LiCl (10 mmol/L, 48 hours), Ad-eIF2Be-F259L transduction, eIF2Be-WT transduction, and Ad-eIF2Be-F259L transduction plus ISO (10 μmol/L, 48 hours). B, Greater magnifications depicting the highlighted areas from the panels in A to evaluate sarcomeric organization. Experiments were conducted 5 times. C, Representative immunostaining of myocytes with α-sarcomeric actinin. Texas Red–conjugated anti-mouse IgG antibody was used as secondary antibody. Myocytes with the following treatment are shown: Control (Cont), ISO (10 μmol/L, 48 hours), Ad-eIF2Be-S535A transduction, and Ad-eIF2Be-F259L transduction plus ISO (10 μmol/L, 48 hours). Slides shown are representative of 11 experiments in A and B and 6 experiments in C.
the observation that overexpression of eIF2B\textsubscript{S535A} more strongly promotes myocyte growth than eIF2B\textsubscript{WT}. Thus, in conjunction with the aforementioned results showing that GSK-3\textsubscript{β} is the predominant kinase phosphorylating S535 eIF2B\textsubscript{S535A}, our results are consistent with the notion that S535 phosphorylation of eIF2B by GSK-3\textsubscript{β} critically regulates cardiac protein synthesis.

**eIF2B\textsubscript{S535A} Differentially Regulates Various Hypertrophic Phenotypes in Response to β-Adrenergic Receptor Stimulation**

Although an increase in protein synthesis is one of the most important features of cardiac hypertrophy, it is also accompanied by other phenotypic changes, including activation of the fetal gene program and cytoskeletal reorganization. Our results suggest that eIF2B\textsubscript{S535A} differentially regulates these other aspects of hypertrophy. Overexpression of eIF2B\textsubscript{S535A} strongly increased protein synthesis, protein content, and...
myocyte size, but it did not significantly induce ANF expression. Also, inhibition of eIF2B failed to inhibit ISO-induced upregulation of ANF. This suggests that induction of ANF expression by ISO is mediated through mechanisms distinct from regulation of protein synthesis. In this regard, we have previously shown that phosphorylation and inhibition of GATA4 plays an important role in mediating inhibition of ANF expression by GSK-3β.6

The effect of eIF2B on cytoskeletal organization has not been reported previously. Overexpression of eIF2B-S535A partially induced actin and sarcomere reorganization in cardiac myocytes and dominant-negative eIF2B reduced the proportion of myocytes with well-organized actin and sarcomere in ISO stimulated myocytes. These results suggest that dephosphorylation of S535 eIF2B may contribute to ISO-induced cytoskeletal reorganization, and that activity of eIF2B is required for full sarcomeric reorganization on β-AR stimulation. These findings are consistent with the notion that each phenotype in β-adrenergic cardiac hypertrophy is mediated by distinct mechanisms. The predominant function of eIF2B in β-adrenergic hypertrophy is regulation of cell size through protein synthesis, whereas eIF2B also contributes to cytoskeletal reorganization, but not ANF expression.

In summary, the results of this study show that S535 phosphorylation of eIF2B is an important mechanism for inhibition of cardiac myocyte hypertrophy by GSK-3β. β-Adrenergic inhibition of GSK-3β decreases S535 phosphorylation, which in turn stimulates protein synthesis. eIF2B has been recognized as a key regulatory step in the process of protein translation initiation. Our results suggest eIF2B to be an important regulatory step for initiation of protein synthesis by β-AR stimulation in cardiac myocytes and therefore make it an attractive target to treat cardiac hypertrophy and heart failure.

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Materials and Methods

Materials

GSK-3β inhibitor-I was purchased from Calbiochem. Prazosin, propranolol and isoproterenol were purchased from Sigma.

Construction of vectors

Total RNA was isolated from the rat brain using Trizol (Invitrogen). The full-length rat eIF2Bε cDNA was obtained by reverse-transcription-PCR (Roche) and subcloned into pcDNA3.1 (Invitrogen). The sequence of the entire clone was confirmed. In addition, two eIF2Bε mutants were constructed using a mutagenesis kit (Stratagene). One mutant has a point mutation in the N-terminal region of eIF2Bε (eIF2Bε-F259L)\(^1\). This mutant has reduced intrinsic guanine nucleotide exchange function without affecting the affinity between eIF2B and eIF2, suggesting that it acts as a dominant negative \(^1\). In another mutant, serine (S) 535 was replaced with alanine (A) (eIF2Bε-S535A), which is the phosphorylation site of GSK-3β in rat eIF2Bε corresponding to S540 in human eIF2Bε.

Adenoviral vectors

Adenovirus harboring GSK-3β (AdGSK-3β) has been described \(^2\). Adenovirus harboring LacZ (Ad-LacZ), eIF2Bε-wildtype (Ad-eIF2Bε-WT), eIF2Bε-F259L (Ad-eIF2Bε-F259L) and eIF2Bε-S535A (Ad-eIF2Bε-S535A) were constructed using AdenoX adenovirus construction kit (BD-Clontech). All vectors have CMV promoter. The method of adenovirus transduction into cardiac myocytes has been described \(^2,3\).
GST- eIF2Bε fusion protein

cDNA encoding the amino acid residues 518-716 of eIF2Bε was generated by PCR and subcloned into pGEX-4T-3. Bacterially expressed GST-eIF2Bε was purified with glutathione-Sepharose, eluted and dialyzed. Another fusion protein was created expressing GST-eIF2Bε-S535A.

Primary culture of neonatal rat ventricular myocytes

Primary cultures of ventricular cardiac myocytes were prepared from 1-day-old BR-Wistar rats (Charles River Laboratories) and purified using Percoll centrifugation as described previously \(^4\). Cells were cultured in cardiac myocyte culture medium containing Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 5% horse serum, 4 µg/ml transferrin, 0.7 ng/ml sodium selenite, 2 g/liter bovine serum albumin (fraction V), 3 mM pyruvic acid, 15 mM HEPES, 100 µM ascorbic acid, 100 µg/ml ampicillin, 5 µg/ml linoleic acid and 100 µM 5-bromo-2’-deoxyuridine. We obtained cultures in which more than 95% of cells were myocytes. Culture media were changed to serum-free at 24 hrs. Myocytes were cultured in a serum-free condition for 48 hrs before experiments. This study was approved by the Institutional Animal Care and Use Committee at New Jersey Medical School.

Phosphorylation of GST-eIF2Bε by GSK-3β

The extent of phosphorylation of GST-eIF2Bε by GSK-3β was measured using immune complex kinase assays. The cells were lysed with 1 ml of lysis buffer (20 mmol/L Tris
(pH 7.5), 25 mmol/L β-glycerophosphate, 100 mmol/L NaCl, 1 mmol/L Na3VO4, 2 mmol/L EGTA, 2 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mmol/L AEBSF) 5. GSK-3β was immunoprecipitated with anti-mouse GSK-3β antibody (Transduction Labs) coupled to protein G-Sepharose. After washing the immune complex three times, the kinase activity was assayed using 1 µg of GST-eIF2βε or GST- eIF2βε-S535A as a substrate in a reaction buffer containing 25 mmol/L β-glycerophosphate, 40 mmol/L HEPES (pH 7.2), 10 mmol/L MgCl2, 1 mmol/L protein kinase inhibitory peptide (rabbit sequence), 50 µM ATP, 5 µCi of [γ32P]ATP. After 20 min of incubation at 30° C, the reaction was terminated by the addition of Laemmli sample buffer on ice. Reaction mixtures were boiled for 5 min and subjected to 12% SDS- PAGE. The gels were dried and the extent of GST-eIF2βε phosphorylation was determined by autoradiography. In a subset of experiments, kinase reaction was performed using whole cell lysates or cold ATP followed by SDS-PAGE and immunoblot analysis using custom-made anti-phospho S535 eIF2βε specific antibody (Biosource International).

**Immunoblot analysis**

For immunoblotting, cultured cells were lysed with the lysis buffer A containing 25 mmol/L NaCl, 25 mmol/L Tris (pH7.5), 0.5 mmol/L EGTA, 10 mmol/L Na-Pyrophosphate, 1 mmol/L Na3VO4, 10 mmol/L NaF, 0.5 mmol/L AEBSF, 0.5 µg/mL leupeptin and 0.5 µg/mL aprotinin. Tissue homogenates were prepared using the RIPA buffer containing 150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 0.1 mmol/L Na3VO4, 1 mmol/L NaF, 0.5 mmol/L AEBSF, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 0.5 µg/mL leupeptin and 0.5 µg/mL aprotinin. Samples containing equal amounts of proteins

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were subjected to SDS-PAGE. Proteins were transferred onto polyvinylidene membranes (Bio Rad) and probed with primary antibodies. These include monoclonal antibody against GSK-3β (Transduction Labs), eIF2Bε (Santa Cruz) and polyclonal antibody against phospho-S535 rat eIF2Bε (Biosource International). Blots were then probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (NEB). Antigen-antibody complexes were visualized by the enhanced chemiluminescence system (ECL, Amersham).

Myocyte cell surface area and sarcomeric organization

Cardiac myocytes were grown on gelatin-coated glass coverslips. Cells were fixed in PBS containing 4% paraformaldehyde, permeabilized in PBS containing 0.3% Triton-X, and blocked with 3% BSA in PBS. Immunostaining was performed by Texas Red-Phalloidin (Molecular Probes) and anti-α sarcomeric actinin antibody (Sigma). Stained slides were analyzed using a fluorescent microscope (Nikon). To determine the myocyte surface area and sarcomeric organization, microscope images were captured by a digital camera and analyses were performed using Adobe Photoshop. Sarcomeric organization was classified as “non-organized” when punctuated staining with random distribution was present, while “organized“ indicated well matured staining forming a clear striated pattern. Myocyte organization was determined to be “partially organized” when a less marked cytoskeletal organization was present with faint striated pattern.
**Measurement of protein synthesis and total protein content**

Cardiac myocytes were plated on six-well plates. As an index of protein synthesis, \[^3\text{H}\text{]phenylalanine incorporation was measured as described previously}^6. Forty-eight hrs after starvation, myocytes were transduced with Ad-eIF2Bε WT, S535A, F259L, or Ad-LacZ. Twenty-four hrs after transduction, some myocytes were stimulated with ISO (10µM) for an additional 12 h. The myocytes were then incubated with 5 µCi/mL of \[^3\text{H}\text{]phenylalanine (120 Ci/mmol) and unlabeled phenylalanine (0.36 mmol/L) added to the medium. The cells were washed with PBS, and 10% TCA was added at 4°C for 60 minutes to precipitate protein. The precipitate was washed three times with 95% ethanol and then resuspended in 0.25N NaOH. Aliquots were counted by a scintillation counter.**

For measurement of protein content each well was rinsed three times with PBS. The cell layer was scraped with 1 ml of 1x standard sodium citrate containing 0.25% (w/v) SDS and vortexed extensively. Total cell protein was determined by the Lowry method, as described previously^6.

**Quantitative real time PCR**

Total RNA was isolated from cultured cardiac myocytes using Trizol. cDNA was obtained by reverse-transcription-PCR (Roche) and quantification of transcripts for atrial natriuretic factor (ANF) and α-myosin heavy chain (α-MHC) was performed by real time PCR (Roche light cycler) using GAPDH as a control. The PCR primers were as follows:

ANF sense ATCTGATGGATTTCAGAACC,

ANF anti-sense CTCTGAGACGGGTTGACTTC;
α-MHC sense GGAAGAGCGAGCGGCGCATCAAGG,
α-MHC anti-sense CTGCTGGACAGGTTATTCCTCA;
GAPDH sense TGCACCACCAACTGCTTA,
GAPDH antisense GGATGCAGGGATGATGTTC \(^7,8\).

**Rat model of myocardial infarction**

For this procedure male Wistar rats with a body weight of 200 g were used. Animals were anesthetized by intraperitoneal injection of ketamin (70 mg/kg) and xylazine (2-5 mg/kg). After orotracheal intubation the thorax was opened left parasternally at a length of 1.5 cm. The left anterior descending artery was ligated just below the left atrial appendage and the quality of the infarction was confirmed visually by the change of the color of the myocardium. The thorax was closed in 2 layers and the animals were extubated. 24 hrs after the intervention animals were anesthetized and the heart was arrested in diastole by injection of KCl. Left ventricular tissue was dissected in divided into infarcted area, adjacent and remote area. Blots were performed using tissue homogenates from the remote area.

**Statistical analysis**

Data are given as mean ± SEM. Statistical analyses were performed using ANOVA. A post hoc test was performed by the method of Tukey. Significance was accepted at the P<0.05 levels.
Online Figure 1  Cardiac myocytes were transduced with Ad-LacZ (30 MOI) or Ad-eIF2Be-S535A (30 MOI). Myocytes were harvested 72 hrs after transduction. Total RNA was subjected to RT-PCR and the level of α-MHC and GAPDH expression was quantitated. The level of α-MHC expression was normalized by that of GAPDH and was expressed as 1 in control virus transduced myocytes. No significant changes in α-MHC expression were observed after transduction of myocytes with Ad-eIF2Be-S535A. The graph shows the mean of 6 individual experiments.
References


