Activation of signaling pathways and regulatory mechanisms of mRNA translation following myocardial ischemia-reperfusion


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MYOCARDIAL ISCHEMIA MAY LEAD to cardiac remodeling (52), a process whereby altered protein expression patterns result in phenotypic changes, including cardiac hypertrophy. Indeed, altered patterns of protein expression are observed shortly after an episode of ischemia and reperfusion in vivo (47). Unfortunately, these changes are ultimately associated with the development of heart failure. Understanding how protein expression patterns are regulated during myocardial ischemia and subsequent reperfusion is therefore of considerable clinical significance.

Presently, there is little information regarding the modulation of regulatory mechanisms of mRNA translation in the heart during ischemia and reperfusion. The mammalian target of rapamycin (mTOR) is a protein kinase that integrates nutritional and mitogenic signals to regulate mRNA translation (50) and ribosome biogenesis (22). As myocardial ischemia promotes delivery of nutrients and hormones from the circulation to the cardiomyocytes, it is likely that mTOR-mediated signaling is responsive to ischemia and reperfusion. This idea is supported by the finding that cardiomyocytes lacking an upstream regulator of mTOR, phosphoinositide-dependent protein kinase-1, are more sensitive to hypoxia compared with control cells (36). The extracellular signal-regulated kinase (ERK) 1/2 and p38 mitogen-activated protein (MAP) kinase signaling pathways also play important roles in regulating mRNA translation (27), and both pathways are activated in cardiomyocytes in response to ischemia-reperfusion (58). In addition, previous in vitro experiments have demonstrated that ATP-to-AMP ratios decrease in hearts subjected to ischemia (24). Reduced ATP-to-AMP ratios stimulate the 5′-AMP-activated protein kinase (15), a signaling protein that modulates mRNA translation via inhibition of both the initiation and elongation phases of translation (19, 26). Finally, ischemia and reperfusion are associated with changes in cellular redox status, calcium homeostasis, and the generation of reactive oxygen species, all of which may affect the regulation of translation initiation (16, 41).

Although both mTOR-dependent and -independent pathways can be affected by cellular events associated with ischemia and reperfusion, their effects on downstream regulatory mechanisms of mRNA translation in vivo have not been elucidated. Therefore, the hypothesis to be tested in this study is that, in heart, hypoxia causes changes in regulatory mechanisms of mRNA translation through modulation of multiple signaling pathways. These studies will be important for furthering our understanding of how ischemia induces changes in protein expression that lead to heart failure.

MATERIALS AND METHODS

Animal care. The animal facilities and experimental protocol used for these studies were approved by the Institutional Animal Care and Use Committee of the Weis Center for Research at Geisinger Medical Center. Adult male Sprague-Dawley rats were maintained on a 12:12-h light-dark cycle with a standard diet (PMI Nutrition International, Brentwood, MO) and water provided ad libitum.

Experimental protocol. Fasted rats were anesthetized with ketamine (60 mg/kg body wt; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (10 mg/kg body wt; Boehringer Ingelheim Vetmedica, St. Joseph, MO). Animals maintained under a heat lamp were intubated and ventilated with room air, and the left main coronary artery was occluded 3–5 mm distal to its origin from the ascending aorta. The entire procedure from the time the chest was opened until...
it was closed took <3 min. Occlusion resulted in an immediate blanching in the apical area, indicating that blood flow to this area was reduced. The model has been described previously (56) and is known to cause a slow, but progressive, decrease in cellular ATP levels (to ~80% of control values after 15 min) and an immediate fall in phosphocreatine levels (to ~60% of control values after 2 min) within the heart during occlusion with similar kinetics for the restoration of ATP and phosphocreatine levels during reperfusion. Animals were administered lidocaine (10 mg/kg body wt; Abbott Laboratories, Chicago, IL) at the time of occlusion to prevent arrhythmias. The heart was then repositioned in the chest, the chest was closed, and the air in the chest cavity was aspirated. This procedure usually took 5–8 min to complete. Following 25 min of occlusion, the chest was reopened, and either a small section well within the previously blanched (ischemic) area was excised on ice and rapidly frozen in liquid nitrogen, or the ligature was cut, resulting in immediate restoration of blood flow to the ischemic area, as demarcated by the return of color to the previously blanched area, and the heart was again repositioned in the chest, which was then closed and the air aspirated. The time point of 25 min was chosen based on previous studies showing that total ischemia of 15–45 min results in reversible cell injury, whereas >45 min results in cell death (23). After 15 min of reperfusion, the chest was opened again, and a small section of the previously blanched area was excised on ice and rapidly frozen in liquid nitrogen. The border area between the ischemic and nonischemic areas was discarded. Control animals underwent the same surgical procedure, except that the coronary artery was not occluded in sham operations and the entire left ventricle was collected 40 min following lidocaine administration.

Sample preparation. Frozen tissue samples were homogenized in seven volumes of homogenization buffer (20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (pH 7.4), 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 50 mM sodium fluoride, 50 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.5 mM sodium vanadate, and 10 μl/ml protease inhibitor cocktail (Sigma, St. Louis, MO) as described previously (7). The homogenate was immediately centrifuged at 1,500 g for 10 min at 4°C, and the resultant supernatant was used for further analysis.

Immunoblot analysis. Tissue contents of proteins associated with signaling pathways and regulatory mechanisms of mRNA translation were evaluated in 1,500-g supernatants by immunoblot analysis. Changes in 4E-BP1 and S6K1 phosphorylation were assessed as described previously (12). Changes in the phosphorylation status of the remaining proteins were assessed by first stripping the membranes of antibody and reanalyzing them with antibodies that specifically recognize Akt phosphorylated on Ser473, GSK3β phosphorylated on Ser9, mTOR phosphorylated on Ser2448, eukaryotic initiation factor 4G phosphorylated on Ser2236, eIF2β phosphorylated on Ser51, eIF2α phosphorylated on Ser1108, p38 MAP kinase phosphorylated on Thr180 and Tyr182, and eukaryotic initiation factor (eIF) 2 phosphorylated on Ser535. Antibodies were from Cell Signaling (Beverly, MA), unless otherwise stated. Proteins were visualized by enhanced chemiluminescence (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK) using a GeneGnome Bioimaging System (Syngene). The amount of protein in the phosphorylated form was normalized to the total amount of the respective protein before data transformation.

Analysis of eIF4E complexes. eIF4E complexes were immunoprecipitated from 1,500-g supernatants, and the association of 4E-BP1 and eIF4G with eIF4E was determined as described previously (25).

Statistical analysis. Data are expressed as means ± SE. Data were analyzed by the InStat version 3 statistical software package (Graph-Pad Software, San Diego, CA). Statistical significance was assessed using a one-way ANOVA and a Student-Newman-Keuls posttest. P values of <0.05 were considered significant.

RESULTS

Many of the mechanisms that regulate mRNA translation are modulated through multiple protein kinase-mediated signaling pathways. To elucidate whether myocardial ischemia and/or reperfusion influences the regulation of mRNA translation through these mechanisms, changes in several relevant signaling pathways were assessed. The phosphoinositide (PI) 3-kinase/Akt/mTOR signaling pathway affects a number of regulatory mechanisms of mRNA translation (32), and, as demonstrated in Fig. 1A, phosphorylation of Akt at Ser473, a residue whose phosphorylation demarcates Akt activation (1), increased slightly during ischemia, and became markedly increased during reperfusion. Likewise, the MAP kinase signaling pathway affects a number of regulatory mechanisms of mRNA translation (4, 46), and, as demonstrated in Fig. 1B, phosphorylation of activating residues on both ERK1/2 increased somewhat during ischemia and became markedly increased during reperfusion. In contrast, phosphorylation of p38 MAP kinase was significantly increased during ischemia and remained elevated during reperfusion (Fig. 1C).

When phosphorylated on its ε-subunit at Ser535, eIF2B-ε-mediated exchange of eIF2B-bound GDP for GTP is inhibited (9, 33), thus preventing the delivery of initiator methionyl tRNA (met-tRNAMet) by eIF2 to the 40S ribosomal subunit during initiation of mRNA translation (18). The observed increase in phosphorylation of eIF2Bε at Ser535 (Fig. 2A) was indicative of this process. Likewise, eIF2α-mediated delivery of tRNAMet can also be inhibited through phosphorylation of eIF2α on its α-subunit at Ser51, as eIF2α acts as a competitive inhibitor of eIF2B when phosphorylated at Ser51 (48). However, phosphorylation of eIF2αε was unchanged during myocardial ischemia, but there was a trend for it to increase during reperfusion (Fig. 2B).

Akt-mediated modulation of regulatory mechanisms of mRNA translation occurs through phosphorylation of mTOR and the GTPase-activating protein TSC2 (29). Phosphorylation of mTOR at Ser2448 was essentially unchanged during ischemia, but increased to ~150% of the control value during reperfusion (Fig. 3A). Activated mTOR phosphorylates the downstream targets 4E-BP1 and the ribosomal protein S6 kinase S6K1 (49). 4E-BP1 inhibits formation of the mRNA transport protein complex, eIF4F, but is unable to do so when highly phosphorylated (13). As demonstrated in Fig. 3B, 4E-BP1 phosphorylation was essentially unchanged during ischemia but increased during reperfusion, although the magnitude of the changes was small. When highly phosphorylated, S6K1 phosphorylates two proteins that facilitate mRNA translation initiation, the ribosomal protein S6 (14) and eIF4B (43). Phosphorylation of S6K1 was unaltered during ischemia but increased during reperfusion (Fig. 3C).

There was no increase in 4E-BP1/eIF4E association (Fig. 4A) during ischemia, but eIF4E assembly, as demarcated by the binding of eIF4G to eIF4E, decreased (Fig. 4B). During reperfusion, the binding of 4E-BP1 to eIF4E tended to decrease and the binding of eIF4G to eIF4E to increase, although eIF4E/eIF4G association was not significantly different than either control or ischemic values (Fig. 4B). The mTOR signaling pathway may also affect eIF4F assembly through phosphorylation of eIF4G, as phosphorylation of eIF4G on Ser1108 is believed to facilitate its association with eIF4E (42). Phos-
phorylation of eIF4G at Ser1108 was unaltered during myocardial ischemia, but increased almost 2.5-fold during reperfusion (Fig. 4C).

The ability of the eIF4F complex to bind mRNA may be regulated by the p38 MAP kinase signaling pathway (37). p38 MAP kinase activates MAP kinase-interacting kinase (MNK)1/2, which subsequently phosphorylates eIF4E (55) and alters eIF4F activity (13). Activation of p38 MAP kinase is demarcated by changes in its phosphorylation (17), and as demonstrated in Fig. 1C the phosphorylation of p38 MAP kinase was increased with myocardial ischemia and reperfusion. These changes in p38 MAP kinase phosphorylation, however, were not associated with alterations in the phosphorylation of eIF4E at Ser209 (Fig. 5).

eEF2 controls polypeptide-chain translocation during the elongation phase of translation and is inhibited through its phosphorylation at Thr56 (5). The phosphorylation of eEF2 at Thr56 was decreased during ischemia and reperfusion (Fig. 6).

DISCUSSION

Protein expression patterns in the heart are significantly altered shortly after an episode of ischemia and reperfusion in vivo (47); however, the signaling pathways and regulatory mechanisms of mRNA translation that contribute to the alterations are not well defined. Translation of mRNA is regulated in part through the PI3-kinase/Akt/mTOR signaling pathway (32), and Akt phosphorylation has been reported to be unaltered by ischemia but increased by reperfusion in vitro (35). Similarly, the data presented herein demonstrate that Akt phosphorylation was increased in response to reperfusion but...
was not significantly changed following ischemia. Moreover, changes in the phosphorylation of mTOR, a downstream target of Akt, indicated that Akt activity was increased in response to reperfusion. In addition, the phosphorylation of three mTOR effectors, S6K1, 4E-BP1, and eIF4G, was increased concomitantly with increased mTOR phosphorylation during reperfusion. Collectively, these changes in protein phosphorylation suggest that the PI3-kinase/Akt/mTOR signaling cascade is activated in the reperfused heart. This finding is of particular interest, given that activation of the mTOR pathway is associated with hypertrophic growth (21). Interestingly, it has also been demonstrated (20) that pressure overload hypertrophy is...
associated with ERK-mediated phosphorylation of S6K1. Therefore, given the observed changes in ERK1/2 and p38 MAP kinase phosphorylation, changes in S6K1 phosphorylation may have also been mediated in part through the MAP kinase signaling pathway. Thus it is tempting to speculate that acute activation of both the mTOR and MAP kinase signaling pathways in the heart following ischemia may have an effect on subsequent cellular hypertrophy. Previous studies have demonstrated that, postischemia, the surviving myocytes undergo 10–15% hypertrophy as demonstrated by increased cardiac length (6) and increased whole cell capacitance, a measure of cell surface area (59, 60).

Delivery of met-tRNAi to the ribosome during the initiation of mRNA translation is regulated by phosphorylation of the α-subunit of eIF2 and its guanine nucleotide exchange protein eIF2B. The activity of eIF2B is regulated by increased phosphorylation of eIF2α, causing it to become a competitive inhibitor of the exchange reaction, and by phosphorylation of the ε-subunit of eIF2B. Under the conditions of the studies presented herein, eIF2α phosphorylation tended to increase, particularly during reperfusion; however, the changes were not significant. In contrast, eIF2Be phosphorylation was increased during both ischemia and reperfusion. However, the observed increases in eIF2Be phosphorylation must have been mediated by a kinase other than GSK3β, whose phosphorylation on Ser9, which is usually associated with inhibition of activity, was increased during both ischemia and reperfusion (unpublished observation). Alternatively, GSK3β activity was increased, despite the observed putative inhibitory changes in GSK3β phosphorylation. Interestingly, the MAP kinase signaling pathway stimulates GSK3β activity via tyrosine phosphorylation (53), and, as has been demonstrated previously in vitro (3, 28), the results presented herein demonstrate that myocardial ischemia and reperfusion stimulate the MAP kinase signaling pathway in vivo. Therefore, it is plausible that GSK3β activity, and thus eIF2Be phosphorylation, was increased in association with the MAP kinase-mediated phosphorylation of GSK3β at residues not investigated in this study. The Ser1 residue on GSK3β is a target of Akt (10), and phosphorylation of this site, which was increased during both ischemia and reperfusion, did tend to correlate with phosphorylation of Ser473 in Akt. Another possibility is that the Ser9 residue on GSK3β was phosphorylation by a kinase other than Akt during ischemia and reperfusion. For example, several isoforms of PKC are activated during ischemia (38), and phosphorylation of Ser9 on GSK3β by PKCβ has been demonstrated in vitro (54).

Phosphorylation of eIF2α is dramatically increased during cerebral reperfusion (8, 31). Therefore, it was surprising that there was only a trend for eIF2α phosphorylation to increase in the reperfused heart. It may be that ischemia- and reperfusion-induced changes in eIF2α phosphorylation in the heart are transient and, therefore, were not observed at the time points selected in the present study. Alternatively, it may be that, unlike in the brain, there is little ischemia- and/or reperfusion-induced activation of eIF2α kinases in the heart. Interestingly, the kinase responsible for eIF2α phosphorylation during cerebral reperfusion, the double-stranded RNA-activated protein kinase-like endoplasmic reticulum-associated protein kinase (PERK), is regulated by changes in intracellular calcium (41). Although PERK is expressed in the heart (51), intracellular calcium levels are normally in flux within cardiomyocytes, and yet there is no indication that PERK is constitutively activated in the heart. As such, it is pertinent to consider how dramatic of an effect ischemia- and reperfusion-induced changes in calcium flux would have on PERK activation in the heart.

In the studies presented herein, signaling of mTOR to 4E-BP1 was increased, particularly during reperfusion. However, changes in the association of 4E-BP1 and eIF4G with eIF4E did not closely correlate with altered 4E-BP1 phosphorylation. Possible explanations for this discrepancy include the observation that the binding of eIF4G to eIF4E can also be regulated through phosphorylation of eIF4G on Ser1108 (42), and a possible role for the two other eIF4E binding proteins, 4E-BP2 and 4E-BP3, in the observed changes in eIF4G·eIF4E association must also be considered.

The MAP kinase signaling pathway modulates mRNA translation through multiple mechanisms, including activation of MNK1/2 and mTOR. MNK1/2 phosphorylates eIF4E on Ser209 (11, 55), which initially was reported to enhance the binding of the protein to the m7GTP cap structure (34). However, more recent studies (reviewed in Ref. 46) have shown that phosphor-
ylation of eIF4E by MNK1/2 reduces its affinity for m7GTP. This idea is supported by studies showing a correlation between decreased phosphorylation of eIF4E and increased rates of protein synthesis (e.g., Ref. 57). The MAP kinase pathway also regulates mRNA translation through phosphorylation of a GTPase activator protein referred to as Tuberin or TSC2 (30). Phosphorylation of Tuberin by ERK represses its GTPase activator protein activity toward the mTOR-binding protein, ras homolog enriched in brain (Rheb). Because Rheb-GDP inhibits signaling through mTOR, phosphorylation of Tuberin by ERK results in enhanced mTOR signaling and increased phosphorylation of proteins involved in regulating mRNA translation, including 4E-BP1, eIF4B, eEF2, and rpS6.

Changes in signaling through mTOR have both acute and protracted effects on mRNA translation. Prolonged inactivation of mTOR (i.e., over a period of hours or days) decreases the capacity for mRNA translation by repressing both ribosome biogenesis and the translation of mRNAs encoding many of the proteins involved in mRNA translation (45). In contrast, acute inhibition of mTOR signaling downregulates the mRNA binding step in translation initiation through decreased assembly of the eIF4F complex and reduced phosphorylation of eIF4B and rpS6 (45). Intuitively, inactivation of the mRNA binding step in translation initiation might be expected to decrease the translation of all mRNAs. However, in both yeast (40) and mammals (44), rapamycin-mediated inhibition of mTOR signaling has little, if any, acute effect on the translation of most mRNAs, but instead preferentially represses the translation of a subset of mRNAs. Such mRNAs typically have distinctive structural features at the 5′ end of the message (39), including terminal oligopyrimidine sequences or sequences predicted to form extensive secondary structure that makes their translation particularly sensitive to changes in eIF4F availability and/or S6K1 activity. Depending on the proportion of the actively translated mRNA population represented by the rapamycin-sensitive mRNAs, changes in global rates of protein synthesis may, or may not, be detectable using methods such as measuring incorporation of radiolabeled amino acids into protein. Thus it would be expected that, in tissues like skeletal muscle (2) that exhibit decreased global rates of protein synthesis in response to acute rapamycin treatment, the proportion of actively translated mRNAs represented by messages with terminal oligopyrimidine sequences or mRNAs with extensive secondary structure in the 5′ untranslated region would be significantly greater than the proportion in a tissue such as liver (44) that does not demonstrate acute rapamycin-dependent changes in global rates of protein synthesis. Although the effect of ischemia and reperfusion on the pattern of mRNA translation in the heart has not been examined previously, based on the changes in eIF4F assembly and S6K1 phosphorylation observed in the present experiment, it is tempting to speculate that such changes might occur. Future studies will be required to establish the validity of this idea.

In conclusion, the studies presented herein demonstrate that myocardial ischemia and reperfusion modulate several signaling pathways and regulatory mechanisms of mRNA translation in vivo. Thus it is reasonable to assume that regulation of mRNA translation plays an important role in determining protein expression in the heart following ischemia and reperfusion. In particular, increased phosphorylation of proteins involved in the mTOR/MAP kinase-mediated signaling pathway with reperfusion is indicative of upregulated mRNA translation initiation and elongation following ischemia. Future studies will be required to elucidate whether reperfusion-mediated activation of the mTOR and MAP kinase signaling pathway play a role in the cardiac remodeling often observed following myocardial ischemia.

**REFERENCES**


ACTIVATION OF mTOR FOLLOWING MYOCARDIAL ISCHEMIA


