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Investigation of the Molecular Mechanisms Leading to Protein Translation Inhibition in Response to Endoplasmic Reticulum Stress

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Abstract: Protein folding is an important process for cellular function. ER is responsible for the synthesis, folding, modification, and quality control of numerous secretory and membrane proteins. The intracellular disturbance caused by different stressors leading to the accumulation of unfolded/ misfolded proteins can all lead to an alteration in ER homeostasis. If the unfolded/misfolded proteins continue to accumulate inside the ER, the unfolded protein response (UPR) is induced to overcome this situation. UPR acts by three different mechanisms:(1) increase the ER protein-folding capacity (Robertsonand Branch, 1987),(2) reduce global protein synthesis, and (3) enhance ER-associated degradation process. The UPR is mediated by three ER transmembrane protein sensors: (1) activating transcription factor 6 (ATF6), (2) inositol requiring kinase 1(IRE1), and (3) double-stranded RNA-activated protein kinase (PKR) like endoplasmic reticulum kinase (PERK). Each sensor of the UPR protein responds to the certain level of unfolded/misfolded protein in the ER. If the cell fails to restore or overcome the protein-folding defect, cell-death signaling pathways are activated**.**

Keywords:ER stress, PERK, Translation, UPR, Protein synthesis, Firefly, Renilla, MIN6 cells.

INTRODUCTION

Translation, the first and most important phase of protein synthesis, involves a process by which mRNA is translated into proteins. The translation of mRNA into proteins involves five major components: ribosomes that perform the process of polypeptide synthesis, tRNA molecules that arrange amino acids in a specific sequence within the mRNA template, aminoacyl-tRNA synthetases that attach amino acids to their tRNA molecules, mRNA that encode the amino acids sequence information for protein synthesis, and protein initiation, elongation and termination factors that facilitate the translation mechanism (Merrick, 2010). mRNA is exported from the nucleus and enters the cytosol as a messenger ribonucleoprotein (mRNP), which is a mRNA molecule coated with RNA binding proteins (Robertson & Branch, 1987). The mRNA can then be translated into protein and there are three main stages to this process: initiation, elongation, and termination (Kapp & Lorsch, 2004). The initiation of translation in eukaryotes is considered the most important stage in the regulation of protein translation. It is also a highly complex step which involves the recruitment of the 80S ribosome and the initiator methionyl-tRNA (Met-tRNAi) on to the start codon (AUG) of the mRNA. This process is facilitated by at least 12 protein initiation translation factors (eIFs) (Hinnebusch, 2011). The initiation phase is completed when the Met-tRNAi base pairs to

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the start codon (AUG) in the P site of the ribosome and is ready to begin the elongation phase of protein synthesis (Hinnebusch & Lorsch, 2012). One of the critical steps during the initiation of translation is the formation of the translational ternary complex (TC) consisting of initiator methionyl-tRNA (Met-tRNAi) and the GTP-bound form of eukaryotic initiation factor 2 (eIF2). Binding of the TC to the 40S subunit requires initiation factors such as eIF1, 1A, 5, and the eIF3 complex. The 43S pre-initiation complex (PIC) binds to mRNA in a process facilitated by eIF3, the poly(A)-binding protein (PABP), eIFs 4B, 4H (in mammals), 4F, a complex consisting of the cap-binding protein eIF4E, eIF4G, and the RNA helicase eIF4A (Hinnebusch, 2011).eIF4E is the critical factor for the formation of eIF4F complex (Duncan & Hershey, 1989), and the binding of eIF4E to eIF4G is inhibited by eIF4E-binding proteins (4E-BPs). eIF4G and 4E-BPs compete for eIF4E binding and as a result of binding eIF4E to 4E-BPs inhibits the formation of eIF4F complex (Haghighat, Mader, Pause, & Sonenberg, 1995). The affinity of eIF4E for 4EBPs is deceased by 4E-BPs phosphorylation which leads to eIF4E free to bind eIF4G (Proud, 2007), (Friedland, Wooten, LaVoy, Hagedorn, & Goss, 2005).
In eukaryotes, the untranslated regions of mRNA have been shown to be essential in the regulation of protein synthesis (Jackson, Hellen, & Pestova, 2010). The 5' leaders can regulate downstream expression through upstream open reading frames (uORF), these 5' leaders structures act as codes so that ribosomes can recognize which transcripts are to be repressed or preferentially translated (Dever et al., 1993)**.**

Internal ribosome entry site (IRES): Eukaryotic cells apply different mechanisms to initiate translation of their mRNAs (Komar & Hatzoglou, 2005). Some viral and eukaryotic cellular mRNA are translated using a cap-independent mechanism such as the IRES mediated mechanism by which the 40S ribosomal subunit is directed to a site 3' of the 5'end often by specific mRNA tertiary structures termed internal ribosome entry sites (IRESs) (Jackson et al., 2010). Poliovirus and encephalomyocarditis virus (EMCV) were the first biological systems found to translate their mRNA by the internal ribosome entry mechanism (Pelletier & Sonenberg, 1988). Later on, many other virus families were also found to use this mechanism for their mRNA translation (Vagner, Galy, & Pyronnet, 2001). It was found that IRES-dependent translation requires a variable number of translation initiation factors depending on the particular IRES (Hellen, 2009). For instant, the hepatitis C virus (HCV) IRES does not require any of the initiation factors of the eIF4 family (Pestova, Shatsky, Fletcher, Jackson, & Hellen, 1998), and the cricket paralysis virus (CrPV) IRES is translated without the requirement for any of the canonical initiation factors including eIF2(Jackson et al., 2010).

Translation control under ER stress: ER plays a central role in protein synthesis. Translation of new proteins takes place on ribosomes associated with the ER. Newly synthesized membrane or secretory proteins are then folded and modified in the ER lumen (Harding & Ron, 2002). Under ER stress condition, protein folding is disrupted which lead to the accumulation of unfolded proteins resulting in activation of unfolded protein response (UPR). In reaction to ER stress, UPR is intended to restore ER homeostasis through decreasing ER load, increasing ER folding capacity and increasing ER associated degradation. This decrease in ER load is classically initiated by the activation of the ER-transmembrane protein PERK, which phosphorylates the translation initiation factor eIF2α resulting in a decrease in global protein synthesis (Back & Kaufman, 2012). It is believed that the inhibition of protein synthesis in response to ER stress gives ER more time to qualifying the loading proteins

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and correctly folded (Evans-Molina, Hatanaka, & Mirmira, 2013). During the ER stress, the kinase PERK phosphorylates eIF2α which leads to translation attenuation to overcome ER stress. Phosphorylation of eIF2α has a dual action during ER stress which inhibits the general mRNA translation and promoting selective translation of specific stress responsive mRNA. (Harding, Zhang, Bertolotti, Zeng, & Ron, 2000). We speculated that additional mechanisms/factors may be involved in repression of protein synthesis in response to ER stress. To investigate this, we exploit the differences in the dependency of viral IRES for translation initiation factors to identify which initiation factors are affected by ER stress and thus may be important in the ER stress response.

General reagents and materials: Thapsigargin was purchased from Merck M, and ^{35}S-L-Methionine was purchased from Perk Almar. Dual-luciferase Reporter Assay System was purchased from Promega. Lipofectamine 2000 was purchased from Invitrogen. Viability/Cytotoxicity Kit was purchased from Molecular Probes. Plasmids were used: pEMCV, pCrpv, Renilla/Firefly (pRF), and pHCV were provided by Professor Martin Bushell-MRC, Leicester.

Cell culture and transfection : Mouse Insulinoma 6 cells Insulinoma (MIN6) were cultured in DMEM media containing 25mM glucose, 15% heat-inactivated FBS, 100µg/ml streptomycin, 100units/ml penicillin, 100units/ml neomycin (PSN), 40mM NaHCO3 and 75µM β-mercaptoethanol and maintained at 37°C and 5%CO_2. Lipofectamine 2000 was used for transfection, prior to transfection, cells were split into 24 sterile multi-well plates. 0.2µg of plasmid was used to transfect one well of 24 wells plate. Transfection were performed as recommended by the manufacturer.

Cell treatment and lysis: Detailed descriptions of treatments are provided in the figure legend. After experimentation, growth medium was aspirated off the cells and the cells washed twice with 1x PBS. For measuring luciferase activity, cells were scrapped into 100µl of passive lysis buffer (Promega) on ice. Lysates were subjected to two freeze-thaw cycles and then centrifuged at 14,000rpm at 4℃ for 10 min. The supernatant was kept for further analysis.

Dual-luciferase reporter assay system: Samples were harvested and prepared to measure luciferase activity using dual-luciferase reporter assay system from Promega. The Luciferase Assay Reagent II (LARII) and Stop&Glo reagent were prepared according to manufactory instruction. After treatment, cells were harvested using ice-cold 50µl passive lysis buffer. Before the measurement, LARII and Stop&Glo reagent should be warmed up to room temperature .10µl of sample lysate was added to 96 well-read plate then 50µl LARII were added to measure firefly luciferase activity using Novastar plate reader to determine luminescence. After that 50µl Stop&Glo was added to measure Renilla luciferase activity using Novastar plate reader to determine luminescence.

TCA Precipitation of protein and protein synthesis measurements: 5µl of cell lysate was spotted onto a 1cm of 3MM Whatman filter papers in a triplicate. Filter papers were boiled for 1 minute in 100 ml of 5% Trichloroacetic acid (TCA) with a pinch of L-methionine. The 5% TCA was discarded and replaced with 100ml of 5% TCA and boiled again for 1 minute. The 5% TCA was discarded again, and the papers were rinsed in 5% TCA followed by washing with absolute ethanol. The papers were dried at 80℃ for one hour. The filter papers were immersed in 3ml of scintillant (Emulsifier-safe, PerkinElmer) and DPM determined by Scintillation counting using a Beckman-Coulter liquid scintillation counter.

Quantification and Statistical Analysis: Statistical differences between different groups were analyzed via single factor analysis of variance (ANOVA) followed by a Bonferroni.

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Comparison between two sets of data was analyzed using Paired t-test or Mann-Whitney non-parametric test. A statistical test was only carried out when the experiments had at least n=3. Statistical significance was only presented when $p \leq 0.05$. Statistical analyses were performed using the GraphPad Prism software.

RESULTS

Bicistronic constructs initiation factors requirement for translation: In order to identify which initiation factors are responsible for the inhibition of protein synthesis in response to ER stress, a series of bicistronic constructs were used in which the translation of the upstream cistron is controlled by a cap-dependent mechanism whereas the downstream cistron (cap-independent) is regulated by a specific viral IRES. These bicistronic constructs have different requirements to initiate translation via their IRES. EMCV IRES can direct translation independently of eIE4E, whereas the CrPV IRES requires no initiation factors, and directly recruits ribosomal subunits. HCV IRES requires all the initiation factors except the eIF4E/4B and 4A (Meijer et al., 2013). pRF which was translated through cap-dependent only and has no IRES was used as the control.

Determination of efficacy of the constructs: The efficacy of the constructs was determined by how protein synthesis is repressed under conditions of ER stress *(Fig. 3.1)* by assessing: 1. the effect of ER stress on cap-dependent renilla expression, and 2. the dependency of the expression of firefly on the presence of an inter-cistronic IRES. To investigate this, MIN6 cells were transfected with pRF and pEMCV. 48 h post-transfection, the cells were incubated for 4 h in the presence or absence of thapsigargin (Tg) and the expression renilla (Ren), and firefly luciferase (FF) was determined by their activity using luminometry.

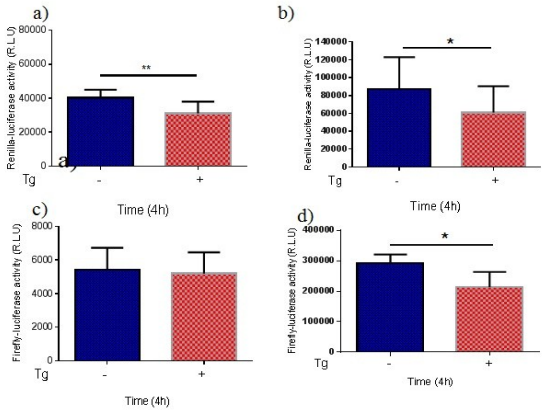

Figure (1). Characterisation of the reporter **constructs.** MIN6 cells transfected with pRF (a,c). Cells transfected with pEMCV (b,d) by using Lipofectamine for 48h, all the cells except the control were incubated with (Tg,1μM) for 4 h at 37 ℃ and 5% CO_2.The luciferase activity of FF and Ren was measured by using Dual-luciferase reporter assay system as a Relative Light Unit (R.L.U). The results are ± s.e.m of n≥3 experiments, data were analyzed by using Paired t test,* P < 0.05.**P<0.01.

Under control conditions, the activities of Ren either pRF or pEMCV were similar. However, treatment with Tg caused a 20-25% decrease in Ren activity compared to untreated cells. Thus, changes in Ren activity in response to ER stress can be used as a readout of ER stress-induced repression of protein synthesis (Fig. 3.1a) and (Fig. 3.1b). The expression of FF in cells transfected with pRF, in either the presence or absence of Tg, was negligible compared to the expression of FF driven by the EMCV IRES in cells transfected with pEMCV (Fig. 3.1c and 3.1d). Thus, the expression of the downstream cistron encoding FF is highly dependent upon the presence of IRES. Our results revealed that the measurement of FF expression from different IRES with different initiation factor requirements can be used to determine the role of specific initiation factors in ER stress-induced repression of protein synthesis.

The repression of protein synthesis in response to ER stress is mediated by the inhibition of the initiation :MIN6 cells were transfected with pCrPV and incubated under standard conditions. post transfection, the cells were treated with Tg and the activity of Ren and FF determined by luminometry (Fig. 3.2). The activity of Ren from cells transfected with

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pCrPV, under control conditions, was (approx. 15,000 RLU). The addition of Tg caused a 30% decrease in Ren activity and readout of ER stress-induced repression of protein synthesis (Fig. 3.2a). The expression of FF in cells transfected with pCrPV in the presence or absence of Tg was unaffected and thus is able to overcome or bypass the effect of ER stress on protein synthesis repression (Fig. 3.2b). In addition, the FF/Ren ratio significantly increased in the presence of Tg (Fig. 3.2c). As a control, the effect of Tg inhibition of protein synthesis was also determined by measuring ^{35}S-Methionine incorporation into protein (Fig. 3.2d). As the translation of CrPVIRES is independent of all initiation factors, this provides evidence that the ER stress-induced repression of protein synthesis is likely caused by the repression of initiation through modulation of one or more initiation factors, but not through the inhibition of elongation phase of protein synthesis.

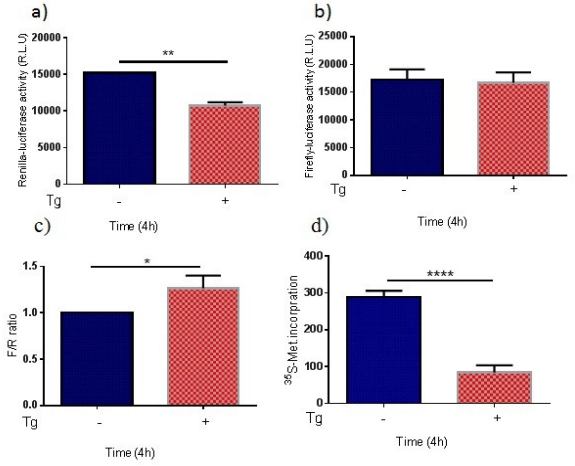

Figure (2). ER stress-induced inhibition of protein synthesis is independent of initiation factors. MIN6 cells transfected with pCrPV by using Lipofectamine for 48 h, all cells treated with (Tg,1µM) except control, [^{35}S]-Methionine was added to all cells include control and then incubated for 4 h at 37℃ and 5% CO_2. The luciferase activity of FF and Ren was measured by using Dual-luciferase reporter assay system as R.L.U (a,b). The F/R ratio was checked (c). Total protein synthesis was determined by measuring TCA perceptible count (d). The results are ± s.e.m of n≥3 experiments, data were analyzed by using Paired t-test, * P < 0.05, **P<0.01. For F/R ratio data were analyzed by using Mann-Whitney non-parametric test.

the repression of protein synthesis in response to ER stress occurs independently of the cap binding complex : As EMCV IRES, driven FF expression is known to occur independently of eIF4E. the results showed that the activity of Ren from cells transfected with pEMCV (Fig. 3.3) was (approx. 90,000RLU). Upon the addition of Tg, there was a 20-25% decrease in Ren activity compared to untreated cells, and thus readout of ER stress-induced repression of protein synthesis (Fig. 3.3a). Similarly, Tg caused a 30% decrease in FF luciferase activity compared to untreated cells (Fig. 3.3b). Thus, there was no significant change in the FF/Ren ratio demonstrating that both cap-dependent and EMCV IRES dependent translation are equally repressed in response to ER stress (Fig. 3.3c). Therefore, ER stress-induced repression of protein synthesis must occur independently of eIF4E. As a control, the inhibition of protein synthesis in cells treated with Tg was also determined by measuring ^{35}S-Methionine incorporation into protein (Fig. 3.3d).

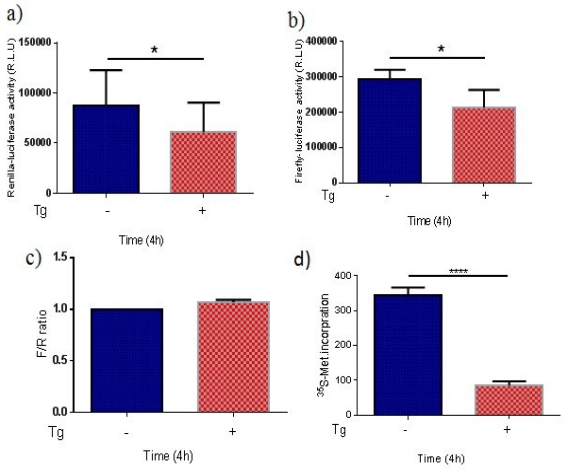

Figure (3). ER stress-induced inhibition of protein synthesis is not dependent on eIF4E.MIN6 cells were transfected with pEMCV (by using Lipofectamine for 48 h, all cells treated with Tg,1µM except the control, and [^{35}S]-Methionine was added to all cells including control cells and then incubated for 4 h at 37℃ and 5% CO_2. The luciferase activity of FF and Ren was measured by using Dual-luciferase reporter assay system as R.L.U (a,b). The F/R ratio was checked (c). Total protein synthesis was determined by measuring TCA perceptible count (d). The results are ± s.e.m of n≥3 experiments, data were analyzed by using Paired t-test, * P < 0.05, ****P<0. 0001. For F/R ratio data were analyzed by using Mann-Whitney non-parametric test.

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The repression of the initiation of protein synthesis in response to ER stress occurs independently of the cap binding complex or the RNA helicase eIF4A: MIN6 cells were transfected with pHCV. Post transfection, the cells were with Tg (Fig.3.4). The activity of Ren from cells transfected with HCV, under control conditions, was (approx. 27,000RLU).The addition of Tg caused a 25-30% decrease in Ren activity, a readout of ER stress-induced repression of protein synthesis (Fig. 3.4a), and a 35-40 % decrease in the expression of FF driven by the HCV IRES (Fig. 3.4b). The FF/Ren ratio showed no significant changes which demonstrate that both upstream and downstream translations are similarly inhibited by Tg (Fig. 3.4c). As the translation from the HCV IRES is independent of eIF4E/4B/4A, this provides evidence that ER stress-induced repression of protein synthesis is independent of eIF4E, eIF4B, and eIF4A. As a control the rate of protein synthesis was determined in cells treated with thapsigargin, and was measuring ^{35}S-Methionine incorporation into protein (Fig. 3.4d).

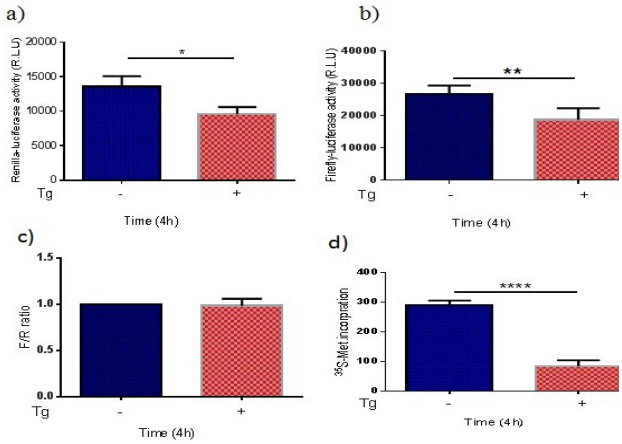
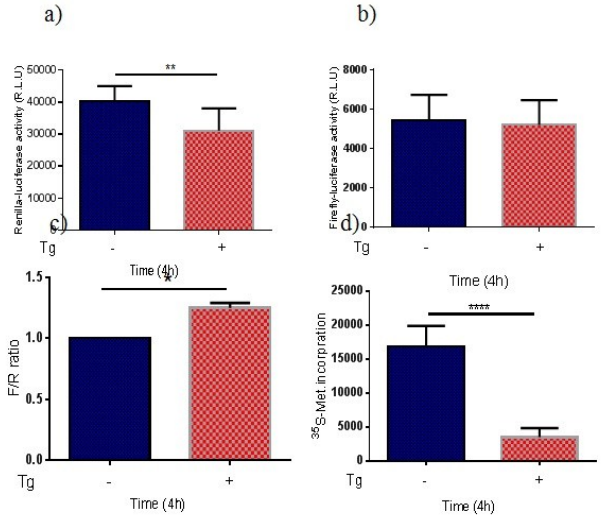


Figure (4). ER stress-induced inhibition of protein synthesis is independent of eIF4E/4B/4A. MIN6 cells were transfected with HCV by using Lipofectamine for 48 h, all the cells treated with (Tg,1μM) except control, [^{35}S]-Methionine was added to all cells including the control and then incubated for 4 h at 37 ℃ and 5% CO_2. The luciferase activity of FF and Ren was measured by using Dual-luciferase reporter assay system as R.L. U (a,b). The F/R ratio was checked (c). Total protein synthesis was determined by measuring TCA perceptible count (d). The results are ± S.E.M of n≥3 experiments, data were analyzed by using Paired t-test, * P < 0.05, **P<0.01, ****P<0.0001. For F/R ratio data were analyzed by using Mann-Whitney non-parametric test.



Figure(5).ER stress-induced inhibition of protein synthesis is cap-dependent only. MIN6 cells were transfected with pRF using Lipofectaminefor 48 h. The cells either treated or untreated with (Tg,1μM), [^{35}S]-Methionine was added to all cells (control cells included). incubation time 4 h at 37 ℃ and 5% CO_2.The luciferase activity of FF and Ren was measured by using Dual-luciferase reporter assay as R.L.U (a,b).The F/R ratio was checked (c). Total protein synthesis was determined by measuring TCA perceptible (d). The results are ± s.e.m of n=3 experiments, data were analyzed by using Paired t-test, * P < 0.05, **P<0.01, ****P<0.001. For F/R ratio data were analyzed by using Mann-Whitney non-parametric test.

Together these results demonstrate that the repression of protein synthesis in response to ER stress is independent on rates of elongation but dependent on changes in the rate of initiation and more specifically changes in initiation factors required for EMCV and HCV IRES mediated translation. One likely initiation factor is eIF2α which is known to be phosphorylated by PERK in response to ER stress resulting in the repression of protein synthesis.

Inhibition of PERK leads to restore protein synthesis in response to ER stress in MIN6 cell: To investigate the role of PERK-dependent eIF2α phosphorylation in the repression of protein synthesis in response to ER stress, MIN6 cells were either treated or untreated with Tg in the presence or absence of PERK inhibitor (GSK2656157), and the rate of total protein synthesis was determined by measuring ^{35}S-L-Methionine incorporation into protein. The results show that upon the addition

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of thapsigargin for 4 h, protein synthesis is inhibited by about 70%. However, in the presence of the PERK inhibitor, recovery of protein synthesis was around 100%. We can, therefore, conclude that the PERK is responsible for the repression of global protein synthesis via its phosphorylation of the α subunit of eIF2 (Fig. 3.6).

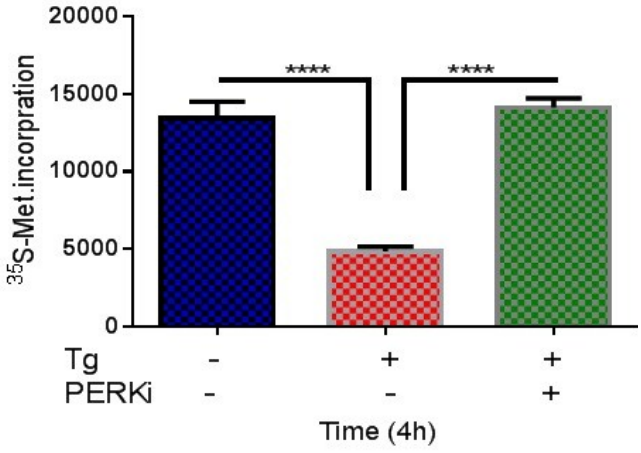

Figure (6). Inhibition of PERK can restore protein synthesis in response to ER stress. MIN6 cells were either treated or untreated (Tg,1μM) with or without PERK inhibitor, GSK2656157 (0.5μM added 30 min prior to thapsigargin). [^{35}S]-Methionine was posted to all cells including control and then incubated for 4 h (37℃ and 5% CO_2). Total protein synthesis was then determined by measuring TCA perceptible count. The results are ± s.e.m of n=3, data were analyzed by using one-way ANOVA and subsequently with Bonferroni's test, **** $P < 0.0001$

DISCUSSION

The results showed that the repression in protein synthesis is independent of a group of initiation factors such as eIF4E/4A and 4B. Ribosome binding to picornavirus IRESes such as EMCV IRES requires the complete set of initiation factors necessary for 5' end cap-dependent translation, except eIF4E (Meijer et al., 2013),(Pestova, Hellen, & Shatsky, 1996). eIF4E has an important role in the translation process since protein synthesis and cellular transformation in human and mouse cells are increased upon overexpression of eIF4E (Herbert & Laybutt, 2016). Under stress condition, the phosphorylation of eIF4E and the increasing in rate of translation are not always correlated. For instant, when cells stressed by arsenite or anisomycin show an increase in eIF4E phosphorylation, however, translation rate is inhibited. Indeed, the inhibition of protein synthesis could be caused by other components of the translational machinery such as eIF2α phosphorylation (Gingras, Raught, & Sonenberg, 1999). In an agreement with previous studies, the results showed that both cap-dependent and EMCV IRES-dependent translation are equally repressed in response to ER stress and there was no significant change in the FF/Ren ratio (Fig. 3.3c), which indicated that the ER stress-induced repression of protein synthesis must occur independently of eIF4E. Regulation of eIF4E activity is through its interaction with a proteins repressor termed the eIF4E-binding proteins (4E-BPs) (Altmann, Schmitz, Berset, & Trachsel, 1997).

4E-BP1 in its hypo phosphory-lation status inhibits the initiation of translation through the interaction with eIF4E, it prevents its binding to other partners of other initiation factor complex including eIF4G. Thus, block the assembly of eIF4F complex. The study provided evidence that ER stress-induced repression of protein synthesis is independent of eIF4E, eIF4B, and eIF4A in HCV IRES. Since the FF/Ren ratio showed no significant changes which demonstrate that both upstream and downstream translations are similarly inhibited by Tg (Fig 3.4). It is believed that the effect of ER stress on translation from HCV IRES is cell-type dependent. In HeLa cells, translation from the HCV IRES is sensitive to the inhibitory effect of ER stress whereas in Huh-7 cells and HEK293T is fairly resistant. HeLa cells are equally sensitive to the inhibitory effects in response to ER stress, which is likely mediated via phosphorylation of eIF2α (MacCallum et al., 2006). These findings are consistent with other studies that observed the degree of repression was similar in cap dependent and cap-independent translation in response to ER stress, resulting in similar IRES/Cap ratio. Moreover, using Tg shows similar suppressive effects on translational activities (Chan & Egan, 2009). It has been shown that expression of a

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phosphorylated eIF2α mimetic in HeLa cells repressed cap-dependent and IRES-mediated translation to a similar extent (MacCallum et al., 2006). In addition, the results showed that the translation of CrPV IRES was not affected by the addition of Tg, and was able to bypass the effects of ER stress on protein synthesis. As the translation from CrPV IRES is independent of all initiation factors, the repression of cap dependent/total protein synthesis is likely mediated by repression of the activity of an initiation factor (Fig 3.2). Moreover, as the efficiency of the translation from the CrPV IRES is unaffected by ER stress, it is unlikely that ER stress inhibits translation elongation. Therefore, the repression of translation could be through inhibition of a number of initiation factors including eIF2α.Accumulation of misfolded proteins in the ER leads to phosphorylation of eIF2α and results in the repression of global protein synthesis. It was reported that the inhibition of cellular translation is mainly caused by phosphorylation of eIF2α (Wek, Jiang, & Anthony, 2006).

In addition to the phosphorylation of eIF2α-dependent mechanisms for translational repression during ER stress, mTORC1 suppression activity was in parallel to the eIF2α-P mechanism. mTORC1 is involved in the regulation of eIF4G, eIF4B and 4EBP1, of which 4EBP1 regulates the function of eIF4E that binds to the 5′mRNA cap structure. Under normal condition, hypophosphorylated 4EBP1 binds tightly to eIF4E. As 4EBP1 competes with eIF4G for binding site on eIF4E, 4EBP1 prevents eIF4G from interacting with eIF4E. However on mTORC1 activation, hyperphosphorylated 4EBP1 dissociates from eIF4E, allowing for the recruitment of eIF4G and eIF4A to the 5′end of a mRNA (Ma & Blenis, 2009). Another mechanism of regulating protein synthesis involves eukaryotic elongation factor 2 (eEF2). Phosphorylation of eEF2 at Thr56 by eEF2 kinase (eEF2K) interferes with the binding of eEF2 to the ribosome and the translocation step during elongation (Browne & Proud, 2002).As the repression of protein synthesis in this work was independent of eIF4E/4A and 4B initiation factors as well as elongation factors. Thus, we can conclude that the repression of protein synthesis is most likely independent of mTORC1 activation and elongation translation. Accumulating evidence has shown the role of PERK-dependent eIF2α phosphorylation in the repression of protein synthesis in response to ER stress. The data showed that there was a complete recovery of protein synthesis in the presenceof the PERK inhibitor *(Fig.3.6)* which gives us a strong evidence for the essential role of PERK in protein synthesis. This result is consistent with other findings that prove PERK is required for both the phosphorylation of eIF2α and the attenuation of translation in response to ER stress (Harding, Novoa, et al., 2000).

There are a number of mechanisms that are involved in the repression of protein synthesis during stress conditions. It was suggested that the regulating of ternary complex formation and subsequently, global translation and protein synthesis is through the competitive inhibition of eIF2B by the phosphorylation of eIF2α (Ma & Blenis, 2009)[30]. Since eIF2 is highly abundant than eIF2B, phosphorylation of only a fraction of eIF2 inhibits eIF2B and leads to block protein synthesis [31]. Phosphorylation of eIF2α under cellular stress leads to inhibition of eIF2B activity [28]. Phosphorylated eIF2 binds tightly to the regulatory subunit of eIF2Bα, β, and δ which lead to inhibit its activity. Moreover, the inhibition of phosphorylated eIF2 is prevented by deletion of eIF2Bα subunit from the complex (Wortham & Proud, 2015). Another mechanism includes the untranslated regions of mRNA, which have been shown an importance in the regulation of protein synthesis. Indeed, specific mRNAs are selectively translated in response to stress conditions when eIF2 is phosphorylated such as mRNA for general control non-depressible 4 (GCN4) in yeast, which is translated in response to amino acid deprivation (Hinnebusch, 2014),

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and in mammals ATF4 mRNA is preferentially translated via eIF2α phosphorylation in response to ER stress which leads to upregulate GADD34, subsequently dephosphorylates eIF2α with aid of protein phosphatase I, resulting in a recovery of protein synthesis(Rojas, Vasconcelos, & Dever, 2015).

The data suggest that the assay system was able to determine the role of specific initiation factors in investigating how protein synthesis is repressed under conditions of ER stress in real-time based on the luciferase activity. The system has previously been used as a reporter assay in mammalian cells to monitor processing of proteins through the secretory pathway and endoplasmic reticulum monitor after treatment with the ER stress inducer Tg(Browne & Proud, 2002). Indeed, the measurement of total protein synthesis using ^{35}S-L-Methionine incorporation as control indicated that the system we used was efficient. Since we showed about 80% decrease in total protein synthesis whereas there was about 30% inhibition in luciferase (Fig. 3.3 and 3.4), which indicated that the inhibition reflected the target protein that meant to be measured.In Summary, this work is an attempt to identify which initiation factor was responsible for the repression of protein synthesis in response to ER stress. The data showed that the eIF2α is likely responsible for the repression of protein synthesis in the presence of ER stress. Also, we showed the importance of PERK activation in repression of protein synthesis in response to ER stress.

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اختبار الآليات الجزيئية المسببة في تثبيط عمليات ترجمة البروتين في حالة إجهاد الشبكة الإندوبلازمية داخل الخلية

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المستخلص: طيّ البروتين هو عملية هامة لوظيفة الخلية. الشبكة الإندوبلازمية في الخلية هي المسؤول عن تصنيع، طيّ، تعديل، ومراقبة الجودة للعديد من البروتينات التي تعمل وتفرز من خلال الغشاء، إن الاضطرابات داخل الخلايا والتي تنجم عن الضغوطات المختلفة قد تؤدي إلى تراكم البروتينات وعدم طيّها بالصورة الصحيحة، والتي يمكن أن تؤدي إلى تغيير أو اختلال في التوازن داخل الشبكة الإندوبلازمية، إذا استمر تراكم البروتينات غير المطوّية بصورة صحيحة داخل الشبكة الإندوبلازمية، يتم تحديث استجابة من الخلية للتغلب على هذا الوضع. وهذه الاستجابة تعمل عن طريق ثلاث آليات مختلفة: (1) زيادة قدرة البروتين للطيّ، (2) تقليل من تخليق البروتين، و(3) تعزيز عملية التخلص من البروتينات غير المطوّية بشكل صحيح، وذلك عن طريق تنشيط عوامل ومسارات مثل(ATF6)،1 (IRE1) و(PERK) وذلك لاستعادة توازن الخلية وفي حالة إذا فشلت الخلية في الاستعادة أو التغلب على عيب الطيّ البروتين، يتم تنشيط مسارات تؤدي إلى موت الخلية.

الكلمات المفتاحية: إجهاد الشبكة البرونوبلازمية، بروتين كيناز شبكية إندوبلازميك، الترجمة، الاستجابة لعدم طيّ البروتين، تخليق البروتين، اليراع، رينيلا، خلايا مشتقة من فأر محوَّرٍ جينيًّا.

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