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Insulin modulates induction of glucose-regulated protein 78 during endoplasmic reticulum stress via augmentation of ATF4 expression in human neuroblastoma cells

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ABSTRACT

The effect of insulin on endoplasmic reticulum (ER) stress was investigated. Insulin protected cell death induced by ER stress and increased glucose-regulated protein 78 (GRP78) mRNA and protein levels. Insulin also significantly increased activating transcription factor-4 (ATF4) protein in the nucleus, which was inhibited by LY294002, a phosphatidylinositol 3-kinase (PI-3 kinase) inhibitor. The increase of ATF4 protein by insulin was not due to transcriptional or translational up-regulation but to a post-translational mechanism. Knockdown of ATF4 by siRNA significantly inhibited GRP78 induction by insulin. These results indicate that insulin modulated ER stress-induced GRP78 expression occurs via ATF4 up-regulation.

1. Introduction

Accumulation of unfolded proteins within the endoplasmic reticulum (ER) lumen induces ER stress, which triggers signaling from the ER to the nucleus [1]. Initial mediators of the ER stress response are three ER transmembrane proteins, inositol-requiring kinase 1α (IRE1α), RNA-dependent protein kinase-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF6) [2,3]. Activated IRE1α and ATF6 stimulate the transcription of chaperone genes. On the other hand, PERK phosphorylates the translation factor eIF2α, which decreases translation and prevents the accumulation of newly synthesized proteins in the ER [4]. Prolonged or severe ER stress leads to apoptotic cell death, which is mediated by caspase activation [5]. A hallmark chaperone induced by ER stress is glucose-regulated protein 78 (GRP78), also known as Bip. GRP78, is crucial for protein folding and assembly in the ER and regulation of ER stress initiation mediators, such as IRE1α, ATF6, and PERK, through a binding-release mechanism [3,6].

The induction of GRP78 by ER stress is mediated by multiple copies of the ER stress response element (ERSE) [7,8]. Both ATF6 and XBP-1 activate GRP78 induction through the ERSE elements, which is not a binding site for ATF4, a transcription factor induced by the PERK-eIF2α signaling pathway [9]. However, induction of GRP78 by ER stress is impaired in PERK knockout cells or eIF2α mutant knockin cells [10,11].

Insulin and insulin-like growth factor I (IGF-I) play a critical role in a number of biological effects, including cell proliferation, differentiation, and protection from apoptosis [12]. Insulin/IGF-I activates downstream pathways, such as the PI3-kinase and the mitogen-activated protein kinase cascades. The effects of insulin/IGF-I have been studied mainly in well-documented insulin target tissues. Recently, it has become clear that insulin also has pivotal effects in the brain, where it regulates neuronal survival [12].

In this study, the effect of insulin signaling on ER stress in human neuroblastoma SH-SY5Y cells was investigated. The present results show that insulin blocks the ER stress-induced activation of caspase-3, consistent with the up-regulation of GRP78, which is considered a survival molecule. Furthermore, the results demonstrate that the augmentation of ATF4 in the nucleus by insulin is responsible for the up-regulation of GRP78 through a mechanism requiring the PI3-kinase pathway.

2. Materials and methods

2.1. Reagents

Thapsigargin and tunicamycin were purchased from Sigma Chemical Co. (St. Louis, MO). LY294002 was purchased from Calbiochem (La Jolla, CA). Ac-DEVD-MCA was purchased from PEPTIDE

Abbreviation: Ac-DEVD-MCA, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid a-(4-methylcoumaryl-7-amide); S.D., standard deviation

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Institute, Inc. (Osaka, Japan). PARP, phospho-eIF2α, and phosphorylation-independent eIF2α antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). ATF4 (sc-200), GRP78 (76-E6), and actin (I-19) antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-DDDDK-Tag (PM020) and Lamin A/C antibodies (612162) were purchased from MBL (Nagoya, Japan) and BD Biosciences, respectively. All siRNAs were purchased from Qiagen (Hilden, Germany).

2.2. Cell culture

SH-SY5Y cells were cultured as described previously [13].

2.3. RT-PCR analysis and real-time PCR analysis

Isolation of total RNAs and RT-PCR were performed as described previously [13]. The primers used for PCR were as follows: GRP78 (5′-AGTTCTTGCCGTTCAAGGTG-3′ and 5′-AGACCGGAACAGATCCATGT-3′), GRP94 (5′-TGGACTCAGATGATCTCCCC-3′ and 5′-GTTGCCAGACCATCCGTACT-3′), and ATF4 (5′-TCAAACCTCATGGG-TTCTCC-3′ and 5′-GTGTCATCCAACGTGGTCAG-3′). The primers for GADD153 and GAPDH were described previously [13]. The density of PCR products was normalized to that of GAPDH. Real-time PCR was performed using ABI 7500 with SYBR green real-time PCR master mix (TOYOBO, Osaka, Japan) using the comparative ΔC_T method. The primers for β-actin were 5′-ATTGCCGACAGGATGCAGAA-3′ and 5′-ACATCTGCTGGAAGGTGGACAG-3′. The ATF4 primers for real-time PCR are the same as those used for classical PCR.

2.4. Western immunoblotting

Nuclear fractions were prepared using a Nuclear Extract kit (Active Motif, Carlsbad, CA). Western immunoblotting was performed as described previously [13].

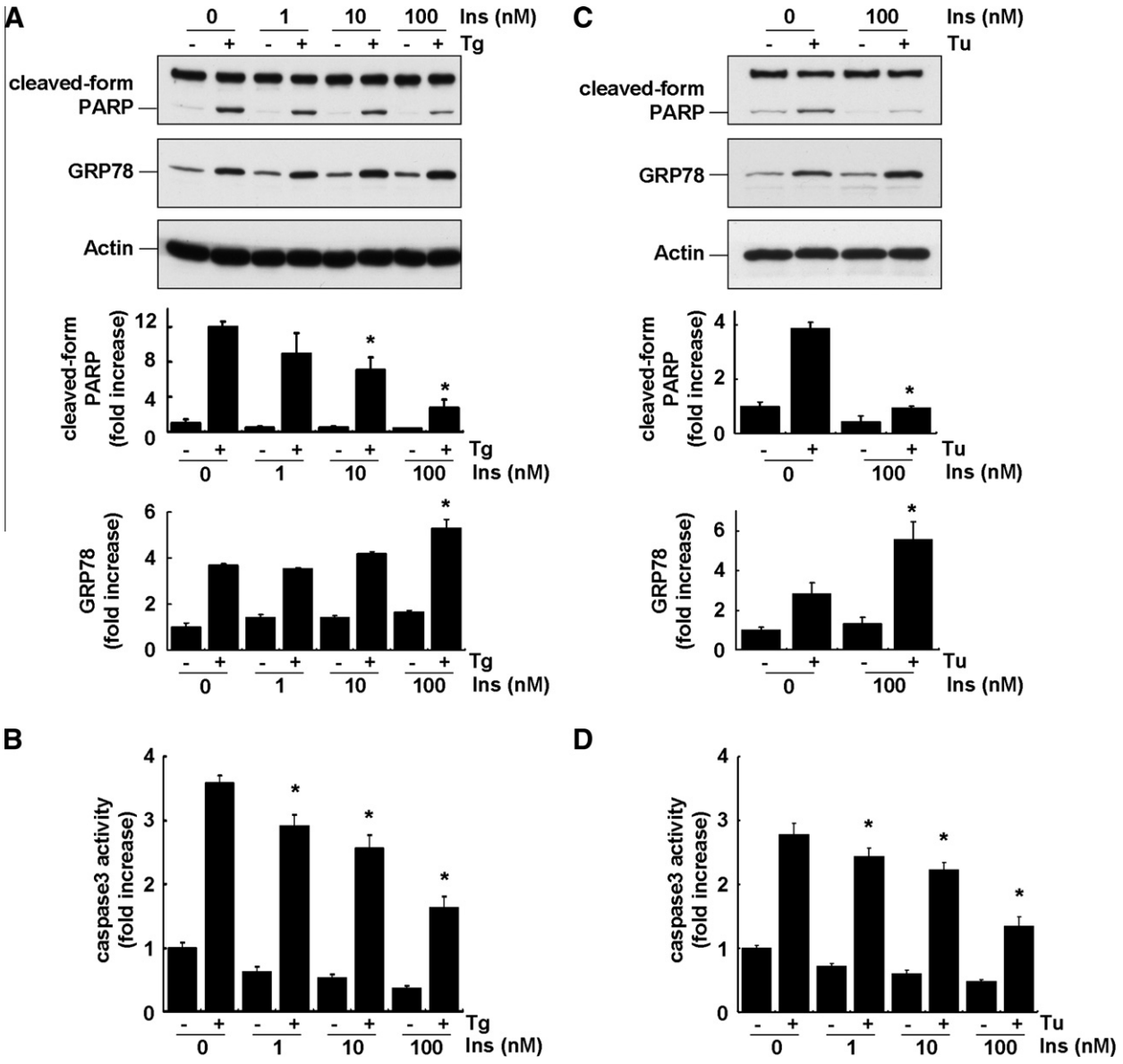

Fig. 1. Insulin up-regulates GRP78 expression by ER stress. SH-SY5Y cells were incubated with 1 μM thapsigargin (Tg), 1 μg/ml tunicamycin (Tu), or 1–100 nM insulin (Ins) for 18 h. (A and C) Whole cell lysates were analyzed by Western blotting. Results of densitometric analysis are shown. The value of DMSO-treated cells was set at 1. The values are mean ± SD ($n = 3$). *$P < 0.05$ vs. control (thapsigargin or tunicamycin alone). (B and D) The caspase-3 activity was determined as described in Section 2. The value of DMSO-treated cells was set at 1. The values are the mean ± SD ($n = 4$). *$P < 0.05$ vs. control (thapsigargin or tunicamycin alone).

2.5. Measurement of caspase-3 activity

After indicated treatments, SH-SY5Y cells were lysed by addition of 30 μl of the reaction buffer (50 mM HEPES (pH 7.4), 10 mM DTT, 100 mM NaCl, 0.1% Triton X-100, 25 μM Ac-DEVD-MCA) and incubated at 37 °C for 2 h. The generation of free AMC was measured at an excitation wavelength of 340 nm and an emission wavelength of 460 nm.

2.6. Reporter assay

Both 3080-bp and 132-bp of the human GRP78 promoter region were amplified by PCR from SH-SY5Y genomic DNA and were cloned into pGL4.16 (Promega, Madison, Inc). For the ATF4 5′UTR -Luc reporter plasmid, human ATF4 5′UTR (which contains uORF1 and uORF2) was isolated by RT-PCR using SH-SY5Y total RNA and was fused to the ATF4 initiation codon with the firefly luciferase gene under the control of a CMV promoter. uORF2 overlaps the ATF4-luciferase fusion gene and contains the stop codon. Transfection was performed using Lipofectamine 2000 (Invitrogen). Luciferase assays were performed using the Dual-Glo luciferase assay kit (Promega). siRNA-mediated gene knockdown was performed using HP validated siRNA (Qiagen) for ATF4 (SI03019345) and ATF6 (SI03019205) and Allstars negative control siRNA. FLAG-2xEGFPpcDNA3.1 contains FLAG-tagged tandem EGFPs driven by a CMV promoter. Full-length ATF4 cDNA and truncated ATF6 cDNA (amino acid 1-373) was isolated from SH-SY5Y total RNA and fused to the C-terminus of FLAG-EGFP in pcDNA3.1.

2.7. Statistical analysis

Results were expressed as mean ± standard deviation (S.D.). The statistical significance was determined by the Bonferroni multiple comparison test using R, version 2.6.2 (www.R-project.org). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Insulin blocks caspase-3 activation by ER stress

Insulin blocked the cleavage of poly(ADP-ribose) polymerase (PARP) and the activation of caspase-3 by thapsigargin in a dose-dependent manner (Fig. 1A and B, respectively). Insulin also had effects on tunicamycin treatment (Fig. 1C and D). Insulin significantly increased the expression of GRP78 in dose-dependent manner. It has been proposed that GRP78 blocks ER stress-induced apoptosis, which suggests that insulin up-regulates GRP78 expression to suppress the activation of caspase-3 by ER stress.

3.2. Effect of insulin on ER stress-induced gene expression

To examine the effect of insulin on ER stress-induced gene expression, RT-PCR analysis was performed. Insulin potentiated the expression of GRP78 mRNA for 4–8 h more than thapsigargin or tunicamycin alone (Fig. 2). Although the induction of ATF4 and GADD153 mRNAs by ER stress was observed, insulin did not significantly enhance their induction. This suggests that, via a transcriptional mechanism, insulin can selectively increase the expression of GRP78, which is already induced by ER stress.

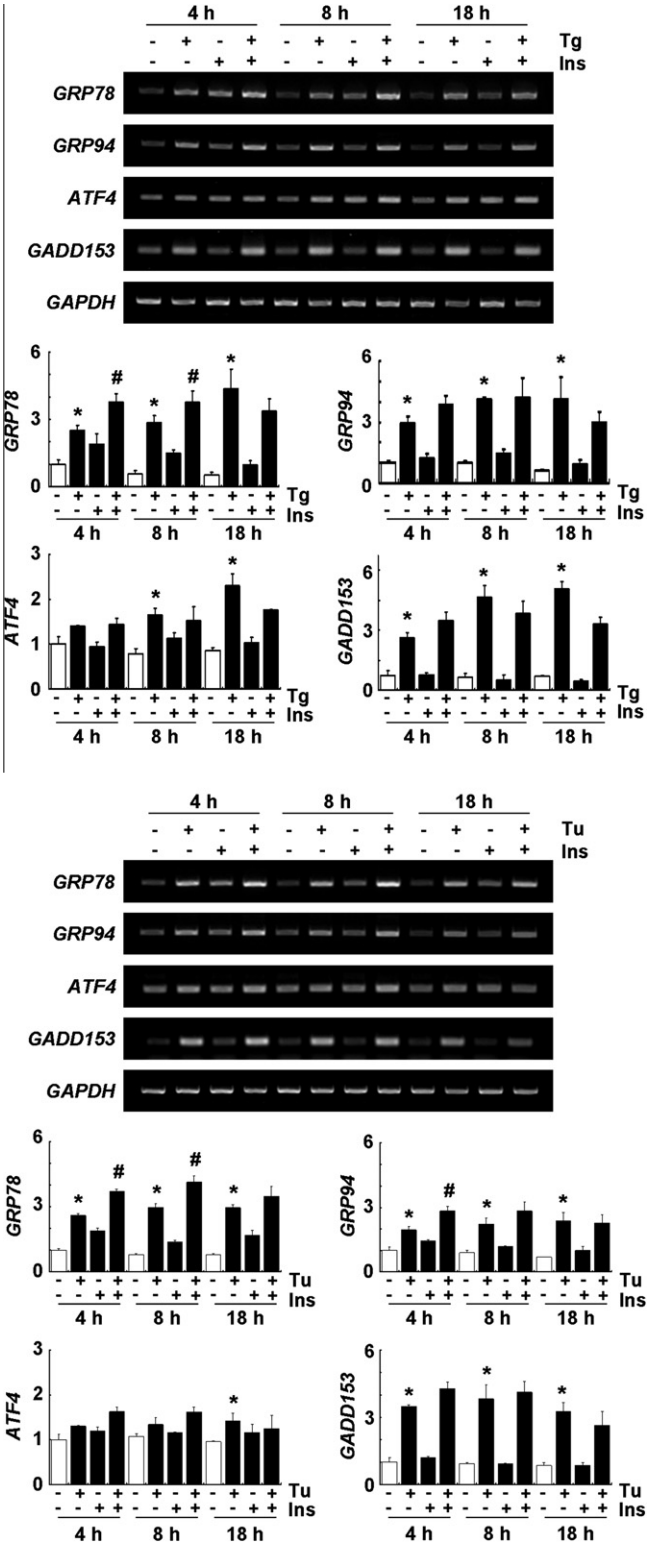

Fig. 2. Effect of insulin on mRNA levels of ER stress responsive genes. SH-SY5Y cells were incubated with 1 μM thapsigargin (Tg), 1 μg/ml tunicamycin (Tu), or 100 nM insulin (Ins) for the indicated times. The mRNA levels were determined by RT-PCR analysis. The value of DMSO-treated cells for 4 h was set at 1. The values are the mean ± SD ($n = 3$). $^{*}P < 0.05$ vs. DMSO-treated samples. $^{\#}P < 0.05$ vs. thapsigargin or tunicamycin-treated cells.

3.3. Insulin stabilizes ATF4 protein

ATF4 is expressed in a preferential translational mechanism during ER stress [10]. To examine whether insulin influenced expression of ATF4 by ER stress, western blotting analysis was performed. Insulin further accumulated ATF4 in the whole cell lysate (2.0- and 2.2-fold, in Fig. 3A) and robustly in the nuclear fraction (6.2- and 7.0-fold, in Fig. 3B) more than thapsigargin or tunicamycin

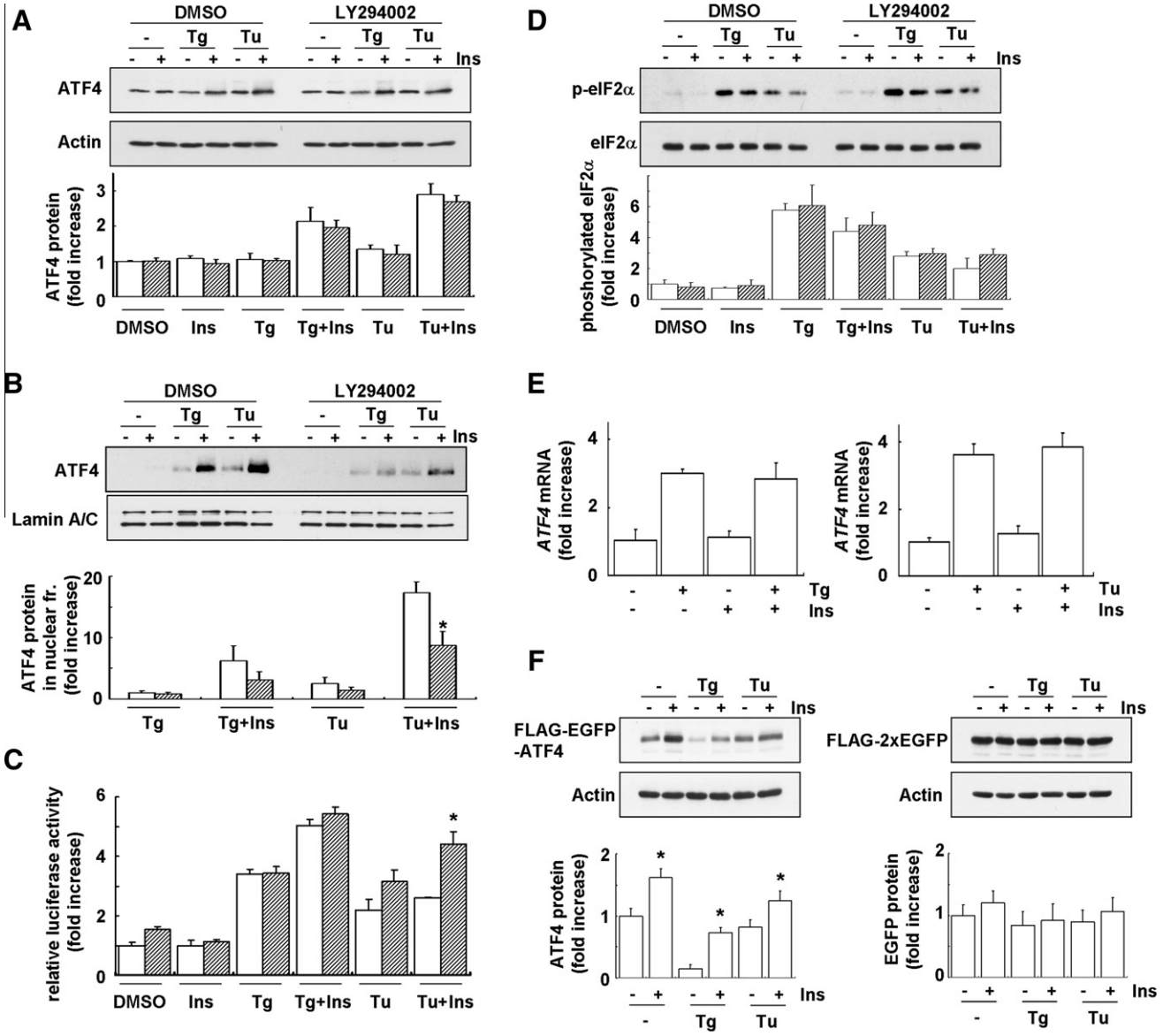

Fig. 3. Effect of Insulin on the induction of ATF4 protein by ER stress. (A and B) SH-SY5Y cells were pretreated with DMSO (open bars) or 10 μM LY294002 (gray bars) for 30 min and further incubated with 1 μM thapsigargin (Tg), 1 μg/ml tunicamycin (Tu), or 100 nM insulin (Ins) for 4 h. Whole cell lysates (A and D) and nuclear fractions (B) were analyzed by Western blotting. The value of DMSO- (A and D) and thapsigargin-treated cells (B) were set at 1. The values are the mean ± SD ($n = 3$). $^*P < 0.05$ vs. DMSO-pretreated cells (open bars). (C) SH-SY5Y cells were co-transfected with 250 ng of ATF4 5′UTR-Luc plasmid and 12.5 ng of *Renilla* luciferase plasmid. The transfected cells were pretreated with DMSO (open bars) or 10 μM LY294002 (gray bar) for 30 min and further incubated with 1 μM thapsigargin (Tg), 1 μg/ml tunicamycin (Tu), or 100 nM insulin (Ins) for 12 h. Relative luciferase activity is a ratio of firefly luciferase activity normalized for *Renilla* luciferase activity, and the value of DMSO-treated cells was set at 1. The values are the mean ± SD ($n = 3$). $^*P < 0.05$ vs. DMSO-pretreated cells (open bars). (E) SH-SY5Y cells were treated for 4 h and real-time PCR analysis was performed. (F) SH-SY5Y cells were transfected with 250 ng of plasmid. FLAG-EGFP-ATF4 fusion protein and FLAG-2xEGFP protein was detected by anti-ATF4 antibody and anti-DDDDK-Tag antibody, respectively.

alone, respectively. The effects of insulin on ATF4 were blocked by pretreatment with LY294002, a PI-3 kinase inhibitor. To verify whether accumulation of ATF4 was dependent on the increase of translation efficacy, the ATF4 5′UTR-Luc reporter assay was performed. As shown in Fig. 3C, thapsigargin or tunicamycin increased reporter activity. Insulin slightly increased reporter activity by thapsigargin but did not increase the reporter activity by tunicamycin. The reporter activity was not inhibited by LY294002 pretreatment in contrast to the ATF4 western blot analysis. Insulin had little effect on the phosphorylation level of eIF2α by ER stress (Fig. 3D) and the level of ATF4 mRNA (Fig. 3E) but significantly increased the level of ATF4 protein under the constitutive expression of ATF4 mRNA (Fig. 3F). Thus, this indicated that insulin stabilized ATF4 through PI-3 kinase signaling.

3.4. ATF4 knockdown attenuates insulin-potentiated GRP78 induction by ER stress

To examine whether ATF4 contributes to the up-regulation of GRP78 expression by insulin, co-transfection of ATF4 and/or ATF6 siRNA with two GRP78 promoter reporters was performed. A CRE/ATF-like site upstream of the ERSEs in the mammalian GRP78 promoter has been described as an ATF4 binding site [14]. In addition to three ERSEs sites, the -3080-Luc reporter has an ATF/CRE-like site (5′-TGACGTGA-3′), which is located at −1835 to −1828 in the human GRP78 promoter. The -132-Luc reporter has three ERSEs without a putative ATF4 binding site. As shown in Fig. 4A and B, thapsigargin and tunicamycin increased the reporter activity. Insulin further amplified the promoter activity compared

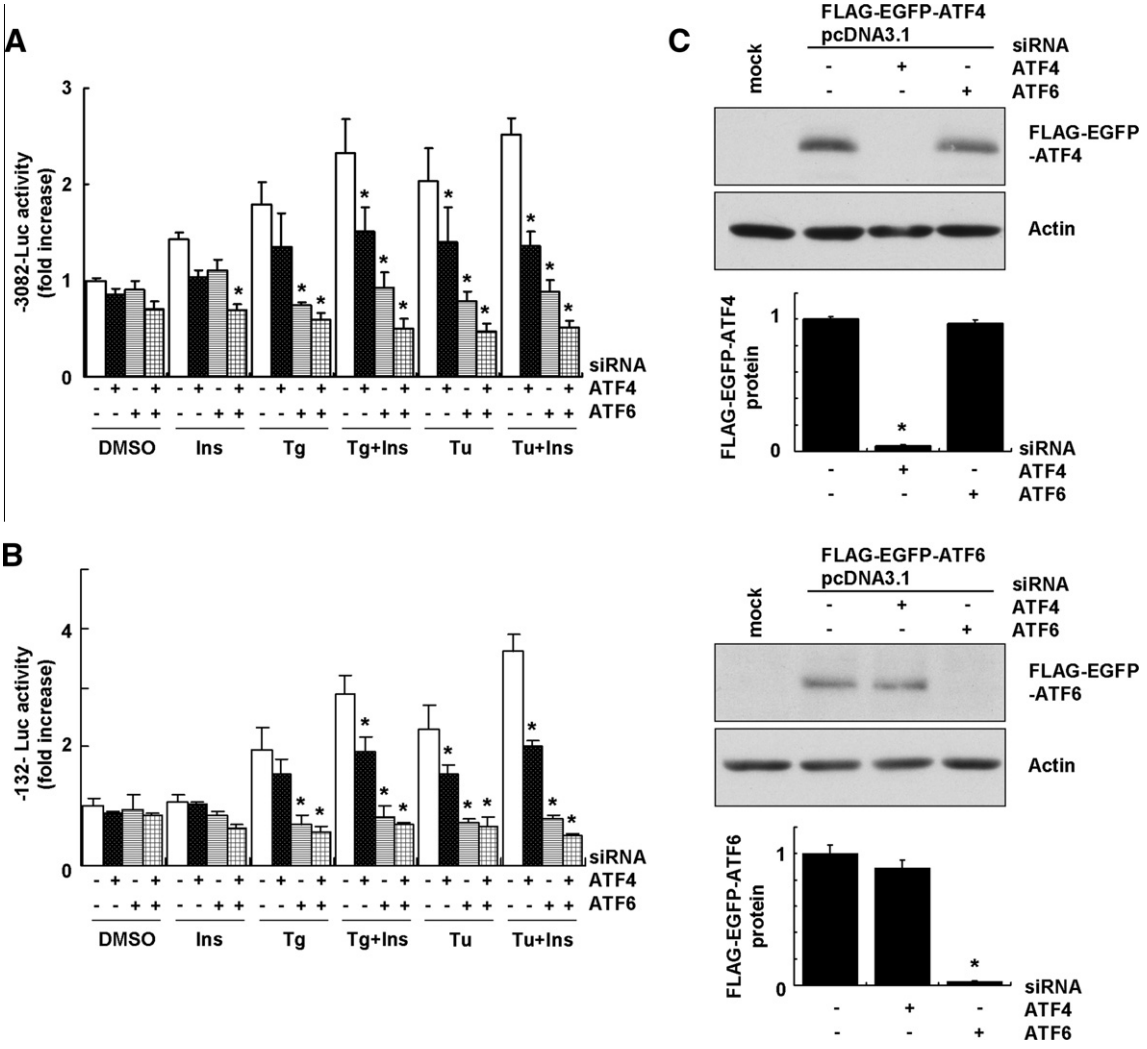

Fig. 4. Effect of ATF4 knockdown on GRP78 promoter activity. SH-SY5Y cells were co-transfected with 2.5 pmol of siRNA as indicated, 250 ng of GRP78 promoter plasmid, and 12.5 ng of *Renilla* luciferase plasmid. GRP78 promoter plasmids were used, -3082-Luc in (A) and -132-Luc in (B). The transfected cells were incubated with 1 μM thapsigargin (Tg), 1 μg/ml tunicamycin (Tu), or 100 nM insulin (Ins) for 12 h. Relative luciferase activity was determined as described in Fig. 3C, and the value of DMSO-treated cells was set at 1. The values are the mean ± SD ($n = 3$). *$P < 0.05$ vs. negative control siRNA transfected cells (open bars).

to thapsigargin or tunicamycin alone, which is consistent with the RT-PCR analysis. ATF4 knockdown significantly inhibited the reporter activity, and combined ATF4 and ATF6 siRNA inhibited the reporter activity more than ATF6 siRNA alone. ATF4 and ATF6 siRNAs specifically reduced the expression of ATF4 and ATF6, respectively (Fig. 4C).

4. Discussion

The present study investigated the effects of insulin on ER stress in human neuroblastoma SH-SY5Y cells. The major findings are as followed: (1) Insulin blocked the cleavage of PARP and caspase-3 activation by ER stress but up-regulated the induction of GRP78 mRNA and protein. (2) Insulin significantly augmented ATF4 protein in the nucleus, which was dependent on PI-3 kinase. (3) Using the reporter assay, the potentiation of ER stress-induced GRP78 expression by insulin was dependent on ATF4. Overexpression of truncated ATF6 can robustly induce GRP78 [15]. In ATF6α knockout cells, the induction of GRP78 by ER stress is greatly diminished but not completely abrogated [16]. ATF4 activates the GRP78 promoter independent of the ERSEs [14]. Our results show that induction of GRP78 by ER stress is mainly dependent on ATF6, and ATF4 plays a critical role in the potentiation of GRP78 induction by insulin. An extrinsic stimulus, such as insulin, can modulate the ER stress induced by intrinsic stress. In this regard, it has been reported that GLP-1 signaling modulates the ER stress in pancreatic β cells [17] and IGF-I protects cells from ER stress-induced apoptosis [18]. How ATF4 activates the GRP78 promoter directly or indirectly by insulin needs further study.

Phosphorylation of eIF2α by ER stress reduces global protein synthesis, but, paradoxically, ATF4 proteins are selectively synthesized through a mechanism of translation reinitiation [10]. Augmentation of ATF4 by insulin signaling does not seem to depend on the above mechanism but is mediated via a post-translational mechanism. Previous studies have demonstrated that the stability and activity of ATF4 is regulated by post-translational modifications [19,20]. It will be important to investigate how insulin increases ATF4 protein expression.

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