Imoxin inhibits tunicamycin-induced endoplasmic reticulum stress and restores insulin signaling in C2C12 myotubes.

Running Title: Imoxin inhibits ER stress and insulin resistance in myotubes

Hyeyoon Eo¹² and Rudy J Valentine¹²*

1. Department of Kinesiology, Iowa State University, Ames, Iowa
2. Interdepartmental Graduate Program in Nutritional Sciences, Iowa State University, Ames, Iowa

*Correspondence to: Rudy J. Valentine

Iowa State University
111C Forker Building, 534 Wallace Rd, Ames, IA 50011
Tel: (515) 294-3867
Fax: (515) 294-8740
Email: rvalenti@iastate.edu

Keywords: imoxin, PKR, tunicamycin, ER stress, insulin signaling
Abstract

Prolonged endoplasmic reticulum (ER) stress can mediate inflammatory myopathies and insulin signaling pathways. The double stranded RNA (dsRNA) activated protein kinase R (PKR) has been implicated in skeletal muscle dysfunction. However, pathological roles of PKR in ER stress in muscle are not fully understood. The current study aimed to investigate the effect of imoxin (IMX), a selective PKR inhibitor, on tunicamycin (TN)-induced promotion of ER stress and suppression of insulin signaling in C2C12 myotubes. Cells were pre-treated with 5 uM IMX for 1 hr, and exposed to 0.5 µg/ml TN for 23 hr. A subset of cells was stimulated with 100 nM insulin for the last 15 min. mRNA expression and protein levels involved in ER stress were measured by RT-PCR and Western blotting, respectively. TN significantly augmented PKR phosphorylation by 231%, which was prevented by IMX. In addition, IMX reduced mRNA and protein levels of ER stress-related markers including CCAAT-enhancer-binding protein homologous protein (CHOP, mRNA: 95% decrease; protein: 98% decrease), activating transcription factor 4 (ATF4, mRNA: 69% decrease; protein: 99% decrease), cleavage of ATF6, and spliced X-box binding protein 1 (XBP-1s, mRNA: 88% decrease; protein: 79% decrease) which were induced by TN. Furthermore, IMX ameliorated TN-induced suppression of phospho-insulin receptor beta (317% increase) and Akt phosphorylation (by 36% at Ser473 and 30% at Thr308) in myotubes, while augmenting insulin-stimulated AS160 phosphorylation and glucose uptake (by ~30%). These findings suggest that IMX may protect against TN-induced skeletal muscle ER stress and insulin resistance, which are potentially mediated by PKR.
INTRODUCTION

The Endoplasmic reticulum (ER) plays a key role in protein folding and calcium homeostasis in many mammalian cell types including skeletal muscle (36). ER stress can be induced when the ER is exposed to physiological or pathological stresses such as accumulation of unfolded/misfolded proteins and imbalanced calcium levels (23, 25). As a result, the cells initiate the unfolded protein response (UPR) to restore ER homeostasis and to sustain cell survival (3). The UPR is mediated by three transmembrane sensors: inositol-requiring protein (IRE)-1, RNA-dependent protein kinase (PKR)-like ER eukaryotic translation initiation factor 2 alpha kinase (PERK), and activating transcription factor (ATF) 6 (33). In absence of stress, these proteins are inactive by binding to an ER chaperone called immunoglobulin heavy chain binding protein/glucose-regulating protein 78 (BiP/GRP78) (2). However, when the cells are under stressful physiological conditions, IRE-1, PERK and ATF6 are released from GRP78 and this alleviates ER stress by modulation of gene expression and protein synthesis in the cells (3). However, failure to recover from ER stress due to disruption of the UPR or prolonged ER stress can promote cell dysfunction and death (3, 33).

In recent years, it has been reported that ER stress is involved in various aspects of skeletal muscle physiology such as skeletal muscle development, regeneration, adaptation, and remodeling (1, 3). Maladaptive UPR can result in skeletal muscle wasting via inhibition of phosphoinositide 3 kinase (PI3K)/protein kinase B (PKB or Akt)/mammalian target of rapamycin (mTOR) cascades related to protein synthesis and promotion of the ubiquitin-proteasome system (UPS) involved in proteolysis (1). Furthermore, ER stress can cause insulin resistance through alteration of insulin signaling molecules and generation of reactive oxygen species (ROS) (13, 35). Specifically, suppression of insulin signaling has been observed in murine muscle cells treated with tunicamycin (TN), an agent that blocks protein glycosylation and strongly induces ER stress (15). As skeletal muscle is one of the main sites of.
postprandial glucose disposal, controlling ER stress in skeletal muscle is crucial to maintain glucose homeostasis as well as muscle health.

The double stranded RNA (dsRNA) activated protein kinase R (PKR) is a serine/threonine protein kinase which can be activated by viral infection, nutrient excess, and inflammatory cytokines (27, 28, 37). PKR is involved in inflammatory signaling (4, 12, 18, 41) and metabolic dysregulation (5, 27, 38) in various cells. Imidazolo-oxindole, or imoxin (IMX), is a pharmacological inhibitor of PKR, blocking its autophosphorylation at Thr446 and Thr451 to suppress its catalytic activity (8, 11). Our group recently reported that IMX attenuated PKR phosphorylation and endotoxin-induced skeletal muscle inflammation (39), as well as glucocorticoid-induced PKR phosphorylation and muscle atrophy (9). In addition, activation of PKR in response to ER stress has been reported (22). However, it has yet to be established whether PKR is involved in ER stress and ER stress-induced insulin resistance in skeletal muscle. In this context, the aim of the current study was to investigate the protective effect of IMX against TN-induced ER stress and insulin resistance in skeletal muscle cells. It was hypothesized that IMX would suppress TN-induced ER stress and restore insulin signaling in the context ER stress.
MATERIALS AND METHODS

Cell Culture and Experimental Design

Mouse C2C12 myoblasts were purchased from American Type Tissue Culture (ATCC CRL-1772), cultured in high-glucose (25mM glucose) Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Waltham, MA) containing 10% fetal bovine serum (Metuchen, NJ), 1% penicillin-streptomycin (Gibco, Waltham, MA) and 1% Glutamax (Gibco, Waltham, MA) in a 5% CO₂ at 37 °C. C2C12 myoblasts (passages < 13) were seeded at 1 × 10⁶ cells/ml in 6-well (for mRNA) or 12-well (for protein) plates. When C2C12 myoblasts were 90% confluent, medium was replaced with differentiation medium (high-glucose DMEM containing 2% horse serum, 1% penicillin-streptomycin, and 1% Glutamax), and replaced every other day. After 5 days of differentiation, myotubes were pre-treated with vehicle [VEH; phosphate buffered saline (PBS)] or 5 µM IMX (EMD Millipore, Burlington, MA) for 1 h, after which, 0.5 µg/ml TN (EMD Millipore, Burlington, MA) or vehicle was added into each well for 23 h. In insulin signaling experiments, 100 nM insulin was added to the myotubes 15 min before harvest. In all experiments, each group had 3 replicates and all the experiments were repeated three times.

Western Blot Analysis

To prepare whole cell lysate, cells were washed with ice-cold PBS and then scraped and lysed with cell lysis buffer (Cat No. 9803, Cell Signaling Technology, Danvers, MA) including Halt™ protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA) and phosphatase inhibitor cocktail 3 (Sigma-Aldrich, St. Louis, MO). The cell lysate was centrifuged for 10 min at 14,000 g at 4°C and the supernatant was transferred for future use. Equal amounts of proteins (5-10 µg) were separated by 4–15% gradient Stain-FreeCriterion TGX gel electrophoresis (Bio-Rad, Hercules, CA), and transferred onto a PVDF (polyvinylidene difluoride) membrane (MilliporeSigma, Burlington, MA). Gels were activated according
to Bio-Rad’s Stain-Free protocol, and total protein on the blot was detected by imaging the membrane under UV light to normalize signal intensity for the protein of interest in each lane to the total protein amount in the same lane. The membrane was then blocked in Tris-buffered saline (pH 7.5) containing 0.1% Tween-20 (Sigma-Aldrich, St. Louis, MO) (TBST) and 5% non-fat dry milk (Lab Scientific Inc., Danvers, MA) for 1 h at room temperature. Next, the membranes were incubated in primary antibodies at a 1:1000 dilution overnight. The primary antibodies were directed against p-PKR<sup>Thr446</sup> (ab32036) purchased from Abcam (Cambridge, United Kingdom); PKR (sc-6282), CHOP (sc-7351), GRP78 (sc-13539) purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX); p-PERK (#3179), ATF4 (#11815), ATF6 (#65880), XBP-1s (#83418), p-insulin receptor beta (p-IRβ<sup>Tyr1150/1151</sup>, #3024), insulin receptor substrate 1 (IRS1; #3407), p-IRS1<sup>Ser636/639</sup> (#2388), p-Akt<sup>Ser473</sup> (#9271), p-Akt<sup>Thr308</sup> (#2965), Akt (#9272), p-AS160 (#8881), and AS160 (#2670) purchased from Cell Signaling Technology (Danvers, MA). Membranes were then washed with TBST, incubated with respective horseradish peroxidase-conjugated secondary antibodies against mouse (#7076, Cell Signaling Technology, Danvers, MA) or rabbit (#7074, Cell Signaling Technology, Danvers, MA) at a 1:2000 dilution, and washed with TBST and Nano-pure water. The membranes were imaged using an enhanced chemiluminescence solution (Thermo Fisher Scientific, Waltham, MA). Immunoreactive bands were captured with the ChemiDoc™ XRS Imaging System (Bio-Rad, Hercules, CA), and densitometry was performed using Image Lab V6.0 (Bio-Rad, Hercules, CA).

**Real-Time Polymerase Chain Reaction (RT-PCR)**

The cells were homogenized in 500 µl Trizol (Sigma-Aldrich, St. Louis, MO). Total RNA was extracted, and concentration was quantified spectrophotometrically using the NanoDrop System (NanoDrop Technologies, Wilmington, DE). Total RNA (1 µg) was reverse transcribed to cDNA using a Verso cDNA Synthesis Kit (Thermo Scientific, Waltham, MA) according to manufacturer’s instructions. Quantitative RT-PCR was performed with 20 ng/reaction using iTaq Universal SYBR Green Supermix
(Bio-Rad, Hercules, CA) and run on a MyiQ PCR system (Bio-Rad). Each gene was normalized against the housekeeping gene 18S using the ΔΔC_T method, and expression of each gene is presented as mean fold-change relative to the non-treated control group. The primer sequences used for each gene are as follows: 18S (forward: TCAAGAACGAAAAGTCCGGAGG, reverse: GGACATCTAAGGGCATCAC), PKR (forward: GATGGAAAATCCGAAGG, reverse: AGGCCAAAAGCAAGCATGCAC), CHOP (forward: CCTAGCTTGCACTGACAGGAG, reverse: TTAAGCCCTTCCTCCTGC), ATF4 (forward: GAGCTTCCTGAACAGGAG, reverse: GGCCACCTCAGAAGGAGGTTC), ATF6 (forward: TAACTTCCAGGGGAGGGTG, reverse: AGAGCCAGGAAGGAGGT), XBP-1µ (forward: AAGAACACGCTTGGGAATGG, reverse: ACTCCCCTGCGCTCCAC), XBP-1s (forward: GAGTCCGGACTGCGGTG, reverse: GTGTCAGAGGTCATC). The experiments were independently repeated in quadruplicates.

2-NBDG Glucose Uptake Assay

Using a fluorescent D-glucose analog 2-[N-(7-nitrobenz-2-oxa1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG, Cat# 11046, Cayman Chemical Company, Ann Arbor, MI), glucose uptake was monitored in C2C12 myotubes. Cells were seeded at 1 × 10^5 cells/ml in 96-well plates. When the cells reached 95% confluent, cells were differentiated for 5 days in differentiation medium as described above. After 5 days of differentiation, myotubes were pre-treated with or without 5 µM IMX for 1 h and then exposed to 0.5 µg/ml TN for 22 h. Cells were washed with sterile PBS twice and then treated with or without 100 nM insulin in glucose-free/serum serum-free DMEM for 30 min. After 30 min of insulin stimulation, 2-NBDG was added to the cells at the final concentration of 100 µg/ml for an additional 30 min. The medium was then washed twice with PBS and then cells were lysed with 100 µl PBS with 0.5% Triton X-100 to stop further metabolism. The fluorescent intensity of cellular 2-NBDG in each well was measured immediately at wavelengths of 475 nm for excitation and 550 nm for emission using a
microplate reader (Fluostar Galaxy, BMG Labtech, Ortenberg, Germany) according to the manufacturer’s instruction.

Resazurin Assay

Cell viability was determined using Resazurin sodium salt (Cat# R7017, Sigma-Aldrich, St. Louis, MO) (29). Cells were seeded at 1 × 10^5 cells/ml in 96-well plates. When the cells reached 95% confluent, cells were differentiated for 5 days in differentiation medium as described above. After 5 days of differentiation, myotubes were pre-treated with or without 5 µM IMX for 1 h and then exposed to 0.5 µg/ml TN for 22 h. Before determination, cells were treated with resazurin reagent at final concentration of 0.025 mg/ml for an additional 1 h. The fluorescent intensity was measured immediately at wavelengths of 560 nm for excitation and 590 nm for emission using a microplate reader (Fluostar Galaxy, BMG Labtech, Ortenberg, Germany) according to the manufacturer’s instruction.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7.0.5 (GraphPad Software Inc., San Diego, CA). Data from three experiments with 3 replicates each are expressed as the mean ± standard error of means (SEM). Each dot in each lane (group) represents the average value of each group from each biological replicate. A one-way analysis of variance (ANOVA) with Tukey’s post-hoc test was performed for multiple comparisons in RT-PCR data of mRNA expression (Fig. 1A and 2). A two-way ANOVA with Tukey’s post-hoc test was performed for comparisons of protein levels (Fig. 1B and 3B – 3F). Student t-test was performed for comparisons between two groups (Fig. 3G and 3H). A three-way ANOVA with Tukey’s post-hoc test was performed for multiple comparisons in levels of proteins involving in insulin signaling pathways (Fig. 4 and 5) and glucose uptake (Fig. 6). The differences
between groups and the main effects of TN, IMX and insulin were considered statistically significant at p<0.05 and shown in each figure.
RESULTS

Effect of TN and IMX on mRNA expression and phosphorylation of PKR in C2C12 myotubes.

The effect of TN and IMX on PKR expression was assessed using RT-PCR (Fig. 1A). TN increased PKR mRNA expression (142% increase) compared with the untreated control group. On the other hand, IMX treatment significantly reduced mRNA expression of PKR (by 95%) in cells exposed to TN compared with the TN alone group. In addition to mRNA expression, the effect of TN and IMX on PKR phosphorylation at Thr446 was evaluated (Fig. 1B). Main effects of TN and IMX, and a TN x IMX interaction were observed (all \( p < 0.01 \)). TN significantly elevated PKR phosphorylation (239% increase) compared with the untreated control group. However, IMX decreased PKR phosphorylation by 54% compared with the TN-only treatment.

Effect of IMX on ER stress signaling molecules induced by TN in C2C12 myotubes.

To investigate the effect of IMX on TN-induced UPR, gene expression related to ER stress signaling molecules was measured using RT-PCR (Fig. 2). As shown in Fig. 2A, 2B and 2C, ER stress markers including CHOP, ATF4, and ATF6 were increased in response to TN treatment (8.4-fold, 2.0-fold, and 2.2-fold, respectively). However, IMX treatment significantly reduced mRNA expression of CHOP and ATF4 induced by TN treatment by 95% and 69%, respectively. In addition, splicing of XBP-1 mRNA was assessed as shown in Fig. 2D, 2E and 2F. TN treatment increased mRNA level of both XBP-1u and XBP-1s (1.5-fold and 3.6-fold, respectively) and XBP-1s/XBP-1u ratio (2.5-fold). However, IMX significantly decreased mRNA levels of XBP-1u and XBP-1s (by 89% and 88%, respectively) compared with TN alone.

In addition to gene expression, protein levels of ER stress markers were measured in C2C12 myotubes (Fig. 3). Significant increases in protein levels of ER stress markers including GRP78 (539%), CHOP...
(1422%), p-PERK (1335%), ATF4 (403%) and XBP-1s (196%) were observed in the cells exposed to TN compared to the untreated control group (Fig. 3B). IMX treatment significantly lowered TN-induced protein levels of ER stress markers including GRP78 (61%), CHOP (98%), p-PERK (88%), ATF4 (99%) and XBP-1s (79%) compared to TN alone (Fig. 3). There were main effects of TN and TN × IMX interactions in the protein levels of GRP78, CHOP, p-PERK, and XBP-1s. Moreover, full-length ATF6 bands at 100 kDa were only detectable in VEH-treated groups (Fig. 3A). However, the cleaved bands at 90 kDa were only observed in response to TN. IMX significantly reduced full-length ATF6 (by 37%) compared to VEH and cleaved ATF6 (by 76%) compared with TN alone, respectively (Fig. 3C and 3D).

Effect of IMX on insulin signaling in C2C12 myotubes.

To evaluate the effects of TN and IMX on the insulin signaling pathway, C2C12 myotubes were treated with IMX and exposed to TN, as described above, and insulin was added. As shown in Fig. 4, TN significantly suppressed protein levels of p-IRβ Tyr1150/1151 (by 68%) and p-IRS1 Ser636/639 (by 36%) compared with untreated control in the presence of insulin. However, IMX treatment augmented insulin-induced phosphorylation of both p-IRβ Tyr1150/1151 (317% increase) and p-IRS1 Ser636/639 (151% increase) in the presence TN (Fig 4). There were main effects of TN, insulin, and IMX in protein levels of p-IRβ Tyr1150/1151 and p-IRS1 Ser636/639 and interactions between TN × IMX, insulin × IMX, and TN × insulin × IMX were found for p-IRβ and p-IRS1 Ser636/639.

In addition to IRβ and IRS1, TN lowered insulin-induced phosphorylation of Akt at Ser473 (20% decrease), which was fully prevented by IMX (Fig. 5A and 5B). Even though TN suppressed insulin-induced Akt phosphorylation at Thr308 (13% decrease), IMX augmented insulin-stimulated phosphorylation of Akt at Thr308 (30% increase, Fig. 5C). Moreover, there was no effect of TN treatment on AS160 phosphorylation, but IMX significantly increased the AS160 phosphorylation in presence of insulin compared to the vehicle (by 46%) and to the TN treatment (by 105%, Figs. 5A and 5D).
Effect of IMX on glucose uptake and cell viability in C2C12 myotubes.

To monitor the glucose uptake in C2C12 myotubes, 2-NBDG assay was performed as shown in Fig. 6A. There were main effects of IMX, TN, INS and TN × INS on glucose uptake. TN suppressed insulin-stimulated glucose uptake compared with VEH (26% decrease). However, IMX significantly increased glucose uptake in presence and absence of TN treatment (26% and 36% increase, respectively). In addition, to evaluate cell viability, resazurin assay was performed in C2C12 myotubes (Fig. 6B and 6C). TN only treatment group had a significant reduction of cell viability by 35% compared to the untreated control group (Fig. 6B). However, IMX did not show significant difference in cell viability regardless of TN treatment (Fig. 6B and 6C).
This study investigated the beneficial effects of IMX on ER stress and ER stress-induced insulin resistance in skeletal muscle myotubes. Our results demonstrate the protective role of IMX on TN-induced ER stress and on TN-induced suppression of the insulin signaling pathway. We found elevation of PKR phosphorylation in the cells exposed to TN, but IMX inhibited PKR gene expression and phosphorylation at Thr446 in presence of TN (Fig. 1). IMX suppressed TN-induced gene expression and protein levels of ER membrane-associated sensors and downstream molecules including GRP78 and CHOP (Fig. 2 and 3). Moreover, we found that IMX promoted insulin signaling which was suppressed by TN (Figs. 4 and 5).

In our recent study, we found that PKR phosphorylation was increased by dexamethasone, and that IMX prevented dexamethasone-induced elevation of muscle-specific E3 ubiquitin ligases including muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx) and subsequent muscle atrophy (9). Moreover, our group demonstrated that IMX attenuated skeletal muscle inflammation induced by lipopolysaccharide in mice (39). Vaughn et al. reported that TN promotes PKR phosphorylation but inhibition of PKR protects TN-induced apoptosis in neuroblastoma cells (40). Similarly, we observed that TN treatment stimulated PKR phosphorylation at Thr446 but IMX treatment prevented TN-induced PKR phosphorylation.

Skeletal muscle is a highly dynamic tissue that plays crucial roles in locomotion, energy production and endocrine function (1). The ER is a cellular organelle involved in protein folding, lipid synthesis and calcium homeostasis in various cell types, including skeletal muscle (1). Skeletal muscle, in particular, contains the sarcoplasmic reticulum (SR) which is an extensive network of ER and plays a key role not only in muscle contraction but in various nuclear and cytosolic signal transduction through regulating cellular calcium level (32). ER stress and maladaptation of UPS are closely related to skeletal muscle atrophy, insulin resistance, inflammation (1). Hence, it is important to control ER stress in skeletal muscle
properly to maintain muscle mass and function as well as to suppress metabolic diseases such as diabetes. During prolonged and uncontrolled UPR GRP78 disassociates from IRE1α, PERK and ATF6 during ER stress because of its higher affinity toward unfolded/misfolded protein than IRE1α, PERK and ATF6 (33). After dissociation from GRP78, IRE1α oligomerization and autophosphorylation promotes splicing of a 26-base intron from X-box binding protein 1 (XBP-1μ) mRNA (1). Spliced XBP-1 (XBP-1s) binds to promoter regions of several genes such as chaperones and components of ER-associated degradation (ERAD) (1, 33). Even though the current study did not evaluate IREα phosphorylation, IMX suppressed not only XBP-1μ and XBP-1s, but also TN-induced XBP-1μ splicing (Fig. 2), suggesting that IMX might modulate IRE1α pathway under ER stress conditions.

Another ER transmembrane sensor, PERK, is also auto-phosphorylated and activated upon ER stress (3). Along with PKR, activated PERK phosphorylates eukaryotic translation initiation factor 2 alpha (eIF2α) on serine 51. Under prolonged ER stress, phosphorylated eIF2α stimulates translation of UPR dependent genes including activating translational factor 4 (ATF4) which is a transcription factor for CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) (3, 16, 17). CHOP is known as growth arrest and DNA damage-inducible protein 153 (GADD153) and is considered the most important mediator of ER stress-induced apoptosis (36, 42). Elevated CHOP expression may down-regulate the expression of Bcl2 which is the anti-apoptotic protein and up-regulate the expression of pro-apoptotic genes such as BIM (26). Furthermore, increased CHOP expression can perturb the cellular redox state by depletion of cellular glutathione (26). Interestingly, under prolonged ER stress, the ER may also directly stimulate the apoptotic pathway through ER stress-mediated calcium leakage into the cytoplasm and this action can cause vicious cycles of mitochondria-mediated apoptosis (14, 34). In the current study, IMX treatment suppressed protein levels of both p-PERK and ATF4 which were induced by TN treatment (Fig. 3D and 3E). Moreover, IMX treatment significantly reduced TN-induced CHOP at both the mRNA and protein level (Fig. 2A and 3B). These data suggest that IMX prevents excessive activation of the PERK/ATF4 axis induced by TN and consequently reduces CHOP expression.
Upon ER stress, ATF6 is also translocated and cleaved by the action of proteases including serine protease [or site-1 protease (S1P)] and metalloprotease [or site-2 proteases (S2P)] in the Golgi apparatus (3, 36). Cleaved ATF6 can be translocated into the nucleus and interact with ATF/cAMP response element (CRE) and ER stress-response elements (ERSE-1) which increase expression of target genes including ER chaperones, XBP-1 and CHOP (24, 36). The current study demonstrated that IMX decreased both full length and cleaved ATF6 regardless of TN treatment (Fig. 3G and 3H). These results suggest that IMX may suppress all the ER stress cascades and consequently reduce expression of CHOP which promotes cellular damage and apoptosis.

The current study indirectly measured mitochondrial mediated apoptosis by performing a resazurin assay which is used for sensitive measurement for the viability of mammalian cells (21). Because living cells can reduce the nonfluorescent dye, resazurin, to the fluorescent dye resorufin through mitochondrial reductase, the lower conversion implies higher cell death (21). Our data demonstrate that TN significantly reduced cell viability. We demonstrated that TN increased the protein level and mRNA expression of CHOP which is a main mediator in ER stress-induced cell death. It has been reported that CHOP can directly stimulate the mitochondrial mediated apoptosis (6). Despite the likely impact this may have on insulin signaling and glucose uptake, IMX lowered ER stress and restored glucose uptake, but IMX did not influence TN-induced cell death as shown in Fig. 6, suggesting TN-induced apoptosis is at least partially independent of ER stress.

In addition to ER stress signaling, the current study evaluated the effect of IMX on TN-induced insulin resistance in C2C12 myotubes. It has been reported that ER stress and UPR pathways play crucial roles in the regulation of insulin signaling in several tissues (10, 19, 20, 31). Among the ER transmembrane sensors, ATF6 is known to impair glucose transporter 4 (GLUT4) expression in rat and human skeletal muscle cells (31). Ozcan et al. reported that ER stress in obesity promotes serine phosphorylation of IRS1 and interferes with insulin signaling (30). Similar to previous studies, the current study found that TN
significantly reduced protein levels of p-IRβ and IRS phosphorylation at Ser636/639. However, IMX

treatment prevented the TN-induced reduction in insulin-stimulated IRβ tyrosine phosphorylation, a key

step in initiating the insulin signaling cascade. Moreover, IMX abolished the suppression of Akt

phosphorylation at Ser473 caused by TN. Interestingly, AS160, a mediator of GLUT4 translocation, was

highly phosphorylated in IMX treated groups regardless of TN exposure. Furthermore, TN-induced

suppression of glucose uptake was significantly ameliorated in the TN-IMX group (Fig. 6A). Collectively,

our data showed that IMX attenuated TN-induced impairments in insulin signaling and glucose uptake.

The results suggest that PKR may promote insulin sensitivity in skeletal muscle. However, ER stress is

known as a contributor to anabolic resistance in skeletal muscle by impairing mTOR complex 1

(mTORC1) activity (7). The current study demonstrated that IMX significantly augmented Akt

phosphorylation which is also important in anabolic signaling. Likewise, we previously found that IMX

augmented anabolic signaling via activation of Akt/mTOR/S6K1 cascades under glucocorticoid-induced

skeletal muscle atrophy (9). Thus, IMX might contribute to combat anabolic resistance caused by ER

stress and/or insulin resistance in skeletal muscle.

In conclusion, the current results provide evidence that TN increased PKR phosphorylation at Thr446 in

skeletal muscle cells, which was prevented by IMX treatment. In addition, IMX attenuated TN-induced

excessive UPR by suppressing arms of the UPR including PERK/ATF4, IRE1/XBP1 and ATF6 in

skeletal muscle myotubes. Furthermore, IMX promoted insulin receptor signaling cascades (IR/IRS/Akt)

and insulin-stimulated glucose uptake which were suppressed by TN. This insulin signaling data provide

insight into a potential role of PKR in glucose homeostasis in skeletal muscle. These findings support the

therapeutic potential of IMX for the improvement of skeletal muscle ER stress and insulin resistance.

GRANTS
DISCLOSURES

The authors declare no actual or perceived conflicts of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed equally to this study. H.E. and R.J.V. conceived and designed research; H.E. performed experiments and analyzed data; H.E. and R.J.V interpreted the results, edited, and revised the manuscript, and approved the final version of manuscript.
1. Afroz D, and Kumar A. ER stress in skeletal muscle remodeling and myopathies. FEBS J 286: 379-398, 2019.
2. Bohnert KR, Gallot YS, Sato S, Xiong G, Hindi SM, and Kumar A. Inhibition of ER stress and unfolding protein response pathways causes skeletal muscle wasting during cancer cachexia. FASEB J 30: 3053-3068, 2016.
3. Bohnert KR, McMillan JD, and Kumar A. Emerging roles of ER stress and unfolded protein response pathways in skeletal muscle health and disease. J Cell Physiol 233: 67-78, 2018.
4. Bonnet MC, Weil R, Dam E, Hovanessian AG, and Meurs EF. PKR stimulates NF-kappaB irrespective of its kinase function by interacting with the I kappaB kinase complex. Mol Cell Biol 20: 4532-4542, 2000.
5. Carvalho-Filho MA, Carvalho BM, Oliveira AG, Guadagnini D, Ueno M, Dias MM, Tsukumo DM, Hirabara SM, Reis LF, Curi R, Carvalheira JBC, and Saad MJA. Double-Stranded RNA-Activated Protein Kinase Is a Key Modulator of Insulin Sensitivity in Physiological Conditions and in Obesity in Mice. Endocrinology 153: 5261-5274, 2012.
6. Deldicque L. Endoplasmic reticulum stress in human skeletal muscle: any contribution to sarcopenia? Front Physiol 4: 236, 2013.
7. Deldicque L, Bertrand L, Patton A, Francaux M, and Baar K. ER stress induces anabolic resistance in muscle cells through PKB-induced blockade of mTORC1. PLoS One 6: e20993, 2011.
8. Eley HL, Russell ST, and Tisdale MJ. Attenuation of muscle atrophy in a murine model of cachexia by inhibition of the dsRNA-dependent protein kinase. Br J Cancer 96: 1216-1222, 2007.
9. Eo H, Reed CH, and Valentine RJ. Imoxin prevents dexamethasone-induced promotion of muscle-specific E3 ubiquitin ligases and stimulates anabolic signaling in C2C12 myotubes. Biomed Pharmacother 128: 110238, 2020.
10. Fonseca SG, Gromada J, and Urano F. Endoplasmic reticulum stress and pancreatic beta-cell death. Trends Endocrinol Metab 22: 266-274, 2011.
11. Gal-Ben-Ari S, Barrera I, Ehrlich M, and Rosenblum K. PKR: A Kinase to Remember. Front Mol Neurosci 11: 480, 2018.
12. Goh KC, deVeer MJ, and Williams BRG. The protein kinase PKR is required for p38 MAPK activation and the innate immune response to bacterial endotoxin. The EMBO Journal 19: 4292-4297, 2000.
13. Gregor MF, Yang L, Fabbrini E, Mohammed BS, Eagon JC, Hotamisligil GS, and Klein S. Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss. Diabetes 58: 693-700, 2009.
14. Guzel E, Arlier S, Guzeloglu-Kayisli O, Tabak MS, Ekiz T, Semerci N, Larsen K, Schatz F, Lockwood CJ, and Kayisli UA. Endoplasmic Reticulum Stress and Homeostasis in Reproductive Physiology and Pathology. Int J Mol Sci 18: 2017.
15. Hage Hassan R, Hainault I, Vilquin JT, Samama C, Lasnier F, Ferré P, Foufelle F, and Hajduch E. Endoplasmic reticulum stress does not mediate palmitate-induced insulin resistance in mouse and human muscle cells. Diabetologia 55: 204-214, 2012.
16. Harding HP, Zhang Y, and Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 397: 271-274, 1999.
17. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat Rev Mol Cell Biol 13: 89-102, 2012.
18. Kang R, and Tang D. PKR-dependent inflammatory signals. Sci Signal 5: pe47, 2012.
19. Khan S, and Wang CH. ER stress in adipocytes and insulin resistance: mechanisms and significance (Review). Mol Med Rep 10: 2234-2240, 2014.

20. Kim OK, Jun W, and Lee J. Mechanism of ER Stress and Inflammation for Hepatic Insulin Resistance in Obesity. Ann Nutr Metab 67: 218-227, 2015.

21. Kuete V, Karaosmanoglu O, and Sivas H. Chapter 10 - Anticancer Activities of African Medicinal Spices and Vegetables. In: Medicinal Spices and Vegetables from Africa, edited by Kuete VA, Academic Press, 2017, p. 271-297.

22. Lee ES, Yoon CH, Kim YS, and Bae YS. The double-strand RNA-dependent protein kinase PKR plays a significant role in a sustained ER stress-induced apoptosis. FEBS Lett 581: 4325-4332, 2007.

23. Lin JH, Walter P, and Yen TS. Endoplasmic reticulum stress in disease pathogenesis. Annu Rev Pathol 3: 399-425, 2008.

24. Liu X, and Green RM. Endoplasmic reticulum stress and liver diseases. Liver Res 3: 55-64, 2019.

25. Malhi H, and Kaufman RJ. Endoplasmic reticulum stress in liver disease. J Hepatol 54: 795-809, 2011.

26. McCullough KD, Martindale JL, Klotz LO, Aw TY, and Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 21: 1249-1259, 2001.

27. Nakamura T, Arduini A, Baccaro B, Furuhashi M, and Hotamisligil GS. Small-molecule inhibitors of PKR improve glucose homeostasis in obese diabetic mice. Diabetes 63: 526-534, 2014.

28. Nakamura T, Furuhashi M, Li P, Cao H, Tuncman G, Sonenberg N, Gorgun CZ, and Hotamisligil GS. Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis. Cell 140: 338-461, 2010.

29. O'Brien J, Wilson I, Orton T, and Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 267: 5421-5426, 2000.

30. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH, and Hotamisligil GS. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 306: 457-461, 2004.

31. Raciti GA, Iadicicco C, Ulaniich L, Vind BF, Gaster M, Andreozzi F, Longo M, Teperino R, Ungaro P, Di Jeso B, Formisano P, Beguinot F, and Miele C. Glucosamine-induced endoplasmic reticulum stress affects GLUT4 expression via activating transcription factor 6 in rat and human skeletal muscle cells. Diabetologia 53: 955-965, 2010.

32. Rayavarapu S, Coley W, and Nagaraju K. Endoplasmic reticulum stress in skeletal muscle homeostasis and disease. Curr Rheumatol Rep 14: 238-243, 2012.

33. Roy A, and Kumar A. ER Stress and Unfolded Protein Response in Cancer Cachexia. Cancers (Basel) 11: 2019.

34. Rozpedek W, Pytel D, Mucha B, Leszcynska H, Diehl JA, and Majsterek I. The Role of the PERK/elf2alpha/ATF4/CHOP Signaling Pathway in Tumor Progression During Endoplasmic Reticulum Stress. Curr Mol Med 16: 533-544, 2016.

35. Salvado L, Palomer X, Barroso E, and Vazquez-Carrera M. Targeting endoplasmic reticulum stress in insulin resistance. Trends Endocrinol Metab 26: 438-448, 2015.

36. Sano R, and Reed JC. ER stress-induced cell death mechanisms. Biochim Biophys Acta 1833: 3460-3470, 2013.

37. Sud N, Rutledge AC, Pan K, and Su Q. Activation of the dsRNA-Activated Protein Kinase PKR in Mitochondrial Dysfunction and Inflammatory Stress in Metabolic Syndrome. Curr Pharm Des 22: 2697-2703, 2016.
38. Udumula MP, Babu MS, Bhat A, Dhar I, Sriram D, and Dhar A. High glucose impairs insulin signaling via activation of PKR pathway in L6 muscle cells. Biochem Biophys Res Commun 486: 645-651, 2017.

39. Valentine RJ, Jefferson MA, Kohut ML, and Eo H. Imoxin attenuates LPS-induced inflammation and MuRF1 expression in mouse skeletal muscle. Physiol Rep 6: e13941, 2018.

40. Vaughn LS, Snee B, and Patel RC. Inhibition of PKR protects against tunicamycin-induced apoptosis in neuroblastoma cells. Gene 536: 90-96, 2014.

41. Yoshida K, Okamura H, Hiroshima Y, Abe K, Kido J-I, Shinohara Y, and Ozaki K. PKR induces the expression of NLRP3 by regulating the NF-κB pathway in Porphyromonas gingivalis-infected osteoblasts. Experimental cell research 354: 57-64, 2017.

42. Zong ZH, Du ZX, Li N, Li C, Zhang Q, Liu BQ, Guan Y, and Wang HQ. Implication of Nrf2 and ATF4 in differential induction of CHOP by proteasome inhibition in thyroid cancer cells. Biochim Biophys Acta 1823: 1395-1404, 2012.

Figure Legends:

Figure 1. Effect of IMX on PKR expression and PKR phosphorylation caused by Tunicamycin.

C2C12 myotubes were pre-treated with vehicle (VEH) or IMX at 5µM for 1 h and then 0.5 µg/ml Tunicamycin (TN) was added for 23 h. (A) PKR mRNA expression was quantified by RT-PCR. (B) p-PKRThr446 and total PKR were measured by western blot. Representative band images are shown on the left panel and quantification is shown on the right panel. Values are presented as mean ± SEM. The cell experiment was independently repeated in triplicates for Western blot and quadruplicates for RT-PCR. The data were analyzed by a one-way (A) or two-way (B) ANOVA followed by the Tukey’s post-hoc test: ***p < 0.001 compared with the untreated control group; ###p < 0.001 compared with the TN only treated group.

Figure 2. Effect of IMX on mRNA expression of ER stress genes induced by TN in C2C12 myotubes.

mRNA expression of (A) CHOP, (B) ATF4, (C) ATF6, (D) XBP-1s, (E) XBP-1µ and (F) XBP-1 splicing. The cells were pre-treated with IMX at 5µM for 1 h and then 0.5 µg/ml TN was added for 23 h. mRNA expression was quantified by RT-PCR. Values are presented as mean ± SEM. The cell experiment was independently repeated in quadruplicates. Data were analyzed by one-way ANOVA followed by the
Tukey’s post-hoc test: *p < 0.05 and ***p < 0.001 compared with the untreated control group; ##p < 0.01 and ###p < 0.001 compared with the TN only group.

**Figure 3. Effect of IMX on protein levels of ER stress-related markers stimulated by TN in C2C12 myotubes.** The cells were pre-treated with IMX at 5µM for 1 h and then 0.5 µg/ml TN was added for 23 h. Protein level of each biomarker was measured by western blot. (A) Representative band images and (B-H) quantification data are shown. Values are presented as mean ± SEM. The cell experiment was independently repeated in triplicates. In Fig. 3B – 3F, main effects and interactions were analyzed by two-way ANOVA (IMX x TN), followed by the Tukey’s post-hoc test: *p < 0.05, **p < 0.01 and ***p < 0.001 compared with the untreated control group; ##p < 0.01 and ###p < 0.001 compared with the TN only treated group. In Fig. 3G and 3H, significance of differences between two groups was analyzed by Student’s t-test.

**Figure 4. Effect of IMX on insulin-stimulated p-IRβ and p-IRS1 in presence or absence of tunicamycin in C2C12 myotubes.** The cells were pre-treated with IMX at 5µM for 1 h and then 0.5 µg/ml TN was added for 23 h, and 100 nM insulin was added for the last 15 min. Protein levels were measured by western blot. (A) Representative band images and (B-C) quantification data of each biomarker are shown. Differences between treatments were analyzed by three-way ANOVA. Values are presented as mean ± SEM. The cell experiment was independently repeated in triplicates. *p < 0.05, **p < 0.01 and ***p < 0.001 by Tukey’s post hoc analysis after three-way ANOVA.

**Figure 5. Effects of Tunicamycin and IMX on insulin-stimulated phosphorylation of Akt and AS160 in C2C12 myotubes.** C2C12 myotubes were pre-treated with IMX at 5µM for 1 h and then 0.5 µg/ml TN was added for 23 h, and 100 nM insulin was added for the final 15 min. Protein levels were measured by western blot. (A) Representative band images and (B-D) quantification results of each biomarker are displayed. Main effects and interactions were analyzed by three-way ANOVA. Values are presented as
mean ± SEM. The cell experiment was independently repeated in triplicates. *p < 0.05, **p < 0.01 and ***p < 0.001 by Tukey’s post hoc analysis after three-way ANOVA.

Figure 6. Effects of Tunicamycin and IMX on cell viability and glucose uptake in C2C12 myotubes.

(A) Glucose uptake monitored by 2-NBDG assay. C2C12 myotubes were pre-treated with or without 5 µM IMX for 1 h and then exposed to 0.5 μg/ml TN for 22 h. To stimulate glucose uptake, cells were washed with sterile PBS twice and then treated with or without 100 nM insulin in serum-free DMEM without glucose for 30 min. After 30 min of insulin stimulation, 2-NBDG was added to cells at the final concentration of 100 ug/ml for another 30 min. Main effects and interactions were analyzed by three-way ANOVA. (B and C) Cell viability was determined by resazurin assay. C2C12 myotubes were pre-treated with IMX at 5µM for 1 h and then 0.5 μg/ml TN was added for 23 h. Values are presented as mean ± SEM. Data were analyzed by one-way ANOVA followed by the Tukey’s post-hoc test: ***p < 0.001 compared with the untreated control group. Values are presented as mean ± SEM. The cell experiments were independently repeated in triplicate. *p < 0.05, **p < 0.01 and ***p < 0.001 by Tukey’s post hoc analysis after three-way ANOVA.
(A) Western blots showing protein levels of GRP78, CHOP, p-PERK, ATF4, XBP-1s, and ATF6 under different conditions. (B) Graphs depicting relative protein levels as a percentage of control for GRP78, CHOP, p-PERK, ATF4, and XBP-1s under various treatments. (C) Bar graph showing the percentage of full-length ATF6 to total protein under VEH and IMX conditions. (D) Bar graph showing the percentage of cleaved ATF6 to total protein under TN and TN-IMX conditions.
(A) 2-NBDG Uptake (% of Control)

- INS
- VEH
- IMX

(B) RFU (% of Control)

IMX (µM) - 1 3 5

(C) RFU (% of Control)

TN (0.5 µg/ml) - + + + +