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Effects of triglyceride on ER stress and insulin resistance

Do-Sung Kim [a,1], Seul-Ki Jeong [b,1], Hyung-Ryong Kim [c], Dal-Sik Kim [d], Soo-Wan Chae [a], Han-Jung Chae [a,*]

[a] *Department of Pharmacology and Institute of Cardiovascular Research, School of Medicine, Chonbuk National University, Jeonju, Chonbuk, Republic of Korea*
[b] *Department of Neurology, Chonbuk National University School of Medicine, Jeonju, Chonbuk, Republic of Korea*
[c] *Department of Dental Pharmacology and Wonkwang Biomaterial Implant Research Institute, School of Dentistry, Wonkwang University, Iksan, Chonbuk, Republic of Korea*
[d] *Department of Laboratory Medicine, School of Medicine, Chonbuk National University, Jeonju, Chonbuk 560-180, Republic of Korea*

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Abstract

This study was conducted to examine the mechanism by which triglyceride induces insulin resistance and ER stress in HepG2 cells. Using *in vitro* study models, we show that triglyceride causes insulin resistance through serine phosphorylation of insulin receptor substrate-1 (IRS-1). In addition, triglyceride induces the expression of endogenous endoplasmic reticulum (ER) stress markers, including GRP 78, IRE-1alpha, XBP-1, p-eIF2alpha, CHOP, and p-JNK. ER stress, in turn, leads to the suppression of insulin receptor signaling through tyrosine dephosphorylation of IRS-1. The results of this study show that triglyceride is a central feature of peripheral insulin resistance, and also suggest that triglyceride-induced ER stress influences insulin resistance. These experiments may be used in the development of an *in vitro* acute obesity model.

Keywords: ER stress; Triglyceride; Insuline resistance; IRS-1; JNK

Chronic elevation in plasma free fatty acid (FFA) levels is commonly associated with impaired insulin-mediated glucose uptake [1,2], and often coexists with obesity and type 2 diabetes [3]. Acute elevationsin plasma FFA levels during triglyceride emulsion infusion impair the insulin-mediated uptake of glucose in rats [4,5] and humans [6–9].

The endoplasmic reticulum (ER) is the key organelle in cells; the most important steps in the folding and modification of proteins, as well as in selection for transport to other compartments, occur in this organelle [10,11]. Certain pathological stress conditions disrupt ER homeostasis and lead to the accumulation of unfolded or misfolded proteins in the ER lumen [12–14]. To cope with this stress, cells activate a signal transduction system that links the ER lumen with the cytoplasm and nucleus; this process is referred to as the unfolded protein response (UPR) [12–14]. The conditions that trigger ER stress include glucose or nutrient deprivation, viral infections, presence of lipids, increased synthesis of secretory proteins, and expression of mutant or misfolded proteins [15–17].

The insulin receptor substrates (IRSs) are a large family of docking proteins that act as an interface between the insulin receptor and a complex network of intracellular-signaling molecules [18]. So far, four members (IRS-1, -2, -3, and -4) of this family have been identified [18]. Insulin stimulates a signaling network that is composed of a number of molecules, which initiate the activation of insulin receptor tyrosine kinase and the phosphorylation of the insulin receptor substrate (IRS) proteins (e.g., IRS-1 and IRS-2) [19]. Among several components of the network, the signaling axis of the IRS proteins and PI3K, which activates downstream serine/threonine kinases such as Akt,

* Corresponding author.
E-mail address: hjchae@chonbuk.ac.kr (H.-J. Chae).
[1] The authors contributed equally to this manuscript.

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regulates most of the metabolic actions of insulin, such as the suppression of hepatic glucose production and the activation of glucose transport in muscle tissue and adipocytes [20]. It is known that this pathway can be impaired at multiple steps via alterations in the protein levels and activities of the signaling molecules, enzymes, and transcription factors of insulin resistance caused by obesity, a state of increased adiposity [20].

A recent study showed that obesity also induces ER stress, and this, in turn, plays a central role in the development of insulin resistance and diabetes by triggering JNK activity via inositol-requiring enzyme-1 (IRE-1) and inhibition of insulin receptor signaling [21]. Subsequently independent studies have also verified the role of ER stress in insulin resistance in several experimental systems [22,23]. In this study, we investigate whether triglyceride could induce ER stress and insulin resistance, and demonstrate a strong and causal relationship between the functional capacity of the ER and the action of insulin.

Materials and methods

Chemical regents. Anti-IRS-1 and anti-phospho-IRS-1 (Ser 307) antibodies were obtained from Upstate Biotechnology (Charlottesville, VA). Antibodies against GRP78, CHOP, JNK, IRE-1alpha, and sliced XBP-1 antibody were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-eIF2alpha, anti-p-eIF2alpha, and anti-p-JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA). The cleaved ATF-6 was obtained from Imgenex Co. (Port Coquitlam, BC). Tunicamycin and JNK inhibitors (SP600125) were acquired from Calbiochem (San Diego, CA). Insulin and other chemicals were purchased from Sigma (St. Louis, MO).

Cell cultures. HepG2 cells were cultured with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS). At 70–80% confluency, cells were maintained in serum-depleted conditions overnight prior to the experiments. JNK inhibitors were added 30 min prior to tunicamycin/triglyceride treatment.

Preparation of triglyceride-rich particles (TGRPs). Triglyceride-rich particles (TGRPs) were isolated from 20% Intra-lipid (Kabi-Vitrum, Stockholm, Sweden) after the removal of multilamellar phospholipid (PL) structures [24]. In brief, 2 ml of 20% Intra-lipid was transferred into a SW41 polyallomer tube and overlayered with KBr–NaC1–Na, EDTA (d 1.006 g/ml). The tubes were centrifuged for 15 min at 25,000 rpm in a Beckman SW41 Ti rotor, and the lipid "cake" at the top was collected and resuspended in KBr–NaC1–Na2, EDTA (d 1.006 g/ml). This procedure was repeated twice in order to remove most of the residual PL. The isolated TGRPs had a TG/PL ratio of 175:1, compared with a ratio of 47:1 in the starting preparation. These ratios indicated that the bulk of the PL was not associated with other particles.

Cell microscopy. For light microscopy examination, HepG2 cells were cultured on cover glasses in complete medium for 4 days, re-fed with serum-free medium for 24 h, and then incubated with TGRPs for 16 h in serum-free medium. The cover glasses were then washed with PBS, fixed for 1 h with 4% formaldehyde in PBS, and then immersed in 60% isopropanol for less then 1 min. Cells were subsequently stained with 1% Oil Red O (Sigma) in 60% isopropanol for 1 h at 4 °C, rinsed in PBS, mounted with glycerol, and processed for evaluation by light microscopy [25].

Determination of triglyceride (TG) contents. Layers of HepG2 cells were washed three times with buffer containing 0.15 mol/L NaC1, 50 mmol/L Tris (pH 7.4), and 0.2% BSA, and were then washed three more times with the same buffer without BSA. Lipids were extracted from cells by the hexane–isopropanol method [26]. In brief, 2 ml of a 3:2 hexane–isopropanol mixture (vol/vol) was added to cell layers for 30 min at room temperature, and the solvent was transferred to a glass tube and dried under nitrogen. Triglycerides were determined enzymatically using a commercial kit (F. Hoffmann-La Roche Ltd., Basel) after the lipids had been dissolved in isopropanol. Cell proteins in the residue obtained after lipid extraction were dissolved in NaOH (0.1 mol/L) and determined according to the Bradford method. Data are expressed as pg lipids/mg protein.

Immunoprecipitation. Cells were washed twice with cold PBS and lysed in cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF). Cell lysates were incubated with anti-IRS-1 antibody (2 μg) for 2 h at 4 °C. Proteins A/G conjugated to agarose beads were added for 1 h. Beads were washed three times with washing buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF) and boiled for 5 min prior to electrophoresis.

Western blot analysis. The immunoprecipitates or total lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes. After blocking with 1.5% BSA, the membranes were incubated with the indicated primary antibody, followed by a secondary antibody. Samples were finally detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK).

Statistical analysis. Data were analyzed by two-tailed Student's *t*-tests. A p-value < 0.05 was considered significant. In each case, the statistical test used is indicated, and the number of experiments is stated individually in the legend of each figure.

Results

Effects of TGRP on the accumulation of triglyceride by HepG2 cells

HepG2 cells were cultured for 16 h in medium alone or in a medium containing 3 mg of TG per ml of TGRPs. Staining of cells with Oil Red O consistently showed a larger number of lipid droplets in the medium in TGRPs than in medium alone (Fig. 1A). Intracellular triacylglycerol levels increased in proportion to the concentrations of TGRP added to the medium (Fig. 1B).

Triglyceride particles induce the activation of ER stress in HepG2 cells

To examine whether triglyceride triggers ER stress in HepG2 cells, we examined the patterns of several molecular indicators of ER stress. Exposure of HepG2 cells to triglyceride resulted in the induction of GRP78, IRE-1alpha, cleaved ATF-6, p-eIF2alpha, XBP-1, and CHOP/GADD 153. Time-lapse experiments revealed that induction of these proteins was observed within 12 h, and was sustained for at least 2–3 h (Fig. 2). In addition, triglyceride increased the activation of c-Jun N-terminal kinase (JNK). Triglyceride-induced activation of JNK was observed at 30 min, and was sustained for 1 h.

Triglyceride induces insulin resistance

To investigate whether triglyceride induces insulin resistance, we treated HepG2 cells with triglyceride, which significantly increased the serine phosphorylation of insulin receptor substrate-1 (IRS-1) (Fig. 3A). Furthermore,

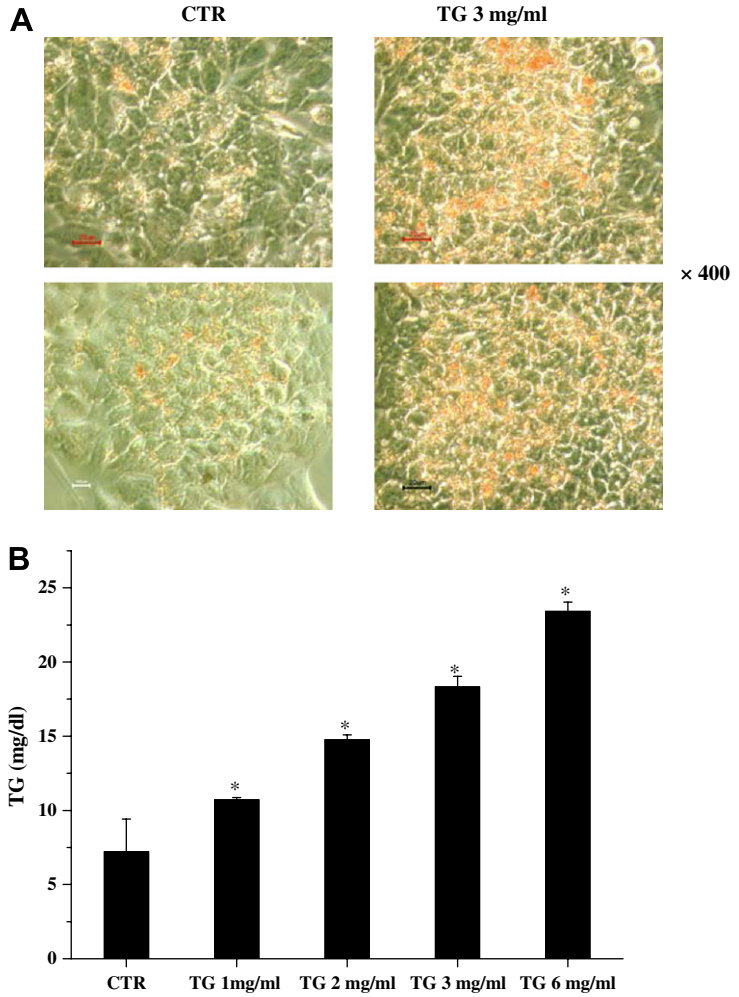

Fig. 1. Effect of triglyceride on intracellular triacylglycerol accumulation in HepG2 cells. (A) Light micrographs of HepG2 cells incubated in the absence (left panel) or in the presence (lower panel) of 3 mg of triglyceride per ml of triglyceride particles. The lipid droplets in the cytoplasm were stained with Oil Red O. (B) HepG2 cells were incubated for 16 h with medium containing increasing concentrations of triglyceride. The medium was then removed and cells were scraped off. Intracellular triglyceride levels were determined in lipid extracts of cells. Data represent means ± SE ($n = 5$). $^{*}p < 0.05$; significantly different from the control cells.

triglyceride significantly decreased insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), and also produced an increase in the molecular weight of IRS-1 (Fig. 3B). These data indicate that triglyceride promotes the serine phosphorylation of IRS-1, which, in turn, inhibits insulin receptor signaling.

ER stress inhibits insulin action in liver cells

To investigate whether ER stress interferes with insulin action, we treated HepG2 cells with tunicamycin, an agent that is commonly used to induce ER stress. Tunicamycin significantly decreased insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) (Fig. 4). We then examined the role of JNK in ER stress-induced IRS-1 serine phosphorylation and inhibition of insulin-stimulated IRS-1 tyrosine phosphorylation. Inhibition of JNK activity with the synthetic inhibitor, SP600125, reversed the ER stress-induced serine phosphorylation of IRS-1 (data not shown). Finally, we investigated the role of JNK in the triglyceride-induced inhibition of insulin-stimulated IRS-1 tyrosine phosphorylation. Inhibition of JNK did not affect the triglyceride-induced inhibition of insulin-stimulated IRS-1 tyrosine phosphorylation. These results indicate that ER stress promotes a JNK-dependent serine phosphorylation of IRS-1, which, in turn, inhibits insulin receptor signaling, but does not inhibit triglyceride-induced insulin receptor signaling.

Discussion

In summary, we have shown that triglyceride activates ER stress, increases serine phosphorylation of insulin receptor substrate-1 (IRS-1), and decreases tyrosine phosphorylation. Furthermore, ER stress decreases the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1).

Reports have recently linked ER stress to the pathogenesis of several conditions, including insulin resistance and

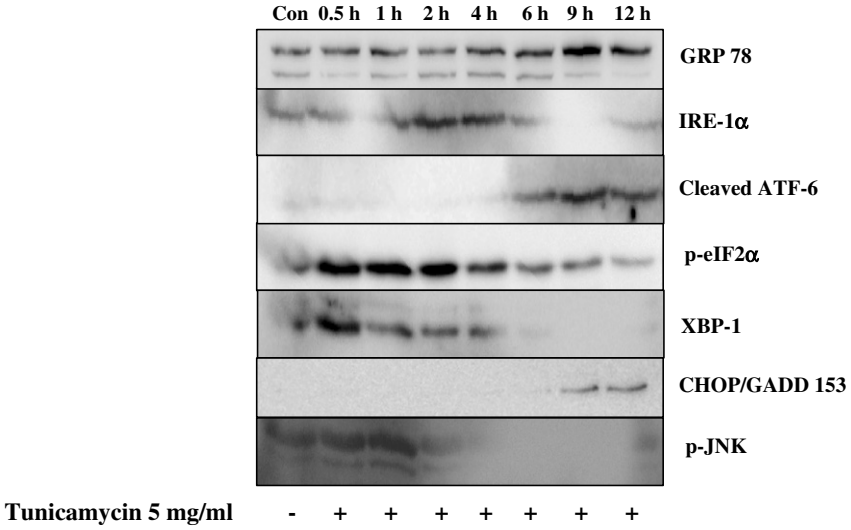

Fig. 2. Effect of triglyceride treatment on the expression of ER stress proteins. HepG2 cells were incubated with 3 mg/ml of triglyceride for the indicated length of time. The ER stress proteins, which included GRP78, ATF-6alpha, XBP-1, p-eIF2alpha, IRE-1apha, CHOP, and p-JNK, were determined by Western blotting.

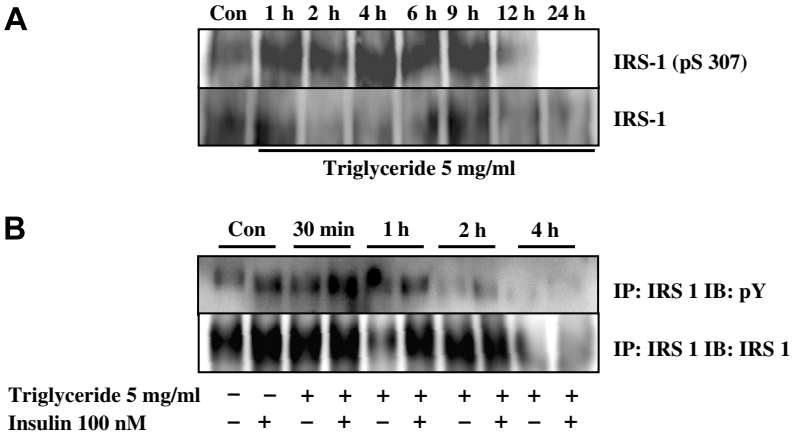

Fig. 3. Inhibition of insulin receptor signaling by triglyceride. HepG2 cells were incubated with 3 mg/ml of triglyceride for the indicated length of time, and were subsequently stimulated with insulin. (A) IRS-1 tyrosine serine phosphorylation (pSer307), (B) phosphorylation (pY), and total protein levels were examined using immunoprecipitation (IP) followed by either immunoblotting (IB) or direct immunoblotting.

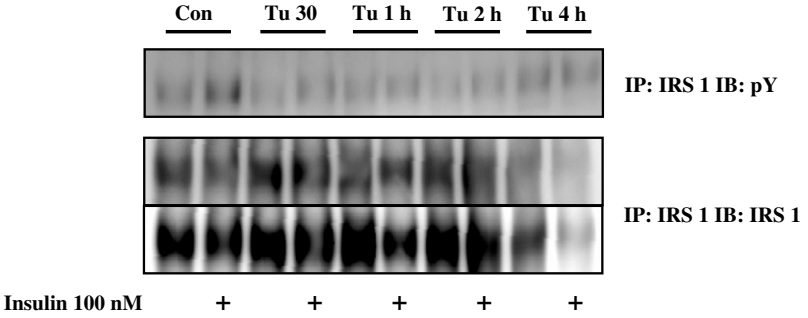

Fig. 4. Inhibition of insulin receptor signaling by tunicamycin-induced ER stress. ER stress was induced in HepG2 cells by treatment with 5 mM tunicamycin for the indicated length of time. The cells were subsequently stimulated with insulin. IRS-1 tyrosine phosphorylation and total protein levels were examined using immunoprecipitation followed by immunoblotting.

type 2 diabetes [27,28]. Although glucose toxicity has been implicated in the induction of ER stress in type 2 diabetes [29], this disease is characterized by pleiotropic metabolic abnormalities, including increased serum TAG and FA levels, which may also be detrimental. In fact, recent studies in cultured pancreatic β-cells [17], hepatocytes [30], and car-

diomyoblasts [31] indicate that FA overload also induces ER stress, often leading to apoptotic cell death. In this study, we show that triglyceride treatment rapidly increases intracellular triglyceride levels, which are associated with the activation of ER stress (Fig. 2). Thus, impairment of the structure and function of ER appears to play an early and important role in the cellular response to fatty acid overload.

There is strong evidence to support the idea that fat diversion from adipose to non-adipose tissues, such as liver and muscle tissues, which are not adapted to TG storage, may lead to insulin resistance and type 2 diabetes [32]. In our study, we sought to examine this relationship using an intra-lipid isolated triglyceride particle-induced hypertriglycemic, insulin-resistant *in vitro* model. FFA has previously been shown to interfere with cellular insulin signaling and induce insulin resistance [32]. In addition, the infusion of lipid emulsions with heparin in order to acutely raise plasma fatty acid concentrations has also been shown to cause profound insulin resistance in rats and human skeletal muscle within 4–6 h [6,33–35]. Therefore, our model may be appropriate for evaluating *in vitro* insulin resistance. As reported previously, in skeletal muscle, fatty acid-induced insulin resistance occurs as a result of intralipid/heparin infusion [6,33]. In the present study, we found that treatment with triglyceride for 1 h also increased serine phosphorylation of IRS-1 and decreased tyrosine phosphorylation of IRS-1 (Fig. 3). The results of triglyceride treatment in our study confirm the association between triglyceride accumulation (Fig. 1) and insulin resistance (Fig. 3).

A recent reported by Ozcan et al. [21] sheds more light on the links between obesity, ER stress, insulin action, and type 2 diabetes. Using cell culture and mouse models, the authors showed that obesity causes ER stress. They observed an elevation of several biochemical indicators of ER stress, c-Jun N-terminal kinase (JNK) activity, and BiP expression in liver and adipose tissues of obese animals compared to their lean counterparts. ER stress led to a significant increase in JNK-mediated serine phosphorylation of insulin receptor substrate-1 (IRS-1), and thus, inhibited insulin action. IRS-1 is a substrate for insulin receptor tyrosine kinase, and serine phosphorylation of IRS-1, particularly that mediated by JNK, reduces insulin receptor signaling [36]. In this study, we demonstrate that triglyceride-induced ER stress and JNK activation (Fig. 2). Furthermore, our study showed that an ER stress agent, tunicamycin, inhibited insulin-stimulated IRS-1 tyrosine phosphorylation (Fig. 4) and a JNK inhibitor (SP600125) reversed ER stress-induced insulin resistance (data not shown). However, our experimental study model showed that triglyceride-induced inhibition of insulin-stimulated IRS-1 tyrosine phosphorylation and ER stress are expressed for similar amounts of time (30 min–1 h). In addition, the JNK inhibitor, SP600125, did not inhibit triglyceride-induced insulin resistance (data not shown). These results indicated that *in vitro* triglyceride-induced insulin resistance is not related to ER stress at an early period of time, but late-occurring insulin resistance may be related to ER stress.

In conclusion, triglyceride individually induced ER stress and insulin resistance, but triglyceride-induced ER stress may then potentiate insulin resistance. Furthermore, the use of triglyceride treatment in HepG2 cells can be utilized as an *in vitro* insulin resistance model.

Acknowledgments

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