EFFECTS OF EXENDIN-4 ON ENDOPLASMIC RETICULUM STRESS-MEDIATED INSULIN RESISTANCE IN 3T3-L1 ADIPOCYTES

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Abstract

Objective. Endoplasmic reticulum stress (ERS) is suspected as an important factor in the initiation of insulin resistance.

Aim. To explore the effects of exendin-4 (Ex-4) on the endoplasmic reticulum stress (ERS)-mediated insulin resistance in 3T3-L1 adipocytes. In our study, 3T3-L1 adipocytes were pre-treated with ERS inhibitors tauroursodeoxycholic acid (TUDCA), Ex-4 and an ERS inducer tunicamycin (TM) then induced insulin resistance. Glucose consumption of the adipocytes was measured. Western blots determined the protein levels of ERS markers and insulin signaling pathway.

Results. TM treatment reduced insulin-stimulated glucose consumption by 19.7% in 3T3-L1 adipocytes. This repression was blunted by 24h pre-treatment with TUDCA or Ex-4. Ex-4 augmented insulin-stimulated glucose consumption in adipocytes by 14.9%. Western blotting showed that TM treatment significantly increased the ER stress markers including p-IRE, p-JNK, p-PERK, p-eIF2 α and ATF6 expression, whereas 24h pre-treatment of adipocytes with TUDCA or Ex-4 alleviated the ER stress. Ex-4 alleviates ERS-induced insulin resistance by upregulating the expression of phosphorylated Akt.

Conclusion. ERs mediates insulin resistance in 3T3-L1 adipocytes, and exendin-4 significantly improves this insulin resistance.

Key words: Exendin-4, ER stress, insulin resistance, 3T3-L1 Adipocytes.

INTRODUCTION

Insulin resistance is a precursor of type 2 diabetes and cardiovascular disease, and is closely associated with obesity, aging, hypertension and hyperlipidemia (1). Adipocyte dysfunction and endoplasmic reticulum stress (ERS) are suspected as two factors in the initiation of insulin resistance, but the underlying mechanisms have yet to be fully elucidated (2).

In eukaryotic cells, the endoplasmic reticulum (ER) is a storage site for intracellular calcium and it is also where proteins translation and synthesis take place and where proteins are folded, stored and transported. In normal cellular function, the ER clears unfolded or misfolded proteins. However, when blood glucose and energy status change, increase in protein synthesis, protein glycosylation disorders, and the disturbance of ER calcium homeostasis can induce protein misfolding, which in turn triggers ERS (3). During ERS, the ER activates a complex series of reactions to regain its normal functions, a process which is called the unfolded protein response (UPR) (4). UPR signalling is predominantly mediated by three sensors in the ER membrane: inositol requiring enzyme-1 (IRE-1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF-6). ERS has been observed in the adipose tissues of obese mice, and interferes with the insulin receptor pathway, suggesting that adipose tissue ERS may be involved in insulin resistance occurrence (2, 5).

Tunicamycin (TM) is an agent known to induce cell ERS (6), by interfering with protein post-translational processing and protein glycosylation modification, thus leading to cellular ERS (7). TM induces ERS in HepG2 cells, it has the effect of decreasing the tyrosine phosphorylation and increasing serine phosphorylation of the insulin signalling pathway protein IRS-1, resulting in a decrease of Akt expression, blockage of the insulin signal transduction pathway, and reduction of the insulin sensitivity in peripheral tissues (2). Tauroursodeoxycholic acid (TUDCA) is a chemical chaperone (5), which correctly folds ER proteins at the cellular level, and improves insulin resistance by reducing ERS. TUDCA also reduces the expression of phosphorylated PERK and IRE-1 then upregulates insulin signal transduction when 3T3-L1 adipocytes are treated with FFA (8). Most important, the treatment of obese mice with TUDCA that alleviate

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ERS improves signaling through the insulin receptor, indicating that the effects of ERS on adipose tissue insulin resistance may be reversible (5).

Glucagon-like peptide 1 (GLP-1) is derived from the posttranslational modification of proglucagon, which is released from the L cells of the small intestine in response to food ingestion (9). It possesses a variety of anti-diabetic functions including the stimulation of glucose-dependent insulin secretion (10), and the inhibition of glucagon release (11). However, in humans GLP-1 treatment has been hampered by the low plasma half-life and fast renal clearance of the natural hormone. Exendin-4 (Ex-4) is a GLP-1 receptor agonist which is not a substrate for cleavage by dipeptidyl peptidase IV. Therefore, it has a longer half-life which makes it more suitable as a therapeutic agent for diabetes. Ex-4 has been documented to promote the activity of insulin-stimulated glucose transport in skeletal myocytes and adipocytes (12), increases insulin sensitivity of peripheral tissues, but the specific signaling mechanisms involved remain unclear. This study investigates the effects of exendin-4 on glucose metabolism in 3T3-L1 adipocytes and on the ERS signaling pathways, to explore the relation between ERS and insulin resistance, and further to gain an insight into the molecular mechanisms by which exendin-4 ameliorates insulin resistance in adipose tissues.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemicals (St. Louis, MO, USA). The insulin solution was purchased from Novo Nordisk (Denmark). Tunicamycin and exendin-4 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methylthiotetrazole (MTT) was purchased from Shanghai Sangon Biotech (Shanghai, China). Glucose oxidase was purchased from Beijing Applygen Technologies Inc. (Beijing, China). Tauroursodeoxycholic Acid was purchased from Calbiochem (Merck, Germany). Primary antibodies, including rabbit polyclonal antibodies to mouse, phospho- and total JNK/eIF2 α /Akt were purchased from Cell Signaling (Beverly, MA, USA). The rabbit polyclonal antibody to mouse phospho- and total IRE1/PERK, ATF6 were provided by Abcam (Cambridge, MA). The western blot Chemiluminescent Detection System (LumiGLO system) was provided by KPL

(Gaithersburg, MD, USA). Other reagents used were all of analytical or cell-culture grade purity.

Cell culture

Fibroblast 3T3-L1 preadipocytes were maintained in DMEM culture medium supplemented with 10% FBS and 4 mmol/L glutamine in an atmosphere of 5% CO₂ at 37°C. 3T3-L1 preadipocytes were grown to confluence on 100- μ m plates and then treated with an adipogenic cocktail (5 μ g/mL insulin, 0.5 mmol/L IBMX, and 10 μ mol/L dexamethasone) for 2 days. This was followed by incubation in an insulin-supplemented medium for additional 2 days. This medium was replaced by DMEM medium at day 6 to maintain the adipocytes (13). On day 8, about 90% of the cells exhibited an adipocytes phenotype and these cells were used in our experiment. The mature adipocytes (day 8) will be used in our experiment.

Oil red O staining

After 3T3-L1 preadipocytes were differentiated into mature adipocytes, cells were fixed in 10 % formalin for 30 min, washed once with 60 % isopropanol, twice with distilled water and incubated with oil red-O solution for 5 min at room temperature. The cells were stained with hematoxylin, washed with distilled water and photographed using an optical microscope.

Glucose consumption

Cells were cultured in 96-well culture plates with some wells left blank. During the experiments, the cells were treated with different reagents as indicated. After these treatments, the culture medium was removed and its glucose concentration was determined using the glucose oxidase method. The volume of culture medium is 100 μ L and the amount of glucose consumption was calculated by subtracting the glucose concentration of the blank wells from the glucose remaining in the wells that contained cells (14).

Cell viability determined using the MTT method

MTT was dissolved at a concentration of 5 mg/mL in sterile phosphate-buffered saline (PBS). After the various treatments on the cell groups were finished, 10 μ L of MTT was added to the 96-well culture plates. After 4 h incubation at 37°C, the MTT medium was replaced with dimethylsulfoxide (DMSO). After shaking at room temperature, the optical densities (OD) were measured at 570nm wavelength using an ELX800 Absorbance Microplate Reader (Bio-Tek).

Western blot analysis

Cell pellets were lysed at 4°C in a buffer containing protease inhibitors (1 mmol/L PMSF) and phosphatase inhibitors (phosphatase inhibitor mixture I). Lysates were cleared by centrifugation at 14,000 x g for 15 min at 4°C. Protein concentrations were determined by a bicinchoninic acid assay using bovine serum albumin as a standard. The cell lysates (40 microgrammes per sample) were then subjected to SDS-PAGE (10%), and transferred to nitrocellulose membranes. The membranes were blocked for 2h at room temperature in 5% nonfat dried milk and then incubated overnight at 4°C with antibodies against p-IRE (1:1,000), IRE (1:1,000), p-JNK (1:1,000), JNK (1:1,000), p-PERK (1:1,000), p-eIF2a (1:1,000), p-Akt (1:1,000), ATF-6(1:1,000), PERK (1:500), eIF2a (1:500), Akt (1:400). The bound antibodies were visualized using anti-rabbit secondary antibodies (1:2000) and the bands were developed onto X-ray film using the ECL chemiluminescence method.

Statistical analysis

Results were presented as means $\pm$ S.E.M. Statistical analyses were performed using Statistical Analysis Software (version 6.04; SAS Institute, Cary, NC, USA). Significance was assessed using analysis of variance (ANOVA) followed by Dunnett's test for the comparison of multiple means within an experiment. P values of less than 0.05 were considered statistically significant.

RESULTS

Effects of insulin on 3T3-L1 adipocyte cell glucose consumption

Differentiated 3T3-L1 were stained with oil red O (Fig. 1). Insulin treatment (10 nM for 30 minutes) significantly increased the glucose consumption of 3T3-L1 adipocyte cells by 22.9% compared with

Figure 1. Staining 3T3-L1 adipocytes with oil red (*400).

control. This demonstrates that 3T3-L1 adipocytes are sensitive to insulin and are an ideal model for the study of insulin resistance (Fig. 2).

Exendin-4 improves ERS mediated insulin resistance in 3T3-L1 adipocytes

TM is a classical agent known to induce cell ERS. Many proofs have shown that ERS correlated with insulin resistance closely (2, 5, 6). Indeed, incubation of 3T3-L1 adipocytes cells with TM (5 microgramme/mL) for 5h decreased insulin-stimulated glucose consumption by 63.2% ($p < 0.05$). Reversely, pre-treatment with TUDCA (1 mmol/L) or exendin-4 (100 nmol/L) for 24h increased insulin-stimulated glucose consumption by 62.2% and 65% ($p < 0.05$) respectively in the TM-treated cells. Exendin-4 had a synergistic action with the insulin and increased insulin-stimulated glucose consumption by 14.9% ($p < 0.05$) (Fig. 2). MTT analysis demonstrated that cell viability of 3T3-L1 adipocytes was not affected with different treatments ($p > 0.05$. Supplement data). Therefore, we suggest that Ex-4 improves ERS mediated insulin resistance in 3T3-L1 adipocytes.

Exendin-4 alleviates ER stress in 3T3-L1 adipocytes

Further, we explore the mechanisms underlying the Ex-4-induced improvement of the ERS-induced insulin resistance in 3T3-L1 adipocytes. Western blot analysis of the harvested cell proteins suggested that the ERS inducer TM significantly increased the expression of the ERS markers including p-IRE, p-JNK, p-PERK, p-eIF2a and ATF-6 (compared with control $p < 0.01$).

Figure 2. Glucose consumption in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pre-treated with TUDCA (1 mmol/L) or exendin-4 (100 nmol/L) for 24 h, followed with or without TM at 5 μg/mL for 5 h. Insulin (10 nM) was added for 30 min in the last step. Glucose consumption was measured as described in "Material and methods" section. The results are shown as mean $\pm$ S.E.M from four independent experiments and two replicates each. *** $p < 0.001$. Con=Control; INS=Insulin; TM=Tunicamycin; TU=Tauroursodeoxycholic acid; EX-4=Exendin-4.

Pre-treatment with exendin-4 (100 nmol/L) for 24h significantly decreased their expression ($p < 0.01$), with a potency equivalent to that of TUDCA (1 mmol/L) pre-treatment. And all treatments had no influence on the total IRE, JNK, PERK and eIF2a expression (Fig. 3A and B). This indicates that Exendin-4 can alleviate ERS in 3T3-L1 adipocytes.

Exendin-4 protects cells from ERS-induced impairment of insulin signalling

ERS has previously been shown to impair insulin signalling. In agreement with others, we found that concurrent with increased ERS, the cellular levels of phosphorylated Akt were markedly reduced in TM-treated 3T3-L1 adipocytes. TUDCA and Ex-4 treatment significantly protected the cells from TM-induced Akt de-phosphorylation (Fig. 4). All treatments had no influence on the expression of Akt. These results indicate that the increase in Akt phosphorylation,

rather than the Akt protein levels, is involved in Ex-4 alleviation of the ERS-induced insulin resistance.

DISCUSSION

Insulin plays an important role in regulating glucose, lipid, and protein metabolism. The liver, skeletal muscle and adipose tissues are the target tissues of insulin. Insulin resistance means the sensitivity of the liver, muscle and adipose tissues response to endogenous or exogenous insulin is reduced. *In vitro* cell models such as skeletal myocytes (L6), hepatocytes (HepG2) and adipocytes (3T3-L1) are used to provide a better understanding of insulin resistance and to enable development of medicines to counteract it (15, 16).

Adipose tissue is an important endocrine organ that secrete large quantities of adipocytokines such as leptin, resistin, adiponectin and TNF- α , which are involved in physiological and pathological processes

Figure 3. Exendin-4 alleviates ER stress in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pre-treated with TUDCA (1 mmol/L) or exendin-4 (100 nmol/L) for 24 h, followed with or without TM at 5 ug/ml for 5 h. Insulin (10 nM) was added for 30 min in the last step. The whole cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and the ER stress markers including the phosphorylation and/or expression of IRE-1, JNK, PERK, eIF-2a, ATF-6 were determined by Western blot analysis with specific antibodies as indicated. β -actin was shown as a loading control. The representative immunoblots are shown from one of three independent experiments, with a quantification analysis presented on the right. All results are presented as the means $\pm$ S.E.M from three independent experiments. ** $P < 0.01$, *** $P < 0.001$. Con=Control; INS=Insulin; TM=Tunicamycin; TU=Tauroursodeoxycholic acid; EX-4=Exendin-4.

Figure 4. Exendin-4 increases insulin sensitivity in 3T3-L1 adipocytes. Immunoblotting for phospho-Akt (Ser473) and Akt from whole cell lysates. β -actin was shown as a loading control. The representative immunoblots are shown from one of three independent experiments, with a quantification analysis presented below. All results are presented as the means $\pm$ S.E.M from three independent experiments. * $P < 0.05$. Con=Control; INS=Insulin; TM=Tunicamycin; TU=Tauroursodeoxycholic acid; EX-4=Exendin-4.

such as inflammation and insulin resistance (17). Recently, obesity has been shown to cause ER stress in both liver and adipose tissue. It has been shown that ER stress is involved in cell apoptosis, inflammation and insulin resistance (18). In peripheral tissues, the relation of ER stress with insulin resistance is more evident than its relation with apoptosis. It has been proved that the unfolded protein response (UPR) plays an important role in ERS-induced insulin resistance in liver cells (2). Obesity causes ER stress in the liver and activates the UPR, then the level of IRE-1 phosphorylation increases, leading to the recruitment of the tumor necrosis factor receptor associated factor-2 (TRAF-2) and the apoptosis signal regulating kinase-1 (ASK-1). These molecules form a composite molecule to co-activate cJUN-N-terminal kinase (JNK), a serine kinase. JNK promotes the phosphorylation of serine residues on the insulin receptor substrate-1 (IRS-1), thereby inhibiting the phosphatidylinositol 3-kinase (PI3K)-mediated insulin signalling pathway, and reducing the insulin sensitivity of the target tissues (19). Therefore, we supposed that activation of IRE-1-JNK signalling is also an important contributor to insulin resistance in adipose tissues. In our study, we found that the level of IRE-1 and JNK phosphorylation increases, which further causes repression of Akt phosphorylation when 3T3-L1 adipocytes were treated with TM. The UPR also stimulates the activity of the nuclear factor κ B (NF- κ B) pathway. PERK phosphorylation leads to I κ B degradation, and the IRE-1 phosphorylation activates NF- κ B signalling by increasing the expression of TRAF-2 (20). Then the activation of NF- κ B increases the expression of inflammatory factors further leading to insulin resistance (21). Therefore, ER stress might represent a link between JNK and IKK-regulated pathways, the two important signalling cascades leading to insulin resistance. The ER stress has also been reported to increase the expression of Trb3, an Akt inhibitor, which is involved in cell nutrient sensing and regulation of cell survival under stressful condition. In 3T3-L1 adipocytes, Trb3 overexpression inhibited the activity of Akt, decreased glucose transporter4 (GLUT-4) translocation, and decreased glucose uptake by 50% (7). Therefore, when ER stress occurs, Trb3 may induce insulin resistance in 3T3-L1 adipocytes. Miller and his colleagues (22) have found that activation of the ER stress response decreases GLUT-4 expression in 3T3-L1 adipocytes, and that this repression is likely mediated through the increase in expression of CHOP10. Their finding suggests that besides defect in insulin-stimulated GLUT-4 translational, repression

of GLUT-4 expression is another mechanism through which activation of the ER stress response induces insulin resistance in obesity. In addition, significant insulin receptor down-regulation was observed in fat tissue of obese human subjects and in 3T3-L1 adipocytes treated with ER stress inducers. Down-regulation of the insulin receptor protein levels in adipose tissue has been shown to correlate with ER stress induced-insulin resistance (23). Taken together, ER stress may impair insulin signalling through different pathways causing insulin resistance in adipocytes.

Glucagon-like peptide-1 (GLP-1) is an incretin. It decreases blood glucose in a glucose-dependent manner and is involved in β -cell differentiation, stimulating pancreatic β -cell growth and survival, and protecting β -cell from apoptosis (24). GLP-1 also stimulates glucose uptake, regulates lipid synthesis and lipolysis in adipocyte, inhibits the expression of inflammatory factors and improves insulin resistance in peripheral tissues (25). Exendin-4, a GLP-1 receptor agonist, is isolated from the saliva of the American venomous lizard and it has a 53 % homology with mammalian GLP-1 (26). It is resistant to DDP-4 cleavage and, therefore, has a long-acting anti-diabetic profile that is suitable for therapeutic use in humans (27). In the aspect of β -cell study, Exendin-4 treatment decreased *in vivo* pancreatic expression of CHOP, XBP1s, and BIP in rodent models (28). It has previously been reported that it improves β -cell survival following ER stress induced by the stressors thapsigargin or tunicamycin (29), suggesting that ER stress is one of the molecular mechanisms underlying β -cell failure. Previous work discovered that Exendin-4 induced cAMP-PKA, extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase, and PI3K-Akt signalling, leading to improved β -cell function through increased insulin biosynthesis and secretion, β -cell proliferation, and decrease of β -cell death (30). In the aspect of peripheral tissues study, previous work from others has shown that Ex-4 augments insulin-stimulated glucose uptake in isolated cells (31, 32), and in Zucker diabetic rats whole-body insulin sensitivity increased after chronic administration of exendin-4 (27). However, the explanation for this insulin-sensitizing effect is still unclear.

In an *in vitro*, Exendin-4 may improve glucose metabolism by up-regulating the expression of IRS-1 and GLUT-4 or by increasing transport efficiency to increase insulin-stimulated glucose uptake in adipocytes (33). In addition, Exendin-4 has been documented to synergistically increase insulin-stimulated glucose

uptake, thus alleviating TNF- α -induced insulin resistance by up-regulating the expression of the tyrosine phosphorylation of IR and IRS-1, then further phosphorylate Protein Kinase B (PKB) and Glycogen synthase kinase 3 (GSK3)(12). In our study, we found that the insulin-stimulated glucose uptake is suppressed by ER stress in 3T3-L1 adipocytes. We used western blot to detect the expression of the ER stress markers such as p-IRE, p-JNK, p-PERK, p-eIF2 α and ATF6 and downstream molecules in the insulin signalling. Our results show that Ex-4 alleviates TM-induced ER stress in 3T3-L1 adipocytes and blunts the repression of insulin-stimulated glucose uptake by ER stress. We suggest that Exendin-4 down-regulates the expression of p-IRE and p-JNK, and then further phosphorylates or up-regulates the expression of downstream molecules in the insulin signalling pathway to improve adipose tissue insulin resistance. Thus, the insulin-sensitizing effect of Exendin-4 in adipocytes may involve the PI3K-Akt signalling pathway. Although we have provided some evidence for the mediation of ERS on the effect of Exendin-4 on insulin sensitivity, further research is needed to provide more evidence. Other mechanisms remain to explore the changes in the MAPK signalling pathway and the potential effects of Exendin-4 on inflammatory signalling.

In conclusion, Exendin-4 improves TM-induced insulin resistance in 3T3-L1 adipocytes, an effect that is also observed with TUDCA. This effect is mediated by reducing ERS. Therefore, our study provides a new insight into the mechanism by which Exendin-4 ameliorates insulin resistance in adipose tissue and suggests that effective ERS inhibitors may serve as candidate agents for the treatment of type 2 diabetes.

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Conflict of interest

The authors declare that they have no conflict of interest.

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