

In vitro treatment of 3T3-L1 adipocytes with recombinant Calcium/calmodulin-dependent Protein Kinase IV (CaMKIV) limits ER stress and improves insulin sensitivity through inhibition of autophagy *via* the mTOR/CREB signaling pathway

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Abstract

Background: CaMKIV has been identified as a potential regulator of skeletal muscle glucose metabolism and insulin gene expression in the pancreas. However, the mechanism of CaMKIV involved in adipose insulin resistance is not fully understood. Autophagy has to be shown as a potential therapeutic target for ER (endoplasmic reticulum) stress and insulin resistance. The purpose of this study is to investigate the effects of CaMKIV on the ER stress, autophagic function and insulin signaling in tunicamycin treated adipocytes.

Methods: In this study, we treated mature 3T3-L1 adipocytes with tunicamycin to induce ER stress. Then recombinant CaMKIV and/or targeted-siRNA of CREB and mTOR were transfected into tunicamycin treated 3T3-L1 adipocytes. The ER stress markers, autophagy activation, mTOR/CREB signaling and insulin sensitivity were analyzed by western blotting or electron microscopy.

Results: CaMKIV not only reversed tunicamycin-induced expression of p-PERK, cleaved-ATF6, Atg7 and LC3II, as well as the reduction of p62 expression, but also improved expression of p-Akt and p-IRS-1. Moreover, CaMKIV inhibited activated ER stress and elevated insulin sensitivity in Atg7 siRNA transfected adipocytes. However, the protective effects of CaMKIV were nullified by suppression of mTOR or CREB in tunicamycin-induced adipocytes.

Conclusion: This study proves recombinant CaMKIV inhibits ER stress and improves insulin signaling by regulation of autophagy. The protective effect of CaMKIV in adipocytes at least partly through mTOR/CREB signaling, which could be regarded as novel opportunities for treatment of obesity and type 2 diabetes.

Background

Obesity induced IR (insulin resistance) is a complex pathological state of inappropriate cellular response to insulin hormone in insulin dependent cells, which is a major risk factor of metabolic disorder associated diseases ^[1]. Adipose is now recognized as not only an energy-storage tissue but also an endocrine tissue that secretes a variety of bioactive substances including adipokines and proinflammatory cytokines ^[2]. Adipose tissue dysfunction is believed to promote insulin resistance and lead to obesity ^[3, 4]. Although considerable progress has been made in understanding the molecular mechanisms underlying these individual disorders, satisfactory treatment modalities remain limited.

Abnormal autophagy has been implicated in a variety of diseases, such as obesity, type 2 diabetes, cancer and cardiovascular disease ^[5, 6]. *Atg7* expression in adipose tissue protects high-fat diet induced obesity and insulin resistance, indicating that autophagy activation contributes to the regulation of fat mass ^[7]. Autophagy activity was upregulated in adipose tissues of obese individuals and inhibition of autophagy enhanced pro-inflammatory gene expression both in adipocytes and adipose tissue explants,

indicating that autophagy might inhibit inflammatory gene expression in adipose tissue during obesity [8, 9]. Recently, autophagic disorder has been suggested a potential link to obesity and ER stress. There are three central ER stress signaling molecules in mammalian cells, namely IRE1 α , PERK, and ATF6 [10]. Several strategies have been proposed to target ER stress as a therapeutic approach for pharmacological intervention in insulin resistance and type 2 diabetes. Thus, reduction of autophagy could be beneficial for adipocytes to dispose of unfolded or misfolded proteins under ER stress and insulin resistance.

CaMKIV is a multifunctional serine/threonine protein kinase encoded by *CaMKIV* gene, it plays a critical role in process of transcriptional regulation of lymphocytes, neurons and male germ cells [11-13]. Recently, CaMKIV has been identified that it plays an essential role in glucose metabolism and insulin genes expression [14, 15]. Moreover, CaMKIV regulates autophagy activation to limit hepatic damage or involved in lipopolysaccharide induced inflammation and acute kidney injury [16, 17]. In recent years, recombinant CaMKIV peptide has been used to several *in vivo* and *in vitro* studies [18, 19]. We supposed that exogenous CaMKIV proteins were transported into intracellular might bind the specific receptors. However, the membrane receptors of CaMKIV still unknown. Signaling through the transmembrane receptor Notch is widely used throughout animal development and is a major regulator of cell proliferation and differentiation [20]. It is interesting to note that CaMKIV enhanced osteoclast differentiation through up-regulating Notch signaling [21]. In addition, it has been also demonstrated that CaMKIV can potentiate Notch-dependent transcription by triggering nuclear export of SMRT (silencing mediator for retinoid and thyroid hormone receptor) [22]. These results gave us a clue that Notch might to be a potential receptor of CaMKIV. As a significant regulator of autophagy, mTOR could be upregulated by CaMKIV [17]. In adipose tissues, autophagy was significantly increased in diabetes compared with non-diabetes, and mTOR expression was decreased in adipose of diabetes cases, indicating autophagy was negatively regulated by mTOR expression in adipose tissues of diabetes [23]. As a basic leucine zipper type transcription factor, CREB is ubiquitously expressed in organs. Its phosphorylation at Ser 133 is initiated by the recruitment of CaMKII and CaMKIV, interestingly, CaMKII can also phosphorylate CREB at Ser 142 and induced negative regulation [24, 25]. It has been suggested CREB regulates expression of IRE1 α and PERK, which suggested CREB regulates the key components of UPR [26]. Rapamycin-induced autophagy against oxidative stress, synaptic/neurotransmission dysfunction, and cognitive deficits in the hippocampus of the rat brain through PI3K/Akt1-mTOR-CREB signaling pathway(s), indicating mTOR/CREB signaling plays a critical role in regulating autophagy [27]. Therefore, we suppose that CaMKIV could regulate mTOR/CREB signaling to inhibit ER stress through reduction of autophagy in adipocytes.

This study was undertaken to test our hypothesis that CaMKIV through decreased autophagy to suppress ER stress and improve insulin resistance by mTOR/CREB signaling. We first tested the insulin sensitivity, ER stress and autophagy function in Tun (tunicamycin)-treated 3T3-L1 cells with or without recombinant CaMKIV. To further identify the mechanism of CaMKIV on insulin resistance, we next analyzed the markers of ER stress, autophagy activity and insulin sensitivity after blockage mTOR/CREB signaling in Tun-treated adipocytes. Our results provided a reciprocal functional interaction among CaMKIV, ER stress,

autophagy and insulin signaling in Tun-treated adipocytes, indicating that CaMKIV regulated autophagy may function as an adaptive role in response to ER stress-induced insulin resistance in type 2 diabetes.

Methods

Antibodies and reagents: The following antibodies were used: Atg7 (Cell Signaling, #2631), p62 (Cell Signaling, #5114), LC3 (Cell Signaling, #4108), CREB (Cell Signaling, #9197), p-CREB (Cell Signaling, #9198), mTOR (Cell Signaling, #2972), p-mTOR (Cell Signaling, #2971), IRS-1 (Cell Signaling, #2382), p-IRS-1 (Cell Signaling, #2381), Akt (Cell Signaling, #4685), p-Akt (Cell Signaling, #4060), p-PERK (Cell Signaling, #3179), PERK (Cell Signaling, #5683), cleaved-ATF-6 (Santa Cruz Biotechnology, #sc-166659), GAPDH (Santa Cruz Biotechnology, #sc-47724) and peroxidase goat anti-rabbit IgG (Santa Cruz Biotechnology, #sc-2768). Insulin were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Mouse recombinant CaMKIV were obtained from Sino Biological (Sino Biological Inc. Wayne, PA, USA). Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate Kit (PerkinElmer Inc., Richmond, CA, USA) was used to detect protein expression. Individual protein bands were quantified by ImageJ software. All other chemicals were obtained from standard resource and were of the highest grade available.

Cell culture and treatment: 3T3-L1 were purchased from American Tissue Culture Collection (ATCC, Manassas, Virginia, USA) and maintained in DMEM (Gibco, Grand Island, New York, USA) with 10% fetal bovine serum (Gibco) at 37°C in a humidified atmosphere with 5% CO₂. The protocol of inducing maturation of 3T3L1 cells was performed as described [28] and which was mini-modified in our present of study. In brief, for adipocytes differentiation, 100% confluent 3T3-L1 were induced with MDI induction media (0.5mM 1-methyl-3-isobutylmethylxanthine, 200nM dexamethasone, 160nM insulin, and DMEM with 10% FBS) (day 0). Two days later media was changed to 10% FBS/ DMEM with 160nM insulin. Cells were then fed with this maintenance medium every 2 days. Full differentiation is usually achieved on the 12th day. The mature 3T3-L1 adipocytes were used in our ongoing experiments. To induce ER stress, mature 3T3-L1 cells were treated with different concentration (0-5µg/ml) of Tun for 4 hours. For the effects of CaMKIV, cells were treated with 100ng/ml CaMKIV for 24 hours. For blocking mTOR or CREB signaling, cells were transfected with 100nM mTOR siRNA or 100nM CREB siRNA for 24 hours, respectively. For insulin signaling, cells were stimulated with 10nM insulin for 10 minutes. Before each experiment, the medium was replaced by fresh medium.

Electron microscopy analysis: Cells were fixed in 4% paraformaldehyde/2% glutaraldehyde/0.1 M sodium cacodylate pH7.3, post-fixed in 1% osmium tetroxide and embedded in epoxy resin (Epon). Ultrathin sections (80nm) were stained with aqueous uranyl acetate and lead citrate and examined with a JEOL 2000FX transmission electron microscope (JEOL). For quantification of autophagolysosome-like vacuoles, the numbers of autophagolysosomal-like vacuoles were counted in each field and normalized by the surface area.

Small interfering RNAs (siRNAs) and transfection: Small interfering RNA (siRNA) for target genes (Atg7: sc-41448; CREB: sc-35111; mTOR: sc-35410, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or scrambled siRNA (CREB, sc-37007, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The transfected cells were cultured in medium containing 10% FBS for 24 hours after transfection. The knockdown efficiency was assessed by western blot.

Western blotting and immunoprecipitation: Cell lysates were extracted by RIPA Lysis and Extraction Buffer (Invitrogen; ThermoFisher Scientific, Inc., MA, USA) which contained 10% protease inhibitor (Thermo Scientific, USA), incubated on ice for 30 min, and then centrifuged at 14000 x g for 15min at 4°C. The protein concentration determined using the BCA kit (Thermo Scientific, USA). The supernatants were mixed with equal volume of 4x SDS-PAGE sample loading buffer and then denatured at 95°C for 10min. The proteins were separated by SDS-PAGE gel, transferred to a polyvinylidene difluoride membranes, incubated with specific primary antibodies at 4°C overnights, and detected with horseradish peroxidase (HRP)-conjugated secondary antibodies by using a VersaDoc Image System (BioRad, Hercules, CA, USA). For immunoprecipitation, the lysate was treated using the Dynabeads™ Protein G Immunoprecipitation Kit (Invitrogen; ThermoFisher Scientific, Inc., MA, USA) according to the protocol. The final precipitated proteins were analyzed via western blotting with the corresponding antibodies.

Statistics: Data were analyzed by the Prism software, version 8.0 (GraphPad Software Inc., San. Diego, CA, US). Characteristics of subjects between 2 groups was performed using Mann Whitney test. Multiple comparisons of quantitative variables among groups were made using Kruskal Wallis test [29]. Data were presented as mean ± SD. N represents the number of animals used. A *P* value of ≤ 0.05 or *P* value of ≤ 0.01 was considered as significantly or highly significantly difference.

Results

Tun-mediated ER stress increases autophagy in mature 3T3-L1 adipocytes. To determine the effects of pharmacological ER stress on autophagic function, an ER stress cell model was built. First, 3T3-L1 cells were induced to differentiate into mature adipocytes. Then the mature adipocytes treated with various dose of Tun (0-5µg/ml) for 4 hours, and ER stress markers were examined by western blotting. The data suggested phosphorylation of PERK expressions and cleaved-ATF6 expressions were increased significantly after Tun exposure with concentration of $\geq 2.5\mu\text{g/ml}$ compared with the control group (Figure 1A). Then we found autophagy markers such as Atg7 and LC3II protein levels were markedly increased as well as a decreased expression of p62 after 4 hours of 2.5 and 5µg/ml Tun treatment group compared with control group (Figure 1B). Hence, we selected 2.5µg/ml Tun to build ER stress cell models and to induce autophagic dysfunction in the ongoing experiments. In addition, the results of EM examination in mature 3T3-L1 adipocytes demonstrated a significant induction of autophagosome/ autolysosome formation in the cells treated with 2.5µg/ml Tun for 4 hours compared with control (Figure 1C). These results suggested that Tun induced ER stress that mediates autophagic dysfunction.

CaMKIV reverses Tun-induced ER stress and autophagic dysfunction and improves impaired insulin sensitivity in adipocytes. To understand the effect of CaMKIV on autophagy and insulin signaling, the markers of ER stress, autophagy and insulin sensitivity were evaluated in mature adipocytes. Cells were pretreated with 2.5µg/ml Tun for 4 hours to induce ER stress and autophagic dysfunction. As the results shown, p-mTOR expression was inhibited by Tun treatment. However, CaMKIV not only induced phosphorylated CREB after CaMKIV incubation with or without Tun pretreated, but also increased the expression of p-mTOR (Figure 2A). These data suggested CaMKIV regulates phosphorylation of mTOR and phosphorylated CREB expressions in Tun-treated adipocytes. In addition, Tun treatment not only elevated the expression of phosphorylation of PERK and cleaved-ATF6, but also induced autophagy, as evidenced by upregulation of Atg7 and LC3-II expression, and downregulation of p62 expression in adipocytes (Figure 2B and 2C). Meanwhile, insulin signaling was impaired, which was identified by the reduction of Akt phosphorylation and IRS-1 tyrosine phosphorylation (Figure 2D). Remarkably, adipocytes treated with recombinant CaMKIV in presence of Tun displayed reduced PERK phosphorylation and cleaved-ATF6 expression and reversed the expression of autophagy indicators such as Atg7, LC3-II and p62 (Figure 2B and 2C). Additionally, the recovery of insulin sensitivity in CaMKIV-treated adipocytes with Tun treatment was also evident, as demonstrated by increased Akt phosphorylation and IRS-1 tyrosine phosphorylation (Figure 2D). These findings suggested that CaMKIV reverses Tun-induced autophagic dysfunction and ER stress, restores insulin signaling and regulates mTOR and CREB expression *in vitro*.

CaMKIV inhibits ER stress and reverses insulin resistance in Atg7 siRNA transfected adipocytes.

Autophagy activation plays an important role in regulating ER stress and insulin resistance^[30], to further clarify the effect of CaMKIV on ER stress and insulin resistance in defective autophagy cells, we used Atg7 siRNA (Atg7si) to build a autophagic dysfunction adipocytes model. Adipocytes were transfected with 100nM Atg7si for 24 hours, which was validated by a reduced Atg7 protein expression (Figure 3A). Notably, downregulation of Atg7 significantly increased phosphorylation of PERK and cleaved-ATF6 expression, as well as decreased the phosphorylation of IRS-1 and Akt (Figure 3B and 3C), indicating impaired autophagy induced ER stress. However, recombinant CaMKIV treatment inhibited ER stress and improved impaired insulin sensitivity in Atg7 siRNA transfected adipocytes, as shown in *figure 3B and 3C*, CaMKIV markedly reduced PERK phosphorylation and cleaved-ATF6 expression, and increased phosphorylation of IRS-1 and Akt. These results not only demonstrated the importance of functional autophagy in maintaining cellular homeostasis, but further indicated CaMKIV reduced ER stress and improved insulin signaling in adipocytes of autophagic dysfunction.

CaMKIV restored insulin sensitivity and autophagic dysfunction through mTOR/CREB signaling in Tun-induced adipocytes. Our results suggested that CaMKIV inhibits ER stress and improves insulin sensitivity through recovery of autophagy. But the underlying mechanism is still unclear. It has been identified that CREB, an important transcriptional factor, involved in CaMKIV-regulated autophagy in hepatic ischemia-reperfusion injury^[16]. And CaMKIV also regulated autophagy through mTOR signaling in lipopolysaccharide-induced inflammation and acute kidney injury^[17]. Therefore, we hypothesized that the protective effects of CaMKIV on autophagy, ER stress and insulin sensitivity through mTOR/CREB

signaling. We then cultured adipocytes in the presence of Tun and/or CaMKIV, with or without the target-siRNA of each signaling pathway such as mTOR siRNA (mTORsi) and CREB siRNA (CREBsi). As the result shown in *Figure 4A*, CaMKIV treatment not only increased phosphorylated CREB expression, but also significantly elevated Tun-induced reduction of mTOR phosphorylation. Meantime, mTORsi treatment not only reduce the mTOR phosphorylation and total mTOR expression but also decreased phosphorylated CREB expression in Tun and CaMKIV incubated cells. Moreover, Tun treatment markedly induced ER stress, increased autophagy and impaired insulin sensitivity in adipocytes. However, CaMKIV treatment under Tun treatment markedly suppressed ER stress, inhibited autophagy, and improved insulin sensitivity. But in adipocytes with Tun and CaMKIV incubation, blockade of mTOR nullified the protective effects of CaMKIV (*Figure 4B-4D*). We next detected the role of CREB in the protective process of CaMKIV. The results suggested CREBsi treatment did not change the phosphorylated mTOR expression. Moreover, in cells treated with CaMKIV and Tun, CREBsi also inhibited phosphorylated CREB and total CREB expression (*Figure 5A*). The addition of CREBsi in the medium of CaMKIV and Tun-cotreated cells markedly reversed the effect of CaMKIV on ER stress, autophagy activation, and insulin signaling, suggesting that CaMKIV inhibited ER stress, suppressed autophagy, and improved impaired insulin signaling through phosphorylation of CREB. Of note, these results indicated that the protective role of CaMKIV on ER stress, autophagy and insulin signaling through mTOR/CREB signaling in adipocytes (*Figure 5B-5D*).

Discussion

In the present of this study, we first proved that recombinant CaMKIV protein inhibited autophagy and ER stress and improved insulin sensitivity in tunicamycin-treated mature 3T3-L1 adipocytes. Next, we further found that the protective effect of CaMKIV was nullified by downregulating mTOR or CREB expression, indicating CaMKIV regulated ER stress, abnormal autophagy and insulin sensitivity through mTOR/CREB signaling. In addition, CaMKIV inhibited ER stress and improved insulin sensitivity in Atg7 siRNA transfected cells. This result further demonstrated the protective effect of CaMKIV on regulating ER stress and insulin signaling.

Recently, autophagy dysfunction and ER stress are recognized as the important cause of insulin resistance^[30]. In response to ER stress, the ER stress sensors IRE1, PERK and ATF6 are activated, resulting in a series of downstream events such as reducing translation and increasing transcription ER chaperones to ensure that normal cell function and viability are maintained^[31]. Autophagy is an evolutionarily conserved lysosomal mechanism that enable cells to conserve and maintain cellular biomass quality and quantity by targeting damaged or unused proteins and even organelles of degradation^[6]. Previous studies have reported ER stress can be trigger by obesity or metabolic factors such as lipids, glucose and cytokines^[32-34]. was common in high-fat feeding, genetic obesity and elderly^[34, 35]. These results demonstrated that autophagic imbalance and ER dysfunction were the main pathway that response to the pathological factor, including lipotoxicity, inflammation and insulin resistance. In the present study, we further provide the evidence that autophagy remarkably associated

with ER stress and insulin resistance. As expected, Tun treatment not only induced ER stress, but also induced abnormal autophagic function and insulin resistance in adipocytes. These results further indicate that ER stress is closely associated with autophagy dysfunction.

CaMKIV is a multifunctional Ser/Thr kinase encoded by the *CAMKIV* gene, which requires Ca^{2+} /CaM for its activity [36]. It is well-known that Ca^{2+} signaling is a major regulator of CaMKIV in cellular, and disruption of Ca^{2+} homeostasis in the ER is well documented to trigger ER stress. According for these finding, we hypothesized that CaMKIV might regulate ER stress. Our results demonstrated that CaMKIV inhibited the protein expressions of ER stress, such as PERK and Cleaved-ATF6, indicating CaMKIV plays a critical role in regulating ER function. Moreover, CaMKIV has been identified as a regulator of glucose metabolism and insulin signaling. Its overexpression in skeletal muscle led to systemic improvements in insulin sensitivity and its activation involved in hepatic and adipose insulin action via increases in myokines released from the skeletal muscle [37]. Our previous study also found that long-term treatment of CaMKIV in mice could improve high-fat diet induced hepatic insulin resistance and autophagic disorder. Our results further demonstrated CaMKIV plays an important role in regulating whole-body insulin sensitivity and hepatic insulin signaling [19].

Recent evidences implicate CaMKIV in the regulation of autophagy in hepatic damage and acute kidney injury. In the regulation process of LPS-induced autophagy, CaMKIV directly augments autophagy by inhibiting GSK3 β activity and FBXW7 recruitment prevents ubiquitin proteosomal degradation of mTOR [17]. However, whether CaMKIV has a role on regulating adipose metabolism and ER stress is still under lineated. The mTOR typically serves as a negative regulator of autophagy, and as a consequence, initiation of autophagy is largely dependent on release of mTOR inhibition [38]. Consistent with this, our finding further suggested Tun induced reduction of phosphorylated mTOR in adipocytes. However, CaMKIV incubation significantly increase the p-mTOR expression. These results suggested that CaMKIV regulated autophagy associated with p-mTOR expression. Our results also demonstrated that treatment of CaMKIV inhibited ER stress and restored insulin sensitivity, indicating CaMKIV could improve the adaptive capacity of the ER and offer novel opportunities for treatment of insulin resistance. Taken together, we concluded that autophagy plays a critical role in the process of CaMKIV inhibits ER stress and improves insulin signaling.

Recent studies strongly suggested several factors including metabolic stressors, obesity, free fatty acid, and inflammatory cytokines could promote autophagic disorder of adipocytes [39, 40]. Disruption of the autophagy pathway by targeted deletion of the *Atg7* gene in adipose tissue protects mice from high-fat diet-induced obesity and insulin resistance, suggesting that activation of the autophagy-mediated pathway could be a mechanism for obesity-induced insulin resistance [7]. In our study, *Atg7* ablation induced ER stress and impaired insulin sensitivity could be reversed by CaMKIV incubation, suggesting the protective role of CaMKIV in autophagy defective adipocytes.

CREB is a transcription factor that integrates growth factors, Ca^{2+} , and cyclic AMP-induced signaling [41]. As a target of the cAMP/PKA pathway, CREB has been found to be activated by Ca^{2+} /calmodulin-dependent protein kinase and to be phosphorylated by kinases of the MAPK pathway [42]. Several groups subsequently showed that CaMKIV phosphorylated CREB at Ser133 *in vitro* and stimulated CREB transcriptional activity *in vivo*, which led to the suggestion that CaMKIV was the principal Ca^{2+} -stimulated CREB kinase [25, 43]. The mTOR/CREB pathway is an intracellular signaling pathway that is important in several normal cellular function that are also critical for regulation of autophagy [44, 45]. Here, we demonstrated that the activation of mTOR/CREB signaling is required for CaMKIV-mediated ER stress, autophagy, and the restoration of insulin signaling. It is interesting to note that activated CREB has been demonstrated in adipose cells under obese conditions, where it promotes insulin resistance by triggering expression of ATF3 and downregulating expression of GLUT4, indicating CREB plays a negative role in obesity induced insulin resistance [46]. However, in our study, ablation of CREB nullified the protective role of CaMKIV in regulation of autophagy, ER stress and insulin resistance. Our study enhances understanding of the mechanisms by which mTOR/CREB contributes to the regulation of CaMKIV-induced modulation in adipose.

This study demonstrated for the first time that the inhibition of ER stress and improvement of insulin signaling is due to the restored autophagy function which improved by recombinant CaMKIV protein rather than the direct intracellular effects of this enzyme. However, our study does have limitation which should be further investigated. Our further prospective studies are needed to find the specific receptor and binding sites. Although further studies are required, this study provided therapeutic implications of CaMKIV for modifying insulin signaling and autophagy function under the condition of ER stress in the adipocytes.

Conclusion

Recombinant CaMKIV protein inhibits ER stress and improves impaired insulin sensitivity by restoration of autophagy in mature 3T3-L1 adipocytes, and the protective effects of CaMKIV on autophagy, ER stress and insulin signaling through regulating mTOR/CREB signaling. Our study contributes to elucidating the potential role of CaMKIV in the pathogenesis of obesity and type 2 diabetes.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent to publish: Not applicable.

Availability of data and materials: The data generated or analyzed during this study are included in this article.

Competing interests: The authors have no competing interests associated with this manuscript.

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Author Contributions: Jiali Liu, Hao Meng and Ruihua Yang processed the samples, analyzed and prepared the data, and were involved in drafting the article. Jiali Liu, Ruihua Yang, and Ting Zhou contributed to data interpretation and revised the article. Jiali Liu and Qian He designed the experiments, interpreted the data, and was primary responsible for writing the manuscript. All authors approved the final version of the manuscript.

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Abbreviations

Akt, protein kinase B; ATF6, activation of transcription factor 6; Atg7, autophagy-related 7; CaMKII, calcium/calmodulin-dependent protein kinase II; CaMKIV, calcium/calmodulin-dependent protein kinase IV; CREB, cyclic AMP-responsive element binding protein; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IgG, immunoglobulin; IRE1 α , inositol-requiring enzyme 1 α ; IRS, insulin receptor substrate; LC3, microtubule associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; p, phosphorylated; PERK, PKR-like ER eIF2 α kinase; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA; UPR, unfolded protein response;

Figures

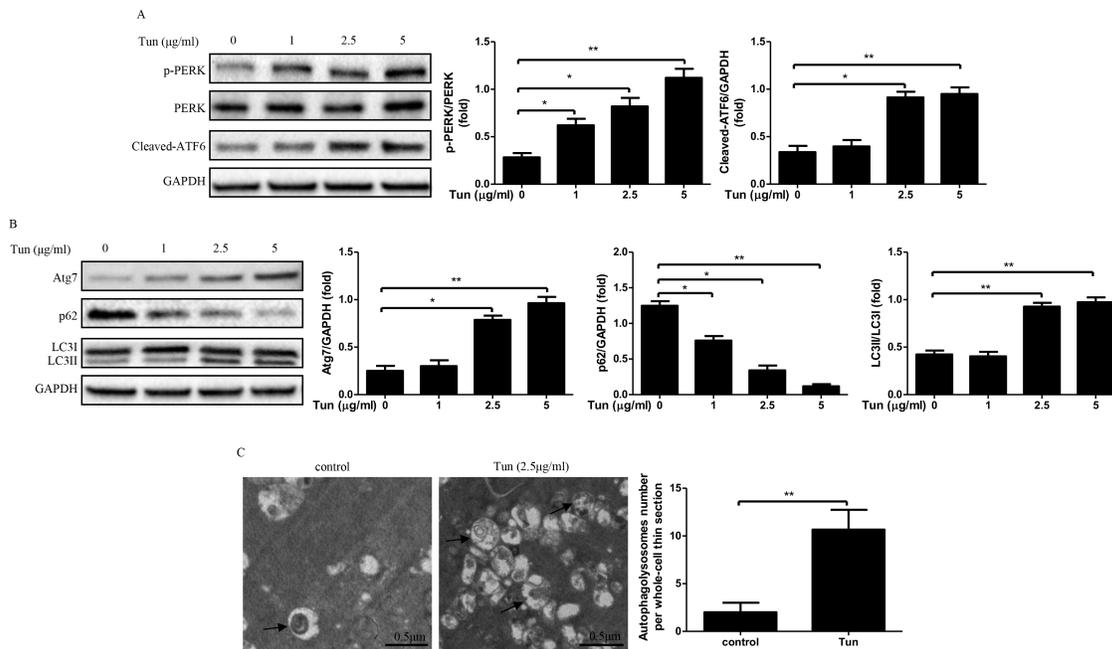


Figure 1

Induction of autophagic dysfunction due to ER stress induced by tunicamycin in adipocytes treated with 0, 1, 2.5 or 5µg/ml Tun for 4 hours. All indicators were measured at protein level. The relative quantity of protein was analyzed using Quantity One software. (A) ER stress markers including PERK phosphorylation (p-PERK) and cleaved-ATF6 in adipocytes. (B) Autophagy-related proteins Atg7, p62 and LC3 in adipocytes treated with 2.5µg/ml Tun for 4 hours. (C) Representation electron micrographs of adipocytes treated with 2.5µg/ml Tun for 4 hours. Quantification of autophagolysosome-like vacuoles per field in the EM images, Scale bars, 0.5µm. Quantitative data are presented as means ± SD from at least 3 independent experiments. * P < 0.05 or ** P < 0.01.

Figure 2

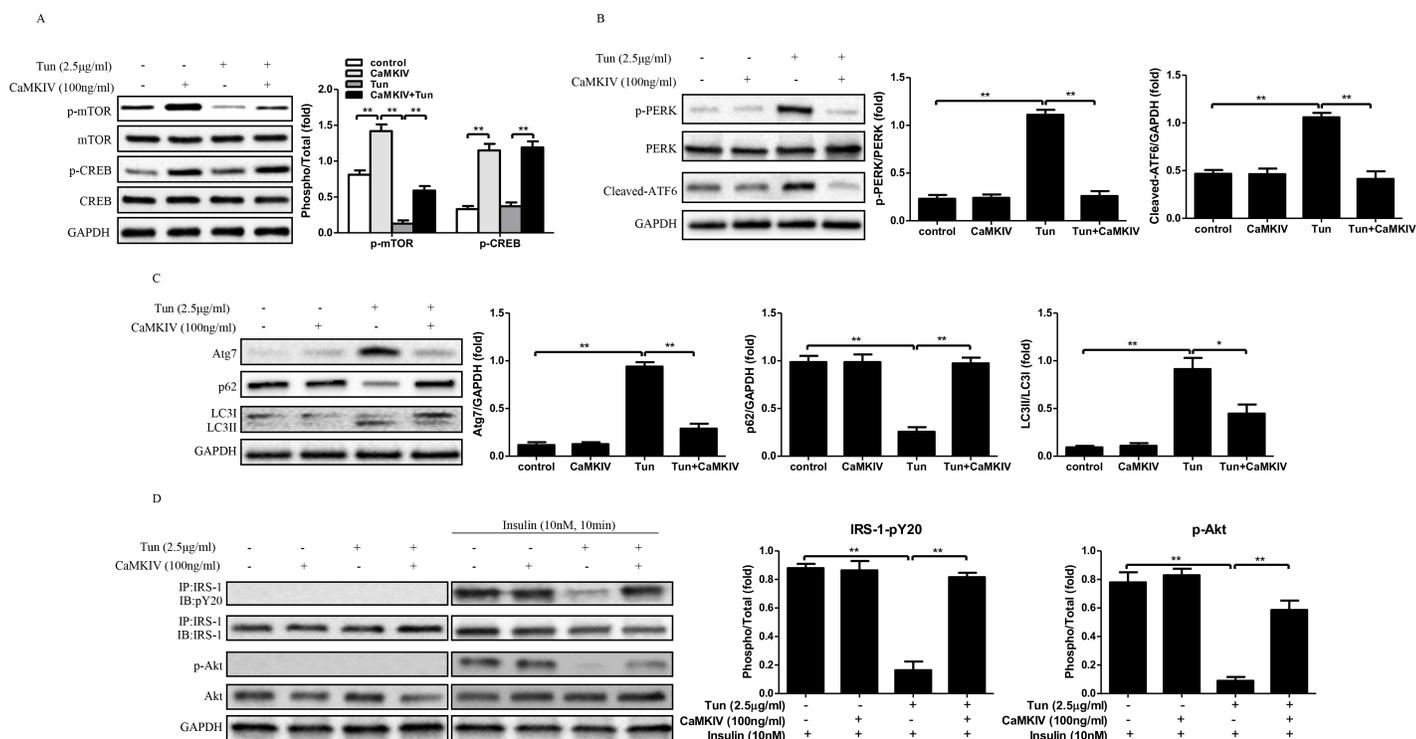


Figure 2

Recombinant CaMKIV incubation reduces ER stress-induced insulin resistance and autophagy dysfunction in mature adipocytes. Adipocytes were pretreated with Tun (2.5µg/ml) for 4 hours, followed by CaMKIV (100ng/ml) for 24 hours. For insulin signaling, cells incubated in the absence or presence of 10nM insulin for 10 min. All indicators were measured at protein level. The relative quantity of protein was analyzed using Quantity One software. (A) p-mTOR, p-CREB expression and their total protein levels. (B) ER stress markers including PERK phosphorylation (p-PERK) and cleaved-ATF6 in adipocytes. (C) Autophagy-related proteins Atg7, p62 and LC3 in adipocytes. (D) IRS-1 tyrosine phosphorylation (pY), Akt serine 473 phosphorylation (p-Akt), and their total protein levels were examined in mice adipocytes either with IP followed by IB or by direct immunoblotting. Quantitative data are presented as means \pm SD from at least 3 independent experiments. IB, immunoblotting; IP, immunoprecipitation. * P < 0.05 or ** P < 0.01.

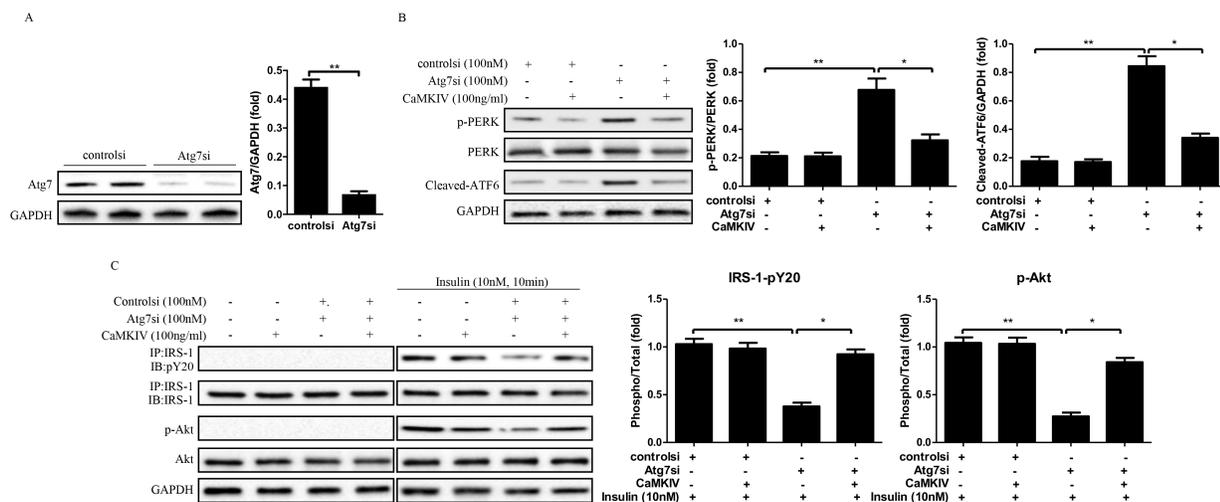


Figure 3

CaMKIV rescues autophagy dysfunction induced ER stress and insulin resistance in mature adipocytes. Adipocytes were pretreated with 100nM Atg7 siRNA (Atg7si) or control siRNA (controls) for 24 hours, followed by 2mM CaMKIV for 24 hours. For insulin signaling, cells incubated in the absence or presence of 10nM insulin for 10 min. All indicators were measured at protein level. The relative quantity of protein was analyzed using Quantity One software. (A) Total protein was isolated from siRNA adipocytes and immunoblotted with antibodies for Atg7. (B) Effect of CaMKIV on ER stress in Atg7^{-/-} adipocytes. (C) IRS-1 tyrosine phosphorylation (pY) and Akt serine 473 phosphorylation (p-Akt) in Atg7si transfected adipocytes with or without CaMKIV. Quantitative data are presented as means ± SD from at least 3 independent experiments. IB, immunoblotting; IP, immunoprecipitation. * P < 0.05 or ** P < 0.01.

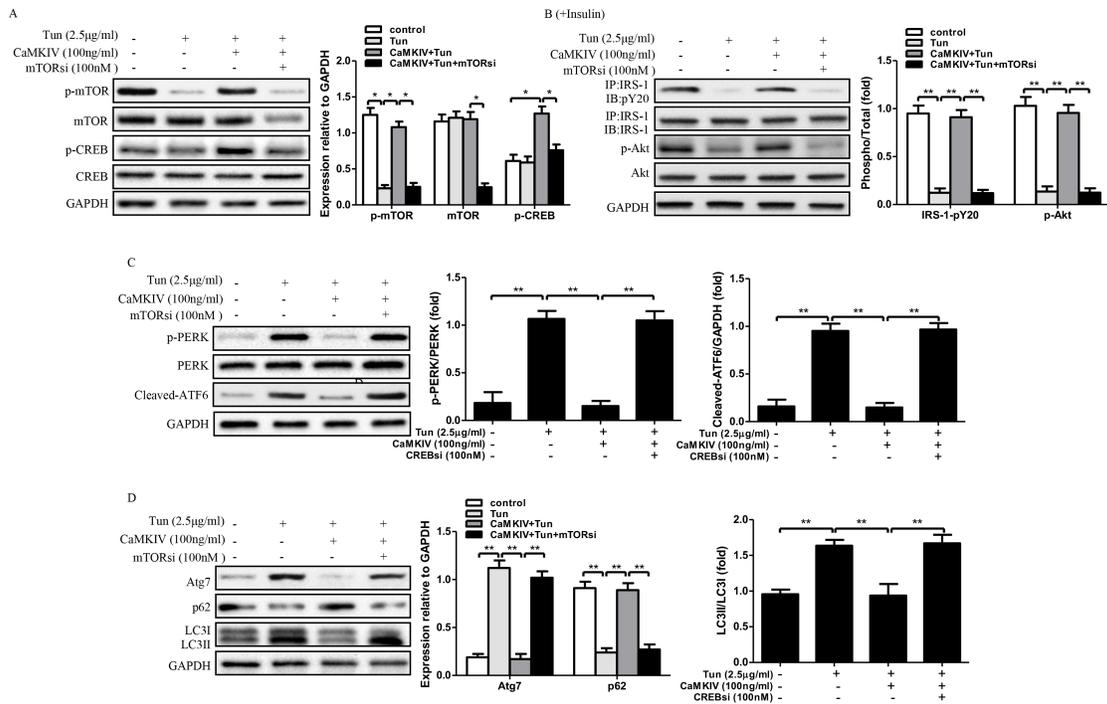


Figure 4

Effect of CaMKIV on ER stress, insulin signaling, and autophagy in mTOR siRNA transfected adipocytes. Cells were cultured with Tun (2.5µg/ml) for inducing insulin resistance. Cells were cultured in the presence or absence of CaMKIV with or without 100nM mTOR siRNA (mTORsi) for 24 hours. For insulin signaling, cells were stimulated with 10nM insulin for 10 min. All indicators were measured at protein levels. The relative quantity of proteins was analyzed using Quantity One Software. (A) p-mTOR, p-CREB expression and their total protein levels. (B) IRS-1 tyrosine phosphorylation (pY), Akt serine 473 phosphorylation (p-Akt), and their total protein levels were examined in mice adipocytes either with IP followed by IB or by direct immunoblotting. (C) ER stress markers including PERK phosphorylation (p-PERK) and cleaved-ATF6 in adipocytes. (D) Autophagy-related proteins Atg7, p62 and LC3 in adipocytes. Quantitative data are presented as means ± SD from at least 3 independent experiments. * P < 0.05 or ** P < 0.01.

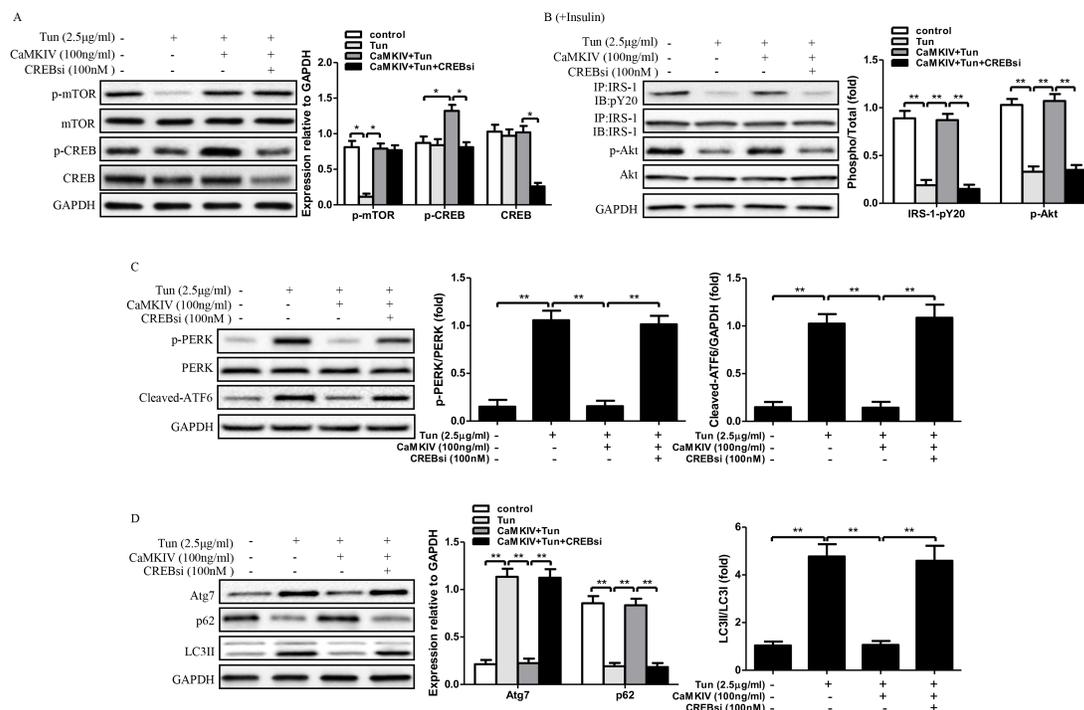


Figure 5

Effect of CaMKIV on ER stress, insulin signaling, and autophagy in CREB siRNA transfected adipocytes. Cells were cultured with Tun (2.5µg/ml) for inducing insulin resistance. Cells were cultured in the presence or absence of CaMKIV with or without 100nM CREB siRNA (CREBsi) for 24 hours. For insulin signaling, cells were stimulated with 10nM insulin for 10 min. All indicators were measured at protein levels. The relative quantity of proteins was analyzed using Quantity One Software. (A) p-mTOR, p-CREB expression and their total protein levels. (B) IRS-1 tyrosine phosphorylation (pY), Akt serine 473 phosphorylation (p-Akt), and their total protein levels were examined in mice adipocytes either with IP followed by IB or by direct immunoblotting. (C) ER stress markers including PERK phosphorylation (p-PERK) and cleaved-ATF6 in adipocytes. (D) Autophagy-related proteins Atg7, p62 and LC3 in adipocytes. Quantitative data are presented as means ± SD from at least 3 independent experiments. * P < 0.05 or ** P < 0.01.