Calcium/calmodulin-dependent Protein Kinase IV Limits ER stress and Insulin Resistance through Inhibition of Autophagy in Adipocytes by mTOR/CREB Signaling

Jiali Liu
Xi'an Jiaotong University Second Affiliated Hospital https://orcid.org/0000-0003-3173-7243
Ruihua Yang
Xi'an Jiaotong University Second Affiliated Hospital
Hao Meng
Xi'an Jiaotong University Second Affiliated Hospital
Ting Zhou
Xi'an Jiaotong University Second Affiliated Hospital
Qian He (qianhexjtu@163.com)
https://orcid.org/0000-0002-5686-9835

Research article

Keywords: CaMKIV, adipose, ER stress, autophagy, insulin resistance

Posted Date: February 17th, 2020

DOI: https://doi.org/10.21203/rs.2.23752/v1

Version of Record: A version of this preprint was published on July 13th, 2020. See the published version at https://doi.org/10.1186/s12902-020-00589-2.

Abstract

Background: CaMKIV has been identified as a potential regulator of skeletal muscle glucose metabolism and insulin gene expression. 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 endoplasmic reticulum (ER) stress and insulin resistance. The purpose of this study is to investigate the effect of CaMKIV on the ER stress, autophagic function and insulin signaling in tunicamycin induced adipocytes.

Methods: In the present study, we first used tunicamycin to establish a cell model of ER stress. Then recombinant CaMKIV and/or targeted-siRNA of CREB and mTOR were added into medium of tunicamycin-induced adipocytes. The ER stress indicators, autophagy activation, mTOR/CREB signaling and insulin sensitivity were analyzed by western blotting or electron microscopy.

Results: The results suggested CaMKIV not only reversed tunicamycin-induced ER stress, but also improved impaired insulin sensitivity through regulating abnormal autophagy in adipocytes. Moreover, CaMKIV inhibited activated ER stress and elevated insulin sensitivity in Atg7-/- adipocytes. However, the protective effects of CaMKIV were nullified by suppression of mTOR or CREB in tunicamycin induce adipocytes, suggesting that CaMKIV could inhibit ER stress, suppress autophagy and restore insulin signaling at least partly through mTOR/CREB signaling.

Conclusion: We conclude that CaMKIV inhibits ER stress and improves insulin signaling through reduction of autophagy in adipocytes viamTOR/CREB signaling, which could be regarded as novel opportunities for treatment of obesity and type 2 diabetes.

Background

Obesity induced insulin resistance (IR) 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 IRE1α, 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, we speculated that reduction of autophagy could be beneficial for cells to dispose of unfolded or misfolded proteins under ER stress.

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]. CREB is a basic leucine zipper type transcription factor and 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 [18, 19]. It has been suggested CREB regulates expression of IRE1a and PERK, which suggested CREB regulates the key components of UPR [20]. Therefore, we suppose that CaMKIV could regulate CREB activation 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-induced cells with or without CaMKIV. To further identify the mechanism of CaMKIV on insulin resistance, we next analyzed the regulators of ER stress, autophagy indicators and insulin sensitivity after blockage mTOR/CREB signaling in Tun-induced adipocytes models. Our results provided the evidences that CaMKIV treatment inhibits ER stress, abnormal autophagy and improves insulin sensitivity in Tun induced adipocytes. Moreover, our results also demonstrated the protective effect of CaMKIV on cell models of Tun-insulin resistance at least partly via mTOR/CREB signaling. Thus, our results provided a potential therapeutic target for the prevention and treatment of obesity and 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℃ in a humidified atmosphere with 5% CO_2. For adipocytes differentiation, 100% confluent 3T3-L1 were induced with MDI induction media (0.5 mM 1-methyl-3-isobutylmethylxanthine, 200 nM dexamethasone, 160 nM insulin, and DMEM with 10% FBS) (day 0). Two days later media was changed to 10% FBS/ DMEM with 160 nM insulin. Cells were then fed with this maintenance medium every 2 days. Full differentiation is usually achieved on the 12th day. To induce ER stress, cells were treated with different concentration (0–5 μg/ml) of Tun for 4 hours. For the effect of CaMKIV, cells were treated with 100 ng/ml CaMKIV for 24 hours. For blocking mTOR signaling, cells were treated with 100 nM mTOR siRNA for 24 hours. For blocking CREB signaling, cells were treated with 100 nM CREB siRNA for 24 hours. For insulin signaling, cells were stimulated with 10 nM 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 tetraoxide and embedded in epoxy resin (Epon). Ultrathin sections (80 nm) 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 transfected 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 48 hours after transfection. The knockdown efficiency was assessed by western blot.

Western blotting and immunoprecipitation

The 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 15 min at 4℃. 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℃ for 10 min. The proteins were separated by SDS-PAGE gel, transferred to a polyvinylidene difluoride membranes, incubated with specific primary antibodies at 4℃ 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 an unpaired, two-tailed Student t test for normally distributed variables. Multiple comparisons of quantitative variables among groups were made using one-way ANOVA testing. 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 difference.

Results

Tun-mediated ER stress increased autophagy in vitro. To determine the effects of pharmacological ER stress on autophagic function, an ER stress cell model was established. First, 3T3-L1 cells were induced to differentiate into mature adipocytes. Then the mature adipocytes treated with various dose of Tun (0–5 μg/ml) for 4 hours, and ER stress proteins were examined by western blotting. The phosphorylation of PERK and the protein levels of cleaved-ATF6 were increased significantly after Tun exposure with concentration of ≧ 2.5 μg/ml compared with the control group (Fig. 1A). To examine the effect of Tun on autophagy, after incubation with 2.5 μg/ml Tun for 4 hours, the autophagy indictor such as Atg7 and LC3II protein levels were markedly increased as well as a decrease in the protein expression of p62 in 2.5 and 5 μg/ml Tun treatment group compared with control group (Fig. 1B). Hence, we used 2.5 μg/ml Tun to establish ER stress cell models and to induce autophagic disorder in the following in vitro experiments. To further identify the effect of ER stress on autophagy, an EM examination in 3T3-L1 adipocytes demonstrated a significant induction of autophagosome/ autolysosome formation in the cells treated with 2.5 μg/ml Tun for 4 hours compared with control (Fig. 1C). These results suggested that Tun induced ER stress that mediates autophagic dysfunction.

CaMKIV reverses Tun-induced ER stress and autophagic disorder, and restores impaired insulin signaling in adipocytes. To understand the effect of CaMKIV on autophagy and insulin signaling in Tun-induced cell models of ER stress, the indicators of ER stress, autophagy and insulin sensitivity were evaluated in mature adipocytes. Cells were pretreated with 2.5 μg/ml Tun for 4 hours to induce ER stress and autophagic dysfunction. As the results shown, CaMKIV not only induced phosphorylated CREB after CaMKIV incubation with or without Tun pretreated, but also increased the Tun-induced reduction of p-mTOR (Fig. 2A). These results suggested CaMKIV regulates phosphorylation of mTOR and phosphorylated CREB in adipocytes. In addition, Tun treatment not only elevated the expression of ER stress indicators such as 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 (Fig. 2B and 2C). Meanwhile, insulin signaling was impaired, which was identified by the reduction of Akt phosphorylation and IRS-1 tyrosine phosphorylation (Fig. 2D). Remarkably, adipocytes treated with CaMKIV in the 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 (Fig. 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 (Fig. 2D). These findings suggested that CaMKIV reverses Tun-induced autophagic disorder and ER stress, restores insulin signaling and regulates mTOR and CREB expression in vitro.

CaMKIV inhibits ER stress and reverses insulin resistance in Atg7 $^{-/-}$ adipocytes. Autophagy activation plays an important role in regulating ER stress and insulin resistance, to further clarify the effect of CaMKIV on ER stress and insulin resistance in defective autophagy cells, we used Atg7 siRNA (Atg7si) to establish autophagic defective adipocytes model. Adipocytes were transfected with 100 nM Atg7si for 24 hours, which was validated by a reduced Atg7 protein expression (Fig. 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 (Fig. 3B and 3C). However, CaMKIV treatment inhibited ER stress and improved impaired insulin sensitivity in Atg7$^{-/-}$ adipocytes, as shown in Fig. 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 disorder.

CaMKIV restored insulin sensitivity and autophagic imbalance 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. And CaMKIV also regulated autophagy through mTOR signaling in lipopolysaccharide-induced inflammation and acute kidney injury. Therefore, our hypothesis is that the protective effect of CaMKIV on autophagy, ER stress and insulin signaling through mTOR/CREB signaling. We then cultured adipocytes in the presence of Tun and/or CaMKIV, with or without specific siRNA of each signaling pathway such as mTOR siRNA (mTORsi) and CREB siRNA (CREBsi). As the result shown in Fig. 4A, CaMKIV treatment not only increased phosphorylation of 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 effect of CaMKIV (Fig. 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 (Fig. 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 (Fig. 5B-5D).

Discussion

In our study, we first demonstrated that CaMKIV inhibited autophagy and ER stress and improved insulin sensitivity in tunicamycin-induced 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 also inhibited autophagic defective-induced ER stress and insulin resistance. 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. 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 [21]. 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 [22–24]. Autophagic dysfunction was common in high-fat feeding, genetic obesity and elderly [24, 25]. 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 autophagic abnormal 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 [26]. 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 [27]. 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 [28].

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 GSK3β 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 [29]. 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 [30, 31]. 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 [32]. 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 [33]. 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 [19, 34]. 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 [35, 36]. 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 and negative role in obesity induced insulin resistance [37]. 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 CaMKIV restored autophagy to attenuates ER stress and reversed insulin resistance through mTOR/CREB signaling in adipocytes. However, our study does have

limitation which should be further investigated. Recombinant CaMKIV peptide has been used to several in vivo and in vitro studies. However, the receptor of CaMKIV still unknown. 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

We have shown that CaMKIV could restore autophagy activation to inhibit ER stress and improve impaired insulin sensitivity in 3T3-L1 adipocytes, and the protective effect of CaMKIV on autophagy, ER stress and insulin signaling through mTOR/CREB signaling. Our study contributes to elucidating the potential role of CaMKIV in the pathogenesis of obesity and T2DM.

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.

Funding: This study was partly supported by funds from the National Natural Science Foundation of China (Program No. 81600619), Natural Science Foundation of Shaanxi Province (No. 708037169021) and Personal Training Special Fund of the Second Affiliated Hospital of Xi'an Jiaotong University [No. RC (XM) 2016010].

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.

Acknowledgments: We appreciate the technical support and materials from the electron microscope center and laboratory animal center of Xi'an Jiaotong University.

Author's information:

Affiliations

- Department of Clinical Laboratory, Xi'an Jiaotong University Second Affiliated Hospital, 157 West 5 Road, Xi'an, Shaanxi, 710004, China

Jiali Liu, Ruihua Yang, Hao Meng, Ting Zhou, Qian He

- Leonard Davis School of Gerontology, University of Southern California, Los Angeles, CA, 90089, USA

Jiali Liu

Corresponding author: Correspondence to Qian He.

References

1. Seidell JCJBJoN: Obesity, insulin resistance and diabetes—a worldwide epidemic. 2000, 83(S1):S5-S8. 2. Hajer GR, van Haeften TW, Visseren FLJEhj: Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. 2008, 29(24):2959-2971. 3. Goossens GHJP, behavior: The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. 2008, 94(2):206-218. 4. Guilherme A, Virbasius JV, Puri V, Czech MPJNrMcb: Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. 2008, 9(5):367. 5. Levine B, Kroemer GJC: Autophagy in the pathogenesis of disease. 2008, 132(1):27-42. 6. Rabinowitz JD, White EJS: Autophagy and metabolism. 2010, 330(6009):1344-1348. 7. Zhang Y, Goldman S, Baerga R, Zhao Y, Komatsu M, Jin SJPotNAoS: Adipose-specific deletion of autophagy-related gene 7 (atg7) in mice reveals a role in adipogenesis. 2009, 106(47):19860-19865. 8. Jansen H, Van Essen P, Koenen T, Joosten L, Netea M, Tack C, Stienstra RJE: Autophagy activity is up-regulated in adipose tissue of obese individuals and modulates proinflammatory cytokine expression. 2012, 153(12):5866-5874. 9. McLaughlin T, Deng A, Gonzales O, Aillaud M, Yee G, Lamendola C, Abbasi F, Connolly AJ, Sherman A, Cushman SW et al: Insulin resistance is associated with a modest increase in inflammation in subcutaneous adipose tissue of moderately obese women. 2008, 51(12):2303-2308. 10. Marciniak SJ, Ron DJPr: Endoplasmic reticulum stress signaling in disease. 2006, 86(4):1133-1149. 11. Winslow MM, Neilson JR, Crabtree GRJCoii: Calcium signalling in lymphocytes. 2003, 15(3):299-307. 12. Blaeser F, Toppari J, Heikinheimo M, Yan W, Wallace M, Ho N, Chatila TAJAJoP-E, Metabolism: CaMKIV/Gr is dispensable for spermatogenesis and CREM-regulated transcription in male germ cells. 2001, 281(5):E931-E937. 13. SÉE V, BOUTILLIER A-L, BITO H, LOEFFLER J-PJTFJ: Calcium/calmodulin-dependent protein kinase type IV (CaMKIV) inhibits apoptosis induced by potassium deprivation in cerebellar granule neurons. 2001, 15(1):134-144. 14. Murao K, Li J, Imachi H, Muraoka T, Masugata H, Zhang G, Kobayashi R, Ishida T, Tokumitsu HJD, Obesity, Metabolism: Exendin-4 regulates glucokinase expression by CaMKK/CaMKIV pathway in pancreatic β-cell line. 2009, 11(10):939-946. 15. Ban N, Yamada Y, Someya Y, Ihara Y, Adachi T, Kubota A, Watanabe R, Kuroe A, Inada A, Miyawaki KJD: Activating transcription factor-2 is a positive regulator in CaM kinase IV-induced human insulin gene expression. 2000, 49(7):1142-1148. 16. Evankovich J, Zhang R, Cardinal JS, Zhang L, Chen J, Huang H, Beer-Stolz D, Billiar TR, Rosengart MR, Tsung AJAJoP-G et al: Calcium/calmodulin-dependent protein

kinase IV limits organ damage in hepatic ischemia-reperfusion injury through induction of autophagy. 2012, 303(2):G189-G198. 17. Zhang X, Howell GM, Guo L, Collage RD, Loughran PA, Zuckerbraun BS, Rosengart MRJTJoI: CaMKIV-dependent preservation of mTOR expression is required for autophagy during lipopolysaccharide-induced inflammation and acute kidney injury. 2014, 193(5):2405-2415. 18. Sheng M, Thompson MA, Greenberg MEJS: CREB: a Ca (2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. 1991, 252(5011):1427-1430. 19. Matthews RP, Guthrie CR, Wailes LM, Zhao X, Means AR, McKnight GSJM, Biology C: Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. 1994, 14(9):6107-6116. 20. Nakayama KJTFJ: CREB regulates the expression of PERK and IRE1a, and controls unfolded protein response under hypoxic conditions. 2016, 30(1_supplement):647.649-647.649. 21. Chen Y, Brandizzi FJTicb: IRE1: ER stress sensor and cell fate executor. 2013, 23(11):547-555. 22. Cnop M, Foufelle F, Velloso LAJTimm: Endoplasmic reticulum stress, obesity and diabetes. 2012, 18(1):59-68. 23. Gregor MF, Hotamisligil GSJJolr: Thematic review series: Adipocyte Biology. Adipocyte stress: the endoplasmic reticulum and metabolic disease. 2007, 48(9):1905-1914. 24. Zhang K, Kaufman RJJN: From endoplasmic-reticulum stress to the inflammatory response. 2008, 454(7203):455. 25. Mejías-Peña Y, Estébanez B, Rodriguez-Miguelez P, Fernandez-Gonzalo R, Almar M, de Paz JA, González-Gallego J, Cuevas MJJA: Impact of resistance training on the autophagy-inflammation-apoptosis crosstalk in elderly subjects. 2017, 9(2):408. 26. Kang H, Sun LD, Atkins CM, Soderling TR, Wilson MA, Tonegawa SJC: An important role of neural activity-dependent CaMKIV signaling in the consolidation of long-term memory. 2001, 106(6):771-783. 27. Lee H-Y, Gattu AK, Camporez J-PG, Kanda S, Guigni B, Kahn M, Zhang D, Galbo T, Birkenfeld AL, Jornayvaz FRJD: Muscle-specific activation of Ca 2+/calmodulin-dependent protein kinase IV increases whole-body insulin action in mice. 2014, 57(6):1232-1241. 28. Liu J, Li Y, Zhou X, Zhang X, Meng H, Liu S, Zhang L, He J, He Q, Geng Y: CaMKIV limits metabolic damage through induction of hepatic autophagy by CREB in obese mice. Journal of Endocrinology 2020, 244(2):353-367. 29. Díaz-Troya S, Pérez-Pérez ME, Florencio FJ, Crespo JLJA: The role of TOR in autophagy regulation from yeast to plants and mammals. 2008, 4(7):851-865. 30. Zhou L, Liu FJA: Autophagy: roles in obesity-induced ER stress and adiponectin downregulation in adipocytes. 2010, 6(8):1196-1197. 31. Stienstra R, Haim Y, Riahi Y, Netea M, Rudich A, Leibowitz GJD: Autophagy in adipose tissue and the beta cell: implications for obesity and diabetes. 2014, 57(8):1505-1516. 32. Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RHJN: Phosphorylated CREB binds specifically to the nuclear protein CBP. 1993, 365(6449):855. 33. Kandel ERJMb: The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. 2012, 5(1):14. 34. Enslen H, Sun P, Brickey D, Soderling SH, Klamo E, Soderling TRJJoBC: Characterization of Ca2+/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. 1994, 269(22):15520-15527. 35. Impey S, Goodman RHJSS: CREB signaling-timing is everything. 2001, 2001(82):pe1-pe1. 36. Wang L, Piguet AC, Schmidt K, Tordjmann T, Dufour JFJH: Activation of CREB by tauroursodeoxycholic acid protects cholangiocytes from apoptosis induced by mTOR inhibition. 2005, 41(6):1241-1251. 37. Qi L, Saberi M, Zmuda E, Wang Y, Altarejos J, Zhang X, Dentin R, Hedrick S, Bandyopadhyay G, Hai TJCm: Adipocyte CREB promotes insulin resistance in obesity. 2009, 9(3):277-286.

Abbreviations

Akt, protein kinase B; ATF6, activation of transcription factor 6; Atg7, autophagy-related 7; CaMKIV, calcium/calmodulin-dependent protein kinase IV; CREB, cyclic AMP-responsive element binding protein; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRS, insulin receptor substrate; LC3, microtubule associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; p, phosphorylated; PERK, PKR-like ER eIF2α kinase; PI3K, phosphoinositide 3-kinase ; siRNA, small interfering RNA

Figures

Figure 1

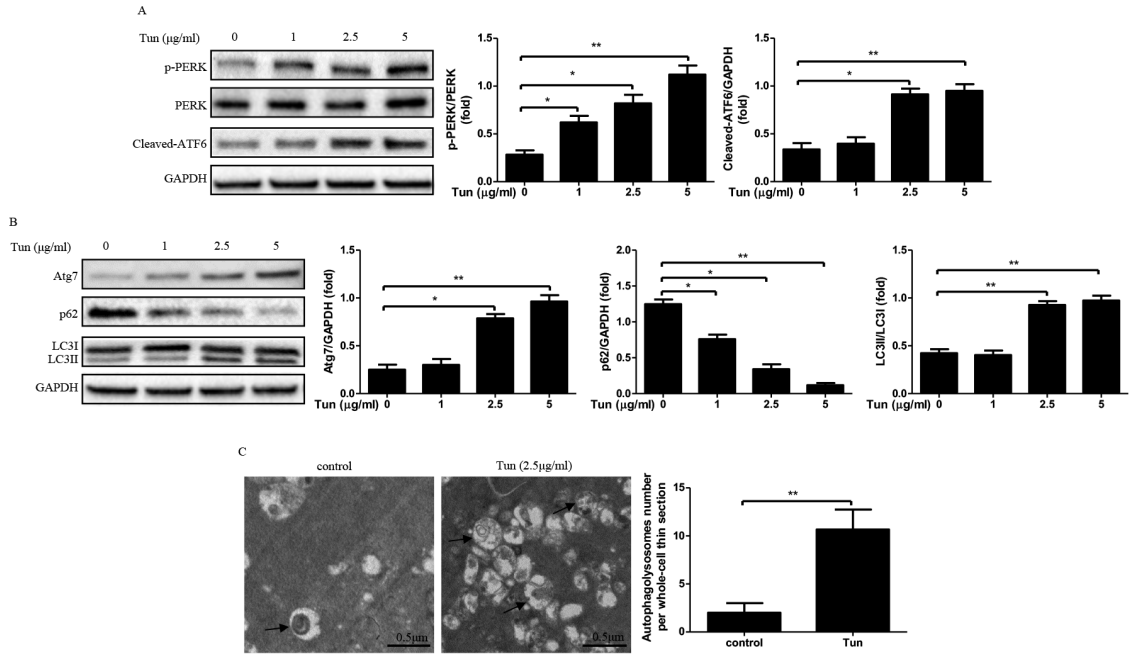

Figure 1

Induction of autophagic imbalance 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.

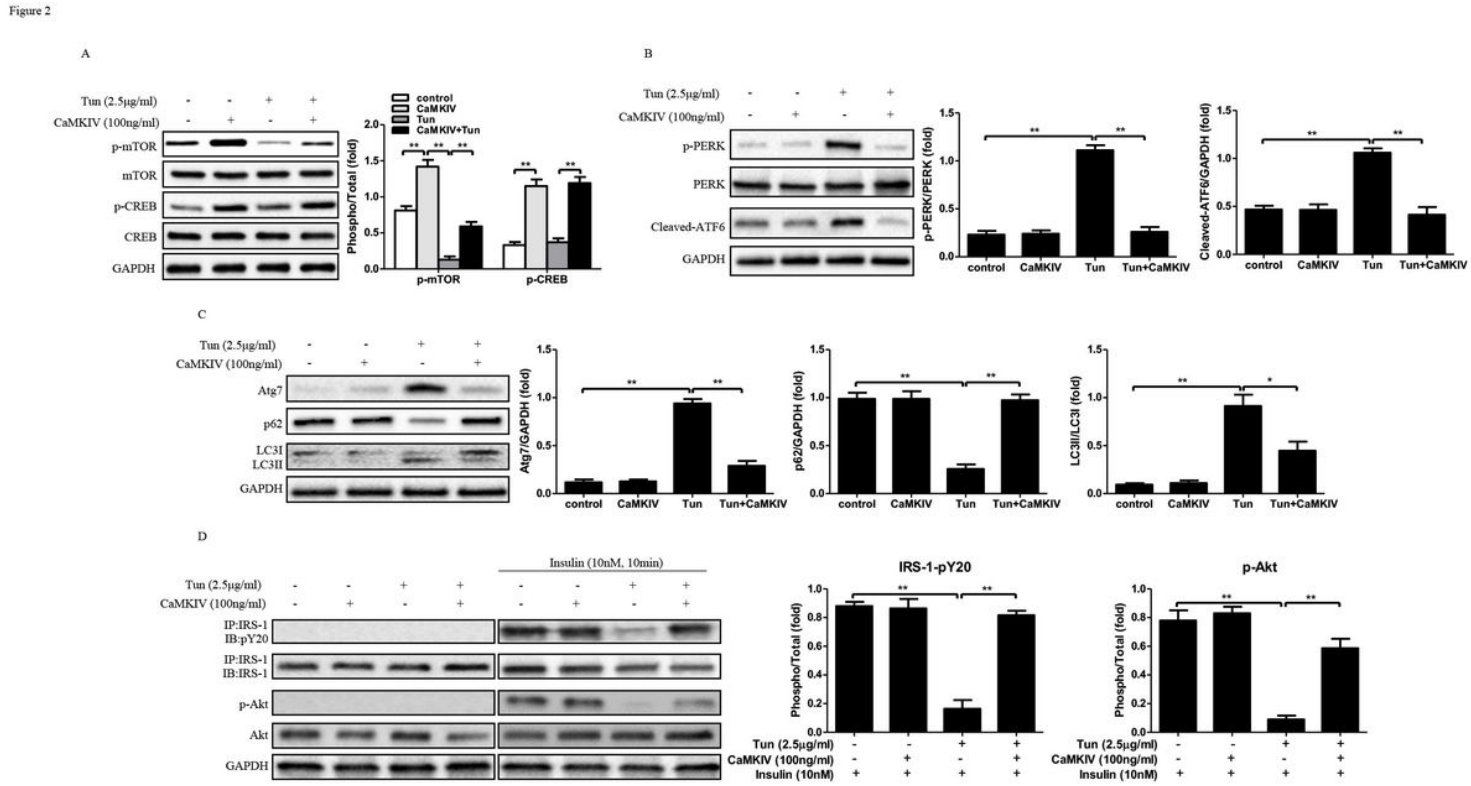

Figure 2

CaMKIV incubation reduces ER stress-induced insulin resistance and elevated autophagy in 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 ± SD from at least 3 independent experiments. IB, immunoblotting; IP, immunoprecipitation. * P < 0.05 or ** P < 0.01.

Figure 3

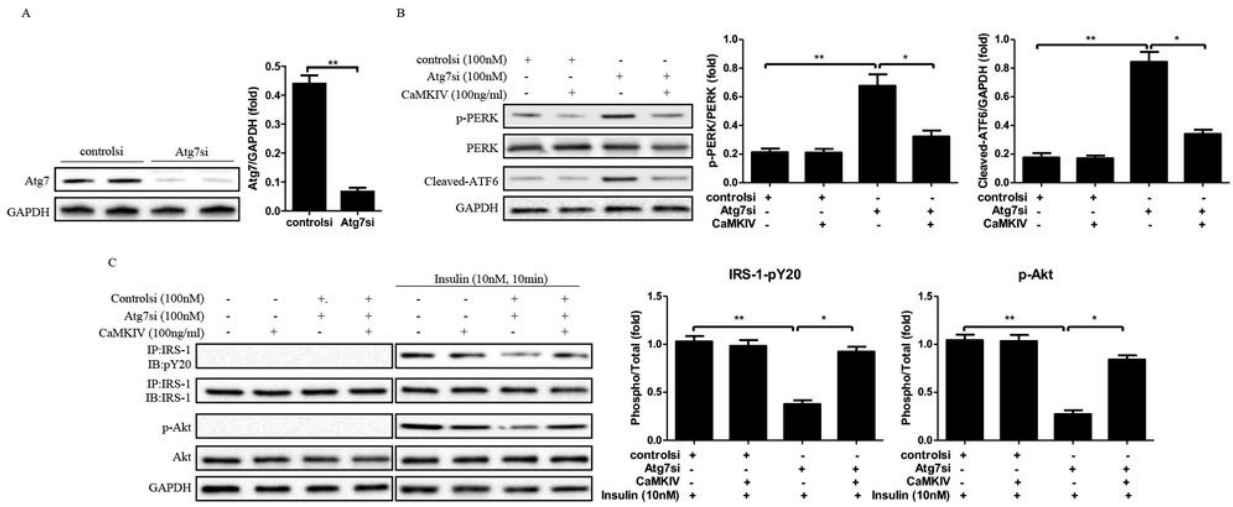

Figure 3

CaMKIV rescues Atg7 deficiency-induced ER stress and insulin resistance in mature adipocytes. Adipocytes were pretreated with 100nM Atg7 siRNA (Atg7si) or control siRNA (controlsi) for 48 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 Atg7-/- 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

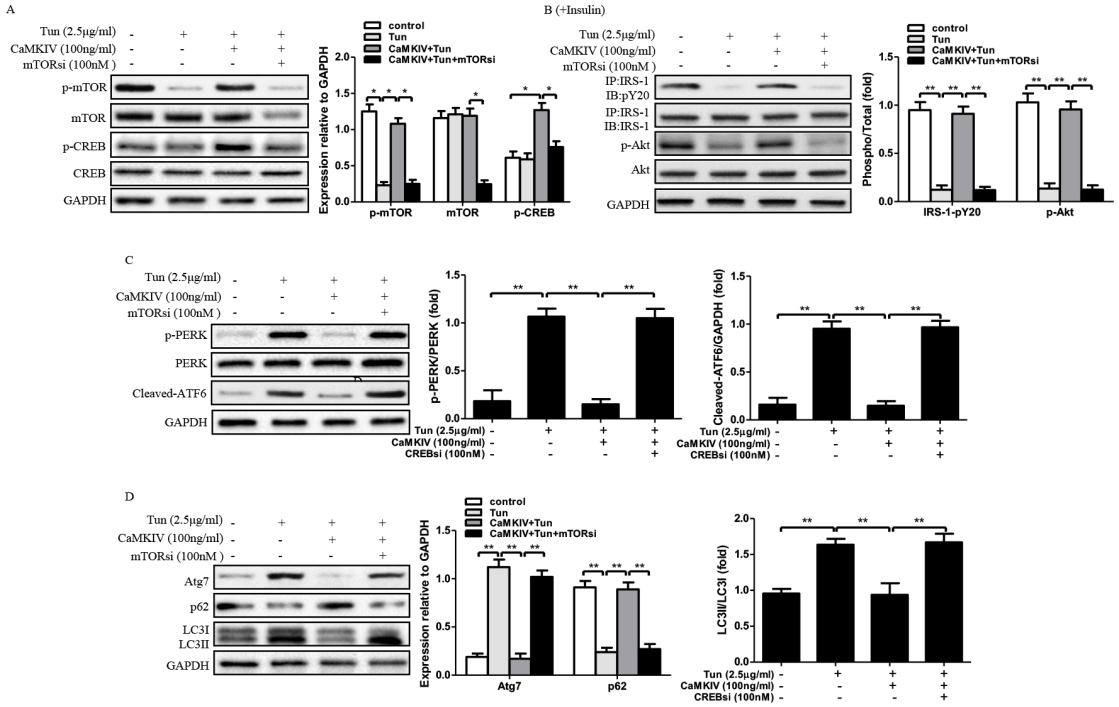

Figure 4

Effect of CaMKIV on ER stress, insulin signaling, and autophagy in mTOR deficient adipocytes. Cells were cultured with Tun (2.5μg/ml) for inducing insulin resistant. 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

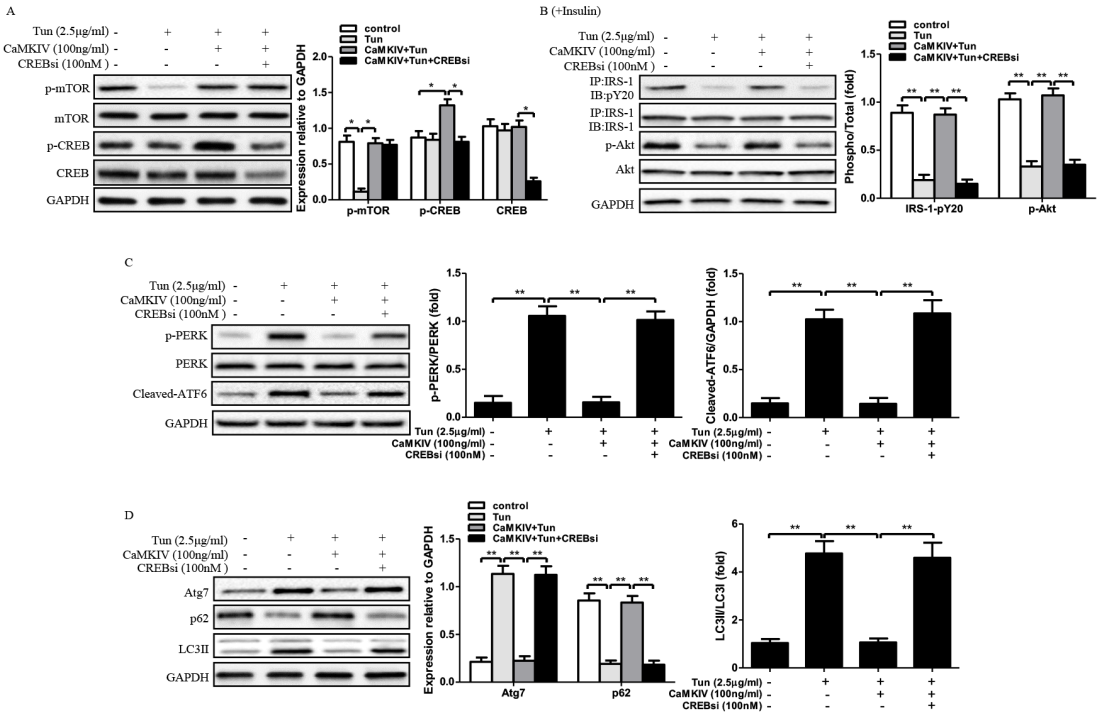

Figure 5

Effect of CaMKIV on ER stress, insulin signaling, and autophagy in CREB deficient adipocytes. Cells were cultured with Tun (2.5μg/ml) for inducing insulin resistant. 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 ** $P < 0.01$.