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CaMKIV limits metabolic damage through induction of hepatic autophagy by CREB in obese mice

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Abstract

High-fat diet (HFD) not only induces insulin resistance in liver, but also causes autophagic imbalance and metabolic disorders, increases chronic inflammatory response and induces mitochondrial dysfunction. Calcium/calmodulin-dependent protein kinase IV (CaMKIV) has recently emerged as an important regulator of glucose metabolism and skeletal muscle insulin action. Its activation has been involved in the improvement of hepatic and adipose insulin action. But the underlying mechanism is not fully understood. In the present study, we aimed to address the direct effects of CaMKIV *in vivo* and to evaluate the potential interaction of impaired insulin sensitivity and autophagic disorders in hepatic insulin resistance. Our results indicated obese mice receiving CaMKIV showed decreased blood glucose and serum insulin and improved insulin sensitivity as well as increased glucose tolerance compared with vehicle injection. Meanwhile, defective hepatic autophagy activity, impaired insulin signaling, increased inflammatory response and mitochondrial dysfunction in liver tissues which are induced by high-fat diet were also effectively alleviated by injection of CaMKIV. Consistent with these results, the addition of CaMKIV to the culture medium of BNL cl.2 hepatocytes markedly restored palmitate-induced hepatic insulin resistance and autophagic imbalance. These effects were nullified by blockade of cyclic AMP response element-binding protein (CREB), indicating the causative role of CREB in action of CaMKIV. Our findings suggested that CaMKIV restores hepatic autophagic imbalance and improves impaired insulin sensitivity via phosphorylated CREB signaling pathway, which may offer novel opportunities for treatment of obesity and diabetes.

Key Words

- ▶ CaMKIV
- ▶ high-fat diet
- ▶ CREB
- ▶ autophagy
- ▶ insulin resistance

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Introduction

In recent years, there has been an increasing appreciation for the significance of insulin resistance, which is a common feature of obesity and predisposes affected individuals to a variety of pathological conditions,

including hypertension, dyslipidemia, cardiovascular disease, and type 2 diabetes (Samuel & Shulman 2012, Odegaard & Chawla 2013, Tangvarasittichai 2015, Craft & Rhoads 2016). Liver is now recognized as not only an

energy control tissue but also a target organ of insulin that contributes to the systemic insulin resistance and metabolic inflammation (Calomeni *et al.* 2013, Koliaki & Roden 2013, Li *et al.* 2014b, Bojsen-Møller *et al.* 2018). Although considerable progress has been made in understanding the molecular mechanisms underlying these individual disorders, satisfactory treatment modalities remain limited. Ca²⁺/calmodulin-dependent protein kinase IV (CaMKIV), a multifunctional serine/threonine protein kinase encoded by the *CaMKIV* gene in human, plays a critical role in the process of transcriptional regulation of lymphocytes, neurons and male germ cells. Of note, CaMKIV has recently emerged as an important regulator in insulin-stimulated glucose metabolism and involved in whole-body improvements of insulin sensitivity (Wu *et al.* 2000, Hook & Means 2001, Lee *et al.* 2014).

CaMKIV, also known as CaMK4, has been shown to regulate various cellular events through phosphorylated transcription factors. It plays an essential role in immune response, inflammation and memory consolidation (Hanissian *et al.* 1993, Kang *et al.* 2001, Fukushima *et al.* 2008, Racioppi & Means 2008). However, recent studies supported a novel function of CaMKIV in regulating the systemic insulin action. For instance, the signal CaMKK/CaMKIV cascade plays an important role in glucose-upregulated transcriptional activation of the insulin gene in both pancreatic beta cells and the insulin-secreting cell line (Yu *et al.* 2004). Activated CaMKIV mediated the stimulatory effect of glucose-dependent insulin secretion through exendin-4 which may improve the function of pancreatic beta cells by the activation of glucokinase (Murao *et al.* 2009). In addition, another research has demonstrated that CaMKIV plays a significant role in CREB-dependent mechanism by which glucose regulates *Irs2* but not *Irs1* gene expression in beta cells, it is interesting to note that in these cells, overproduction of a dominant negative form of CREB completely suppressed glucose and CaMKIV stimulation of *Irs2* expression (Persaud *et al.* 2011). Lee and colleagues assessed insulin sensitivity in both wild-type (WT) mice and the novel transgenic mice (CaMKIV). The results suggested that the expression of an active form of CaMKIV in muscle improved insulin-stimulated muscle glucose uptake, with increases in GLUT4 content and improvements in insulin signaling. Also, CaMKIV activation could improve hepatic and adipose insulin action via increase in myokines released from the skeletal muscle (Lee *et al.* 2014).

Although the role of CaMKIV in insulin signaling has only recently been identified, the functional basis for the effect in the autophagy-mediated insulin signaling

pathway remains elusive. However, the relevance of CaMKIV to autophagic response has been implicated that CaMKIV activates autophagy *in vitro* and prevents oxidative stress-induced cell death, and augmentation of autophagy in CaMKIV KO mice restored organ damage to WT levels (Evankovich *et al.* 2012). Moreover, another study suggested that CaMKIV serves a permissive role of LPS-induced autophagy in Mφ and renal tubular cells by inhibiting GSK3β and FBXW7-mediated ubiquitination and proteasomal degradation of mTOR and titrates basal mTOR expression (Zhang *et al.* 2014). In addition, phosphorylation of CREB, a critical transcription factor for several forms of experience-dependent plasticity in a range of species, is commonly activated by CaMKIV. We therefore hypothesized that CaMKIV could affect liver functions, such as regulation of insulin signaling and autophagy activity. In this study, we aimed to address the direct effects of CaMKIV *in vivo* and to evaluate the potential interaction of impaired insulin sensitivity and autophagic disturbance during this process. Our results support the therapeutic potential of this new player in the regulation of hepatic insulin resistance and metabolic disorders.

Materials and methods

Materials

The following reagents were purchased from Sigma (Sigma-Aldrich): Palmitate (FFA), insulin, chloroquine, 3-MA and 666-15. Mouse recombinant CaMKIV was obtained from Sino Biological (Sino Biological Inc. Wayne, PA, USA). The following antibodies were used: anti-Atg7 (1:1000, #2631), anti-p62 (1:500, #5114), and anti-LC3 (1:500, #4108), anti-CREB (1:5000, #9197), anti-p-CREB (1:1000, #9198) (all from Cell Signaling Technology Inc.); anti-CaMKIV (1:1000, sc-55501), anti-p-IRS-1 (1:300, sc-17200), anti-pY20 (1:300, sc-508), anti-Akt (1:500, sc-5298), anti-p-Akt (1:400, sc-377556), anti-GAPDH (1:5000, sc-47724) and peroxidase goat anti-rabbit IgG (1:3000, sc-2768) and peroxidase goat anti-mouse IgG (1:3000, sc-2357) (Santa Cruz Biotechnology Inc.). For detection, a Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate Kit (PerkinElmer Inc.) was used. Individual protein bands were quantified by ImageJ software. All other chemicals were obtained from standard resource and were of the highest grade available.

Animals and treatment

All studies were approved by the Xi'an Jiaotong University Laboratory Animal Administration Committee and

performed according to the Xi'an Jiaotong University Guidelines for Animal Experimentation. For *in vivo* experiments, 4-week-old C57BL/6J male mice were obtained from Medical Experimental Animal Center of Xi'an Jiaotong University. Mice were maintained in a temperature- (22°C), humidity-, and light- (12 h of light, 12 h of darkness, darkness from 19:30 h to 7:30 h)-controlled environment. Recombinant mouse CaMKIV was freshly diluted in saline solution (0.9% NaCl) at a concentration of 1 µg/µL. Mice were distributed in four groups (15 mice/group): (1) ND+vehicle (normal diet+saline solution intraperitoneally i.p.); (2) ND+CaMKIV (normal diet+recombinant mouse CaMKIV, i.p. 1 µg/g body weight/day); (3) HFD+vehicle (diet containing 60% of energy as fat, 5.24 kcal/g+saline solution intraperitoneally i.p.); (4) HFD+CaMKIV (diet containing 60% of energy as fat, 5.24 kcal/g+recombinant mouse CaMKIV, i.p. 1 µg/g body weight/day). At the end of the 12-week study period, mice received vehicle or an intraperitoneal injection of insulin at a dosage of 2 IU/kg for the insulin signaling test; 15 min after the injection, all animals were killed and their liver tissues and blood samples were obtained and stored at -80°C for subsequent analysis.

Cell culture and treatment

BNL cl.2 cells were purchased from American Tissue Culture Collection (ATCC) and maintained at 37°C in a humidified atmosphere of 5% CO₂ in DMEM (Gibco) with 10% fetal bovine serum (Gibco). To induce insulin resistance, cells were treated with 500 µM of palmitate (FFA) for 16 h. For the effect of CaMKIV, cells were treated with 100 ng/mL of CaMKIV for 20 h. For blocking CREB signaling, cells were treated with 100 nM of CREB inhibitor 666-15 for 24 h. For insulin signaling, cells were stimulated with 10 nM insulin for 10 min. Before each experiment, the medium was replaced by fresh medium.

Measurement of serum parameters and metabolic tests

After collection, serum was stored at -80°C before analysis. Serum triglycerides were measured by colorimetric enzymatic method using commercial kits (SGM Italia, Italy and Randox Laboratories Ltd., UK). Serum FFA was measured using an ELISA kit (Abcam) according to the manufacturer's instructions. Serum TNF-α, IL-6, MCP-1 and FGF-21 were measured using the commercially available ELISA kit (R&D Systems, Inc.). Glucose

tolerance testing (GTT) was performed after the mice were fasted overnight. Insulin tolerance testing (ITT) was performed after mice were fasted for 6 h. Metabolic tests were performed using a standard protocol as described previously (Zhou *et al.* 2013). Hepatic glycogen content was measured using an EnzyChrom™ Glycogen Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions.

Gene silencing

siRNA for target genes (CaMKIV: sc-29903, CREB: sc-29903, Atg7: sc-41448, Santa Cruz Biotechnology, Inc.) or scrambled siRNA (Control, sc-37007, Santa Cruz Biotechnology, Inc.) were transfected using Lipofectamine™ 2000 Transfection Reagent (Invitrogen). The transfected cells were cultured in DMEM containing 10% FBS for 48 h after transfection.

mRNA isolation and analysis by real-time PCR

Total RNA was prepared from isolated liver tissues or BNL cl.2 cells by TRIzol extraction (Qiagen) according to the manufacturer's recommendations. Total RNA (1 µg) was converted into cDNA using a cDNA synthesis kit (Invitrogen). β-Actin was used as an internal control and tests were performed in triplicate for each sample. The relative quantity was calculated using the 2^{-ΔΔCt} method. Quantitative real-time PCR analysis was performed as described previously (Liu *et al.* 2015). The specific primer sequences were shown in Table 1.

Liver tissues histomorphometry

As described previously (Zhou *et al.* 2013), liver tissues were fixed in solution and washed. Samples were next sectioned at 10 µm using a cryostat. The sections were stained with Oil Red O kit (Abcam) according to the manufacturer's instructions. The Oil Red O-positive area over the total area was quantified using ImageJ software.

Electron microscopy analysis

The isolated muscle tissues, adipose tissues and liver tissues were first fixed in a solution comprising 4% paraformaldehyde, 2% glutaraldehyde, and 0.1 M sodium cacodylate (pH 7.3), post fixed in 1% osmium tetroxide and then embedded in epoxy resin (Epon). Ultrathin sections (80 nm) were stained with aqueous uranyl acetate and lead citrate and then examined with

Table 1 The sequences of primers for real-time PCR.

Target gene	Sense	Anti-sense
TNF- α	5'-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3'	5'-GTATGAGATAGCAAATC GGCTGACGGTGTGGG-3'
IL-6	5'-AACGATGATGCACTTGCAAG-3'	5'-GAGCATTGGAATTGGGGTA-3'
MCP-1	5'-CCCACTCACCTGCTGCTACT-3'	5'-TCTGGACCCATTCTTCTTG-3'
Fgf-21	5'-AGATCAGGGAGGATGGAACA-3'	5'-ATCAAAGTGAGGCGATCCATA-3'
p62	5'-TGTGGAACATGGAGGGAAGAG-3'	5'-TGTGCCTGTGCTGGAACCTTC-3'
Srebf1c	5'-TGGAGACATCGCAACAAG-3'	5'-GGTAGACAACAGCCGCATC-3'
Pparg	5'-GACATCAAGCCTTTACCACA-3'	5'-GCTCTTGTGAATGGAATGTCTTC-3'
Fasn	5'-GCAGCAAGTGCCACCAACAA-3'	5'-CTCATCGGAGCGCAGGATAGA-3'
Acaca	5'-GAGTGACTGCCGAAACATCTCTG-3'	5'-GCCTCTTCTGACAAACGAGT-3'
Scd1	5'-CTGCCTCTTCGGGATTTTCTACT-3'	5'-GCCCATTCGTACACGTGATTC-3'
Dgat2	5'-GAGGGGTCTGGGCGATGGGGCACT-3'	5'-CGACGGTGGTGTGGGCTTGGAGT-3'
G6pc	5'-CGACTCGCTATCTCCAAGTGA-3'	5'-GTTGAACCACTCTCCGACCA-3'
Pck1	5'-GTGCTGGAGTGGATGTTTCGG-3'	5'-CTGGCTGATTCTGTTCAGG-3'
β -Actin	5'-TGTCACCTTCAGCAGATGT-3'	5'-AGCTCAGTAACAGTCCGCCTAGA-3'
GAPDH	5'-ACCCAGAAGACTGTGGATGG-3'	5'-CACATTGGGGGTAGGAACAC-3'

a transmission electron microscope (2000FX. JEOL, Peabody, MA, USA). Sixteen electron micrographs of each mouse were digitized, and the area and number of clearly distinguishable mitochondria were analyzed using image-analysis software (Osteomeasure; OsteoMetrics, Decatur, GA, USA).

Autophagy flux analysis

Chloroquine experiment was described as previously (Zois *et al.* 2011, Cho *et al.* 2014). Chloroquine dissolved in PBS was injected s.c. at a dose of 50 mg/kg body weight, and mice were killed 24 h following the injection. Vehicle-treated mice were injected s.c. with an equal volume of sterile PBS solution. The injection dose of chloroquine was determined based on previous reports and our preliminary study, which had no detectable effect on liver function. After the dissection, the liver tissues were immediately homogenized in lysis buffer for Western blotting analysis.

Western blot and immunoprecipitation

Western blotting and immunoprecipitation were performed using a standard protocol as described previously (Li *et al.* 2014a). Positive Western blot loading control was used to identify the stability of the system. A standard sample was run in each blot to compare density values between blots by expressing each band density value as fold of this standard sample. Immunocomplexes were resolved by means of SDS-PAGE and immunoblotted with the indicated antibodies. The relative quantity of proteins was analyzed using the ImageJ software.

Statistics

Data were analyzed by the Prism software, version 5.0 (GraphPad Software Inc.). Characteristics of subjects between two 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 $\pm$ s.d. *n* represents the number of animals used. A *P* value of <0.05 or *P* value of <0.01 was considered as significantly different.

Results

CaMKIV administration protects from the glucose intolerance and insulin resistance induced by high-fat feeding mice

To address the effects of CaMKIV on glucose metabolism and insulin sensitivity *in vivo*, mice were administered recombinant mouse CaMKIV i.p daily at the dosage of 1 μ g/g body weight under a normal diet condition or HFD condition started from 4th to 12th week. The mean body weight of all mice groups, measured during the whole experimental period, shown in Fig. 1A. HFD feeding clearly led to a significant increase in body weight. These data were confirmed by the area under the curve (AUC) of body weight, calculated during the period (4th to 12th week) (Fig. 1B). The groups on HFD showed a similar body weight much higher than ND-fed mice. Compared with mice cotreated with ND and vehicle injection, after 12-week study period, obese mice with vehicle treatment exhibited obesity and decreased glucose tolerance and insulin sensitivity as measured by GTT and

Figure 1

Effect of recombinant mouse CaMKIV on glucose metabolism and insulin sensitivity in HFD-fed mice. All analyses compared age- and sex-matched mice fed a normal diet (ND) or high-fat diet (HFD) for 12 weeks and injected with CaMKIV (1 µg/g/day) or vehicle in the last 8 weeks. (A) Body weight. (B) AUC of body weight during time (4th–12th week). (C) Blood glucose. (D) Serum insulin. (E) GTT. (F) ITT. Data are expressed as means $\pm$ s.d. in each bar graph from 12 to 15 mice per group. * $P < 0.05$ or ** $P < 0.01$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0251>.

ITT, respectively. But administration of CaMKIV could improve the HFD-induced glucose intolerance and insulin resistance (Fig. 1E and F). Meanwhile, obese mice with vehicle injection also showed elevated blood glucose and higher serum insulin level. However, injection of CaMKIV could reverse this phenomenon in obese mice (Fig. 1C and D). Taken together, these results indicated obese mice with CaMKIV treatment had an improved capability on glucose metabolism *in vivo*.

Effect of CaMKIV on hepatic glucose and lipid metabolism

As the major organ regulates glucose and lipid metabolism in the body, liver also plays a central role in the synthesis and degradation of fatty acids. To address the role of CaMKIV on glucose and lipid metabolism, mice were fasted 16 h before killing. We first analyzed mice liver weight and steatosis. These results showed there was no difference in liver weight and lipid accumulation between obese mice with CaMKIV or vehicle injection (Fig. 2A and B). However, serum free fatty acid was found markedly decreased in obese mice with CaMKIV treatment compared with vehicle injection (Fig. 2C). Similar results were also observed in serum triglyceride (Fig. 2D). In addition, the vehicle injection in obese mice showed increased levels of glycogen in liver tissues, whereas the levels were significantly decreased after CaMKIV injection (Fig. 2E). Consistent with these data, the expression of hepatic glucoregulatory genes *G6pc* and *Pck1* in mRNA (Fig. 2F)

and their protein levels (Supplementary Fig. 1A, see section on [supplementary materials](#) given at the end of this article) were markedly increased in obese mice with vehicle treatment. However, CaMKIV treatment in obese mice showed a reduced mRNA and protein expression of *G6pc* and *Pck1* compared with obese mice treated with vehicle. To further insight into the liver fatty acid and triacylglycerol synthesis, we therefore decided to evaluate the expression of some lipogenic genes in mRNA and protein levels. The results demonstrated the expression of these genes were markedly upregulated in obese mice with vehicle treatment (Fig. 2G and Supplementary Fig. 1B). However, CaMKIV treated with obese mice showed a significant decrease in liver tissues (Fig. 2G and Supplementary Fig. 1B), indicating the improved hepatic glucose and lipid metabolism may contribute to the CaMKIV-induced effects.

Effect of CaMKIV on mitochondrial functions and efficiency

Numerous studies in human and animal models have shown that obesity or insulin resistance is frequently associated with reduced mitochondrial mass and impaired mitochondrial function in insulin sensitivity tissues (Petersen *et al.* 2004, Koliaki *et al.* 2015, Montgomery & Turner 2015). To clarify the mediator of effect of CaMKIV on mitochondrial function, we performed molecular and histomorphometric analysis in mice. Our results demonstrated the critical genes which are involved in

Figure 2

Effects of CaMKIV on hepatic glucose and lipid metabolism *in vivo*. All analyses compared age- and sex-matched mice fed a normal diet (ND) or high-fat diet (HFD) for 12 weeks and injected with CaMKIV (1 μ g/g/day) or vehicle in the last 8 weeks. (A) Liver weight. (B) Oil O staining of liver sections (magnification, $\times 100$). Scale bar, 20 μ m. (C) Serum free fatty acid. (D) Serum triglyceride. (E) Glycogen content, results are expressed per gram of liver tissues. (F) Relative mRNA expression of gluconeogenesis-associated genes in liver tissues. (G) Relative mRNA expression of lipogenic genes in liver tissues. mRNA levels were examined by quantitative real-time PCR analysis in liver tissues obtained from all the groups. Results are presented as gene expression levels normalized to the average levels of the mean-centered housekeeping gene β -actin. Data are expressed as means $\pm$ s.d. in each bar graph from 12 to 15 mice per group. * $P < 0.05$ or ** $P < 0.01$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0251>.

mitochondrial biogenesis both in mRNA and protein levels were significantly decreased in obese mice with vehicle treatment. However, CaMKIV injection significantly induced the mRNA and protein expression of these genes in obese mice (Fig. 3A and B). The alteration of these indicators suggested that mitochondrial biogenesis was improved in obese mice with CaMKIV injection. Consistent with these results, the histomorphometric analysis of liver tissues, adipose tissues and skeletal muscle using electron microscopy showed both mitochondrial area and number were significantly increased in obese mice injected with CaMKIV compared with vehicle injection (Fig. 3C, D and E). These results demonstrated the protective effect of CaMKIV on liver is due to elevated peripheral mitochondrial activity.

Injection of CaMKIV involved in chronic inflammatory response

To further investigate alleviation of the hepatosteatotic phenotype, we next analyzed key metabolic and inflammatory myokines in serum and their mRNA and protein expression in mice liver tissues. The results indicated serum MCP-1, Fgf21, TNF- α and IL-6 were significantly increased in obese mice with vehicle injection (Fig. 4A, B, C and D). Nevertheless, the increased serum MCP-1 was markedly suppressed in obese mice with CaMKIV treatment (Fig. 4A). Moreover, except serum TNF- α and IL-6, a decrease in trend was also observed in serum Fgf21 in obese mice

treated with CaMKIV (Fig. 4B), though the changes did not seem to be remarkable compared with the vehicle ($P = 0.057$), implicating the decreased inflammatory response may contribute to CaMKIV effects. In addition, we found the *Mcp-1*, a critical gene which involved in mitochondrial biogenesis, *Fgf21*, *Tnf- α* and *Il-6* mRNA expression were significantly increased in obese mice with vehicle injection, indicating activated inflammatory response which is induced by obesity (Fig. 4E, F, G and H). Remarkably, the results also showed, except *Il-6*, the expression of *Mcp-1*, *Fgf21* and *Tnf- α* markedly decreased after CaMKIV treatment in obese mice. Because CaMKIV regulated the mRNA expression of *Mcp-1*, *Fgf21* and *Tnf- α* , we further analyzed the protein expression of these genes. The results showed MCP-1, Fgf21, and TNF- α protein expression were significantly increased in obese mice treated with vehicle (Supplementary Fig. 1C). CaMKIV injection in obese mice significantly inhibited the expression of MCP-1 and Fgf21 (Supplementary Fig. 1C). These results further demonstrated that the effect of CaMKIV on the improvement of obesity-induced hepatic insulin resistance at least partly by reducing the chronic inflammatory response *in vivo*.

Regulation of CaMKIV in hepatic autophagy and insulin receptor signaling *in vivo*

It has been reported that increased hepatic autophagy improves HFD-induced insulin resistance (Codogno &

Figure 3

Effect of CaMKIV on mitochondrial function *in vivo*. All analyses compared age- and sex-matched mice fed a normal diet (ND) or high-fat diet (HFD) for 12 weeks and injected with CaMKIV (1 μ g/g/day) or vehicle for in last 8 weeks. (A) Relative mRNA expression of mitochondrial function-associated genes in liver tissues. (B) Protein expression of mitochondrial function-associated genes in liver tissues. (C) Representative electron micrographs (40,000 $\times$) of mitochondria in liver tissues, adipose tissues and skeletal muscle. Scale bar, 1 μ m. (D and E) Mitochondria number and mitochondria area in liver tissues, adipose tissues, and skeletal muscle, respectively. mRNA levels were examined by quantitative real-time PCR analysis in liver tissues obtained from all the groups. Results are presented as gene expression levels normalized to the average levels of the mean-centered housekeeping gene β -actin. Data are expressed as means $\pm$ s.d. in each bar graph from 12 to 15 mice per group. * P < 0.05 or ** P < 0.01.

Meijer 2010, Yang *et al.* 2010). To further explore the mechanism by which CaMKIV improves diet-induced insulin resistance, we first analyzed liver protein expression of autophagy by Western blotting. As shown

in Fig. 5A, there was a remarkable inhibition of autophagy as evidenced by downregulation of LC3II and particularly Atg7. Meanwhile, p62 expression in protein and mRNA levels were elevated in liver tissues of obese mice with

Figure 4

Effect of CaMKIV on inflammatory response in mice. All analyses compared age- and sex-matched mice fed a normal diet (ND) or high-fat diet (HFD) for 12 weeks and injected with CaMKIV (1 μ g/g/day) or vehicle in the last 8 weeks. (A) Serum MCP-1 level. (B) Serum Fgf21 level. (C) Serum TNF- α level. (D) Serum IL-6 level. (E) *Mcp-1* expression. (F) *Fgf21* expression. (G) *Tnf- α* expression. (H) *Il-6* expression. mRNA levels were examined by quantitative real-time PCR analysis in liver tissues obtained from all the groups. Results are presented as gene expression levels normalized to the average levels of the mean-centered housekeeping gene β -actin. Data are expressed as means $\pm$ s.d. in each bar graph. * P < 0.05 or ** P < 0.01.

vehicle treatment. However, as a result of CaMKIV injection, obese mice showed an increased Atg7 and LC3II expression (Fig. 5A) and a decreased expression of p62 in protein and mRNA levels compared with vehicle injection (Fig. 5A and B). In addition, long-term injection of CaMKIV in mice showed an increased expression of CaMKIV compared with the vehicle injection (Fig. 5A). We next used lysosomal protease inhibitor chloroquine to evaluate the autophagic flux. Although an increase of LC3II level was readily observable with the treatment of chloroquine, the additional CaMKIV caused a significant difference in the LC3II expression in liver tissues with chloroquine (Fig. 5C). Consistently, the insulin sensitivity was also markedly increased in obese mice with CaMKIV injection compared with vehicle injection as assessed by the phosphorylation of IRS-1 and Akt (Nakatani *et al.* 2005) (Fig. 5D). These results supported a clear tendency toward the relevance of CaMKIV to hepatic insulin signaling and autophagy.

Effect of CaMKIV on autophagy activity and insulin signaling in hepatocytes

To elucidate the effects of CaMKIV on autophagy and insulin sensitivity *in vitro*, we used 100 ng/mL recombinant mouse CaMKIV at different time points (0, 10, 20 and 40 h)

to determine whether overexpression of CaMKIV restores autophagic imbalance and improves insulin signaling on FFA-induced BNL cl.2 hepatocyte models of insulin resistance. As shown in Fig. 6A and C, administered with CaMKIV for 20 h or 40 h caused a time-dependent increase in protein expression of Atg7 and LC3II as well as a reduction in protein and mRNA expression of p62 in the presence of FFA. It also revealed an increased protein expression of CaMKIV in CaMKIV-treated cells (Fig. 6A). Immunofluorescence for endogenous LC3B revealed decreased puncta in obese mice treated with vehicle but increased puncta in CaMKIV (Fig. 6B), demonstrating the restoration of autophagosome formation. Moreover, it has been reported that abnormal autophagy was implicated in the Akt signaling pathway (Xu *et al.* 2012); therefore, we postulated that CaMKIV might affect insulin sensitivity in hepatocytes. As expected, improved insulin receptor signaling was observed by the treatment of CaMKIV for 20 h in hepatocytes, as assessed by phosphorylation of IRS-1 and Akt (Fig. 6D). These results indicated CaMKIV is strongly associated with autophagy activity and insulin receptor signaling at cell level.

To further understand the involvement of CaMKIV in autophagy and insulin signaling *in vitro*, we used siRNA against CaMKIV to determine whether ablation of CaMKIV could decrease autophagy and impair insulin sensitivity. CaMKIV siRNA (CaMKIVsi) was validated by

Figure 5

Effect of CaMKIV on hepatic autophagy and insulin sensitivity *in vivo*. All analyses compared age- and sex-matched mice fed a normal diet (ND) or high-fat diet (HFD) for 12 weeks and injected with CaMKIV (1 µg/g/day) or vehicle for in last 8 weeks. For insulin signaling, animals were injected with 2 IU/kg of insulin. (A) Atg7, p62, LC3II, and CaMKIV expression in liver tissues. (B) p62 expression. (C) Determination of autophagic flux in mice receiving CaMKIV in the absence or presence of chloroquine. Liver tissue lysates were analyzed by Western blot using anti-LC3 antibody. Results are given as fold increase normalized to internal control GAPDH. (D) IRS-1 phosphorylation and Akt phosphorylation in liver tissues. Quantification was performed on three different fields per animals. Data are expressed as means $\pm$ s.d. in each bar graph from 12 to 15 mice per group. IB, immunoblotting; IP, immunoprecipitation. * $P < 0.05$ or ** $P < 0.01$.

Figure 6

Effect of CaMKIV on autophagy and insulin receptor signaling in FFA-treated hepatocytes. Cells were cultured with or without mouse recombinant CaMKIV (100 ng/mL). For inducing insulin resistance, cells were cultured with 500 μ M FFA for 16 h. For insulin signaling, cells were stimulated with 10 nM of insulin for 10 min. Indicators of autophagy and insulin receptor signaling were measured at protein levels. (A) Time course for autophagy indicators and CaMKIV expression in hepatocytes following FFA and/or CaMKIV treatment. (B) Immunofluorescence for endogenous LC3B. Scale bar, 20 μ m. Cells were treated with CaMKIV for 20 h in FFA-induced insulin resistance. (C) p62 expression. (D) Phosphorylation of IRS-1 and Akt in hepatocyte. Data expressed as means $\pm$ s.d. in each bar graph represent the average of three independent experiments. IB, immunoblotting; IP, immunoprecipitation. * P < 0.05 or ** P < 0.01.

the measurement of reduced CaMKIV protein expression in CaMKIVsi-transfected BNL cl.2 cells (Fig. 7A). We next detected the indicators of autophagy and insulin receptor signaling after CaMKIVsi transfected hepatocytes for 48 h. The results showed that autophagy was significantly inhibited as evidenced by the decreased expression of Atg7 and LC3II and induced mRNA and protein expression of p62 in hepatocytes with CaMKIVsi treatment (Fig. 7B and C). Consistent with these results, immunofluorescence of LC3II also suggested decreased number of autophagosome formation in CaMKIV^{-/-} hepatocytes (Fig. 7D). The insulin-simulated phosphorylation of both IRS-1 and Akt was decreased in CaMKIV-knockdown BNL cl.2 hepatocytes (Fig. 7E). These findings demonstrated CaMKIV significantly corrected autophagic imbalance and enhanced insulin sensitivity in hepatocytes.

Effect of CaMKIV on autophagy activity and insulin sensitivity is mediated through phosphorylated CREB in hepatocytes

It has been reported that activated CaMKIV regulates CREB phosphorylation, a director downstream substrate

of CaMKIV, significantly involved in numerous cellular processes (Marie *et al.* 2005, Sato *et al.* 2006, Ding *et al.* 2017). To investigate whether CaMKIV involved in insulin receptor signaling and induced autophagy via phosphorylated CREB, we first observed an induction of CREB phosphorylation and an increased expression of CaMKIV after CaMKIV incubation for 20 h (Supplementary Fig. 2A and B). In contrast, the decreased of p-CREB and reduced CaMKIV expression were observed after CaMKIVsi treatment for 48 h (Supplementary Fig. 2C and D). These results indicated the CaMKIV expression involved in CREB phosphorylation through in hepatocytes. We then sought to explore the molecular mechanism of CaMKIV on metabolic regulation in BNL cl.2 cells. Different strategies (CREB siRNA (CREBsi) or CREB inhibitor 666-15) were used to downregulate CREB in cultured hepatocytes. We first treated cells with FFA to induce insulin resistance. Then the cells were cultured with 100 nM 666-15 or 100 nM CREBsi to block the CREB expression and then add 100 ng/mL CaMKIV into the medium. Consistent with the previous results, an increased Atg7 and LC3II expression and decreased p62 mRNA and protein expression were

Figure 7

Ablation of CaMKIV decreased autophagy and impaired insulin signaling in hepatocytes. Cells were cultured with or without 100 nM CaMKIVsi for 48 h. For insulin signaling, cells were stimulated with 10 nM of insulin for 10 min. Indicators of autophagy and insulin receptor signaling were measured at protein levels. (A) CaMKIVsi was validated by reduction of CaMKIV protein level. (B) Autophagy indicators in hepatocytes. (C) p62 expression. (D) Immunofluorescence for LC3B. Scale bar, 20 μ m. (E) Phosphorylation of IRS-1 and Akt in hepatocytes. Data expressed as means $\pm$ s.d. in each bar graph represent the average of three independent experiments. IB, immunoblotting; IP, immunoprecipitation. * $P < 0.05$ or ** $P < 0.01$.

observed after cells treated with CaMKIV in FFA-induced insulin resistance (Fig. 8A and E). Moreover, CaMKIV also enhanced the IRS-1 and Akt phosphorylation in FFA-treated cells (Fig. 8C). IB analysis showed that insulin induced CREB phosphorylation without altering the total CREB levels and CaMKIV levels, and addition of CaMKIV or CREBsi changed the effect (Fig. 8C). Of note, CaMKIV treatment had no effect on autophagic expression and insulin sensitivity after CREBsi-treated cells. Similar results were observed in 666-15-treated cells, the effect of CaMKIV in induction autophagy (Fig. 8B and F) and improvement of insulin sensitivity were blocked (Fig. 8D). Immunofluorescence for LC3II also demonstrated CaMKIV through p-CREB to regulate autophagy activity in FFA-induced cells (Supplementary Fig. 3). Meantime, our supplemental results further demonstrated that overexpression of CREB enhances the protective effect of CaMKIV on autophagic imbalance and impaired insulin signaling in FFA-induced cell models of insulin resistance (Supplementary Fig. 5). Taken together, these results indicated CREB phosphorylation is required for CaMKIV to reverse autophagic imbalance and improve impaired insulin signaling in FFA-induced insulin resistance models.

Discussion

Understanding the mechanism of CaMKIV in the progress of insulin sensitivity is of both scientific and clinical significance. CaMKIV is a multifunctional enzyme

encoded by the *CaMKIV* gene in human, its function was best understood in neurons, where it inhibits apoptosis and stimulates growth in a calcium- and CREB-dependent manner (Sée *et al.* 2001). Earlier study has shown that CaMKIV was also expressed by hepatocytes, but its role in hepatocytes has not been fully defined (Miyano *et al.* 1992). To our knowledge, this study was the first to investigate the association of exogenous CaMKIV on glucose tolerance, insulin resistance, hepatic autophagy and mitochondrial function in HFD-induced obese mice. To date, all the *in vivo* or *in vitro* studies that examined the effect of CaMKIV on insulin secretion, glucose metabolism, and autophagy activity have been conducted in CaMKIV KO or transgenic mice fed with a normal chow (Evankovich *et al.* 2012, Lee *et al.* 2014). According to these previous works, our data clearly demonstrated that long-term administration of CaMKIV improved obese-induced insulin resistance. However, much remains to be elucidated regarding the mechanism of CaMKIV action before its putative potential as therapeutic target can be realized.

Recent studies have implicated the activity of CaMKIV not only improved insulin-stimulated muscle glucose uptake in muscle, but also improved liver and adipose insulin action via increases in myokines released from skeletal muscle (Lee *et al.* 2014). In the present study, mice developed signs of insulin resistance and defective hepatic autophagy upon long-term HFD treatment, and these effects were ameliorated when administrated with recombinant CaMKIV for 8 weeks

Figure 8

CaMKIV-dependent CREB phosphorylation in hepatocytes. Cells were cultured with 500 μ M FFA for 16 h to induce insulin resistance. For blocking CREB, cells were treated with CREB siRNA (100 nM) for 48 h or 666-15 (100 nM) for 24 h. Then cells were treated with or without 100 ng/mL CaMKIV for 20 h. For insulin signaling, cells were stimulated with 10 nM of insulin for 10 min. Indicators of autophagy and insulin receptor signaling were measured at protein levels. (A and B) Expression autophagy indicators, p-CREB and CaMKIV in hepatocytes. (C and D) Phosphorylation of IRS-1 and Akt. (E and F) p62 expression. Data expressed as means $\pm$ s.e. in each bar graph represent the average of three independent experiments. IB, immunoblotting; IP, immunoprecipitation. * $P < 0.05$ or ** $P < 0.01$.

simultaneously. Consistent with these *in vivo* findings, CaMKIV also effectively increased autophagy and restored insulin sensitivity through phosphorylation of CREB in hepatocytes. Collectively, these results support the notion that CaMKIV functions as a novel opportunity for treatment of obesity and diabetes.

It has been reported that autophagy plays a significant role in regulating insulin sensitivity in obesity and the development of type 2 diabetes. In obese mice, the early inflammatory response in metabolic diseases, such as diabetes and obesity, was recently shown to result from autophagy disorder (Wang *et al.* 2013). Most metabolic inflammasome promotes autophagy, induction of which would be expected to serve as a negative-feedback mechanism that limits ER stress and disease progression in adipose tissue (Zhou & Liu 2010). In contrast, defective autophagy in hepatic showed an increased ER stress and insulin resistance (Yang *et al.* 2010). Although several mechanisms have been implicated in glucose metabolism and insulin action, the role of CaMKIV as a protective process has been unclear. The exact mechanisms CaMKIV increased autophagy are not fully understood but probably

involved in multiple signals. The preliminary study has indicated CaMKIV prevents ubiquitin proteasomal degradation of mTOR by inhibiting GSK3 β activity and FBXW7 recruitment, thereby directly augmenting autophagy in both the M ϕ and the kidney. These CaMKIV-dependent processes lending support to the hypothesis that CaMKIV signaling mediated the autophagic response to sepsis (Zhang *et al.* 2014). More recently, CaMKIV was found to be a protective mechanism in hepatic ischemia-reperfusion (I/R) by activating the CREB-mediated autophagy in hepatocytes (Evankovich *et al.* 2012). Moreover, CaMKIV has been implicated in the progress of some inflammatory diseases. Inhibition of CaMKIV substantially reduced interstitial mononuclear infiltration and subsequent renal fibrosis in a murine model of focal segmental glomerulosclerosis (Ao *et al.* 2013). However, another study has suggested that CaMKIV prevents HMGB1 release through the regulation of HDAC1 *in vivo* during liver ischemic-reperfusion injury and in cultured hepatocytes exposed to hypoxia, these results indicated a protective role of CaMKIV in liver during ischemic stress by counterbalancing the inflammatory signals

(Evankovich *et al.* 2010). Interestingly, our present study demonstrated that injection of CaMKIV *in vivo* suppresses the serum MCP-1 level and inhibits inflammatory cytokines including MCP-1, Fgf21, and TNF- α expression on mRNA and/or protein level, although some of these recoveries did not seem to be the most remarkable. These findings might suggest a tissue-specific pattern of CaMKIV in the regulation of chronic inflammatory response. In our study, because CaMKIV is administered systemically, the observed findings cannot be exclusively contributed to CaMKIV itself. The restored hepatic autophagic function could be a reason for the observation. Therefore, the occurrence of decreased inflammatory cytokines suggests that inhibition of chronic inflammatory response in hepatic is most likely not a direct consequence of CaMKIV, but rather could be associated with the restoration of autophagy.

In another aspect, recent evidence indicated that CREB as a transcription regulator participated in autophagy activation (Settembre & Ballabio 2014). The previous study has identified that downregulation of CREB decreased the expression of autophagy genes and reduced the number of GFP-LC3 puncta in hepatocytes. In addition, overexpression of CREB could reverse the inhibition of autophagy mediated by downregulation of Atg7 (Seok *et al.* 2014). In our study, we also demonstrated that knockdown of CREB resulted in BNL cl.2 hepatocytes autophagic imbalance (Supplementary Fig. 4), indicating a critical role of CREB in mediating CaMKIV function. These results suggested transcriptional regulation by CREB is probably independent of the acutely acting mTOR, is slower and more persistent. CREB should effectively sustain regulation of autophagy for longer period of times. These several mechanisms showed the critical role of CREB in autophagy. In addition, CREB is one of the most important transcription factors that are phosphorylated and activated by CaMKIV which is involved in different cellular processes such as proliferation, differentiation, apoptosis and survival (Berridge *et al.* 2000, Finkbeiner 2000, Shankar & Sakamoto 2004), while no studies have investigated this pathway in hepatic insulin resistance. Therefore, we hypothesized that CaMKIV might improve the impaired hepatic autophagy to increase insulin sensitivity through p-CREB. In this study, we demonstrated p-CREB expression level changed at the presence of insulin, indicating the link between CaMKIV and CREB in liver is present in the insulin-induced insulin receptor and IRS-1 tyrosine phosphorylation. Our results further demonstrated that CaMKIV through p-CREB enhances the autophagy

activity and improves insulin signaling and might act as an antidiabetic modality with potential application in the treatment of type 2 diabetes.

Although our results demonstrated CaMKIV-induced autophagy appeared causal to hepatic insulin resistance, a definitive link could be established if restoration autophagy could rescue the impaired insulin sensitivity. Therefore, our study provided the evidence that reconstitution of Atg7 results in enhanced autophagic activity and improved insulin signaling in hepatocytes (Supplementary Fig. 6). Atg7 not only encodes ubiquitin-activating enzyme (E1)-like enzyme but also has been central for autophagosome formation responsible for both Atg12-Atg5 conjugation and LC3 conversion. Previous study showed that the reduced insulin level in obese mice fails to recover Atg7 deficiency, but the suppression of Atg7 directly results in hepatic insulin resistance in lean mice (Kotoulas *et al.* 2006, Yang *et al.* 2010). It is nevertheless possible that CaMKIV may contribute to the initiation of autophagic machinery, which would further restore organelle dysfunction and metabolic disorder.

Increasing evidence indicated that decreased mitochondrial number and destroyed mitochondrial function was closely associated with human aging and metabolic diseases, such as type 2 diabetes and obesity (Heinonen *et al.* 2017, Kauppi *et al.* 2017, Morio *et al.* 2019). Thus, improvement of mitochondrial function by exercise training, calories restriction, or resveratrol supplementation has been associated with improved metabolic health in humans. Our *in vivo* findings provided the evidence that CaMKIV played a significant role in HFD-induced hepatic mitochondrial dysfunction. In our study, obese mice with CaMKIV treatment showed an improvement of GTT and ITT and a restored hepatic mitochondrial function. The exact mechanism by which CaMKIV stimulated mitochondrial mass remains to be determined, but the recent research has reported that CaMKIV could stimulate *Pgc-1 α* gene expression which was involved in mitochondrial biogenesis in skeletal muscle (Lagouge *et al.* 2006). Earlier studies have reported PGC-1 α as a key regulator of mitochondrial biogenesis was stimulated by CaMKIV expression, and reduced PGC-1 α level seems to be involved in insulin resistance and type 2 diabetes (Wenz 2011). Recent studies also suggested *Pgc-1 α* gene upregulation could balance the production of ATP between mitochondria and nonmitochondria, it could also stimulate the other mitochondria biogenesis-related genes (such as *Nrf1* and *Nrf2* expression), and

coactivate several mitochondrial regulatory factors including *Mef2* and *Ppars* (Czubryt *et al.* 2003, Wenz 2011, 2013). In addition, NRF2 has been implicated in the control of *Tfam* expression which is well known to regulate mtDNA transcription and replication. MFN2 was identified as an important factor to regulate mitochondrial fusion and protect mitochondrial function (Chen *et al.* 2003). Remarkably, our findings showed that CaMKIV upregulated the expression of *Pgc-1 α* , *Sirt1*, *Mfn2*, *Nrf-1* and *Tfam* mRNA expression in liver tissues of obese mice. These results indicated that CaMKIV effectively restored mitochondrial function through upregulation of mitochondrial biogenesis-associated genes. Therefore, the effect of CaMKIV on improvement of insulin sensitivity might have contributed to the restoration of hepatic mitochondrial function.

In conclusion, our study revealed that administration of CaMKIV in obese mice improved hepatic insulin sensitivity and suggested a causal link between CaMKIV, impaired hepatic insulin signaling, and defective hepatic autophagy. Our findings may implicate modulated CaMKIV secretion/action as a promising therapeutic approach in metabolic disorders.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0251>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

L J L, L Y, L S Y and Z X processed the samples, analyzed and prepared the data, and were involved in drafting the article. L J L, Z X Y, Z L, H J T and M H contributed to data interpretation and revised the article. L J L, H Q and G Y 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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