CTSK inhibitor exert its anti-obesity effects through regulating adipocyte differentiation in high-fat diet induced obese mice

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Abstract. Obesity is associated with increased risk of developing numerous adverse health conditions. Cathepsin k (CTSK) is highly expressed in adipose tissues of obese patients and animal models. Although CTSK has been demonstrated to promote adipocyte differentiation in 3T3-L1 cells, the effects of CTSK selective inhibitor (CKSI) on weight gain and insulin resistance have not been well examined. High-fat diet (HFD) induced obese male C57BL/6 mice were fed a diet with or without CKSI for 8 weeks. The HFD induced increase in adipose tissue weight gain, increase in homeostasis model assessment (HOMA) index as well as accumulation of large adipocytes. After CKSI treatment, all these effects were blunted compared with the HFD control group. A study of the mechanism demonstrated a role for CKSI in significantly down-regulating the expression of two key transcription factors, peroxisome proliferators-activated receptor-γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα), which are markers of adipogenic differentiation. These results indicated that the CKSI possesses an anti-obesity effect, possibly involving the inhibition of adipocyte differentiation. CTSK is likely to be a new target of therapeutic intervention for the treatment of obesity.

Key words: Cathepsin k, CKSI, Obesity, Adipocyte differentiation

OBESITY is a major public health problem in both industrialized and developing countries, which increases one’s risk of developing insulin resistance and metabolic syndromes [1]. Within the next five years, the World Health Organization projections indicate approximately 2.3 billion adults will be overweight and more than 700 million adults will be obese. In recent years despite advances in understanding the molecular basis of obesity, anti-obesity drugs lack physiological specificity and have side-effects [2].

At the cellular level, obesity may occur through adipocyte differentiation of precursor cells present in adipose tissue [3]. Adipogenesis has been investigated extensively using 3T3-L1 cells. Adipocyte differentiation is governed by an elaborate network of transcription factors including two major ones, peroxisome proliferators-activated receptor-γ (PPARγ) and CCAAT/Enhancer-Binding Protein α (C/EBPα). PPARγ has been shown to be sufficient to induce growth arrest and trigger the entire program of adipogenesis. C/EBPα could bind and transactivate the promoters of several adipocyte genes, including adipocyte Protein 2 (Ap2), glucose transporter type 4 (GLUT4), leptin and the insulin receptor.

Cathepsin k (CTSK) was initially identified as a papain-like cysteine peptidase expressed in high levels in osteoclasts. Its physiological role is to degrade type I collagen. Several CTK inhibitors have been developed to reduce the excessive bone matrix loss associated with osteoporosis. Besides osteoclasts, CTSK was also expressed in white adipose tissue first reported by Soukas et al. [4]. Our previous study demonstrated that expression of CTSK was increased in visceral adi-
pose tissues (VAT) of obese patients and CTSK protein content in VAT was proportional to patient’s BMI as well [5]. In cultured 3T3-L1 preadipocytes, inhibition of CTSK reduced cell adipogenesis by preventing type I collagen degradation, one important component of extracellular matrixes [6]. These data supported CTSK may play a role in the onset of obesity and inhibit CTSK using CKSI should prevent obesity in vivo. To test this hypothesis, in this study, we investigate the direct effect of CKSI in mice fed on high-fat diet (HFD).

**Materials and Methods**

Animals Male C57BL/6 mice (aged 6 weeks) were purchased from the Slac Laboratory Animal CO. LTD (Shanghai, China). Animal care and all experiments were conducted under the ethical guidelines of the Institutional Animal Care and Use Committee of Shanghai Science and Technology. After one week acclimatization, the normal group of 8 mice was fed with a standard diet (SD), whereas the model group including 40 mice was fed with a HFD (5.2kcal/g, 20% of energy in carbohydrate, 21% in protein, and 59% in fat). After eight weeks, the resulting obese mice were subdivided randomly into five groups consisting of eight mice each: HFD group (injected with phosphate buffered saline), backed to standard diet and was named normal diet group (ND, 3.8kcal/g, 55% of energy in carbohydrate, 21% in protein, and 14% in fat) and three HFD +CKSI groups (injected intraperitoneally with different concentrations of CKSI every other day; 0.1µmol/kg, 1.0µmol/kg and 10µmol/kg respectively). All animals had free access to diets and water under a controlled environment (19-22°C, 30-40% humidity, and 12:12-h light-dark cycle). Mouse body weight and food intake were monitored every two weeks. After 8 weeks, visceral adipose tissue and subcutaneous adipose tissue were harvested and stored at -70°C for further analysis. Blood samples were taken from the retro-orbital sinus to determine the plasma biomarkers.

Reagents CTSK selective inhibitor (CKSI) was purchased from Calbiochem (EMD Millipore). It inhibits preferentially CTSK with $K(i)=22$nmol/L, whereas it inhibits cathepsin S with $K(i)=890$nmol/L, L with $K(i)=340$nmol/L. CKSI solution was prepared using PBS.

Biochemical Analysis After overnight fasting, mice were anesthetized with diethyl ether and sacrificed. Blood samples were drawn from the retro-orbital sinus and allowed to clot at room temperature for 30 min. Serum samples were then collected by centrifugation at 1000×g for 15 min. CTSK, insulin concentrations and blood glucose in serum were assayed using commercial kits (Wuhan EIAab Science Co, China; Linco Research, USA) and an automatic biochemical analyzer (Hitachi 7600-020, Japan’s Hitachi Ltd) separately.

Histology After blood had been drained, subcutaneous and visceral adipose tissue was fixed in 10% neutral formalin solution for 48 h. The tissue was subsequently dehydrated by graded ethanol series (75—100%) and embedded in paraffin wax. The embedded tissue was sectioned, stained with hematoxylin and eosin (H&E). Then it was examined by light microscopy (Olympus Optical, Japan) and photographed.

Western Blot Analysis Adipose tissue was homogenized in ice-cold lysis buffer. Protein concentration was determined using a protein determination kit (Bio-Rad, Hercules, CA, USA). Proteins (50μg) were separated by SDS/PAGE on 10% (w/v) polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Darmstadt, Germany). The membranes were incubated with the primary antibody (PPARγ, sc7273; C/EBPα, sc-61; Actin, sc-8432; Santa Cruz Biotechnology, CA, U.S.A.) and immunoreactive proteins were revealed by enhanced chemiluminescent method of western blotting, ECM Kit (Pierce, IL, USA). High molecular weight calibration kit (Pharmatia Biotech, USA) was used as standards. Electrophoregram densities were analyzed using software for three times.

RNA Preparation and Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Total RNA was extracted by Trizol reagent (Invitrogen Life Technologies, USA) from disrupting adipose tissue. For RT-PCR, first strand cDNA was generated by reverse transcription system (Invitrogen Life Technologies, USA) from total RNA. PCR primers specific to each gene are as follows:

- **C/EBPα(713bp):** 5'-GGGGCATCTTCCGCCAAGCTG-3' 5'-TCACCCAGGAAACCTGGCCTG-3';
- **β-actin(345bp):** 5'-CGGTTCCAGTGCCCCTGAGGC-3' 5'-GGAGGGCCGAGAATGACTGTG-3';
- **PPARγ(397bp):** 5'-CAGAGCATGGTGCCTTCGCTGA T-3' 5'-TCCCCACAGACTCGGCACTCA-3'.

PCR was then performed using Taq DNA polymerase according to the manufacturer’s protocol. PCR products were analyzed by electrophoresis on a 1% agarose gel. Expression levels of C/EBPα and PPARγ were normalized to that of β-actin. Data was analyzed
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Induced obesity (Fig. 1B). Insulin was less effective in lowering glucose level in HFD animals as insulin resistance index (HOMA-IR) was increased 279.3% after 8 weeks, which was significantly higher than normal diet ($p<0.001$, Fig. 1C).

**CKSI treatment prevented HFD-induced body-weight gain and obesity**

Increased CTSK expression in human and mouse adipose tissue could be one of the hallmarks of obesity. To examine the direct participation of CTSK in obesity, mice fed on HFD were treated with various concentrations of CKSI for 8 weeks. Food intake did not significantly differ among the groups (Fig. 2A). As shown in (Fig. 2B), with 0.1-10μmol/kg of CKSI administration, concentrations that inhibited CTSK, but not other major Cathepsins, the increase of body weights was significantly suppressed compared with control group only fed on HFD (CON-HFD). HFD-CKSI mice weighted 45.6±0.548g, 43.8±1.304g, 41.6±0.548g

**Results**

**High-fat diet induced obesity and insulin resistance**

After eight weeks, mice fed on SD weighted 29.8±0.837g, while mice in HFD group weighted 39.2±0.837g. There is no difference about food intake between two groups (Fig. 1A). Compared with mice fed on SD, body weight gain in mice fed on HFD increased by 31.5%, which confirmed the HFD induced obesity (Fig. 1B). Insulin was less effective in lowering glucose level in HFD animals as insulin resistance index (HOMA-IR) was increased 279.3% after 8 weeks, which was significantly higher than normal diet ($p<0.001$, Fig. 1C).

![Fig. 1](image)

**Fig. 1** Body weight and insulin resistance index (HOMA-IR) were induced after feeding with High-fat diet for 8 weeks. (A) There is no difference about food intake between two groups. (B) Time course of body weights over 8 weeks in mice fed a SD and HFD. (C) Comparison of HOMA-IR change of mice fed a SD and HFD. HOMA-IR = [insulin×glucose]/22.5 Data are expressed as the means±S.D. (SD means standard diet group, n=8; HFD means high fat diet group, n=40). **$p<0.01$, significant differences between SD and HFD groups.**
Fig. 2  CKSI administration prevented HFD-induced body-weight gain.
(A) Food intake did not significantly differ among the groups. (B) Time course of body weights over 8 weeks in mice fed with HFD and intraperitoneally injected with PBS (n=8, HFD), CKSI0.1µmol/kg (n=8, HFD CKSI0.1), CKSI1.0µmol/kg (n=8, HFD CKSI1), CKSI10µmol/kg (n=8, HFD CKSI10), and backed to standard diet in one group and was named normal diet group(n=8, ND). (C) Comparison of the body weight change after CKSI administration for 8 weeks. (D) Weights of visceral adipose tissue. (E) Weights of subcutaneous adipose tissue. Results are shown as means±S.D. T test was performed, **p<0.01, significant differences compared to HFD group.

(n=8) respectively, while HFD-CON mice weighted 48.6±1.140g (n=8). Meanwhile, CKSI blocked body weight gain in a dose-dependent manner (from 2.8g to 7.2g) (Fig. 2C). Adipose tissue weight was also examined. As shown in Fig. 2D and E, not only visceral but also subcutaneous adipose tissue displayed significant reduced weight gain as compared with those of HFD control, by 33.1-67.7% and 33.3-83.3% respectively. These data suggested that the lower body weight of the mice treated with CKSI is largely attributed to a reduction in white adipose tissue (WAT) weight gain and therefore the alleviated obesity in CKSI-treated mice is due to the reduced adiposity in the white adipose tissues.

**CKSI treatment improved glucose disposal and insulin sensitivity**

Visceral obesity is often associated with increased serum glucose and insulin level [7]. To evaluate the functional outcome of long-term CKSI supplementation on glucose homeostasis, oral glucose tolerance test
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At baseline, HFD feeding increased the fasting serum CTSK level, as showed in Fig. 4A. CKSI treatment for additional 8 weeks significantly reduced the levels of CTSK in a dose-dependent manner (by 1.424 pg/mL for CKSI0.1, -4.824 pg/mL for CKSI1.0, and -13.344 pg/mL for CKSI10, Fig. 4B) with weight loss. Meanwhile, correlation analysis indicated that levels of CTSK concentrations positively correlated with weight of both visceral adipose tissue (r=0.811, p<0.001, Fig. 4C) and subcutaneous adipose tissue (r=0.780, p<0.001, Fig. 4D).

CKSI treatment decreased adipocyte size

We reasoned that the decrease in adipose tissue mass can be a result of a decrease in adipocyte size. To test this hypothesis, we analyzed hematoxylin/eosin (H&E)-stained histological sections from the epididymal fat pads of HFD and CKSI treated mice. Histological analysis demonstrated that adipocyte size was increased in the HFD group as compared with the

(OGTT) were conducted at the end of 8-weeks’ treatment period. As showed in Fig. 3A, hyperglycemia was observed in HFD fed animals. CKSI treatment improved glucose intolerance in HFD animals 30 minutes after glucose challenge, although no statistical significance was reached. Lower amount of insulin were also detected (Fig. 3B: HFD-CON: 1.419±0.126 vs HFD-CKSI0.1: 0.572±0.193; HFD-CKSI1.0: 0.338±0.198; HFD-CKSI10: 0.253±0.042; p<0.01). Furthermore, CKSI supplementation efficiently reduced the increase of insulin resistance index HOMA induced by HFD (Fig. 3C: HFD-CON: 0.143±0.096 vs HFD-CKSI0.1: 0.050±0.217; HFD-CKSI1.0: 0.005±0.028; HFD-CKSI10: 0.100±0.005), suggesting higher insulin sensitivity.

Fig. 3 CKSI improves glucose disposal and insulin sensitivity. (A) Assessment of glucose metabolism in mice with OGTT. (B) Levels of serum insulin in different groups. (C) Comparison of HOMA-IR change of mice. HOMA-IR= [insulin×glucose]/22.5. Results are shown as means±S.D. T test was performed, *p<0.05, **p<0.01, significant differences compared to HFD group.

We next examined the effect of CKSI on serum CTSK in HFD mice. At baseline, HFD feeding increased the fasting serum CTSK level, as showed in Fig. 4A. CKSI treatment for additional 8 weeks significantly reduced the levels of CTSK in a dose-dependent manner (by 1.424 pg/mL for CKSI0.1, -4.824 pg/mL for CKSI1.0, and -13.344 pg/mL for CKSI10, Fig. 4B) with weight loss. Meanwhile, correlation analysis indicated that levels of CTSK concentrations positively correlated with weight of both visceral adipose tissue (r=0.811, p<0.001, Fig. 4C) and subcutaneous adipose tissue (r=0.780, p<0.001, Fig. 4D).

CKSI treatment decreased the level of serum cathepsin k

Increased CTSK expression in human and mouse adipose tissue could be one of the hallmarks of obesity. We next examined the effect of CKSI on serum CTSK in HFD mice. At baseline, HFD feeding increased the fasting serum CTSK level, as showed in Fig. 4A. CKSI treatment for additional 8 weeks significantly reduced the levels of CTSK in a dose-dependent manner (by 1.424 pg/mL for CKSI0.1, -4.824 pg/mL for CKSI1.0, and -13.344 pg/mL for CKSI10, Fig. 4B) with weight loss. Meanwhile, correlation analysis indicated that levels of CTSK concentrations positively correlated with weight of both visceral adipose tissue (r=0.811, p<0.001, Fig. 4C) and subcutaneous adipose tissue (r=0.780, p<0.001, Fig. 4D).
The effect was significantly detected at 0.1µmol/kg and was maximal at 10µmol/kg. Both the subcutaneous and visceral adipose tissue showed the same trend. These data confirmed the inhibitory effect of CKSI on adipocyte differentiation.

Discussion

In this study, we showed that 8 weeks treatment of mice with CKSI effectively reduced body fat mass and improved insulin resistance. These effects may be mediated by its inhibiting adipogenesis via down-regulating expression of transcription factors PPARγ and C/EBPα. These data, for the first time, underscore the therapeutic potential of CKSI in combating obesity and related disorders.
Fig. 5  Effect of CKSI on adipocyte cell size of epididymal adipose tissue. (A) Hematoxylin and eosin-stained photomicrographs of epididymal adipose sections are shown. The data shown are representative of three independent experiments. (B) Mean adipocyte size was calculated in different groups. Results are shown as means±S.D. T test was performed, *p<0.05, **p<0.01, significant differences compared to HFD group.

Fig. 6  CKSI decreased the expression of adipogenic transcription factors. (A) Total lysates and RNA were obtained from both visceral and subcutaneous adipose tissue in different groups. Protein levels were immunoblotted with PPARγ antibodies and relative PPARγ mRNA level was determined by RT-PCR. (B) Total lysates and RNA were obtained from both visceral and subcutaneous adipose tissue in different groups. Protein levels were immunoblotted with C/EBPα antibodies and relative C/EBPα mRNA level was determined by RT-PCR. Representative autoradiography from three independent experiments was shown.
CTSK is a member of the papain family of cysteine proteases and present in osteoclasts, human ovary, heart, skeletal muscle, lung, placenta, testis, small intestine and colon [10]. Previous research focused on the role of CTSK in the occurrence of osteoporosis. The expression of CTSK mRNA was first reported in WAT by Soukas et al. Chiellini et al. [11] reported that CTSK mRNA expression in WAT of obese mouse models was higher than that of wild types, which highlights its function in the process. In 2008, Min Yang found body weight gain in CTSK −/− mice was reduced after HFD for 16 weeks. Consistent with this, total visceral and subcutaneous fat weight were significantly reduced compared with those of the CTSK+/+ mice. Our previous results indicated that CTSK inhibitor E-64 could block the conversion from preadipocytes into mature adipocytes [5]. Recent studies suggest that cathepsins (CTSS) promotes human preadipocyte differentiation. CKSI was a newly CTSK selective inhibitor and the concentration used in experiments would not have effects on CTSS.

In this study, our data are in line with previous studies. Body weight gain, adipose tissue weight, and HOMA index were significantly lowered in CKSI-treated mice compared to the HFD group, with no change in food intake. Moreover, histological analysis revealed that epididymal adipose tissue of the HFD+CKSI group exhibited fewer large cells. Further studies have shown that CKSI decreased expressions of adipogenic transcription factors PPARγ and C/EBPα in adipose tissue.

To the best of our knowledge, this is the first study indicated that circulating CTSK concentrations were elevated in HFD mice and changes of CTSK concentrations positive correlated to both visceral adipose tissue and subcutaneous adipose tissue. So CTSK can be used as serum marker to follow up the degree of obesity. Most importantly, CTSK levels decreased in a parallel manner to insulin resistance index HOMA which suggested that CTSK was likely to be involved in the pathogenesis of insulin resistance, although there was no difference in glucose circulating levels. An explanation for this finding may involve visceral adipose tissue although exact mechanism was not clear. Min Yang demonstrated that CTSK was one of thio-proteases responsible for insulin receptor β-chain processing and absence of CTSK resulted in accumulation of insulin receptor β-chain [12]. Among the potential candidates, increased GLUT4 expression could contribute to improve insulin effect on glucose utilization.

It has been suggested that CTSK participates in adipogenesis by degrading fibronectin [13]. Recently many groups reported that CTSK readily degrades type I collagen, the major component of the organic bone matrix [14]. Our previous in vitro study found that type I collagen, another extracellular matrix, was an important downstream molecular target of CTSK [6]. The collagenolytic activity of CTSK is detected both on the outside of the helical region of the molecule and at various sites inside the helical region [15]. Type I collagen down-regulates expression of lipogenic proteins and thereby interferes with morphological changes necessary for new gene expression. However, it remains to be determined whether CKSI could effectively prevent type I collagen degradation in adipose tissue and subsequently inhibit the process of adipogenesis.

There are some potential limitations of the current study. First, It is not clear whether the same results would be achieved in hereditary obese mice treated with CKSI. Second, detailed mechanism studies, e.g. the exact roles of PPARγ and C/EBPα in regulating adipocyte differentiation by CKSI, are warranted further pursue. Finally, more accurate techniques, such as high glucose clamp test, should be performed to assess the indicators. CTSK inhibitors are presently under evaluation in clinical trials for the treatment of osteoporosis, osteoarthritis and metastatic bone disease [16]. This will enable the metabolic effects to be more fully explored clinically in humans. CTSK was distributed in lung, thyroid follicular epithelium, and endometrium in addition to adipose tissue. The side effects of CKSI should be studied in further investigation.

In all, our results uncover the novel function of CTSK, which plays important roles in obesity development and the accompanied insulin resistance by regulating adipocyte differentiation. Therefore, CTSK activity may represent a potential therapeutic target for obesity and the adjunct insulin resistance.

Acknowledgement

This work was supported by the grant from the Project of National Natural Science Foundation of China (NSFC; 81200564).

Author Disclosure Summary

All the authors of this paper have nothing to declare.
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