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Cathepsin K Regulates Adipocyte Differentiation: Possible Involvement of Type I Collagen Degradation

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Abstract. We previously found that cathepsin K (CTSK) played an important role in adipocyte differentiation. However, the underlying molecular mechanism is not clear. Through the time window study, it was observed that CTSK activities were required mainly in the early phases of adipogenic process. At the same time, the expression of type I collagen disappeared. However, type I collagen can still be observed during the whole process when the CTSK inhibitor-E64 was added. The mRNA levels of peroxisome proliferator-activated receptor γ (PPAR-γ) and CCAAT/enhancer binding protein α (C/EBP-α) was also declining. These imply that CTSK may play a role in adipogenesis in early differentiation phases and produce an effect at least partly by degrading type I collagen, which may provides a basis for developing novel therapeutic approaches to treat obesity and the diseases associated with it.

Key words: Cathepsin k, Adipocyte differentiation, Type I collagen, PPAR-γ, C/EBP-α, Obesity

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POST-CONFLUENT mouse 3T3-L1 preadipocytes differentiate into adipocytes when treated with the inducing mixture including insulin (Ins), dexamethasone (Dex) and isobutylmethylxanthine (IBMX) [1]. The sequence of the events in the differentiation leads to expression of a number of adipocyte-specific genes in these cells. Transcription factors such as PPAR-γ and C/EBPα play a key role in the complex transcriptional cascade during adipogenesis. Many signals affect adipocyte differentiation in a positive or negative manner, while components involved in cell-cell or cell-matrix interactions are also pivotal in regulating the differentiation process.

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Extracellular matrix (ECM) components are synthesized and degraded during the process of adipocyte differentiation [2]. Matrix metalloproteinases (MMPs), especially MMP-2 and -9, have been shown to be necessary for this event [3]. CTSK was a cysteine protease involved in degradating the ECM of osteoclast, whose mRNA expression has been previously reported in white adipocyte tissue (WAT) from ob/ob animals and wild types by Soukas [4] and Chiellini *et al.* [5]. In our previous study [6], we provided the first evidence that: The CTSK mRNA and protein levels were up-regulated in the WAT of obese patients and there is a significantly positive correlation between CTSK protein level and patient body mass index (BMI). *In vitro* studies, the mRNA level, protein level and enzyme activity of CTSK gradually rose up along with the conversion of preadipocytes to mature adipocytes. Moreover, since CTSK inhibitor has a blockade effect on this process, we concluded that CTSK could be directly involved in regulating adipocyte differentiation.

The aim of the present study is to investigate that it is at what point of its differentiation program that CTSK regulates the differentiating preadipocytes. We

Table 1. PCR Primers

gene	Positive-sense strand	Anti-sense strand	Expected size
Collagen type I	5'ttctcctggtgaagctggtc3'	5'ggaaacctctctcgcctctt 3'	482 bp
Collagen type II	5'gctggtgaagaaggaaaacg3'	5'gaagtccctggaaccctgat3'	550 bp
Collagen type III	5'ctcctggattgaagggtgaa3'	5'ctccatttgcaccaggttct3'	549 bp
Collagen type IV	5'gctgcttttgtggaagtcat3'	5'agtgttgcaaaccctgaaaa3'	396 bp
Entactin	5' atgtgaacctggacgtggat3'	5'gtgtaagagcgtccgtggtt3'	577 bp
Fibronectin	5' agagcacacccgttttcatc3'	5'cctttcacagccatcaaggt3'	550 bp
Tubulin	5'gccctacaattccatcctca3'	5'ctggatggtacgcttggtct3'	478 bp
β-Actin	5' ctgggacgatatggagaaga 3'	5' agaggcatacagggacaaca 3'	202 bp

are also interested in the changes of ECM to elucidate the CTSK action during the differentiation process.

Materials and Methods

3T3-L1 cell culture and inhibitor experiment

3T3-L1 cells (obtained from ATCC No. CL-173) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Life Technologies), 100 units/ml penicillin, and 100 mg/ml streptomycin at 37°C with 5% CO_2. Setting the day of confluence as Day 0, differentiation was induced from Day 2 by adding a hormonal cocktail containing 1.7 μM insulin, 1 μM dexamethasone and 0.5 mM isobutylmethylxanthine. On Day 4 the media was replaced by DMEM supplemented with 1.7 μM insulin only. Typically by Day 10, 95% of preadipocytes had differentiated into adipocytes as determined by lipid accumulation visualized with Oil Red O staining. In inhibitor experiments, cells were induced to differentiate by the method mentioned above, in the presence or absence of E-64 (Sigma, St. Louise, MO, USA). It was dissolved in dimethyl sulfoxide (DMSO) and added freshly as described. The final concentration of DMSO was less than 0.1%.

MTT Assay

The MTT assay was performed according to the method as following. 3T3-L1 preadipocytes were plated into 96-well micro titer plates at a density of 2×10^3 cells/well. After 24 hours, the culture medium was replaced by 200 μL serial dilutions (0.5–5 μm/L) of E64 and the cells were incubated for 72 hours. Culture solutions were then removed and replaced by 90 μL of culture medium without FCS. Ten micro liters of a sterile, filtered MTT solution (5 mg/mL) in phosphate-buffered saline (PBS, pH = 7.4) was added to each well to reach a final concentration of 0.5 mg/mL. After 4 hours, the unreacted dye was removed, and then the insoluble formazan crystals were dissolved in DMSO (100 μL/well) and measured spectrophotometrically in an Elx800 Universal Microplate Reader (Bio-Tek Instuments, Inc, USA) at 570 nm. The cell population was expressed as the absolute optical density (OD) 550 absorbance rates.

Oil Red O staining of cultured 3T3-L1cells

Cells were fixed with 10% buffered neutral formalin, rinsed with PBS, and stained with 1ml of Oil Red O dye (0.25%, 10 min). After washing again with ethanol (60%) and water, cells were counterstained with alum hematoxylin solution (2 min) and mounted (glycerin).

Adipogenesis assay

3T3-L1 cells were cultured to a concentration of 6×10^5 cells per well until 5 days after accumulation of intracellular lipid droplets. Adipogenesis was measured by an adipogenesis assay kit (Calbiochem, San Diego, CA, USA) according to the manufacture's instructions. Oil Red O was quantified at its absorbance of 490 nm (Model 680 microplate reader, Bio-Rad).

Isolation and analysis of RNA

Total RNA was extracted by Trizol reagent (Invitrogen Life Technologies, USA) from 3T3-L1 cells. For RT-PCR, first strand cDNA was generated by reverse transcription system (Invitrogen Life Technologies,

USA) from total RNA. PCR was then performed using Taq DNA polymerase according to the manufacturer's protocol. PCR primers specific to each gene are as follows:

The PCR products were analyzed by electrophoresis on a 1% agarose gel.

Zymographic analysis

3T3-L1 cell monolayers in 12-well plates were rinsed then incubated for 20 hours in 1 ml of serum-free medium. The media were collected and concentrated by centrifugation at 3000 g for 15 min through Centricon YM-30 membranes (Millipore, Bedford, MA, U.S.A.). Aliquots (normalized according to the corresponding initial volume) were electrophoresed under nonreducing conditions on 10% (w/v) polyacrylamide gels containing 0.1% (w/v) gelatin. The gels were washed in 1 × Zymogram Renaturing Buffer for 0.5 h and incubated at 37°C for 16 h in 1 × Zymogram Developing Buffer (50 mM Tris/HCl, pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% (w/v) NaN_2). They were then stained with SimplyBlue^{TM}SafeStain (Invitrogen Life Technologies).

Western blot analysis

For Collagen type I experiments, the cells were washed with PBS and proteins were extracted in 1 ml of PBS/2% (v/v) Nonidet P40. The protein concentration was determined using a protein determination kit (Bio-Rad, Hercules, CA, USA). Proteins (50 μg) were separated by SDS/PAGE on 10% (w/v) polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). The membranes were incubated with an anti- Collagen type I antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and immunoreactive proteins were revealed by Enhanced Chemiluminecent Method (ECM) Kit of Western Blotting (Pierce, IL, USA). To equalize protein loadings, a preliminary protein gel was stained with Coomassie Brilliant Blue R-250 for a visual estimation of the quantity of proteins in the cellular extracts. High molecular weight calibration kit (Pharmatia Biotech, USA) was used as standards. The lytic zones were analysed densitometrically using software for measurements of electrophoregram densities.

Immunohistochemistry study

Cells were grown to confluence on microscope slides, then induced to differentiate in the presence or absence of CTSK inhibitors (E64). At Day 10 of the differentiation process, they were rinsed with cooled PBS and fixed in cooled (30 : 70, v/v) Methanol/acetone mix for 15 min. The cells were rehydrated in PBS for 15 min and treated with 2% (w/v) BSA in PBS for 30 min to block non-specific binding sites. They were then incubated with anti- Collagen type I antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at 22°C, then washed three times with PBS for 5 min. Finally the cells were incubated with fluorescein HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 10 min at 22°C and examined by immunofluorescence microscopy.

Real-time quantitative PCR

Total RNA was reverse transcribed using random hexamers as primers and Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). 10 ng of cDNA was amplified with the ABsolute^{TM}QPCR Mixes (ABgene House Epsom Surrey KT19 9AP, UK) plus gene-specific upstream and downstream primers during 45 cycles on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Each cycle consisted of denaturation at 95°C for 15 s, annealing at 58°C for 60 s. The specific primer sequences were as follows: C/EBP-α forward primer: **gagccgagataaagccaaac**; reverse primer: **cttgaccaag gagctctcag**; PPAR-γ forward primer: **gccagtttcgatc cgtagaa**; reverse primer: **gaggccagcatcgtgtagat**; β-Actin forward primer: **ctgggacgatatggagaaga**; reverse primer: **agaggcatacagggacaaca**. Amplified β-Actin expression was used as standard control to normalize the differences in individual samples. The relative expression of mRNA species was calculated using the comparative CT method for separate tube amplification as described previously [7]. The results are expressed as a normalized ratio.

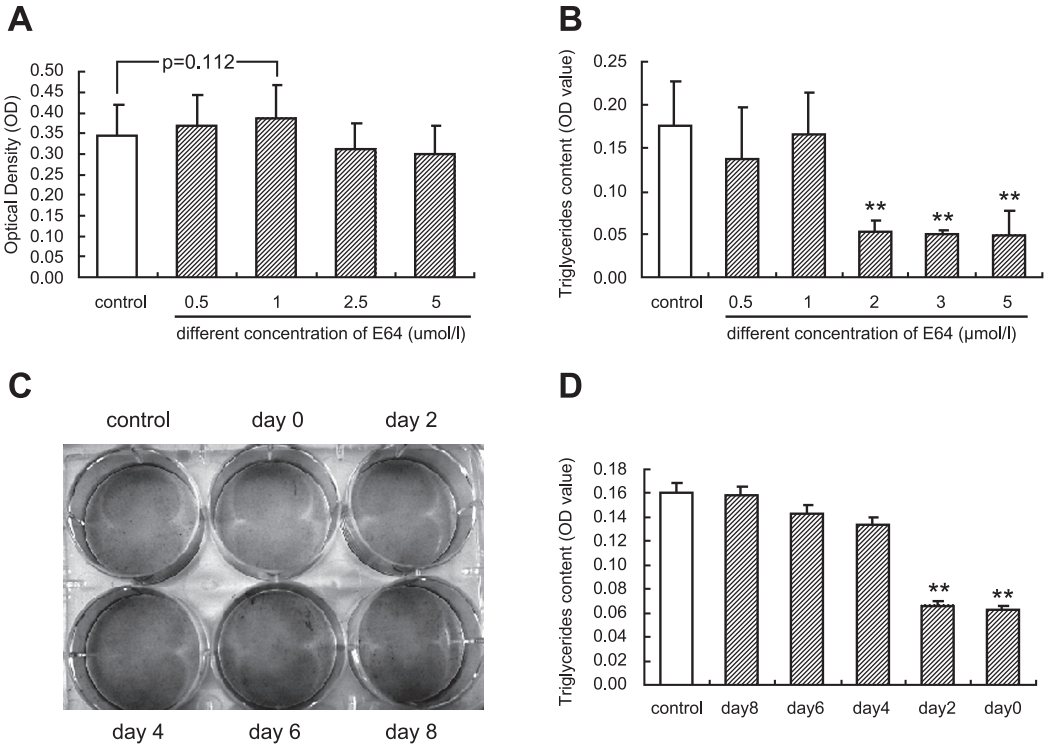

Fig. 1. (A) Effect of CTSK inhibitor E64 activation on the proliferation of 3T3-L1 preadipocytes. Cell number was assessed by the MTT assay. Each column represents the mean ± SE of 24 wells. (B) Effects of different concentration of E64 on accumulation of cytoplasmic triglycerides. Levels of triglycerides were measured by adipogenesis assay kit according to the manufacture's instructions. (C) The time-course effects of CTSK inhibitor E64 treatment on adipocyte differentiation. Cells were treated with 5.0 umol/l E64 at confluence (day 0), at the time of Dex/IBMX addition (day 2, 4) or two (day 6) or four (day 8) days after inducing mixture. In this experiment E64 was added at every medium change. Cultures were fixed and stained with Oil Red O. (D) Effects of different time of 5.0 umol/l E64 on accumulation of cytoplasmic triglycerides. ** <0.01 vs control group (without E64 addition).

Results

CTSK inhibitor blocks adipocyte differentiation in early phases

In our previous study, CTSK activities were directly involved in regulating the adipocyte differentiation. Now we need to find out whether there is a time window in the differentiation program when CTSK activities were involved. First, to determine whether E64 stimulated cell growth or cell death on 3T3-L1 cells, 3T3-L1 cells were incubated with different concentrations of E64 from 0 (vehicle control) to 5.0 μmol/l in DMEM and then were evaluated for cell density using the MTT method. As Fig. 1A shows, the number of 3T3-L1 preadipocyte increased at an E64 concentration of 1.0 μmol/ L,while it decreased when the concentration of E64 went up to 5.0 μmol/L. However, there were no statistical differences between control group and any groups treated with E64 ($P>0.05$). These data illustrate that E64 does not affect 3T3-L1 cell number in any concentration ranged from 0.5 to 5.0 μmol/L. Accumulation of cytoplasmic triglycerides was assessed and the results (Fig. 1B) showed that incubation with E-64 blocked adipocyte conversion in a concentration-dependent manner. Statistical significance was showed when the concentration is no less than 2.0 μmol/l ($P<0.01$).Then Oil Red O staining was used to detect the time window of CTSK. As shown in Fig. 1C, treating the cells with E64 only at Day 0 and 2 of the differentiation protocol (0–2) could block adipocytic conversion as effectively as treating them with E64 during the whole protocol (0–10). E64 had a slight inhibitory effect when treating from Day 4 (Days 4–10). In contrast, the inhibitors had no visible effect when added to the cells during the later differen-

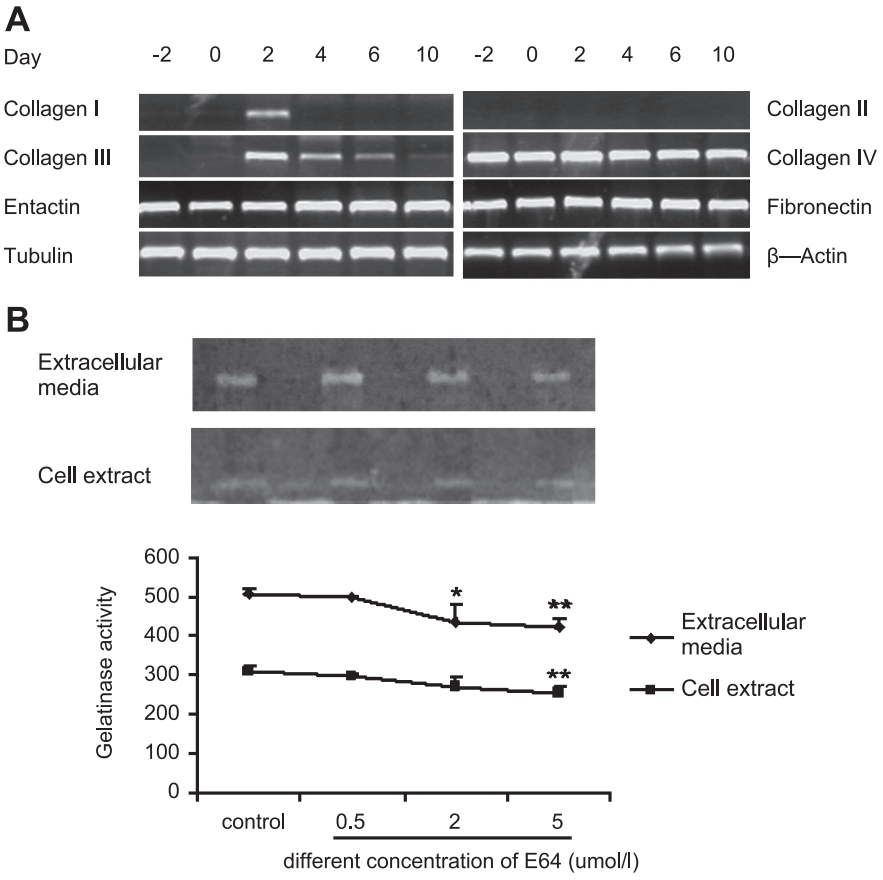

Fig. 2. (A) mRNA expression of extracellular matrix during adipocyte differentiation. Total RNA was harvested at different time and RT-PCR was performed. Similar data were obtained in three different experiments. (B) Gelatinase activities of CTSK were examined by zymography. Cell were washed and maintained in serum-deprived medium supplemented with different concentration of E64 and 0.1% BSA for 24 h.Media and cell extracts were then collected and analyzed in gelatin zymography. A representative Coomassie blue staining of electrophoresis is shown. Densitometric analysis of the lytic area was performed. Values are means ± SE of three independent experiments. * <0.05, ** <0.01 vs control group (without E64 addition).

tiation protocol (Day 6–10, 8–10). It was also demonstrated lipid accumulation was significantly lower when treated with E64 at Day 0 and 2 of the protocol (Fig. 1D). These results suggest that CTSK activities are necessary for adipocytic conversion only in the early phases of the process.

Expression of ECM during adipocytic differentiation of 3T3-L1 cells

During adipocyte differentiation, there are drastic changes in cell morphology and the level and type of ECM components. Changes of ECM during the adipocytic process in 3T3-L1 cells have not been determined. Total RNA was isolated at six different time points (Day –2, 0, 2, 4, 6 and 10), of which cells were not induced at Day –2 and 0, induced at Day 2 and 4, and post differentiated at Day 6 and 10. Type II collagen transcript levels were undetectable during the whole process. Type I, III and IV collagen following a similar pattern were negatively correlated with the adipocytic differentiation, whereas fibronectin and entactin accompanied the adipocyte differentiation process. There were no changes in the expression of tubulin and β-actin. Type III collagen was only detected after induction (Day 2). Parallel results showed that expression of type I collagen was only observed in Day 2 (Fig. 2A).

We are interested in whether CTSK, a cysteine protease, also displays collagenase and gelatinase activities. At Day 2 of the differentiation process, the gelatinase activities were detected in the 3T3-L1 con-

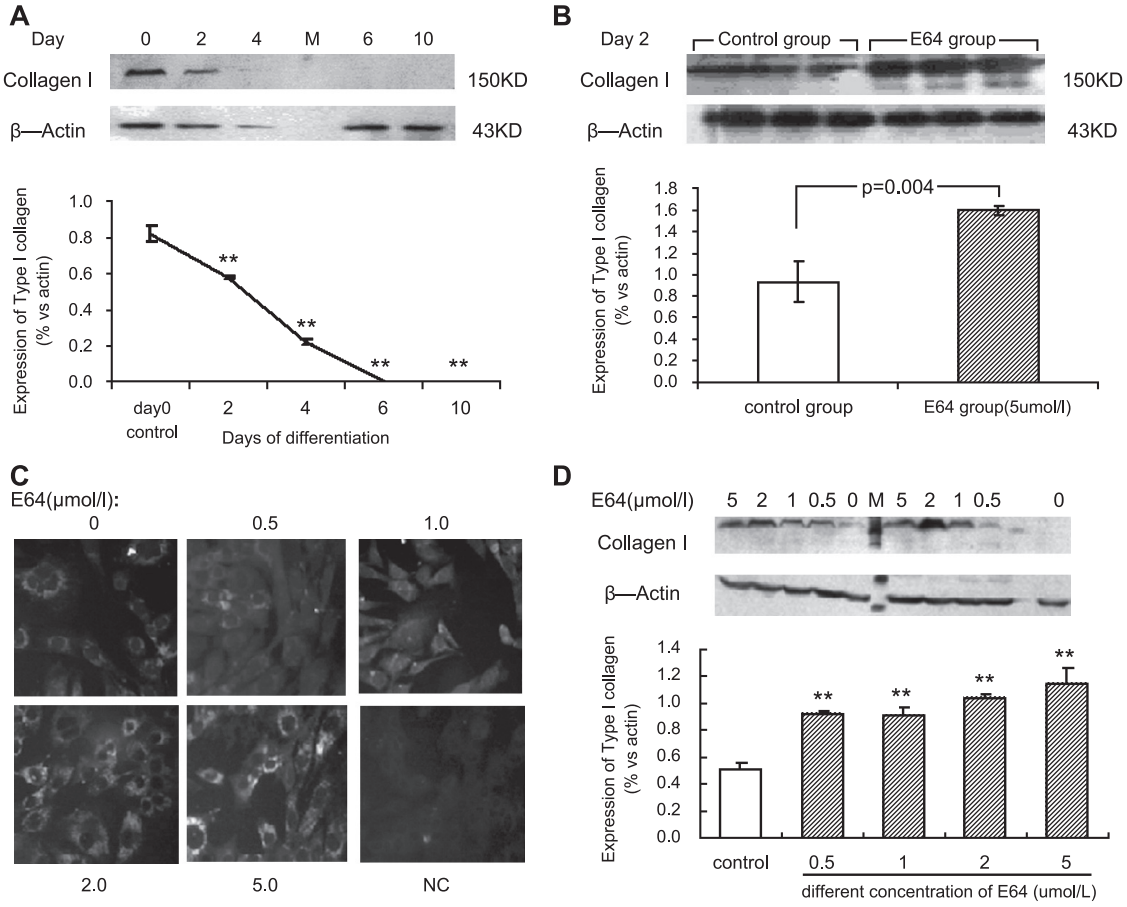

Fig. 3. (A) Expression of type I collagen during 3T3-L1 preadipocyte differentiation. At the indicated time, cells were washed and maintained in serum-deprived medium supplemented with 0.1% BSA for 24 h. Media were then collected, concentrated, and analyzed by Western blot using specific antibodies directed against type I collagen. Representative autoradiograghies from six independent experiments are shown. (B) Effect of CTSK inhibitor on the expression of type I collagen in day 2. Western blot analysis was performed on total protein extracts after 2 days of CTSK inhibitor E64 (5 umol/l) treatment on cells maintained for 24 h in serum-deprived medium. p = 0.004 VS control group. (C–D) Effect of CTSK inhibitor on the expression of type I collagen after 10 days of treatment. Immunocytochemistry and Western blot analysis using an anti-collagen antibody were performed. Representative autoradiographies are shown from three independent experiments. NC, negative control; ** <0.01 vs control group (without E64 addition).

ditioned media and in the cell extract by zymography. Lytic zones in the area confirmed the active enzyme effects of CTSK on gelatine substrate, furthermore the density of lytic zones decreased when cells treated with E64 in a concentration-dependent manner ($P<0.01$ when E64 no less than 2.0 μmol/l). As expected, the gelatinase activities in the cell extracts were much lower than the conditioned media (Fig. 2B).

CTSK changes the expression of type I collagen during adipocyte differentiation

It was shown that expression of type I collagen was decreased during adipocytic differentiation by western-blot. At Day 10, the type I collagen-rich ECM degraded and disappeared when maturing adipocytes are round (Fig. 3A). We next studied the changes of type I collagen level when cells treated with CTSK inhibitor E64 at Day 2. Protein level of type I collagen was elevated after 5 umol/l E64 treatment to 170% compared to control group (Fig. 3B, $P = 0.004$). We wondered whether this effect could be sustained during the dif-

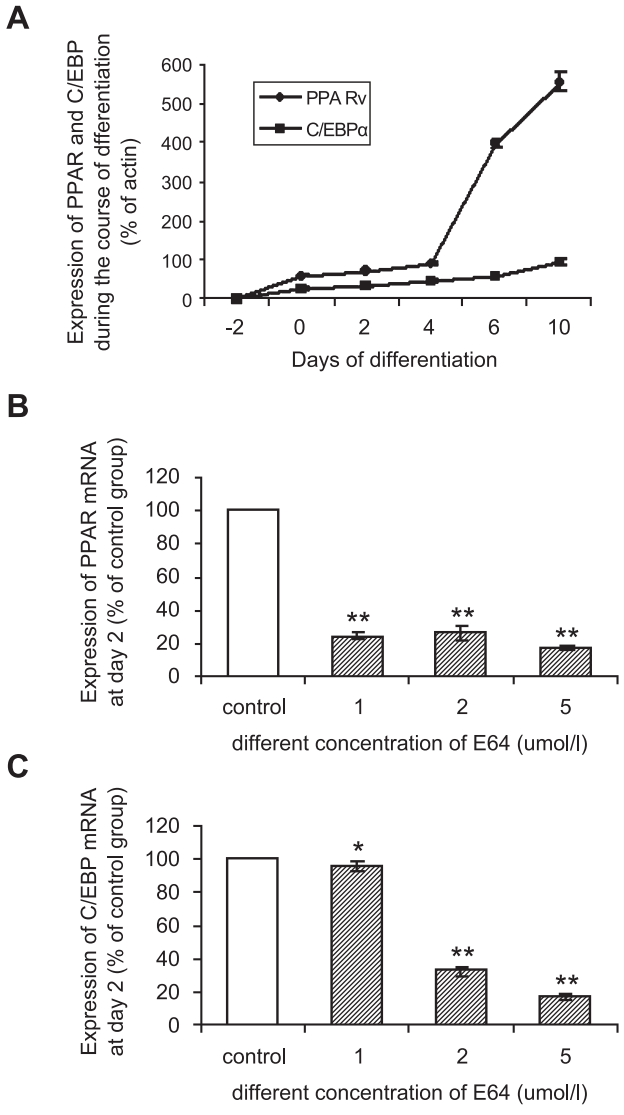

Fig. 4. Effects of CTSK inhibitors E64 on PPARγ and C/EBPαmRNA expression. (A) Preadipocytes were treated in adipogenic medium and Total RNA was harvested at different time and real time-PCR was performed. The relative concentration of PPARγ and C/EBPα mRNA was increased during the differentiation process. (B–C) At day 2, 3T3-L1 preadipocytes were cultured in adipogenic medium in the presence of different concentration of CTSK inhibitor E64 (from 1 to 5 umol/l). Then cells were washed and Real-time PCR was performed. PPARγ and C/EBPα mRNA levels of control group were set as 100%. * <0.05, ** <0.01 vs control group (without E64 addition).

ferentiation process. Then at Day 10 the level of type I collagen was examined with immunocytochemistry. It could still be observed and increased in a concentration-dependent manner (Fig. 3C). The same result was shown in the immunoblotting experiments (Fig. 3D). This suggests that the prevention of type I degradation may be one mechanism through which CTSK inhibitors block adipocytic differentiation.

CTSK inhibitor-induced adipogenic block is associated with reduced expression of PPARγ and C/EBPα

Since C/EBPα and PPARγ were known to be the powerful transcription factors involved in regulating of adipocyte differentiation, whether they involved in CTSK inhibitor-induced adipogenic block was unknown. We measured the levels of transcripts for PPARγ and C/EBPα in vehicle- and E64 treating cells separately by Real-time quantitative PCR. In physiological condition, the levels of PPARγ and C/EBPα mRNA transcripts markedly increased after adipogenic induction (Fig. 4A). In contrast, when cells incubated with E64, the level of PPARγ and C/EBPα transcripts significantly decreased (Fig. 4B–C, $P<0.05$).

Discussion

The present study, for the first time, provides the evidence that CTSK activities present in cell culture medium play a critical role during the initial stages of adipocyte differentiation. Furthermore, the effect of CTSK may be mediated by the type I collagen degradation. As C/EBPα and PPARγ are known to be the powerful transcription factors, our results indicate that CTSK affects the whole process of adipocyte differentiation by reducing the C/EBPα and PPARγ mRNA expression.

Our results indicate that when treating the cells with CTSK inhibitor at initial stages, adipocytic conversion was blocked as effectively as the whole length of the differentiation protocol. However E64 did not inhibit the mitotic clonal expansion preceding differentiation, in accordance with our observation that they do not affect cell proliferation. It suggests CTSK have a critical role in the early steps of adipogenesis. We know that the organization of ECM into a suitable structure is required for adipocyte differentiation. Whether the role of CTSK is related to degradate ECM components, it has never been explored in 3T3-L1 cells. Fortunatly we observed the main change of ECM during the process: type I collagen was only expressed at Day 2 when CTSK activity was mostly required. However,

protein expression of type I collagen gene was increasing until the end of differentiation process while CTSK inhibitor was added. We also observed that there was an important change of type I collagen network after blocking CTSK activity. Collagen is one of the most abundant components of ECM and serves as substratum for cell adhesion, migration, spreading, and anchorage-dependent growth [8, 9]. Type I collagen downregulates gene expression of lipogenic proteins and thereby interferes with morphological changes necessary for new gene expression [10]. Our study demonstrated that adipocyte differentiation was blocked by inhibiting CTSK activity through up-regulating type I collagen expression. 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. Because of this activity, CTSK has been implicated in diseases involving bone and cartilage destruction [11, 12] and the invasive potential of breast and prostate cancer [13]. We also observed changes of other ECM components such as type III, IV collagen, fibronectin and entactin. Whether they act as the substrate of CTSK, futher investigation is needed.

Several classes of transcription factors have been shown to be important in adipocyte conversion. Of these, PPARγ and C/EBPα have been demonstrated as key regulators both in adipocyte differentiation and in maintaining adipocyte phenotype. PPARγ is strongly induced during adipocyte differentiation and activates several adipocyte-specific genes [14]. C/EBPα binds to and transactivates the promoters of many genes that are specifically expressed during adipocyte differentiation [15, 16]. Although these findings revealed that PPAR and C/EBP families are crucial for adipocyte differentiation, it is unlikely that they affect transcriptional expression of type I collagen gene. First, our work showed that type I collagen mRNA significantly decreased within 24 hours whereas PPARγ and C/EBPα were observed at later stage and their expressions reached to the maximum at the terminal stage. The same results were also observed by MacDougald OM and Cowherd RM [17, 18]. Secondly, transient transfection showed that overexpression of PPARγ and its heterodimer partner RXRα actually did not affect the promoter activity of type I collagen gene. Therefore, it would not be governed by these master regulatory proteins promoting the adipocyte differentiation. In contrast, several studies demonstrated that PPARγ and C/EBPα cDNA was under the transcriptional control of type I collagen gene promoter and enhancer sequences [19, 20].

CTSK deficient animals (CTSK–/–) results in reducing the body fat content under conditions with a rapid accumulation of fat storage [21]. This appeared to occur by an increased release and/or utilization of FFA and by an augmented ratio of lipolysis or lipogenesis. In contrast, our study showed that with CTSK inhibitor E64, lipid storage and glycerol release decreased. The difference could be partly explained that the activity of CTSK could facilitate this process in which other proteases also participate since its function may be compensated by others. Metalloproteases (MMP-2 and MMP 9) are present in WAT, where they play a role in adipogenesis [22]. Recent work by Taleb *et al.* [23] shows that the cysteine protease cathepsin S is expressed in WAT where it promotes adipogenesis potentially via degradating fibronectin.

In conclusion, our results showed that CTSK promotes the early step of preadipocyte differentiation. This effect may be mediated by degradating type I collagen. This knowledge provides a basis for understanding the physiological and pathophysiological mechanisms that underlie adipose tissue formation and for the development of novel and sound therapeutic approaches to treat obesity and the diseases related.

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