Caffeic Acid Phenethyl Ester Inhibits Differentiation to Adipocytes in 3T3-L1 Mouse Fibroblasts

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We investigated the inhibitory effect of caffeic acid phenethyl ester (CAPE) on the differentiation of 3T3-L1 mouse fibroblasts to adipocytes. 3T3-L1 cells were differentiated for adipocytes given high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 μg dexamethasone (DEX), 500 μg isobutylmethylxanthine (IBMX), and 5 μg/ml insulin for 7 days. After differentiation, cells were stained with Oil-Red-O to detect oil droplets in adipocytes. Additionally, the cells were lysed and measured for triglyceride contents. Total RNA was isolated from differentiated cells on day 0, 4 and 7. Then, RNA was analyzed using reverse transcription (RT)-polymerase chain reaction (PCR). CAPE dose-dependently suppressed oil droplet accumulation and reduced the droplet size. These findings showed that CAPE at concentrations of 25 to 50 μg/ml could significantly inhibit triglyceride deposition (p<0.05). Treatment of 3T3-L1 with CAPE reduced the mRNA levels of peroxisome proliferator-activated receptor (PPAR) gamma and CCAAT/enhancer-binding protein (C/EBP) alpha. Fatty acid synthase (Fas) and adipocyte-specific fatty acid binding protein (aP2) are known to be associated with lipid metabolism in adipocytes, and both Fas mRNA and aP2 mRNA were significantly suppressed by CAPE treatment. These findings suggested that CAPE suppresses 3T3-L1 differentiation to adipocytes through inhibition of PPARgamma, C/EBPalpha, Fas and aP2 expression.

Key words caffeic acid phenethyl ester; adipocyte differentiation; fatty acid synthase; peroxisome proliferator-activated receptor gamma; CCAAT/enhancer-binding protein alpha; adipocyte-specific fatty acid binding protein

MATERIALS AND METHODS

Materials

3T3-L1 mouse fibroblast cells were obtained from The Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM) and Penicilin Streptomycin mixed solution were obtained from Nacalai Tesque (Kyoto, Japan). High glucose (HG)-DMEM was obtained from GIBCO (NY, U.S.A.). Fetal bovine serum (FBS) was purchased from Hyclone-QB Perbio (Logan, UT, U.S.A.). Insulin and dexamethasone (DEX) were obtained from Wako Pure Chemicals (Osaka, Japan). Dexamethasone (DEX), isobutylmethylxanthine (IBMX), CAPE and Triglyceride E test WAKO were purchased from Wako Pure Chemicals.

The Quant-iT™ dsDNA HS Assay kit was obtained from Invitrogen (CA, U.S.A.). High-Capacity cDNA Reverse Transcription Kits were purchased from Applied Biosystems Japan (Tokyo, Japan). Quick Taq™ HS DyeMix was obtained from TOYOBO (Tokyo, Japan). The BIO-RAD PROTEIN ASSAY was obtained from Bio-Rad Laboratories.

Methods

Cell Culture and Differentiation

3T3-L1 cells were seeded in 6-well plate and grown to confluence at 5% CO2 in DMEM with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Two days after confluence, cells were fed a HG-DMEM supplemented with 10% FBS, 1 μM DEX, 500 μM IBMX, 5 μg/ml insulin for 3 days (Day 0—2). Medium was then changed to a HG-DMEM with 10% FBS, 5 μg/ml insulin with various concentrations (10, 25, 50 μM) of CAPE for 2 days (Day 3—4) in respective cultures. In addition, after two days the medium was changed to only HG-DMEM with or without CAPE for the last 3 days (Day 5—7).
**Oil-Red-O Staining** After differentiation, cells were stained with Oil-Red-O to detect oil droplets in adipocytes. Cells were washed 3 times with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 2 min and then stained with 3.3 mg/ml Oil-Red-O in 75% isopropanol for 30 min. Cells were washed with PBS 3 times and observed under a microscope (Olympus CKX41, Japan). Stained oil droplets in the cells were dissolved containing 4% (v/v) Nonidet-P40 in isopropanol with gentle agitation for 5 min. Supernatant was measured with a spectrophotometer at 500 nm.

**Triglyceride Determination** Differentiated cells were lysed with Lysis buffer (50 mM Tris, 0.15 M NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Tween-20, pH 7.5 with HCl) and measured for triglyceride content on day 4 and 7. The triglyceride content in the cell lysates were quantified using the Triglyceride E test WAKO. The concentration was corrected using DNA as an internal standard. DNA was quantified using a Quant-iT™ dsDNA HS Assay kit.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** To detect mRNA expression levels, total RNA was extracted from differentiated cells on day 0, 4 and 7 with an RNeasy minikit. cDNA was generated from 2 μg of total RNA. RT reaction was performed using a High-Capacity cDNA Reverse Transcription Kit. The resulting cDNA was amplified with Quick Taq™ HS DyeMix. The primers and amplification conditions used are summarized in Table 1. The PCR conditions were as follows: for peroxisome proliferator-activated receptor (PPAR)gamma, adipocyte-specific fatty acid binding protein (p2), fatty acid synthase (Fas) and glyceraldyehde-3-phosphate dehydrogenase (GAPDH), 25 cycles of 94°C for 30 s, 55°C for 30 s, 68°C for 30 s; for CCAAT/enhancer-binding protein (C/EBP)alpha, 30 cycles of 94°C for 30 s, 55°C for 30 s, 68°C for 30 s. The amplified products were separated by electrophoresis on 1.5% agarose gel. The mRNA levels were corrected relative to that of GAPDH mRNA. The imaging analysis of mRNA expression was done using Scion-image (MD, U.S.A.).

**Statistical Analysis** Results in this study are shown as the mean±S.D. of at least three independent experiments. Statistical significance was examined by William’s test or Dunnett’s test for multiple comparisons. A value of \( p < 0.05 \) was considered significant.

**RESULTS**

**Morphology of Inhibition by CAPE for Differentiated 3T3-L1 to Adipocytes** 3T3-L1 differentiation to adipocytes gradually occurred with lipid accumulation by day 4. On day 7, 3T3-L1 cells were differentiated to mature adipocytes which were charged with a quantity of oil droplets. The addition of CAPE during adipocyte differentiation caused a reduction in intracellular lipid accumulation as compared with the control. Staining with Oil-Red-O on day 4 and day 7 cells (Fig. 1A). At day 4 cells began to accumulation of the lipid droplets into cells. At day 7 cells showed CAPE dose-dependently suppressed oil droplet accumulation and reduced the size of the droplets. As for the quantity of intracellular lipids, the treatment of CAPE cells were significantly and dose-dependently suppressed lipid accumulation (Fig. 1B).

**CAPE Inhibited Inter cellular Triglyceride Accumulation with 3T3-L1 Adipogenesis** Following the induction of differentiation, 3T3-L1 cells were treated with or without CAPE to day 7, then harvested of cell lysate and measured the triglyceride content on day 4 and 7. At day 4 differentiated cells started reserve of triglyceride into cells, and the triglyceride content increased on day 7 differentiated cells. The presence of CAPE cells were significantly and dose-dependently suppressed the triglyceride deposition on day 4 and 7 (Fig. 2A). DNA quantity was unaffected by CAPE at day 4 and 7 (Fig. 2B). These findings showed that CAPE at concentrations of 25 to 50 μM could significantly inhibited triglyceride deposition (\( p < 0.05 \)).

**Effects of CAPE for Gene Expression Associated with Adipogenesis in 3T3-L1 Cells** Total RNA was extracted on day 0, 4 and 7. Effects of gene expression on associated with adipogenesis on RT-PCR were investigated.

PPARgamma and C/EBPalpha expression increased over time as the differentiation advanced in control cells. Treatment of 3T3-L1 with CAPE reduced the mRNA levels of PPARgamma. Its expression was suppressed by CAPE in a dose-dependent manner compared to without CAPE cells on days 4 and 7 (Fig. 3A). CAPE treatment also suppressed the expression of C/EBPalpha on day 4 and 7 (Fig. 3B). In CAPE treatment, the increased Fas and p2 mRNA expressions were significantly suppressed the expression of Fas and p2 on day 4 and 7 (Figs. 4A, B).

**DISCUSSION**

We observed that CAPE treatment dose-dependently suppressed oil droplet accumulation, reduced the size of droplets and could significantly inhibited triglyceride deposition. To our knowledge, this study is the first to demonstrate reduced triglyceride deposition after CAPE treatment. These results indicate that CAPE administration may be effective for adipocytes differentiation.

Adipocytes precursor cells exposed to high-glucose DMEM supplemented with 10% FBS, 1 μM DEX, 500 μM IBMX, and 5 μg/ml insulin were able to undergo full maturation into adipocytes. At this time, a major adipocyte marker protein, PPARgamma and C/EBPalpha mRNA were more highly expressed. PPARgamma and C/EBPalpha as the key transcription factors for adipocyte gene activation and differentiation have been pivotal to our understanding of adipogen-

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**Table 1. The Primers Sequence Used for PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer (5‘→3’)</th>
<th>Reverse primer (5‘→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARgamma</td>
<td>TTCAGAAGTGCTGTGTCGTT</td>
<td>TCTTTCTGGTCAAGATCGCC</td>
</tr>
<tr>
<td>C/EBPalpha</td>
<td>ATCCAGAGGCGAGATGTT</td>
<td>AAGTCTTAGCAAGGAAGGC</td>
</tr>
<tr>
<td>Fas</td>
<td>CAGTATAGGCCTGCGCCAA</td>
<td>TAGCCCTCACCAGACTCAC</td>
</tr>
<tr>
<td>apo2</td>
<td>AAATCAGCGAGAGCAGACAG</td>
<td>AAATTTCACTCCAGGCTCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAGGCCAGGTTGTCCTCTG</td>
<td>TGTGAGGGAGATGCTATG</td>
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</tbody>
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PPARgamma induces the transcription of many adipocyte genes encoding proteins and enzymes involved in creating and maintaining the adipocyte phenotype. In 3T3-L1 cells, studies of adipocytes differentiation provide extensive evidence for C/EBPalpha function. Our studies also showed that CAPE inhibits 3T3-L1 adipogenesis by regulating the expression of PPARgamma and C/EBPalpha mRNA. These results indicate the inhibitory effect on the 3T3-L1 differentiation is caused by decreased PPARgamma and C/EBPalpha expression.

CAPE significantly suppressed the up-regulation of PPARgamma and C/EBPalpha in 3T3-L1 differentiation,
suggesting the inhibition of late adipogenic markers, such as Fas and aP2. Fas is relate to the differentiation and lipid ac-
cumulation in 3T3-L1 preadipocytes.\textsuperscript{14)} Furthermore, Fas
plays an important role in lipogenesis in mammals.\textsuperscript{15)} An in-
crease in Fas mRNA expression during 3T3-L1 adipocytes differenciation occurred both due to increase in Fas gene
transcription and stabilization of Fas mRNA.\textsuperscript{16,17)} In this
study, increased expression of Fas induced by adipogenic
cocktails was significantly inhibited by CAPE treatment, im-
plying CAPE blocking of adipocytes differenciation in 3T3-
A. Fas

B. aP2

Fig. 4. Effects of CAPE on Adipocyte-Specific Gene Expression during 3T3-L1 Adipogenesis

Following the induction of differentiation by DEX, IBMX, and Insulin, 3T3-L1 cells were treated with or without CAPE up to day 7. Total RNA was extracted on days 0, 4, and 7, and investigated for effects of mRNA levels of Fas (A) and aP2 (B) using RT-PCR. The levels of mRNA were corrected relative to that of GAPDH mRNA. Relative densitometric units were determined using Scion images. Values given for these relative mRNA levels are the mean±S.D. for three experiments. * Significant at p<0.05 using Williams’ test.

L1 preadipocytes were partly related with down-regulation of Fas expression.

aP2, one of fatty acid binding proteins, is first detected in 3T3-L1 adipocytes through the inhibition of PPARgamma, C/EBPalpha and late adipogenic markers, such as Fas and aP2 expression. This study shows that CAPE has an anti-adipogenic effect and has potential in preventive and therapeutic agents. In a future study, we will further investigate the inhibition by CAPE on the differentiation to adipocytes in vivo.

REFERENCES