Dehydrodiconiferyl Alcohol Isolated from *Cucurbita moschata* Shows Anti-adipogenic and Anti-lipogenic Effects in 3T3-L1 Cells and Primary Mouse Embryonic Fibroblasts

**Background:** A water-soluble extract, prepared from *Cucurbita moschata*, has potent anti-obesity activities.

**Results:** Dehydrodiconiferyl alcohol (DHCA), isolated from the extract, inhibited the mitotic clonal expansion by suppressing the DNA binding activity of C/EBPβ and directly inhibited the expression of the regulators of lipogenesis in 3T3-L1 and primary embryonic fibroblasts.

**Conclusion:** DHCA may contain the anti-adipogenesis as well as anti-lipogenesis.

**Significance:** DHCA might have potential as an anti-obesity therapeutic.

A water-soluble extract from the stems of *Cucurbita moschata*, code named PG105, was previously found to contain strong anti-obesity activities in a high fat diet-induced obesity mouse model. One of its biological characteristics is that it inhibits 3T3-L1 adipocyte differentiation. To isolate the biologically active compound(s), conventional solvent fractionation was performed, and the various fractions were tested for anti-adipogenic activity using Oil Red O staining method. A single spot on thin layer chromatography of the chloroform fraction showed a potent anti-adipogenic activity. When purified, the structure of its major component was resolved as dehydrodiconiferyl alcohol (DHCA), a lignan, by NMR and mass spectrometry analysis. In 3T3-L1 cells, synthesized DHCA significantly reduced the expression of several adipocyte marker genes, including peroxisome proliferator-activated receptor γ (*Pparg*), CCAAT/enhancer-binding protein α (*Cebpα*), fatty acid-binding protein 4 (*Fabp4*), sterol response element-binding protein 1c (*Srebp1c*), and stearoyl-coenzyme A desaturase-1 (*Scd*), and decreased lipid accumulation without affecting cell viability. DHCA also suppressed the mitotic clonal expansion of preadipocytes (an early event of adipogenesis), probably by suppressing the DNA binding activity of C/EBPβ, and lowered the production level of cyclinA and cyclin-dependent kinase 2 (Cdk2), coinciding with the decrease in DNA synthesis and cell division. In addition, DHCA directly inhibited the expression of SREBP-1c and SCD-1. Similar observations were made, using primary mouse embryonic fibroblasts. Taken together, our data indicate that DHCA may contain dual activities, affecting both adipogenesis and lipogenesis.

Obesity, a major health concern of the 21st century, serves as a risk factor for various diseases, including diabetes, hypertension, arthritis, and coronary artery disease (1–4). Obesity can be described as a state of excessive growth of adipose tissue mass. Adipocyte is a major cellular component in adipose tissue, and thus, the mechanism regulating adipocyte size and number has been an important target for obesity research (5–8). Because excessive energy is stored in adipocytes as triglyceride, the expansion of adipocyte size (hypertrophy) is proportional to the amount of intracellular lipid accumulation, and it is dependent on adipocyte lipogenic activity (9, 10). However, adipocytes cannot be enlarged and accumulate lipids indefinitely. Consequently, hypertrophy is an initial event of obesity development, whereas an increase in cell number (hyperplasia) becomes a more influential factor in the chronic state of obesity (1, 9, 10).

Adipocyte hyperplasia depends on adipogenesis, the generation of new adipocytes from precursor cells, and research on the area of adipogenesis has been facilitated by the establishment of the murine preadipocyte cell line 3T3-L1, which can be induced to differentiate into mature adipocytes *in vitro* (11–13). Preadipocyte differentiation program involves several stages (14–16). Upon reaching confluence, proliferative preadipocytes become growth-arrested by contact inhibition (17, 18). When exposed to the differentiation inducer consisting of insulin at a non-physiologically high concentration, dexamethasone (a glucocorticoid) and 1-methyl-3-isobutylxanthine (MIX); a cAMP
phosphodiesterase inhibitor that increases intracellular cAMP concentration) cells re-enter the cell cycle (18–20). After this proliferation stage, referred to as a mitotic clonal expansion, the cells undergo terminal differentiation. Whether mitotic clonal expansion is required for adipocyte differentiation remains controversial (16, 19, 21); however, it is generally accepted that DNA unwinds, and remodeling during the mitotic clonal expansion stage is involved in the initiation of expression of various adipogenic genes (22, 23).

Many reagents have been found or developed to modulate adipogenesis (24, 25). For example, U0126, a MEK inhibitor, decreases the expression of cell cycle markers such as cyclinA and Cdk2, followed by inhibition of mitotic clonal expansion. It also suppresses the expression of major adipogenic genes such as Pparg and Cebpa and subsequently inhibits adipocyte differentiation (10, 19). Roscovitine, a Cdk inhibitor, suppresses DNA replication and cell proliferation, which inhibits mitotic clonal expansion and consequently results in blocking the progression of the adipogenesis program (16, 19). In addition to these agents targeting early event of adipogenesis, anti-adipogenic compounds inhibiting terminal differentiation, a late event of adipogenesis program, have also been reported. The protein-tyrosine phosphatase inhibitor vanadate is known to specifically inhibit terminal differentiation by decreasing expression of adipogenic genes and reducing the accumulation of cytoplasmic triglyceride (26, 27).

Previously, we observed that PG105, a water-soluble extract from the stem parts of Cucurbita moschata, suppresses 3T3-L1 cell differentiation (28). In this study, we show that dehydrodiconiferyl alcohol (DHCA), isolated from the chloroform fraction of PG105, has potent anti-adipogenic and anti-lipogenic activities. DHCA is a type of lignan belonging to the phytoestrogen family (29, 30). Although many lignans have been shown to possess a variety of biological activities and are described as anti-oxidant and anti-fungal compounds (31–33), their working mechanism is largely unknown. In this study, we found that DHCA inhibits adipocyte differentiation and intracellular lipid accumulation in the 3T3-L1 cell culture system and primary mouse embryonic fibroblasts (MEFs); furthermore, it affects the expression of various genes involved in adipogenesis or lipogenesis at the RNA level. DHCA also regulated the production of selective cell cycle markers affecting mitotic clonal expansion by suppressing the DNA binding activity of C/EBPβ. These data suggested that DHCA might have a potential to be developed as a reagent controlling the fat accumulation.

**EXPERIMENTAL PROCEDURES**

**Solvent Extraction**—Dried stems of C. moschata (2 kg) purchased from a farm (Jinju, Korea) were crushed and extracted with boiling water for 3 h three times. It was then concentrated and freeze-dried to obtain PG105. The yield of PG105 from the dried stems was estimated to be 14%. PG105 (200 g) was dissolved in sterile distilled water and extracted with n-hexane, chloroform, ethyl acetate, and n-butyl alcohol in a sequential manner. Each solvent-soluble fraction and the final aqueous residue were filtered, concentrated, freeze-dried, and dissolved in ethanol at a concentration of 100 mg/ml. All preparations were stored at −70 °C until use. The isolation scheme and the yield of each fraction from the dried stems are shown in Fig. 1.

**Isolation and Purification of Active Compound**—Chloroform-soluble fraction was loaded onto a silica gel column (130 g, 3 × 27 cm, Merck), and 11 fractions were eluted with a mixture of n-hexane, chloroform, and methanol (hexane/CHCl3/MeOH, 16:15:1 (v/v/v)). The purity of each fraction was checked by TLC. Two types of solvent systems (n-hexane/CHCl3/MeOH, 10:9:1 (v/v/v), and n-hexane/CHCl3/MeOH, 4:3:1 (v/v/v)) were used. The spots were visualized by exposure to 10% H2SO4 spray. Chloroform fraction number 9 was re-chromatographed using the silica gel column and eluted with a mixture of chloroform and methanol (CHCl3/MeOH, 30:1 (v/v)), obtaining three different fractions. The third fraction with the highest bioactivity was further purified by semi-preparative HPLC using µ-Bondapak C18 column (7.8 × 300 mm, Waters), and its final eluate was named CMC-9. The mobile phase consisted of 40% methanol in distilled water, and its flow rate was 2 ml/min. The purity of the major compound of CMC-9 was established by HPLC using Inertsil ODS-2 column (4.5 × 150 mm, GL Sciences, Torrance, CA). The mobile phase consisted of 20% methyl cyanide in distilled water. The major peak was detected at 15.12 min by a 254-nm UV detector, and its purity was estimated to be 93% by peak area. The structure of active compound was elucidated as dehydrodiconiferyl alcohol by the analyses of NMR, mass, and IR spectral data. The molecular formula was deduced as C20H22O6 by high resolution electron ionization mass data. The 1H and 13C NMR data showed characteristic signals of a lignan skeleton having 2 units of phe-nylpropanoid with two methoxy groups in the aromatic rings. The link between two aromatic rings was confirmed by HMBC spectra.

**Cell Culture and Differentiation of 3T3-L1 Cells**—Cell culture and differentiation method followed the protocol described previously (28). Briefly, 3T3-L1 preadipocytes obtained from American Type Culture Collection (Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) calf serum. Cells in passages 3–9 were used in all studies. To induce differentiation, 2-day post-confluent 3T3-L1 (designated day 0) was incubated in differentiation medium consisting of MDI (5 µg/ml insulin, 1 µM dexamethasone, 0.2 µM MIX), and 10% FBS in DMEM for 2 days. The cells were then incubated in DMEM supplemented with 10% FBS and 1 µg/ml insulin for another 2 days, and the medium was replenished every other day for an additional 4 days. To study the effects of various fractions of C. moschata on adipocyte differentiation, different concentrations of the fractions were added along with the culture media over the entire time of the experiment. Synthetic DHCA was prepared as 1000 stocks in ethanol and added to cells. Troglitazone and SB203580 purchased from Calbiochem were used as a negative and positive controls in various experiments, respectively.

**Preparation and Differentiation of MEFs**—Primary MEFs were prepared and used for cell differentiation experiments as described previously (47). Briefly, embryos at day 14 post-coitum were obtained, at which point the brains and dark red organs were removed. Embryos were then finely minced and
digested with trypsin/EDTA (37 °C) at 250 μl per embryo with
gentle shaking for 30 min. The reaction was stopped by adding
an equal volume of cold PBS with 50% FBS. The solution was
filtered through a Falcon 40-μm cell strainer and then collected
by centrifugation (1500 rpm for 2 min). Cells were washed twice
with culture media ((DMEM) containing 10% FBS (Cellgro))
and then plated with warm media. The medium was changed
3 h later to remove unattached cells. Remaining cells were cul-
tured and frozen for later use. To induce differentiation, 2-day
postconfluent MEFs (designated day 0) were incubated in dif-
erentiation medium containing MDI (5 μg/ml insulin, 1 μM
dexamethasone, 0.5 mM MIX), and 10% FBS in DMEM for 3
days. The cells were then incubated in the same DMEM, but
lacking dexamethasone and MIX, for another 2 days, and the
medium was replenished every other day for an additional 4
days. DHCA was added along with the culture media over the
entire period of differentiation.

Oil Red O Staining—After the induction of differentiation,
cells were washed with phosphate-buffered saline (PBS) and
fixed with 10% formalin in PBS for 1 h, then washed an addi-
tional three times with water, and finally air-dried. Cells were
stained with Oil Red O (6 parts of saturated Oil Red O dye
(0.6%) in isopropyl alcohol and 4 parts of water) for 15 min.
Excess of stain was removed by washing with 70% ethanol,
and stained cells were then washed with water. To quantify the
intracellular lipids, spectrophotometric quantification of the
stain was performed by dissolving the stained lipid droplets
with 4% Nonidet P-40 in isopropyl alcohol for 5 min. The
absorbance of extracted dye was then measured at 520 nm.

LDH Assay—Cytotoxicity of DHCA was evaluated by color-
imetric assay based on the measurement of LDH activity.
Briefly, after various types of cells were treated with DHCA or
Triton X-100 for 2 days, an aliquot of medium was taken and
centrifuged at 2000 rpm for 10 min. Supernatant (100 μl) was
added to the LDH detection reagent (Takara Bio, Shiga, Japan)
to make a total volume of 200 μl. Spectrophotometric analysis
was performed at room temperature (20–24 °C) using an
ELISA microplate reader measuring absorbance at 490 nm.
LDH release value was determined by calculating the average
absorbance value of the triplicates and subtracting from each of
these the absorbance value obtained in the background control
(media only). The equation used for IC50 calculation was as
follows: viability (%) = (1 – (experiment value – background
value))/(Triton X-100 value – background value)) × 100.

RNA Preparation and Northern Blot Analysis—cDNA probes
for mouse PPARγ, C/EBPα, aP2, preadipocyte factor-1 (Pref-1),
SREBP-1c, SCD-1, and 28 S rRNA were prepared by RT-PCR
with 4% Nonidet P-40 in isopropyl alcohol for 5 min. The
stain was performed by dissolving the stained lipid droplets
were washed with 0.1% SDS added in 0.2× SSC at
55 °C for 30 min, and exposed to Reflection NEF 496 film
(PerkinElmer Life Sciences) with an intensifying screen at
−80 °C.

Immunoblocting—After induction of differentiation, cells
were washed with cold PBS, scattered into PBS, and then cen-
trifuged at 2000 rpm at 4 °C for 3 min. The cell pellets were resus-
pended in 250 μl of lysis buffer (25 mM Tris-HCl, pH 7.9, 50 mM
NaCl, 2% Nonidet P-40, 0.2% SDS, 1 mM PMSF, and 0.5 mM
DTT) and centrifuged at 12,000 rpm at 4 °C for 5 min to collect
200 μl of supernatant. Total protein contents in the superna-
tant were determined by Bradford assay. Twenty micrograms of
protein from the supernatant were heated at 100 °C for 3 min
and clarified by centrifugation. Protein was separated by 12%
SDS-PAGE and electrophoretically transferred to nitrocel-
lose membrane. To block nonspecific binding, membrane was
incubated in TBST (150 mM NaCl, 10 mM Tris-HCl, 0.09%
Tween 20, pH 8.0) containing 5% skim milk at room tempera-
ture for 1 h. The blot was then incubated in TBST containing
5% skim milk with primary antibody for cyclinA (1:200, Santa
Cruz Biotechnology, Santa Cruz, CA), Cdk2 (1:2500, BD Bio-
sciences), IGF-1R (1:200, Santa Cruz Biotechnology), phospho-
insulin Rβ (for phospho-IGF-1R; 1:200, Santa Cruz Bio-
technology), phospho-IRS-1 (1:200, Santa Cruz Biotech-
nology), and β-actin (1:5000, Sigma) at room temperature for
2 h or at 4 °C for overnight. After washing three times with TBST for 15 min,
the membrane was incubated in TBST with secondary anti-
body, HRP-conjugated anti-mouse or anti-rabbit IgG (1:1000,
Amersham Biosciences), at room temperature for 1 h. After
thrice washes with TBST, the blot was developed by the
enhanced chemiluminescence detection system (Pierce) and
visualized by exposure to autoradiography film.

Electrophoretic Mobility Shift Assay (EMSA)—The CRE oli-
gonucleotide (Santa Cruz Biotechnology) was radiolabeled with
32P-labeled ATP (25 μCi/ml, PerkinElmer Life Sciences) by
incubating with T4 polynucleotide kinase (Takara Bio) for
10 min at 37 °C. After inactivation for 10 min at 65 °C, a 32P-end-
labeled CRE probe was prepared by removing free isotope using mini Quick Spin Columns (Roche Applied Science). Nuclear proteins were extracted from 3T3-L1 cells using a NucBusterTM extraction kit (Novagen, San Diego), quantified by the Bradford method, and incubated with the radiolabeled CRE probes and reagents composed of an EMSA accessory kit (Novagen) for 30 min at 4 °C, according to the manufacturer’s instructions. The DNA-protein complex was resolved in a 7% native polyacrylamide gel, and the radioactive bands on the gels were visualized by autoradiography film. An unlabeled (cold) CRE probe and a CRE mutant oligonucleotide (Santa Cruz Biotechnology; 100-fold) were used to check the specificity of the probes. Oligomer sequences used to generate the radiolabeled probe and to check the specificity of the probe are as follows: CRE wild type oligonucleotide, 5’-AGAGATTGCGCTGACGT-CAGAGAGCTAG-3’ (underline indicates CRE); CRE mutant type oligonucleotide, 5’-AGAGATTGCGCTGAGTCAGAGCTAG-3’ (boldface indicates mutated bases).

**Confocal Microscopy and Immunofluorescence Assay**—For confocal microscopy, cells were grown on a coverslip. For immunofluorescence assay, cells were cultured in a 24-well microplate using the differentiation medium with or without DHCA for 16 h, fixed using 4% paraformaldehyde in PBS for 20 min at room temperature, and permeabilized by incubating in methanol for 7 min at −20 °C. After washing three times in PBS and blocking in 10% BSA-containing PBS, cells were incubated for 1 h at room temperature with anti-cyclinA antibody (rabbit polyclonal, cross-reacts with mouse cyclinA, 1:100) or anti-C/EBPβ antibody (1:100) diluted in blocking buffer. For detection, FITC-conjugated anti-rabbit IgG or Alexa588-conjugated anti-rabbit IgG (1:200) was diluted in blocking buffer containing 1 ng/ml DAPI (4’, 6-diamidino-2-phenylindole; Molecular Probes, Eugene, OR) or Hoechst (Polyscience, Warrington, PA). For confocal microscopy, cells were incubated with secondary antibody mixture for 1 h at room temperature, washed three times in PBS, and covered with Permount mounting medium (Fisher). Slides were analyzed by confocal microscopy (MRC-1024 Laser Scanning Confocal Image System, Bio-Rad). For immunofluorescence assay, cells were incubated with secondary antibody mixture for 1 h at room temperature, washed three times in PBS, and observed by inverted immunofluorescence microscopy (Axiovert 200 M, Zeiss, Göttingen, Germany).

**Two-dimensional Isoelectric Focusing/SDS-PAGE**—3T3-L1 and primary MEFs were prepared at 0 or 16 h after MDI treatment and lysed in buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) diethiothreitol (DTT), 2% (v/v) Phosphate-lyte, and 1 mM benzamidine. The lysates were subjected to IPG strips (pI 3–10) and isoelectrofocused using the Multiphor II system (Amersham Biosciences). Proteins were then further resolved by 10% SDS-PAGE, and C/EBPβ was detected by using antibody for C/EBPβ.

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIPs were performed using the ChIP-IT™ express kit (Active Motif, Carlsbad, CA). Briefly, 3T3-L1 and primary MEFs were fixed by adding 37% formaldehyde to media and lysed according to the manufacturer’s instructions. Chromatin sheared by sonication were precipitated with anti-C/EBPβ antibody. PCR amplifications were performed with input DNA or precipitated DNA and specific primers encompassing the C/EBP-binding site in the proximal promoter of C/EBPα, PPARγ, and aP2 genes. The amplified products were resolved by electrophoresis in agarose gel. The primer sequences used to amplify the specific fragment of the promoter are as follows: C/EBPα, forward 5’-TCC CTA GTG TTG CCT GGA AG-3’ and reverse 5’-CAG TAG GAT GGT GCC TGC TG-3’; PPARγ, forward 5’-TTG AGA TGT GTG ATT AGG AG-3’ and reverse 5’-AGA CTT GGT ACA TTA CAA GG-3’; and aP2, forward 5’-CCT CCA CAA TGA GGC AAA TC-3’ and reverse 5’-CTG AAG TCC AGA TAG CTC.

**Statistical Analysis**—All values are expressed as means ± S.E., and differences between values were analyzed by unpaired Student’s t test by using SigmaPlot software (version 9.0; SYSTAT Software Inc., Point Richmond, CA). Dose-response relations were evaluated by way of regression analysis. p values less than 0.05, which were calculated as one-tailed p values, were considered to be statistically significant.

**RESULTS**

**Preparation of Anti-adipogenic Fractions from PG105**—We previously showed that PG105, a water-soluble extract from the stems of *C. moschata*, contains strong anti-obesity activities in a high fat diet-induced obesity mouse model (28). One of its biological characteristics is the inhibition of the differentiation of 3T3-L1 cells to mature adipocytes. Using this property, we attempted to identify the biologically active fraction and compound(s) responsible for this activity. Initially, a total water-soluble extract (PG105) was prepared from the dried stems of *C. moschata* and sequentially extracted with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol (Fig. 1). 3T3-L1 cells were treated with 100 μg/ml of one of four obtained fractions, together with MDI, and then the extent of adipocyte differentiation was determined by the Oil Red O staining method. Among four fractions, chloroform fraction showing a maximum activity (Fig. 1) was chosen for further purification.

The chloroform fraction was subjected to the column chromatography using silica gel, and 11 subfractions were eluted by a mixture of *n*-hexane/chloroform/methanol (16:15:1). Each fraction was concentrated by heat evaporation and freeze-drying, dissolved in ethanol, and added to 3T3-L1 cell culture media at the concentration of 25 μg/ml and simultaneously with MDI. After 8 days of culture, the cells were stained with Oil Red O dye to observe the presence of lipid droplets. Chloroform fraction number 9 produced the highest activity among the 11 fractions in terms of the inhibition of adipocyte differentiation (Fig. 2).

**Purification and Identification of DHCA**—Various column fractions were concentrated under reduced pressure, and the homogeneity of each fraction was determined by TLC. Chloroform fraction number 9 showing a single spot on TLC plate (supplemental Fig. S1A) was subjected to silica gel column chromatography again followed by eluting with a mixture of chloroform/methanol (30:1). The third among the obtained three fractions was most effective in inhibiting adipocyte differentiation at a concentration of 2.5 μg/ml (Fig. 2), indicating that anti-adipogenic activities might have been enriched in this frac-
Inhibition of Adipocyte Differentiation by DHCA

Effects of Synthetic DHCA on RNA Levels of Genes Involved in Adipocyte Differentiation—Because adipocyte differentiation accompanies the changes in expression of various adipogenic and lipogenic genes (18, 19, 23, 25), effects of DHCA were examined on the mRNA levels of genes involved in adipocyte differentiation and lipid synthesis in 3T3-L1 cells by Northern blot analysis. Confluently cultured 3T3-L1 preadipocytes were stimulated with MDI and treated with 0.1% ethanol (vehicle control) or three different concentrations of DHCA (0.7, 7, and 70 μM) for 8 days. As shown in Fig. 5A, the mRNA level of PPARγ and C/EBPα was decreased by DHCA treatment. The amount of C/EBPα RNA was decreased by DHCA treatment in a dose-dependent manner and was almost undetectable at 70 μM. The expression of preadipocyte marker, Pref-1, was decreased by MDI stimulation (Fig. 5A, compare lanes 1 and 2) but slightly increased upon treatment with DHCA (Fig. 5A, lanes 3–5). The mRNA level of sterol regulatory element-binding protein-1c (SREBP-1c) and stearoyl-CoA desaturase-1 (SCD-1), the major regulators of lipogenesis, was markedly increased by MDI (Fig. 5A, compare lanes 1 and 2). However, DHCA treatment virtually shut down their gene expression even at the lowest concentration, 0.7 μM (Fig. 5A, lanes 3–5). These data suggested that lipogenic gene expression is more sensitive to DHCA, as compared with adipogenic marker genes expression. These results are consistent with the observation that the accumulation of cytoplasmic triglyceride was prevented by DHCA treatment as shown in Fig. 4.
Inhibition of Adipocyte Differentiation by DHCA

Total RNAs were prepared at various time points as indicated in Fig. 5B. All genes tested, except for Pref-1, were highly activated by MDI, and their RNA levels were significantly increased as the MDI treatment time increased (Fig. 5B, compare lanes 1, 3, 5, 7, and 9), but DHCA effectively suppressed the expression of these genes to the background or an undetectable level at all time points (for example, compare lanes 7 and 8 in Fig. 5B). Pref-1 was the exceptional case; the expression level of this preadipocyte marker gene was already high before MDI stimulation, and its level gradually decreased to an undetectable level on day 11 (Fig. 5B, lane 9). It is interesting to note that the RNA level of Pref-1 was a lot higher at day 11 in DHCA-treated cells as compared with untreated control cells (Fig. 5B, compare lanes 9 and 10), suggesting that this lignan molecule might influence the production or stability of Pref-1 RNA. Whatever the detailed molecular action mechanism is, these results suggested that DHCA exerts suppressive effects on genes involved in adipogenesis.

DHCA also decreased the RNA levels of adipogenic and lipogenic genes in primary MEFs. MEFs were cultured, stimulated with MDI, and treated with various concentrations of DHCA (7, 35, and 70 μM) for 8 days. After differentiation, total RNAs were isolated and examined by Northern blot analysis. As shown in Fig. 5C, the RNA levels of PPARγ, C/EBPα, and SREBP-1c were increased by MDI stimulation, whereas DHCA significantly down-regulated these expression in a dose-dependent manner.

Effects of Synthetic DHCA on Mitotic Clonal Expansion and Expression of Cell Cycle Markers—Adipogenesis is divided into two stages, early and late, each characterized by mitotic clonal expansion and terminal differentiation, respectively (18, 23). To study the working mechanism underlying inhibitory effects of DHCA on adipocyte differentiation as shown above, we examined its influence on mitotic clonal expansion. Cells were stimulated with MDI, simultaneously treated with different concentrations of DHCA, and analyzed for the cell number and DNA replication on days 2 and 4. DHCA suppressed cell division and DNA synthesis in a dose-dependent manner as shown in Fig. 6, A and B. The cell number and the level of 3H-labeled thymidine incorporation were ~2-fold lower in 70 μM DHCA-treated cells than the untreated control, indicating the delayed progression of the
cell cycle. This anti-proliferative effect seems to be specific for MDI-stimulated cells, as DHCA alone, without MDI stimulation, minimally influenced the cell division and DNA synthesis in the cells (Fig. 6, A and B).

To test whether such anti-proliferative effect of DHCA is restricted to 3T3-L1 cells, 3 different cell lines (Raw264.7, HepG2, and ECV304 cells) were treated with diverse concentrations of DHCA, and after 2 and 4 days, the cell number and 3H-labeled thymidine incorporation were measured in the same way. As shown in Fig. 6, C and D, the proliferation rate varied between different cell lines, but at a given cell line, there was no significant difference between DHCA-treated and untreated cells (Fig. 6, C and D). These data suggested that the inhibitory activity of DHCA on the cell cycle might be specific to the differentiated 3T3-L1 preadipocytes.

To confirm the inhibitory effect of DHCA on mitotic clonal expansion at the molecular level, the changes in cell cycle marker gene expression were examined. MDI-stimulated 3T3-L1 cells were cultured in the presence or absence of 70 μM DHCA. Total cell lysates were prepared from the cells every 8 h after MDI stimulation, followed by Western blot analysis using antibodies against cyclinA and Cdk2, which are required for the progression from G1 to S in most cell types (36). As shown in Fig. 7A, the level of cyclinA was increased by MDI stimulation until 24 h, at which point it then decreased (compare lanes 1, 2, 4, 6, and 8). The expression of Cdk2 was also activated as differentiation progressed, reaching a peak at 24–32 h (Fig. 7A, lanes 6 and 8). However, DHCA treatment lowered the level of cyclinA and Cdk2 (for example, compare lanes 6 and 7 in Fig. 7A), indicating that the progression of the 3T3-L1 cell cycle might have been blocked by DHCA treatment.

Similar results were obtained using primary MEFs. MEFs were cultured and stimulated with MDI in the presence or absence of DHCA for 24 h. Total cell lysates were prepared, followed by Western blot analysis, using antibodies to cyclinA and Cdk2. The expression of cyclinA and Cdk2 was up-regulated by MDI, but DHCA significantly lowered their levels, indicating that MDI-mediated proliferation of primary MEFs could also be effectively inhibited by this lignan molecule (Fig. 7C).

These findings are supported by the cells immunostained with antibody to cyclinA at 16 h during the adipocyte differentiation. The signal intensity of cyclinA in DHCA-treated 3T3-L1 cells was
visibly lower than that in untreated cells (Fig. 7B). In DAPI staining analysis of the same cells, no cytotoxic signs were observed such as an apoptotic body in these DAPI-stained nuclei (Fig. 7B). These results suggested that DHCA might inhibit mitotic clonal expansion by regulating the expression of cell cycle marker genes, rather than simply damaging the cells.

**FIGURE 5. Effects of synthetic DHCA on RNA level of adipocyte marker genes.** A, dose-dependent suppression of gene expression involved in adipogenesis or lipogenesis by DHCA treatment. 3T3-L1 cells were cultured in differentiation medium in the absence or presence of DHCA at concentrations of 0.7, 7, and 70 μM. Eight days after treatment, total cellular RNA was extracted, and 20 μg of RNA was used for Northern blot analysis. 28 S rRNA was used as a loading control. B, time course of DHCA effects. 70 μM DHCA was added into differentiation medium at day 0, and total cellular RNA was extracted at appropriate time points followed by Northern blot analysis. Cell viability, as determined by direct cell counting, was unaffected during the whole experimental period. C, effect of DHCA on the RNA level of adipogenic or lipogenic genes in primary MEFs. MEFs were cultured in differentiation medium in the presence of various concentrations of DHCA as indicated (7, 35, and 70 μM). Eight days after treatment, total RNAs were extracted, and 20 μg of RNA was used for Northern blot analysis. GAPDH was used as a loading control.

**FIGURE 6. Effects of synthetic DHCA on mitotic clonal expansion.** A and C, cell number counting. The same number of 3T3-L1 cells was seeded in a 6-well plate and incubated in culture medium or differentiation medium in the presence or absence of DHCA for 4 days (A). The same number of Raw264.7, HepG2, and ECV304 cells were cultured with or without DHCA at the concentrations of 0.7, 7, and 70 μM for 4 days (C). Cell numbers were counted using a trypan blue-staining method on days 2 and 4. B and D, [3H]thymidine incorporation assay. 3T3-L1 (B), Raw264.7, HepG2, and ECV304 (D) cells were cultured in the presence of 1 μCi/ml [3H]thymidine for 4 days. The amount of [3H]thymidine incorporated into the cells was determined with a liquid scintillation counter on days 2 and 4. Relative fold changes were calculated based on the result obtained from the cells incubated in MDI-exclusive culture medium without DHCA treatment.
Inhibition of Adipocyte Differentiation by DHCA

Effects of Synthetic DHCA on Signaling Molecules and Transcription Factors for Adipocyte Differentiation—The inhibitory activity of DHCA on mitotic clonal expansion indicated that this lignan molecule might suppress specific signals involved in the re-entry of growth-arrested preadipocytes into the cell cycle. To identify the underlying molecular mechanism, the effect of DHCA was examined on various proteins responsible for the initiation of the cell cycle during the early stage of adipocyte differentiation. 3T3-L1 cells were stimulated with MDI for 30 min in the absence or presence of DHCA, and the total cell lysates were prepared, followed by Western blot analysis to determine the status of insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor substrate (IRS)-1, which are involved in the initial signaling cascade induced by insulin (37–39). As shown in Fig. 8A, MDI and DHCA did not affect the protein expression of IGF-1R but MDI dramatically increased the phosphorylation of IGF-1R and IRS-1, as compared with that of unstimulated control cells (compare lanes 1 and 2 in Fig. 8A). However, treatment with DHCA did not have any significant effect on MDI-mediated phosphorylation of IGF-1R and IRS-1 (Fig. 8A, compare lanes 2 and 3).

We also investigated the time course of DHCA effect on the status of ERK1 and ERK2, both playing key roles in one of the important downstream cascades under the insulin signaling pathway (40, 41). 3T3-L1 cells were stimulated with MDI, followed by treatment with DHCA, and then total lysates were extracted at various time points and subjected to Western blot analysis with antibodies against ERK1/2 and phosphorylated ERK1/2 (Fig. 8B). The total amount of ERK1/2 was not affected by MDI stimulation as well as by the treatment with DHCA (Fig. 8B). When the culture medium was supplemented with FBS as a negative control, the amount of phosphorylated ERK1/2 was slightly increased at 30 min, but thereafter, it gradually decreased to an undetectable level (Fig. 8B, compare lanes 1, 2, 5, 8, and 11). The phosphorylation was dramatically increased in MDI-treated cells, with a kinetics similar to that observed in negative control cells, and the presence of DHCA had no effect on it (for example, compare lanes 5–7 in Fig. 8B).

Insulin signaling has been also reported to increase the activity of the cAMP-response element-binding protein (CREB) through the PI3K pathway (8, 42). To test whether DHCA affected the DNA binding activity of CREB, 3T3-L1 cells were stimulated with MDI and simultaneously treated with DHCA for 1 h, and nuclear proteins were analyzed by EMSA with CREB-specific binding oligonucleotide. The result showed that the DNA binding activity of CREB was induced by the treatment with MDI (Fig. 8C, compare lanes 1 and 2), and DHCA slightly increased the amount of the specific DNA-protein complex (Fig. 8C, compare lanes 2–4).

Among the downstream targets of CREB, C/EBPβ is one of the critical transcription factors involved in the initiation of mitotic clonal expansion and the induction of the expression of pleiotropic transcription factors, such as C/EBPα and PPARγ (16, 18, 43–45). We investigated the effect of DHCA on the expression of C/EBPβ, using the same cell lysates employed to detect ERK1/2. The level of C/EBPβ protein was increased as the adipocyte differentiation progressed, reaching a maximum level at 3 h (Fig. 8D, compare lanes 1, 3, 6, 9, and 12). However, there was little difference between DHCA-treated and

FIGURE 7. Effects of synthetic DHCA on cell cycle marker expression. A, Western blot analysis of cell cycle-regulating proteins. 3T3-L1 cells were cultured in differentiation medium in the absence or presence of DHCA for 24 h. Total cell lysates were extracted, and 30 μg of protein was subjected to SDS-PAGE and immunoblotted with antibodies against cyclinA, Cdk2, and β-actin. B, confocal microscopic analysis of the cells immunostained with antibodies against the cell cycle marker CyclinA. 3T3-L1 cells were incubated for 24 h. Total cell lysates were extracted, and 30 μg of protein was subjected to SDS-PAGE and immunoblotted with antibodies against cyclinA, Cdk2, or β-actin. The β-actin protein was used as a loading control.
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A

| MDI 70 μM DHCA | - | + | + |
|----------------|---|---|---|
| p-IRS-1        |   |   |   |
| p-IGF-1R       |   |   |   |
| IGF-1R         |   |   |   |

Lane 1 2 3

B

| Hours | 0 | 0.5 | 1 | 3 | 6 |
|-------|---|-----|---|---|---|
| MDI 70 μM DHCA | - | - | + | - | + |
| p-ERK1/2       |   |   |   |   |   |
| ERK1/2         |   |   |   |   |   |

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13

C

| DHCA (μM) | - | 7 | 70 | - |
| Cold competitor | WT | MT |
| CREB       |   |   |   |   |

Lane 1 2 3 4 5 6

D

| Hours | 0 | 0.5 | 1 | 3 | 6 |
|-------|---|-----|---|---|---|
| MDI 70 μM DHCA | - | - | + | - | + |
| C/EBPβ       |   |   |   |   |   |
| β-actin      |   |   |   |   |   |

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13

E

3T3-L1

C/EBPβ Hoechst Merge C/EBPβ Hoechst Merge

NC

MDI

MDI + DHCA

Primary MEF

F

| 5% Input | IP: C/EBPβ |
|----------|------------|
| MDI 70μM DHCA | - | + | + | - | + |
| C/EBPα PPARy 422/aP2 |

Lane 1 2 3 4 5 6

| 5% Input | IP: C/EBPβ |
|----------|------------|
| MDI 70μM DHCA | - | + | + | - | + |
| C/EBPα PPARy 422/aP2 |

Lane 1 2 3 4 5 6

G

3T3-L1

NC MDI MDI + DHCA

Primary MEF

NC MDI MDI MDI + DHCA
untreated control cells (for example, compare lanes 9 and 10 in Fig. 8D).

It had been reported that the expression level of C/EBPβ increases immediately upon the induction of differentiation, whereas its DNA binding activity is observed at later times, for example between 12 and 16 h after MDI stimulation, concurrent with the entry of preadipocytes into S phase at the onset of mitotic clonal expansion (16, 41, 45). To examine whether the DNA binding activity of C/EBPβ was affected by DHCA, 3T3-L1 and primary MEFs were stimulated with MDI in the absence or presence of DHCA for 16 h and then treated with Hoechst stain and antibody against C/EBPβ. In this immunofluorescence assay, the C/EBPβ protein was localized in the nucleus, and in particular, the “punctate” pattern of C/EBPβ was observed when cells were treated with MDI only (Fig. 8E, compare large boxes). The punctate pattern indicates that C/EBPβ has acquired DNA binding activity and become localized to the centromeres (43, 45). In the presence of DHCA, however, the C/EBPβ protein was dispersed in the nucleus (Fig. 8E).

To confirm this result, the ChIP experiment was performed on cell lysates taken for the immunofluorescence assay. The data from these experiments showed that MDI treatment induced the binding of C/EBPβ to chromatin with the C/EBP regulatory element present in the promoters for C/EBPα, PPARγ, and αP2 (Fig. 8F, compare lanes 4 and 5), whereas these interactions were disrupted in DHCA-treated cells (Fig. 8F, compare lanes 5 and 6).

It was previously reported that sequential phosphorylation of C/EBPβ, initially by MAPK (on Thr-188) and later by glycogen synthase kinase 3β (GSK3β) (on Ser-184 or Thr-179), is necessary for its DNA binding activity (41, 46). To verify whether DHCA affects the status of phosphorylated C/EBPβ, cell lysates were prepared in the same way as ChIP assay, subjected to twodimensional gel electrophoresis, and then immunoblotted using anti-C/EBPβ antibody. The results showed that 16 h after MDI treatment, C/EBPβ exhibited lower isoelectric points as compared with that from unstimulated cells (Fig. 8G). However, DHCA treatment highly inhibited MDI-mediated conversion of C/EBPβ to more acidic (pl) forms, resulting in a large amount of the protein with more basic pl values. This result indicated that DHCA blocked the phosphorylation of C/EBPβ, consistent with the data showing the DHCA-mediated decrease in DNA binding activity of C/EBPβ as measured by above for immunofluorescence and ChIP assays.

Taken together, DHCA could suppress the mitotic clonal expansion by blocking the phosphorylation as well as the DNA binding activity of C/EBPβ, resulting in the inhibition of adipogenic differentiation without affecting the expression of C/EBPβ and its upstream signaling pathways.

Effects of Synthetic DHCA Added at Different Times of MDI Treatment—The above data indicated that DHCA blocks adipogenesis by suppressing the clonal expansion of preadipocytes at the early stage of adipogenesis. To test whether the effects of DHCA are restricted to the early stage of adipocyte differentiation, cells were treated with DHCA at various time points during adipogenesis. Seventy μM DHCA was added to the cells, simultaneously with MDI or 1-4 days after MDI stimulation, and the RNA level of various genes was determined by Northern blot analysis. Consistent with the data shown in Fig. 5, simultaneous treatment with MDI and DHCA strongly inhibited the adipogenic gene expression (Fig. 9, lane 2). However, when cells were treated with DHCA 1 day after MDI stimulation, the expression of PPARγ and C/EBPα could not be completely suppressed (Fig. 9, lane 3). Such a reduced effect became clearer when the DHCA treatment time was more delayed (Fig. 9, compare from lane 2 to lane 6). On the contrary, the delayed treatment of DHCA was still effective in decreasing the RNA level of SREBP-1c and SCD-1, even 4 days after MDI stimulation (Fig. 9, lanes 3–6). The data indicated that DHCA could regulate the late stage of adipogenesis by directly inhibiting the expression of lipogenic genes, even after the adipogenic genes were sufficiently induced.

**DISCUSSION**

We previously found that PG105, a water-soluble extract from stem parts of *C. moschata*, not only inhibits the adipocyte
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differentiation in vitro but also reduces the adiposity of high fat diet-fed obese mice (28). In an effort to identify the biologically active compound(s), the conventional solvent fractionation was performed using PG105, and a specific subfraction containing a potent inhibitory effect on adipocyte differentiation was isolated from the chloroform fraction. The structural analysis revealed that a major component of the active fraction is DHCA.

To test whether DHCA actually has an anti-adipogenic activity, DHCA was chemically synthesized, and its biological activities were tested using 3T3-L1 and primary MEFs. DHCA suppressed the adipocyte differentiation and the accumulation of intracellular triglyceride in a dose-dependent manner, coinciding with the decreased expression of major adipogenic transcription factors, PPARγ and C/EBPα, as well as a lipogenic transcription factor, SREBP-1c. These data indicated that DHCA regulates adipocyte differentiation and related biochemical factors by controlling the key transcription factors involved in the pathways.

The mitotic clonal expansion is known to occur prior to the activation of various adipocyte-specific genes (16, 18, 21). Our data indicated that DHCA might suppress mitotic clonal expansion by inhibiting the proliferation and DNA replication of preadipocytes through the regulation of the production and stability of cell cycle markers such as cyclinA and Cdk2. It is noteworthy that this anti-proliferative effect of DHCA was restricted to MDI-stimulated 3T3-L1 cells or primary MEFs because the cell number and DNA synthesis of unstimulated 3T3-L1 cells as well as Raw264.7, ECV304, and HepG2 cells were little affected by DHCA treatment even at the highest concentration of 70 µM. These results suggested that DHCA might interfere with the signals prior to mitotic clonal expansion induced by MDI, which is essential for the initiation of the adipogenic program.

It has previously been reported that C/EBPβ is expressed early in the adipocyte differentiation, and its Thr188 site is immediately phosphorylated by ERK1/2, which is required but is not sufficient for its DNA binding activity. Following a period of lengthy delay, GSK3β translocates from the cytoplasm into the nucleus and additionally phosphorylates Ser184 or Thr179 of C/EBPβ, resulting in the conformational change and the acquisition of DNA binding activity. At this time, dually phosphorylated C/EBPβ becomes localized to C/EBP consensus-binding sites in centromeric satellite DNA, concurrent with synchronous re-entry of preadipocytes into the cell cycle (43, 45). Our data showed that DHCA could inhibit the phosphorylation, DNA binding, and centromeric localization of C/EBPβ. Although it still remains to be clarified how DHCA affects the phosphorylation of C/EBPβ, it is clear that C/EBPβ is a key target molecule of DHCA for its anti-adipogenic activity in 3T3-L1 and primary MEFs.

Preadipocytes that have passed the stage of mitotic clonal expansion are known to enter terminal differentiation and become characteristic mature adipocytes (16, 25, 27). To investigate the roles of DHCA in adipocyte terminal differentiation, 3T3-L1 cells were treated with DHCA after the mitotic clonal expansion was fully initiated. Under this experimental condition, adipocyte differentiation or the expression of PPARγ and C/EBPα was not affected by DHCA, although the expression of SREBP-1c and SCD-1 was effectively suppressed. These data indicated that the observed effects of DHCA on adipogenesis may be the combined results of its actions on the mitotic clonal expansion and lipogenic genes.

DHCA contains the dual functions controlling both adipogenesis and lipogenesis by regulating key transcription factors. Taken together, our data indicate that DHCA might be a useful and safe reagent that can control the adipose tissue mass, and thus have potential as an anti-obesity therapeutic.

REFERENCES
1. Deedwania, P. C., and Gupta, R. (2006) Management issues in the metabolic syndrome. J. Assoc. Physicians India 54, 797–810
2. Friedman, J. M. (2000) Obesity in the new millennium. Nature 404, 632–634
3. Kopelman, P. G. (2000) Obesity as a medical problem. Nature 404, 635–643
4. Byers, T., and Sedjo, R. L. (2007) Public health response to the obesity epidemic. Too soon or too late? J. Nutr. 137, 488–492
5. Hirsch, J., and Han, P. W. (1969) Cellularity of rat adipose tissue. Effects of growth, starvation, and obesity. J. Lipid Res. 10, 77–82
6. Greenwood, M. R., and Johnson, P. R. (1977) Adipose tissue cellularity and its relationship to the development of obesity in females. Curr. Concepts Nutr. 5, 119–135
7. Heine, P. A., Taylor, J. A., Iwamoto, G. A., Lubahn, D. B., and Cooke, P. S. (2000) Increased adipose tissue in male and female estrogen receptor-α knockout mice. Proc. Natl. Acad. Sci. U.S.A. 97, 12729–12734
8. Cook, A., and Cowan, C. (2008) Adipose. StemBook. http://www.stembook.org/node/561
9. Knittle, J. L., Timmers, K., Ginsberg-Fellner, F., Brown, R. E., and Katz, D. P. (1979) The growth of adipose tissue in children and adolescents. Cross-sectional and longitudinal studies of adipose cell number and size. J. Clin. Invest. 63, 239–246
10. Liu, K., Guan, Y., MacNicol, M. C., MacNicol, A. M., and McGeehe, R. E., Jr. (2002) Early expression of p107 is associated with 3T3-L1 adipocyte differentiation. Mol. Cell. Endocrinol. 194, 51–61
11. Shepherd, P. R., Gnudi, L., Tozzo, E., Yang, H., Leach, F., and Kahn, B. B. (1993) Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue. J. Biol. Chem. 268, 22243–22246
12. Gnudi, L., Shepherd, P. R., and Kahn, B. B. (1996) Overexpression of GLUT4 selectively in adipose tissue in transgenic mice. Implications for nutrient partitioning. Proc. Nutr. Soc. 55, 191–199
13. Wolins, N. E., Quaynor, B. K., Skinner, J. R., Tzekov, A., Park, C., Choi, K., and Bickel, P. E. (2006) OP9 mouse stromal cells rapidly differentiate into adipocytes. Characterization of a useful new model of adipogenesis. J. Lipid Res. 47, 450–460
14. Dani, C. (1999) Embryonic stem cell-derived adipogenesis. Cells Tissues Organs 165, 173–180
15. Soukas, A., Socci, N. D., Saatkamp, B. D., Novelli, S., and Friedman, J. M. (2001) Distinct transcriptional profiles of adipogenesis in vivo and in vitro. J. Biol. Chem. 276, 34167–34174
16. Tang, Q. Q., Otto, T. C., and Lane, M. D. (2003) Mitotic clonal expansion. A synchronous process required for adipogenesis. Proc. Natl. Acad. Sci. U.S.A. 100, 44–49
17. Greigore, F. M. (2001) Adipocyte differentiation. From fibroblast to endocrine cell. Exp. Biol. Med. 226, 997–1002
18. Fajas, L. (2003) Adipogenesis. A cross-talk between cell proliferation and cell differentiation. Annu. Med. 35, 79–85
19. Otto, T. C., and Lane, M. D. (2005) Adipocyte development. From stem cell to adipocyte. Crit. Rev. Biochem. Mol. Biol. 40, 229–242
20. Tchoukalova, Y. D., Hausman, D. B., Dean, R. G., and Hausman, G. J. (2000) Enhancing effect of troglitazone on porcine adipocyte differentiation in primary culture: a comparison with dexamethasone. Obes. Res. 8, 664–672
Inhibition of Adipocyte Differentiation by DHCA

21. Qiu, Z., Wei, Y., Chen, N., Jiang, M., Wu, J., and Liao, K. (2001) DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 preadipocyte differentiation into adipocytes. J. Biol. Chem. 276, 11988–11995

22. Liu, J., DeYoung, S. M., Zhang, M., Zhang, M., Cheng, A., and Saltiel, A. R. (2005) Changes in integrin expression during adipocyte differentiation. Cell Metab. 2, 165–177

23. Nambi, J. M., and Young-Cheul, K. (2000) Adipocyte differentiation and gene expression. J. Nutr. 130, 31225–31265

24. Harp, J. B. (2004) New insights into inhibitors of adipogenesis. Curr. Opin. Lipidol. 15, 303–307

25. Koutnikova, H., and Auwerx, J. (2001) Regulation of adipocyte differentiation. Ann. Med. 33, 556–561

26. Jin, S., Zhai, B., Qiu, Z., Wu, J., Lane, M. D., and Liao, K. (2000) c-Crk, a substrate of the insulin-like growth factor-1 receptor tyrosine kinase, functions as an early signal mediator in the adipocyte differentiation process. J. Biol. Chem. 275, 34344–34352

27. Yeh, W. C., Cao, Z., Classon, M., and McKnight, S. L. (1995) Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev. 9, 168–181

28. Choi, H., Eo, H., Park, K., Jin, M., Park, F. I., Kim, S. H., Park, J. E., and Kim, S. (2007) A water-soluble extract from Cucurbita moschata shows anti-obesity effects by controlling lipid metabolism in a high fat diet-induced obesity mouse model. Biochem. Biophys. Res. Commun. 359, 419–425

29. Fuhrstenberg, K. (1965) Lignin. Its constitution and formation from p-hydroxycinnamyl alcohols. Lignin is duplicated by dehydrogenation of these alcohols; intermediates explain formation and structure. Science 148, 595–600

30. Lewis, N. G., and Yamamoto, M. (1995) Towards the specification of consecutive steps in biodegradation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 455–496

31. Barceló, A. R., Pomar, F., López-Serrano, M., and Pedreño, M. A. (2003) Peroxidase: a multifunctional enzyme in grapevines. Funct. Plant Biol. 30, 577–591

32. Gang, D. R., Costa, M. A., Fujita, M., Dinkova-Kostova, A. T., Wang, H. B., Burlat, V., Martin, W., Sarkanen, S., Davin, L. B., and Lewis, N. G. (1999) Regiochemical control of monolignol radical coupling. A new paradigm for lignin and lignan biosynthesis. Chem. Biol. 6, 143–151

33. Nose, M., Bernards, M. A., Furlan, M., Zajicek, J., Eberhardt, T. L., and Lewis, N. G. (1995) Towards the specification of consecutive steps in macromolecular lignin assembly. Phytochemistry 39, 71–79

34. Shimano, H., Horton, I. D., Shimomura, I., Hammer, R. E., Brown, M. S., and Goldstein, J. L. (1997) Isoform 1c of sterol regulatory element-binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. J. Clin. Invest. 99, 846–854

35. Hu, K., and Jeong, J. H. (2006) A convenient synthesis of an anti-Helicobacter pylori agent, dehydrodiconiferyl alcohol. Arch. Pharm. Res. 29, 563–565

36. Sherr, C. J., and Roberts, J. M. (1999) CDK inhibitors. Positive and negative regulators of G1-phase progression. Genes Dev. 13, 1501–1512

37. Smith, P. J., Wise, L. S., Berkowitz, R., Wan, C., and Rubin, C. S. (1988) Insulin-like growth factor-1 is an essential regulator of the differentiation of 3T3-L1 adipocytes. J. Biol. Chem. 263, 9402–9408

38. MacDougald, O. A., and Lane, M. D. (1995) Transcriptional regulation of gene expression during adipocyte differentiation. Annu. Rev. Biochem. 64, 345–373

39. Dupont, J., Khan, J., Qu, B. H., Metzler, P., Helman, L., and LeRoith, D. (2001) Insulin and IGF-1 induce different patterns of gene expression in mouse fibroblast NIH-3T3 cells. Identification by cDNA microarray analysis. Endocrinology 142, 4969–4975

40. Auld, C. A., Caccita, C. D., and Morrison, R. F. (2007) Hormonal induction of adipogenesis induces Skp2 expression through PI3K and MAPK pathways. J. Cell. Biochem. 100, 204–216

41. Tang, Q. Q., Grebner, M., Huang, H., Kim, J. W., Otto, T. C., Pandey, A., and Lane, M. D. (2005) Sequential phosphorylation of CCAAT enhancer-binding protein β by MAPK and glycogen synthase kinase 3β is required for adipogenesis. Proc. Natl. Acad. Sci. U.S.A. 102, 9766–9771

42. Alberts, A. S., Montminy, M., Shenolikar, S., and Feramisco, J. R. (1994) Expression of a peptide inhibitor of protein phosphatase 1 increases phosphorylation and activity of CREB in NIH 3T3 fibroblasts. Mol. Cell. Biol. 14, 4398–4407

43. Lee, H., Lee, Y. J., Choi, H., Ko, E. H., and Kim, J. W. (2009) Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. J. Biol. Chem. 284, 10601–10609

44. Lane, M. D., Tang, Q. Q., and Jiang, M. S. (1999) Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. Biochem. Biophys. Res. Commun. 266, 677–683

45. Tang, Q. Q., and Lane, M. D. (1999) Activation and centromeric localization of CCAAT/enhancer–binding proteins during the mitotic clonal expansion of adipocyte differentiation. Genes Dev. 13, 2231–2241

46. Kim, J. W., Tang, Q. Q., Li, X., and Lane, M. D. (2007) Effect of phosphorylation and S–S bond-induced dimerization on DNA binding and transcriptional activation by C/EBPβ. Proc. Natl. Acad. Sci. U.S.A. 104, 1800–1804

47. Teng, R., Gavrilova, O., Suzuki, N., Chanturiya, T., Schimel, D., Hugendubler, L., Lammen, S., Yver, D. R., Cushman, S. W., Mueller, E., Yamamoto, M., Hsu, L. L., and Noguchi, C. T. (2011) Disrupted erythropeoitin signalling promotes obesity and alters hypothalamic proopiomelanocortin production. Nat. Commun. 2, 520