Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 21 (2010) 1170–1177
Journal of Nutritional Biochemistry

An active extract of *Lindera obtusiloba* inhibits adipogenesis via sustained Wnt signaling and exerts anti-inflammatory effects in the 3T3-L1 preadipocytes

Christian Freise[a], Ulrike Erben[a], Ulf Neuman[b], Kiyoung Kim[c], Martin Zeitz[a], Rajan Somasundaram[a], Martin Ruehl[a,*]

[a]*Department of Gastroenterology, Infectiology and Rheumatology, Campus Benjamin Franklin, Charité-Universitätsmedizin Berlin, 12200 Berlin, Germany*
[b]*Department of General, Visceral and Transplantation Surgery, Campus Virchow Klinikum, Charité-Universitätsmedizin Berlin, 13353 Berlin, Germany*
[c]*Faculty of Beauty Design, Human Environmental Science College, Wonkwang University, 570-749 Iksan City, South Korea*

Received 27 May 2009; received in revised form 23 September 2009; accepted 24 September 2009

Abstract

Obesity, the related metabolic syndrome and associated liver diseases represent an epidemic problem and demand for effective therapeutic strategies. In this regard, natural compounds derived from Oriental medicine such as green tea polyphenols influencing adipogenesis attract growing attention. In Korea, an aqueous extract from the Japanese spice bush *Lindera obtusiloba* is traditionally used for treatment of inflammation and prevention of liver damage. We here investigated effects of the *L. obtusiloba* extract on cell growth, apoptosis, Wnt signaling and differentiation of (im)mature adipocytes using 3T3-L1, an established cell line for studying adipogenesis. *L. obtusiloba* extract reduced the de novo DNA synthesis of 3T3-L1 preadipocytes in a concentration dependent manner with an IC_{50} of ~135 μg/ml paralleled by induction of caspase 3/7 mediated apoptosis. Hormone-induced 3T3 L1 differentiation in the presence of *L. obtusiloba* extract resulted in a reduced accumulation of intracellular lipid droplets by 70%, in down-regulated expression of the adipogenesis-associated proteins glucose transporter-4 and vascular endothelial growth factor, in reduced secretion of the proadipogenic matrix metalloproteinase-2, and in dampened phosphorylation of the Wnt pathway effector protein β-catenin with subsequent diminished expression of the peroxisome proliferator-activated receptor-γ. Treatment of mature adipocytes with *L. obtusiloba* extract also significantly reduced intracellular lipid droplets. In addition to this strong interference of *L. obtusiloba* extract with adipogenesis, *L. obtusiloba* extract exerted anti-inflammatory effects. *L. obtusiloba* extract significantly attenuated lipopolysaccharide- and tumor necrosis factor α-induced secretion of IL-6 by preadipocytes, thus influencing insulin resistance and inflammatory state characterizing obesity. In conclusion, extracts of *L. obtusiloba* should be evaluated as a potential complementary treatment option for obesity associated with the metabolic syndrome.

Keywords: *Lindera obtusiloba*; Adipogenesis; Inflammation; 3T3 L1

1. Introduction

In recent years, obesity developed into a pandemic problem with a continuously rising incidence [1]. Overweight and obesity are associated with major health risks such as cardiovascular disease, diabetes, non-alcoholic fatty liver disease and cancer. Adipocyte hyperplasia, or adipogenesis, occurs throughout life, e.g., in response to the need for additional fat mass stores that arises when caloric intake exceeds nutritional requirements [2]. Extensive modifications in adipose tissue composition involving adipogenesis, angiogenesis and extracellular matrix synthesis and proteolysis occur during development of obesity [3–5]. The adipogenic process is tightly controlled by multiple inhibitory and stimulatory signaling events mediated by cell cycle and differentiation factors [6]. It involves two major events — the recruitment and proliferation of adipocyte precursor cells, called preadipocytes, followed by their differentiation into mature fat cells [7]. Moreover, adipogenesis involves the integration of many different signaling pathways and transcription factors such as the Wnt pathway and the peroxisome proliferator-activated receptors (PPAR) [8].

An important feature of obesity is its linkage to chronic inflammation. Adipose tissue is the largest endocrine organ in the body and is characterised by cytokine and chemokine production and acute-phase inflammatory signaling [9–11]. As an established (pre) adipocyte model cell line 3T3-L1 cells comprise main features of macrophages and intact features relating to innate immunity. Furthermore, it secretes immunomodulatory molecules [12] such as the proinflammatory IL-6 which, by impairing insulin signaling, induces insulin resistance [13,14]. Prevention and treatment of obesity and diabetes should therefore combine anti-obesity and anti-inflammatory effects.

* Corresponding author. Department of Gastroenterology, Charité-Campus Benjamin Franklin, 12203 Berlin, Germany. Tel.: +49 30 8445 4017; fax: +49 30 8445 4017.
E-mail address: martin.ruehl@charite.de (M. Ruehl).

0955-2863/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2009.09.013

Current anti-obesity medications target cells, receptors and signaling molecules such as leptin within the central nervous system with the intention to suppress appetite or to stimulate adipocyte metabolism [15–19]. Emerging combinatory therapies direct several targets e.g. hormonal satiety signals and induction of apoptosis in adipocytes [20,21]. As shown in vivo, adipose tissue can be reduced by apoptosis of preadipocytes and adipocytes [6]. Natural compounds such as genistein, linoleic acid or the green tea derived epigallocatechin gallate were identified to down-regulate adipogenesis by inhibition of differentiation [22,23] and to induce apoptosis in adipocytes [24,25].

The traditional Oriental medicine comprise a rich source of potential drugs hitherto unknown to Western medicine. In Korea, decoctions of wood and bark from *Lindera obtusiloba* are traditionally used for the treatment of inflammation and for the improvement of blood circulation [26], and herbal infusions of *L. obtusiloba* are applied to treat chronic liver diseases (not published K. Kim). Bioactive components derived from the leaves of *L. obtusiloba* were found to belong to the groups of lignans and butanolides. They exert antitumor activity against non–small-cell lung cancer cell line A549, the ovarian cancer cell line SK-OV-3, the skin cancer cell line SK-MEL-2, the central nervous system cell line XF498 and the colon cancer cell line HCT15 [27,28]. In addition, we previously demonstrated that the aqueous extract of *L. obtusiloba* derived from wood and bark exerted antifibrotic effects in the liver due to antioxidative and antiproteolytic activity [29]. Accumulating data strengthen the assumption, that obesity and insulin resistance are closely associated with liver fibrosis [30]. We here investigated direct effects of *L. obtusiloba* extract on the proliferation, the apoptosis, the signaling pathways involved in adipogenic differentiation and the storage of intracellular lipids in mature adipocytes, as well as the effect on IL-6 release induced by proinflammatory stimuli in the murine adipocyte model cell line 3T3-L1.

2. Methods and materials

2.1. Materials and reagents

Tissue culture plates and polystyrene microtiter for enzyme-linked immunosorbent assay (ELISA) as well as for fluorimetric analysis were from Nunc (Roskilde, Denmark) and Dynex (Chantilly, VA, USA), respectively. If not stated otherwise, all reagents were purchased from Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany) and were of the highest purity available. Cell culture media and solutions were purchased from Invitrogen (Karlsruhe, Germany) or Biochrom (Berlin, Germany).

2.2. Preparation of L. obtusiloba extract

Freeze-dried extracts of *L. obtusiloba* were obtained as described previously [29]. To obtain stock solutions (10 mg/ml) powder was redissolved in sterile phosphate-buffered saline (PBS) at 60°C for 30 min. Final concentrations corresponded to 400 mg/ml of the original twig amount. All concentrations given in the text refer to the dissolved powder. Aliquots were stored at −20°C.

2.3. Cell culture

Murine 3T3-L1 preadipocytes were purchased from the American Type Culture Collection (Manassas, VA, USA; American Type Culture Collection No. CL-173) and were cultured in a humidified atmosphere at 37°C and 5% CO_2. Standard culture medium consisted of DMEM (Dulbecco's modified eagle medium) with 862 mg/l L-alanyl-L-glutamine, 4.5 g/l glucose, 50 μg/ml streptomycin, 50 units/ml penicillin, 50 μg/ml L-ascorbic acid, supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cell layers were detached with 0.05% trypsin/0.02% EDTA solution. Cell morphology in culture was directly examined by inverse phase contrast microscopy (Zeiss, Oberkochen, Germany).

2.4. 3T3-L1 cell proliferation

3T3-L1 preadipocytes (5×10^3) were seeded in 96-well tissue culture plates in 100 μl standard culture medium. After 24 h, cells were cell cycle synchronized in 100 μl culture medium containing 0.2% FBS for additional 24 h. Cultures were treated with up to 270 μg/ml *L. obtusiloba* extract as indicated for 20 h. Proliferation was determined by adding 0.5 μCi per well [^{3}H] thymidine (GE Healthcare, Munich, Germany) for 4 h. Cells were fixed with 10% TCA (trichloroacetic acid), and the DNA was solubilized with 200 mM sodium hydroxide, neutralized with an equal volume of 800 mM hydrochloric acid and transferred to glass filter pads. Radioactive decay was monitored by liquid β-scintillation counting (LKB Wallac, Turku, Finland).

2.5. Cell viability

Viability of 3T3-L1 cells was determined using the two-colour fluorescence Live/Dead Viability/Cytotoxicity Kit according to the manufactures instructions (Molecular Probes, Eugene, OR, USA). Briefly, cells were seeded and grown for 20 h as described above. After washing with PBS, cells were incubated with 200 μl PBS containing 1 μM calcein AM for 45 min at 25°C in the dark. Viable cells were assessed by measurement of the conversion of the nonfluorescent calcein AM to the fluorescent calcein by intracellular esterases using a Spectramax Gemini EM microplate reader (λ_{ex}: 494 nm, λ_{em}: 517 nm; Molecular Probes, Sunnyvale, CA, USA).

2.6. Preadipocyte differentiation

3T3-L1 preadipocytes in confluent cell layers were cultured for additional two days. Cells were incubated with a differentiation cocktail (MDI) containing 1.7 μM insulin, 0.25 mM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine in standard culture medium for 48 h, followed by additional 48 h with standard culture medium containing insulin alone. On Day 5, medium was removed and the standard culture medium was replaced every second day. On days 9–12, at least 80% of all 3T3-L1 cells in the culture displayed visible intracellular lipid droplets.

2.7. Oil-Red-O staining

Dye solution was prepared as follows: 530 mg Oil-Red-O were dissolved overnight in 150 ml isopropanol; the solution was filtered before 112.5 ml bidistilled water was added. After 16 h at 4°C, the precipitate was removed by filtration and the supernatant was stored at room temperature. Adipocyte cell layers were washed with PBS, fixed with 4% formaldehyde in PBS for 15 min, stained with the Oil-Red-O dye solution for 1 h and washed with 70% ethanol. Cells were checked by phase contrast microscopy (Zeiss Microimaging, Oberkochen, Germany). To quantify accumulated lipid droplets, cell-incorporated dye was redissolved in isopropanol and measured using a MR5000 ELISA micro plate reader (Dynatech, Billinghurst, UK) at a wavelength of 490 nm.

2.8. Apoptosis by caspase 3/7 activity

3T3-L1 preadipocytes (2×10^5) were seeded to 24-well tissue culture plates in standard culture medium. Confluent cell layers were thoroughly washed with DMEM and subsequently incubated with culture medium containing 0.2% FBS for 24 h. In parallel, preadipocytes were differentiated to adipocytes as described above. 3T3-L1 cells were then treated for 24 h with 135 μg/ml *L. obtusiloba* extract or with 100 nM staurosporine in culture medium containing 0.2% FBS. Apoptosis was determined using the SensoLyte Homogenous AFC Caspase-3/7 Assay Kit (AnaSpec, San Jose, CA, USA) according to the manufacturer instructions. In brief, cells were lysed in 200 μl lysis buffer for 1 h at 4°C. The clear supernatant obtained after centrifugation at 2,500×g for 30 min was stored at −80°C until measurement. Caspase-3/7-mediated conversion of the substrate N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin was monitored fluorometrically using the Spectramax microplate reader (λ_{ex}: 380 nm, λ_{em}: 500 nm).

2.9. Whole cell extracts

3T3-L1 cells, cultured and treated in six-well tissue culture plates as described above were rinsed with ice-cold PBS and lysed with a buffer containing 14 mM Tris–HCl, pH 6.8; 2.25 M urea; 1.4% sodium dodecyl sulfate (SDS); 100 mM DTT and, per 10 ml buffer, one tablet of Complete Mini Protease Inhibitor cocktail (Roche, Penzberg, Germany). Aliquots of 333-μl lysate were transferred to 0.5 ml reaction tubes and frozen at −80°C. Protein content was determined using the Nano Orange Protein Assay Kit (Molecular Devices) according to the manufactures instructions.

2.10. Western-blot

From each cell lysate, 25 μg protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Bio-Rad, Munich, Germany) using a tank blot apparatus (Hoefer, Holliston, MA, USA). Membranes blocked for 1 h with 5% skim milk powder in a buffer containing 10 mM Tris, 154 mM NaCl, 0.1% Tween 20 were incubated for 2 h with the respective primary antibodies. Monoclonal anti-rat vascular endothelial growth factor (VEGF; 1:1,000; R&D Systems, Wiesbaden, Germany), anti protein glucose transporter-4 (GLUT4; 1:1,000; Biogenesis, Poole, UK), anti-β-actin (1:10,000), anti-β-catenin (1:1,250; Cell Signaling, Beverly, MA, USA) and polyclonal antibodies specific for Thr41/Ser45 phospho-β-catenin (1:1,250; Cell Signaling) and PPARγ (1:2,000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) were used. After washing, membranes were incubated 1 h with rabbit or goat IgG-specific horseradish peroxidase-labeled secondary antibodies (1:2,500; Dako, Hamburg, Germany). Bands were detected by enhanced chemiluminescence (GE Healthcare) and digitized using a flat bed scanner

(Plustek, Norderstedt, Germany). Band intensities were quantified using Image J (version 1.41; National Institutes of Health, Bethesda, MD, USA).

2.11. SDS-PAGE substrate zymography

On day 9, supernatants from cultures of 3T3-L1 cells in six-well tissue culture plates were replaced by 300 µl DMEM. After 4 h, aliquots of the supernatants were stored at 80°C. The release and degree of matrix metalloproteinase (MMP)-2 activation was checked by SDS-PAGE gelatin zymography. Adjusted to the respective cell numbers in the cultures, 8-14 µl of the supernatants were mixed with 5 µl nonreducing zymogram sample buffer (Bio-Rad) and subjected to SDS-PAGE with 1 mg/ml gelatine. After washing three times for 10 min with 2.5% Triton X-100, gels were incubated for 20 h at 37°C in MMP-activity buffer containing 50 mM Tris–HCl pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij 35 and were finally stained with Coomassie Brilliant Blue R 250. Areas of proteolytic activity appeared white against a blue background and band intensities were quantified from inverted grey pictures as described in the western-blot section.

2.12. Interleukin-6 production

3T3-L1 preadipocytes cultured in a 24-well tissue culture plate were treated with 1 µg/ml lipopolysaccharide (LPS) or 10 ng/ml tumor necrosis factor α (TNFα) (Peprotech, London, UK), *L. obtusiloba* extract alone and combinations of *L. obtusiloba* extract with LPS/TNFα. After 24 h, 1 ml cell-free culture supernatant was stored at −80°C. Concentrations of interleukin-6 (IL-6) were determined by specific ELISA (BD Pharmingen) within a range of 15–1000 pg/ml. Briefly, 96-well MaxiSorp microplates coated overnight with rabbit anti-mouse IL-6 antibody were washed three times with PBS. Samples were added to the wells and plates were incubated for 2 h at room temperature. After four times washing, plates were incubated with a horseradish peroxidase-labeled anti-rabbit for 1 h at room temperature and again thoroughly washed five times. The substrate was added and the reaction stopped with 1 M sulfuric acid after 30 min at room temperature. Absorbance at a wavelength of 450 nm was measured using the ELISA microplate reader.

2.13. Statistical Analysis

One-way analyses of variance/Tukey tests were performed using SigmaStat for Windows (version 2.03; Systat, San Jose, CA, USA). $P<.05$ was considered significantly different.

3. Results

3.1. L. obtusiloba extract reduces proliferation of 3T3-L1 preadipocytes without exerting cytotoxic effects

Measuring the de novo DNA synthesis of cell cycle-synchronized and serum-starved cells allows determination of subtle effects on toxicity, apoptosis or cellular quiescence. *L. obtusiloba* extract inhibits de novo DNA synthesis of 3T3-L1 preadipocytes in a concentration dependent manner (Fig. 1A). At concentrations as low as 34 µg/ml, *L. obtusiloba* extract significantly reduced the [^{3}H]-thymidine uptake compared to the control without the extract. The IC_{50} range of *L. obtusiloba* extract was 130–140 µg/ml. Further studies focused on this concentration range using 135 µg/ml *L. obtusiloba* extract. To distinguish between specific growth inhibition and possible cytotoxic effects of *L. obtusiloba* extract, cytoplasmic esterase activity as a measure of cell viability was determined in 3T3-L1 preadipocytes. Analog to the proliferation assay procedure, 3T3-L1 preadipocytes were incubated with the *L. obtusiloba* extract at various concentrations. At IC_{50} of *L. obtusiloba* extract for 3T3-L1 preadipocyte proliferation cell viability was not significantly different to untreated cells (Fig. 1B). Concentrations up to 270 µg/ml *L. obtusiloba* extract were not found to negatively affect the cell viability. Thus, the growth-inhibitory effects of *L. obtusiloba* extract in 3T3-L1 preadipocytes are not due to cytotoxicity.

3.2. L. obtusiloba extract induces apoptosis in 3T3-L1 preadipocytes and adipocytes

Next, we tested whether induction of apoptosis in 3T3-L1 cells was responsible for the reduced de novo DNA-synthesis in the presence of *L. obtusiloba* extract. At a very low concentration of

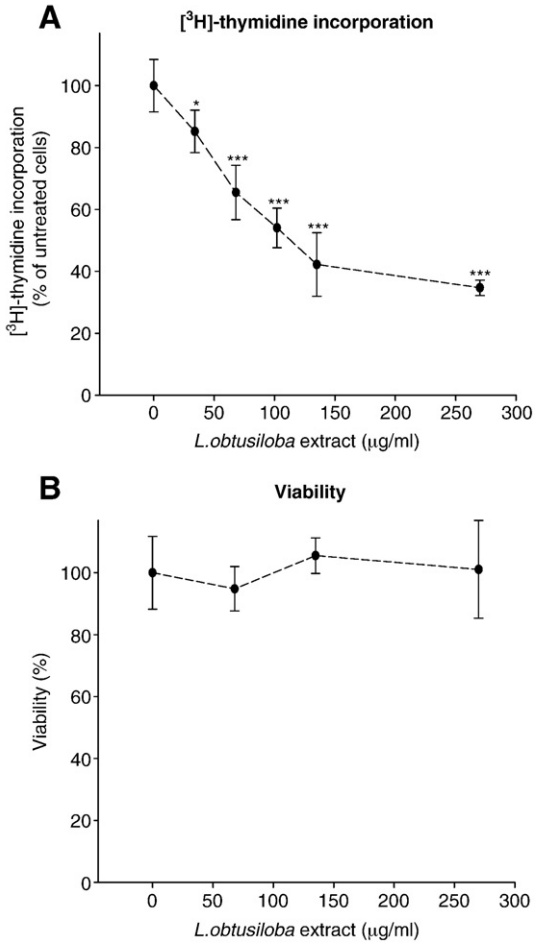

Fig. 1. Proliferation and viability of 3T3-L1 preadipocytes treated with *L. obtusiloba* extract. Cell cycle-synchronized 3T3-L1 preadipocytes were treated with up to 270 µg/ml *L. obtusiloba* extract for 24 h. Cultures without *L. obtusiloba* extract served as controls. (A) Proliferation as determined by [^{3}H]-thymidine incorporation was normalized to data obtained from untreated cells (100%). (B) Cell viability was quantified fluorimetrically by intracellular esterase-mediated conversion of calcein AM and was calculated in relation to untreated cells (100%). Shown are mean values and S.D. of five parallel measurements. *$P<.05$; ***$P<.001$.

100 nM, the protein kinase inhibitor staurosporine was a potent inducer of apoptosis as shown by 3.9fold and 2.7fold enhanced activities of caspase-3/7 in preadipocytes and in differentiated adipocytes, respectively (Fig. 2). *L. obtusiloba* extract (135 µg/ml) also significantly increased caspase 3/7 activitiy in both, preadipocytes as well as adipocytes, compared to the respective untreated 3T3-L1 cells. With 80% caspase-3/7 activity of the staurosporine control, differentiated 3T3-L1 cells were even more prone to apoptosis than 3T3-L1 preadipocytes that reached about 64% of the respective control.

3.3. L. obtusiloba extract impairs lipid accumulation in differentiating 3T3-L1 cells and induces a loss of intracellular lipids in mature adipocytes

Reduction of the de novo DNA synthesis of 3T3-L1 preadipocytes combined with unaffected cell viability pointed to an impact of *L. obtusiloba* extract on cellular quiescence and subsequent adipocyte maturation. We therefore tested effects of *L. obtusiloba* extract on 3T3-L1 preadipocyte differentiation induced by the hormone cocktail as demonstrated microscopically by accumulation of intracellular fat droplets and spectrophotometrically by high Oil-Red-O contents

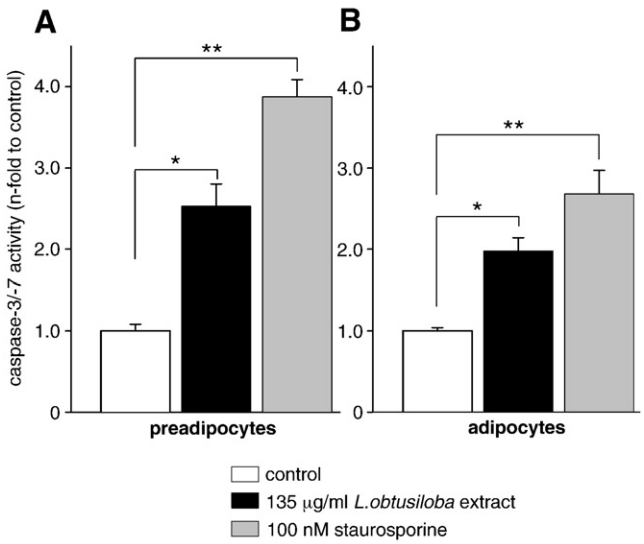

Fig. 2. Apoptosis of 3T3-L1 preadipocytes and adipocytes in the presence of *L. obtusiloba* extract. 3T3-L1 preadipocytes from standard culture and cells fully differentiated by treatment with the MDI cocktail for 9 days were incubated with 135 µg/ml *L. obtusiloba* extract for 24 h. Cultures without additions or with 100 nM staurosporine served as negative and positive controls. Enzymatic activities of caspase-3/7 were determined from cell lysates by fluorogenic substrate conversion. Shown are mean values and S.D. of four parallel measurements from one representative experiment out of three. *$P<.05$; ***$P<.001$.

(Fig. 3). In the presence of 135 µg/ml *L. obtusiloba* extract lipid accumulation in differentiating 3T3-L1 cells was reduced by 70%. Consequently, we asked for specific effects of *L. obtusiloba* extract on the metabolism of intracellular lipids in 3T3-L1 adipocytes. 3T3-L1 cells fully differentiated to adipocytes by the MDI cocktail were treated with 135 µg/ml *L. obtusiloba* extract for up to 48 h (Fig. 4). Intracellular lipid accumulation was quantified to adjusted cell numbers at the end of the treatment. *L. obtusiloba* extract not only impaired lipid droplet formation during differentiation but reduced lipid content in adipocytes significantly within 48 h by about 30% compared to fully differentiated 3T3-L1 cells (Fig. 4A). Since 3T3-L1 cell numbers in all cultures were comparable independent of the presence of *L. obtusiloba* extract (Fig. 4B), we concluded that the extract reduced the lipid content at single cell level. Therefore we further specified the impact of *L. obtusiloba* extract on expression of proteins specific for adipogenesis and for adipocytes.

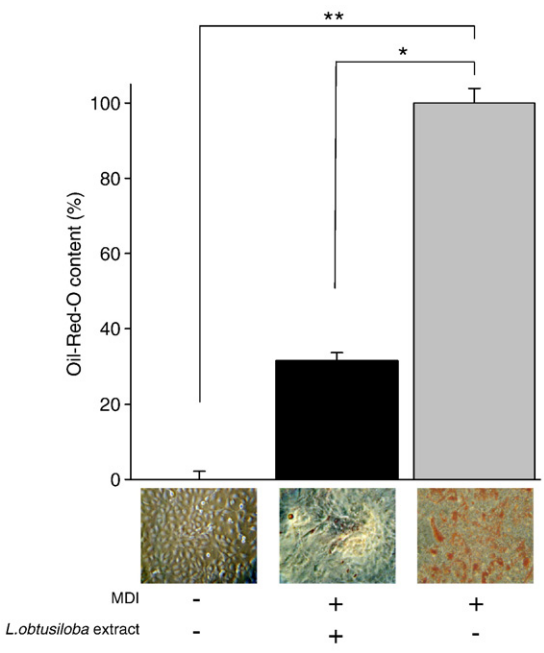

Fig. 3. Effects of *L. obtusiloba* extract on lipid accumulation in 3T3-L1 preadipocyte differentiation. 3T3-L1 preadipocytes were differentiated with MDI alone or in the presence of 135 µg/ml *L. obtusiloba* extract for 9 days. 3T3-L1 preadipocytes served as control. Intracellular lipid droplets were stained with Oil-Red-O and cell layers were assessed by phase contrast microscopy (original magnification ×100). After cell lysis, Oil-Red-O amounts were quantified spectrophotometrically. Shown are mean values and S.D. of three independent experiments with four parallel measurements. *$P<.05$; **$P<.01$.

3.4. *L. obtusiloba* extract attenuates the 3T3-L1 differentiation dependent expression of GLUT4 and VEGF and stabilizes Wnt signaling

Studying the effects of *L. obtusiloba* extract on the differentiation-associated expression of GLUT4 and VEGF preadipocytes were differentiated using the differentiation cocktail in combination with *L. obtusiloba* extract. As shown by western-blot analysis, both marker proteins GLUT4 and VEGF were strongly expressed by mature adipocytes and expression levels correlated with the degree of differentiation. At the IC_{50} for 3T3-L1 preadipocyte proliferation, *L. obtusiloba* extract almost inhibited the expression of GLUT4 and VEGF that were reduced by 90% compared to fully differentiated cells (Fig. 5). These finding pointed to attenuated adipogenesis.

As for signaling pathways involved, we investigated if altered Wnt signaling inhibited 3T3-L1 adipogenesis. Following stimulation of the Wnt-pathway, the central downstream mediator β-catenin translocates into the nucleus. Inhibition of Wnt-signaling occurs via destabilization of cytoplasmic β-catenin by phosphorylation and proteasomal degradation.

We hypothesized that the anti-adipogenic effects of *L. obtusiloba* extract might be due to diminished phosphorylation of β-catenin, hence promoting Wnt signaling. In 3T3-L1 adipocytes β-catenin was highly phosphorylated whereas in the preadipocytes no

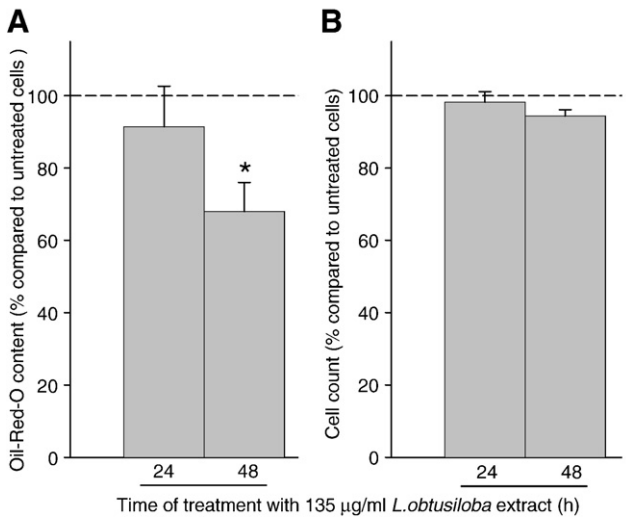

Fig. 4. Intracellular lipids in 3T3-L1 adipocytes after treatment with *L. obtusiloba* extract. 3T3-L1 fully differentiated to adipocytes by MDI were treated with 135 µg/ml *L. obtusiloba* extract for 24 and 48 h or were left untreated. (A) Intracellular lipid droplets were quantified spectrometrically after Oil-Red-O staining. (B) In parallel culture settings, cell numbers were determined. Lipid accumulation and cell numbers were calculated in relation to the respective control without *L. obtusiloba* extract (100%). Shown are mean values and S.D. of three independent experiments with four parallel measurements. *$P<.05$.

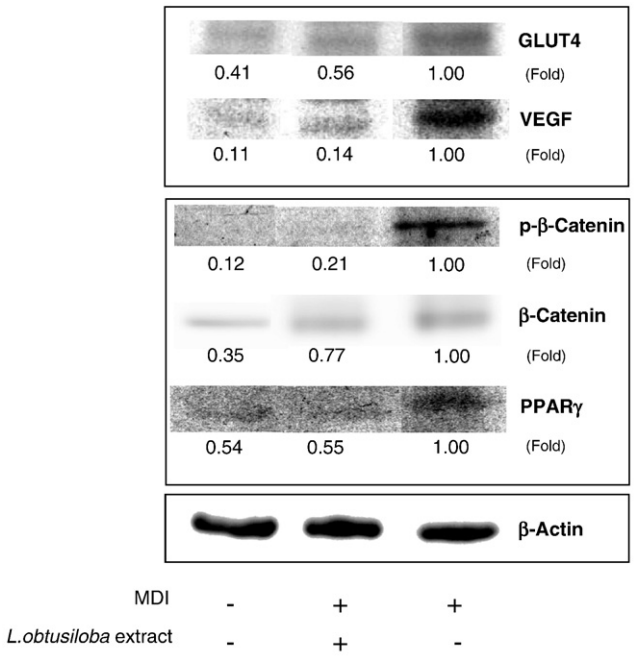

Fig. 5. Influence of *L. obtusiloba* extract on adipogenesis-related proteins in 3T3-L1 preadipocyte differentiation. 3T3-L1 preadipocytes were differentiated to adipocytes by MDI with or without 135 μg/ml *L. obtusiloba* extract. Whole cell lysates from these cells and from 3T3-L1 preadipocytes as control, were analyzed by Western blot specific for adipogenesis-related GLUT4 and VEGF, for the Wnt pathway protein β-catenin including its phosphorylation state, for the transcription factor PPARγ, and for β-actin as equal loading control. Blots are representative for three independent experiments.

phosphorylated β-catenin was detected by specific Western blot (Fig. 5). Addition of 135 μg/ml *L. obtusiloba* extract during treatment with the differentiating hormone cocktail resulted in β-catenin phosphorylation reduced by 80% and pointed to sustained Wnt signaling due to *L. obtusiloba* extract. Since Wnt signaling also suppresses adipogenic transcription factors [31], we tested the effects of *L. obtusiloba* extract on the expression of the PPARγ. 3T3-L1 preadipocytes did not and fully differentiated adipocytes strongly expressed PPARγ. In the presence of *L. obtusiloba* extract during the MDI treatment of 3T3-L1 cells PPARγ expression remained at the same low levels as in the 3T3-L1 preadipocyte cultures. Taken together, *L. obtusiloba* extract promotes Wnt signaling in 3T3-L1 resulting in the inhibition of the adipogenic process by attenuated PPARγ expression.

3.5. L. obtusiloba extract impairs the secretion of the gelatinase MMP-2

Besides the Wnt pathway, proteolytic activity of gelatinases is considered important in the development of adipose tissue [32]. Thus, we tested the effects of *L. obtusiloba* extract on the secretion of MMP-2 and MMP-9 by 3T3-L1 preadipocytes and adipocyte differentiation. Neither the gelatinase MMP-9 nor proMMP-9 were found in the supernatants of 3T3-L1 (pre)adipocytes (data not shown). The 3T3-L1 preadipocytes secreted only small amounts of MMP-2. The enzymatically inactive proform was not detected (Fig. 6). In contrast, supernatants of fully differentiated 3T3-L1 adipocytes contained high amounts of secreted MMP-2 and an additional band of pro-MMP-2. The presence of only 68 μg/ml *L. obtusiloba* extract during 3T3-L1 differentiation resulted in a MMP-2 secretion decreased by ~60% confirming the previous results pointing to inhibition of the adipogenic process by *L. obtusiloba* extract.

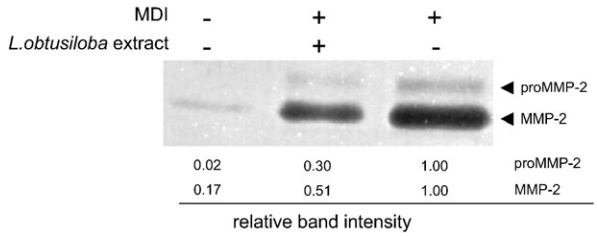

Fig. 6. *L. obtusiloba* extract reduces MMP-2 secretion during 3T3-L1 preadipocyte differentiation. 3T3-L1 preadipocytes were differentiated with MDI to adipocytes without or in the presence of 68 μg/ml *L. obtusiloba* extract. FBS-free supernatants adjusted to the cell numbers were subjected to gelatin zymography and MMP-2 enzymatic activity was visualized by Coomassie Brilliant Blue R-250 staining. Zymographies are representative of three independent experiments.

3.6. L. obtusiloba extract blocks toll-like receptor 4 and TNF receptor mediated IL-6 production by 3T3-L1 preadipocytes

Obesity links to chronic inflammation and pro-inflammatory cytokine secretion by preadipocytes contributes to insulin resistance. Thus, we finally investigated the impact of *L. obtusiloba* extract on the production of the key cytokine IL-6 by 3T3-L1 cells. *L. obtusiloba*

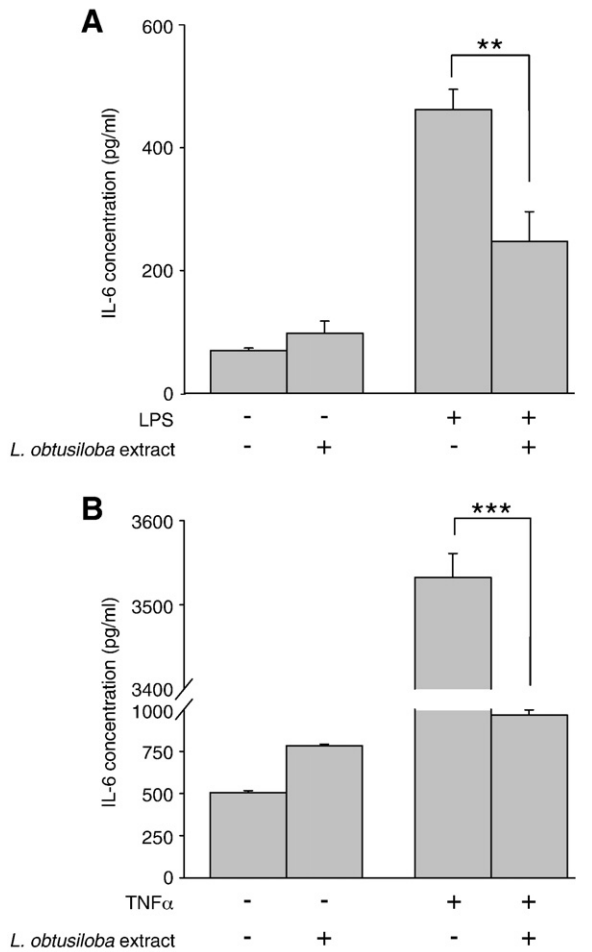

Fig. 7. LPS- and TNFα-dependent IL-6 production by 3T3-L1 preadipocytes in the presence of *L. obtusiloba* extract. 3T3-L1 preadipocytes were treated with 1 μg/ml LPS (A) or with 10 ng/ml TNFα (B) alone or in the presence of 135 μg/ml *L. obtusiloba* extract. After 24 h, the proinflammatory IL-6 in the supernatants was determined by ELISA. Shown are mean values and SD from three independent experiments with four parallel measurements. $^{**}P<.01$; $^{***}P<.001$.

extract alone had no effect on the IL-6 production of the preadipocytes as analysed by ELISA (Fig. 7). If treated with 1 μg/ml LPS or with 10 ng/ml TNFα, 3T3 L1 preadipocytes released high amounts of IL-6. Concomitant presence of *L. obtusiloba* extract and LPS- or TNFα led to significantly lower IL-6 concentrations in supernatants of 3T3-L1 preadipocytes compared to the respective controls with signaling via toll-like receptor (TLR)-4 or the TNF receptor only. We concluded here that *L. obtusiloba* extract has not only direct anti-adipogenic but additional strong anti-inflammatory activities.

4. Discussion

Physiologic remodeling of adipose tissue involves adipogenesis and apoptosis in preadipocytes and in mature adipocytes [2,33,34]. To maintain the function of adipose tissue to store excess energy, it provides sufficient fat cell numbers by adipogenesis, accompanied by enlargement of existing adipocytes. Both, size and number of adipocytes affect obesity and insulin resistance associated with the metabolic syndrome [6,35]. Thus, deletion of preadipocytes and adipocytes via apoptosis and inhibition of adipogenesis are therapeutic targets for obesity related diseases [23,25,36].

Here, we used the established murine preadipocyte cell line 3T3-L1 [37,38] to study anti-adipogenic effects of an aqueous extract derived from wood and bark of the Japanese spice bush *L. obtusiloba*. We demonstrate that the *L. obtusiloba* extract significantly suppresses proliferation, slightly induces apoptosis in 3T3-L1 preadipocytes and blocks their hormone-induced maturation into adipocytes. The latter comes along with reduced lipid droplet accumulation and with lowered expression of GLUT4 and VEGF. In adipocytes, *L. obtusiloba* extract also induces apoptosis and decreases the amount of intracellular lipid droplets. Effects of *L. obtusiloba* extract involve sustained Wnt pathway signaling eventually leading to down-regulation of PPARγ expression and reduced secretion of MMP-2. Regarding insulin resistance and the inflammatory state characterizing obesity, we show anti-inflammatory effects of *L. obtusiloba* extract, by inhibition of LPS- and TNFα-induced IL-6 production by preadipocytes.

Proliferation of adipocytes can not be determined due to the known cessation of mitotic growth by the CCAAT-enhancer binding protein in these cells [39]. On the other hand, proliferation of adipocyte precursor cells represents the basic process for adipocyte differentiation. Thus, inhibition of preadipocyte proliferation is an early target to prevent adipogenesis [35]. We show that *L. obtusiloba* extract indeed inhibits the proliferation of 3T3-L1 preadipocytes with an IC_{50} of 130–140 μg/ml (Fig. 1). Suppression of proliferation in preadipocytes after *L. obtusiloba* extract treatment for 24 h is not due to toxicity (Fig. 1). Deletion of adipocytes by apoptosis is a relatively recent concept [2,40] which is supported by several animal studies [41,42]. Since the degree of apoptosis induced by *L. obtusiloba* extract is comparable in adipocytes and preadipocytes (Fig. 2), *L. obtusiloba* extract might directly affect the regulation of adipose tissue homeostasis. Recently various natural compounds such as the coumarin derivative esculetin [43], the soy isoflavone genistein and the active substance in guggulipid guggulsterone [44], the polyphenol epigallocatechin gallate derived from green tea [23] or the phytoalexin resveratrol found in red wine and grape juice [25,45] were also hypothesized to exert anti-obesity effects by inhibiting differentiation of preadipocytes. Interestingly, *L. obtusiloba* extract effectively blocks the generation of intracellular lipids during hormone-induced adipocyte differentiation (Fig. 3A). Further evidence of impaired adipogenesis was obtained by Western blot analysis of GLUT4, representing a marker gene for late-stage preadipocyte differentiation [46,47] and of VEGF which is abundantly expressed in mature adipocytes and in minor amounts in preadipocytes [48]. The presence of *L. obtusiloba* extract during 3T3-L1 preadipocyte differentiation reduced the expression of GLUT4 and of VEGF (Fig. 5), confirming not only the inhibition of adipogenesis but also implicating an anti-angiogenic effect of *L. obtusiloba* extract.

To get deeper insights in the mode of action of *L. obtusiloba* extract, we next addressed cellular signal transduction in 3T3-L1 cells and focused on the Wnt pathway. This pathway mediates key cell-cell signaling events during embryogenesis, is required for adult tissue maintenance and is mandatory in adipogenesis [49,50]. Sustained overexpression of Wnt 1 or Wnt 10b blocks adipogenesis in preadipocytes via inhibition of CCAAT/enhancer binding protein-a and of PPARγ, two adipogenic transcription factors [51–53]. Conversely, inhibition of Wnt signaling in preadipocytes promotes their differentiation into adipocytes [31]. The key mediator of the Wnt pathway is β-catenin. While unphosphorylated β-catenin accumulates in the nucleus, phosphorylation by GSK-3 and the casein kinase-1α marks the protein for proteasomal degradation [54–56]. Thus, in adipocytes high levels of β-catenin are phosphorylated. If present during 3T3-L1 differentiation, 135 μg/ml *L. obtusiloba* extract effectively reduced the phosphorylation of β-catenin, thus stabilizing β-catenin and blocking adipogenesis. The expression of the transcription factor PPARγ represents a downstream target of Wnt signaling and is a developmental regulator of preadipocyte differentiation [46,57]. Inhibition of adipogenesis by the *L. obtusiloba* extract was associated with the inhibition of PPARγ expression as described earlier for catechin [58].

The expression of PPARγ was shown to impact the survival of adipocytes. Conditional ablation of PPARγ in mature adipocytes led to cell death and repopulation by PPARγ-positive preadipocytes [59]. Whether the induction of apoptosis in mature adipocytes by the *L. obtusiloba* extract (Fig. 2B) is independent from or related to the reduction of PPARγ-expression remains to be investigated. Thitherto, we prefer the assumption that the induction of apoptosis in mature adipocytes relates to the reduction of PPARγ-expression by the *L. obtusiloba* extract and address this relationship in further studies.

In a previous study we reported that antifibrotic effects of *L. obtusiloba* extract were due to reduction of enzymatic activity of the gelatinases MMP-2 and MMP-9 [29]. These enzymes are produced and released by human and murine (pre)adipocytes and are required for the initiation of adipogenesis [32,60,61], and inhibition of MMPs blocks conversion of preadipocytes to adipocytes [32,60,62]. The overall reduced secretion of MMP-2 in 3T3-L1 preadipocytes, due to the presence of *L. obtusiloba* extract during the hormone cocktail treatment (Fig. 6), confirms that the extract impairs the adipogenic differentiation in 3T3-L1.

Aiming for possible therapeutic intervention in obesity, we investigated effects of *L. obtusiloba* extract on intracellular lipids in mature fat cells. Treatment of fully differentiated 3T3-L1 adipocytes with *L. obtusiloba* extract for 48 h significantly reduced intracellular lipids (Fig. 4) pointing to the induction of lipolysis by *L. obtusiloba* extract. This supports the notions of other reports on mechanisms for anti-obesity effects of natural compounds [63,64].

Recent studies closely link obesity and the metabolic syndrome to inflammation [10,13,14]. Proinflammatory stimuli like leptin, TNFα and IL-6 overproduced during obesity contribute to the pathogenesis of insulin resistance [65,66]. Thus, our finding that *L. obtusiloba* extract significantly reduces the IL-6 release from preadipocytes treated with the TLR-4 ligand LPS and with the cytokine TNFα (Fig. 7), refer to an additional anti-inflammatory effect of *L. obtusiloba* extract involving principles form the innate as well as from the acquired immune system.

On one hand, our results provide evidence for mechanisms underlying the general beneficial effects of *L. obtusiloba* extract used in traditional Korean medicine. On the other hand, our findings in vitro strongly suggest *L. obtusiloba* extract as a specific compound to complement treatment of the metabolic syndrome

suppressing the obesity-related immune response and development of insulin resistance.

References

[1] Roth J, Qiang X, Marban SL, Redelt H, Lowell BC. The obesity pandemic: where have we been and where are we going? Obes Res 2004;12:88S–101S.
[2] Prins JB, O'Rahilly S. Regulation of adipose cell number in man. Clin Sci (Lond) 1997;92:3–11.
[3] Christiaens V, Scroyen I, Lijnen HR. Role of proteolysis in development of murine adipose tissue. Thromb Haemost 2008;99:290–4.
[4] Nakajima I, Muroya S, Tanabe R, Chikuni K. Extracellular matrix development during differentiation into adipocytes with a unique increase in type V and VI collagen. Biol Cell 2002;94:197–203.
[5] Nakajima I, Yamaguchi T, Ozutsumi K, Aso H. Adipose tissue extracellular matrix: newly organized by adipocytes during differentiation. Differentiation 1998;63: 193–200.
[6] Sorisky A, Gagnon A. Clinical implications of adipose tissue remodelling: adipogenesis and apoptosis. Can J Diabetes 2002;26:232–40.
[7] Avram MM, Avram AS, James WD. Subcutaneous fat in normal and diseased states 3. Adipogenesis: from stem cell to fat cell. J Am Acad Dermatol 2007;56:472–92.
[8] Rosen ED, MacDougald OA. Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol 2006;7:885–96.
[9] Schaffler A, Scholmerich J, Salzberger B. Adipose tissue as an immunological organ: Toll-like receptors, C1q/TNFs and CTRPs. Trends Immunol 2007;28:393–9 [Epub 2007 Aug 2002].
[10] Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. J Clin Invest 2003;112:1785–8.
[11] Trayhurn P. Endocrine and signalling role of adipose tissue: new perspectives on fat. Acta Physiol Scand 2005;184:285–93.
[12] Lin Y, Lee H, Berg AH, Lisanti MP, Shapiro L, Scherer PE. The lipopolysaccharide-activated toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. J Biol Chem 2000;275:24255–63.
[13] Sopasakis VR, Sandqvist M, Gustafson B, Hammarstedt A, Schmelz M, Yang X, et al. High local concentrations and effects on differentiation implicate interleukin-6 as a paracrine regulator. Obes Res 2004;12:454–60.
[14] Rotter V, Nagaev I, Smith U. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. J Biol Chem 2003;278:45777–84 [Epub 42003 Sep 45772].
[15] Batterham RL, Ffytche DH, Rosenthal JM, Zelaya FO, Barker GJ, Withers DJ, et al. PYY modulation of cortical and hypothalamic brain areas predicts feeding behaviour in humans. Nature 2007;450:106–9 [Epub 2007 Oct 2014].
[16] Christensen R, Kristensen PK, Bartels EM, Bliddal H, Astrup A. Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials. Lancet 2007;370:1706–13.
[17] Oh IS, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, et al. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature 2006;443:709–12 [Epub 2006 Oct 2001].
[18] Bachman ES, Dhillon H, Zhang CY, Cinti S, Bianco AC, Kobilka BK, et al. betaAR signaling required for diet-induced thermogenesis and obesity resistance. Science 2002;297:843–5.
[19] Bjorbaek C, Kahn BB. Leptin signaling in the central nervous system and the periphery. Recent Prog Horm Res 2004;59:305–31.
[20] Kushner RF. Anti-obesity drugs. Expert Opin Pharmacother 2008;9:1339–50.
[21] Beglinger C, Degen L. Gastrointestinal satiety signals in humans–physiologic roles for GLP-1 and PYY? Physiol Behav 2006;89:460–4 [Epub 2006 Jul 2007].
[22] Kim HK, Nelson-Dooley C, Della-Fera MA, Yang JY, Zhang W, Duan J, et al. Genistein decreases food intake, body weight, and fat pad weight and causes adipose tissue apoptosis in ovariectomized female mice. J Nutr 2006;136:409–14.
[23] Lin J, Della-Fera MA, Baile CA. Green tea polyphenol epigallocatechin gallate inhibits adipogenesis and induces apoptosis in 3T3-L1 adipocytes. Obes Res 2005; 13:982–90.
[24] Hargrave KM, Meyer BJ, Li C, Azain MJ, Baile CA, Miner JL. Influence of dietary conjugated linoleic Acid and fat source on body fat and apoptosis in mice. Obes Res 2004;12:1435–44.
[25] Rayalam S, Yang JY, Ambati S, Della-Fera MA, Baile CA. Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes. Phytother Res 2008;7:7.
[26] Yook C. Lindera obtusiloba. In: Yook CS, editor. Medical Plants of Korea. Seoul: Jinmyeong Publishing Co; 1989. p. 184.
[27] Kwon HC, Baek NI, Choi SU, Lee KR. New cytotoxic butanolides from Lindera obtusiloba BLUME. Chem Pharm Bull (Tokyo) 2000;48:614–6.
[28] Kwon HC, Choi SU, Lee JO, Bae KH, Zee OP, Lee KR. Two new lignans from Lindera obtusiloba blume. Arch Pharm Res 1999;22:417–22.
[29] Ruehl M, Erben U, Kim K, Freise C, Dagdelen T, Eisele S, et al. Extracts of Lindera obtusiloba induce antifibrotic effects in hepatic stellate cells via suppression of a TGF-beta-mediated profibrotic gene expression pattern. J Nutr Biochem 2008; 26:26.
[30] Bertolani C, Marra F. The role of adipokines in liver fibrosis. Pathophysiology 2008; 3:3.
[31] Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, et al. Inhibition of adipogenesis by Wnt signaling. Science 2000;289:950–3.
[32] Croissandeau G, Chretien M, Mbikay M. Involvement of matrix metalloproteinases in the adipose conversion of 3T3-L1 preadipocytes. Biochem J 2002;364: 739–46.
[33] Prins JB, Walker NI, Winterford CM, Cameron DP. Apoptosis of human adipocytes in vitro. Biochem Biophys Res Commun 1994;201:500–7.
[34] Qian H, Hausman DB, Compton MM, Martin RJ, Della-Fera MA, Hartzell DL, et al. TNFalpha induces and insulin inhibits caspase 3-dependent adipocyte apoptosis. Biochem Biophys Res Commun 2001;284:1176–83.
[35] Rayalam S, Della-Fera MA, Baile CA. Phytochemicals and regulation of the adipocyte life cycle. J Nutr Biochem 2008;19:19.
[36] Hsu CL, Yen GC. Effects of capsaicin on induction of apoptosis and inhibition of adipogenesis in 3T3-L1 cells. J Agric Food Chem 2007;55:1730–6 [Epub 2007 Feb 1713].
[37] Green H, Kehinde O. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 1975;5:19–27.
[38] Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. Physiol Rev 1998;78:783–809.
[39] Umek RM, Friedman AD, McKnight SL. CCAAT- enhancer binding protein: a component of a differentiation switch. Science 1991;251:288–92.
[40] Della-Fera MA, Qian H, Baile CA. Adipocyte apoptosis in the regulation of body fat mass by leptin. Diabetes Obes Metab 2001;3:299–310.
[41] Magun R, Boone DL, Tsang BK, Sorisky A. The effect of adipocyte differentiation on the capacity of 3T3-L1 cells to undergo apoptosis in response to growth factor deprivation. Int J Obes Relat Metab Disord 1998;22:567–71.
[42] Geloen A, Roy PE, Bukowiecki LJ. Regression of white adipose tissue in diabetic rats. Am J Physiol 1989;257:E547–E553.
[43] Yang JY, Della-Fera MA, Hartzell DL, Nelson-Dooley C, Hausman DB, Baile CA. Esculetin induces apoptosis and inhibits adipogenesis in 3T3-L1 cells. Obesity (Silver Spring) 2006;14:1691–9.
[44] Yang JY, Della-Fera MA, Rayalam S, Ambati S, Baile CA. Enhanced pro-apoptotic and anti-adipogenic effects of genistein plus guggulsterone in 3T3-L1 adipocytes. Biofactors 2007;30:159–69.
[45] Rayalam S, Della-Fera MA, Yang JY, Park HJ, Ambati S, Baile CA. Resveratrol potentiates genistein's antiadipogenic and proapoptotic effects in 3T3-L1 adipocytes. J Nutr 2007;137:2668–73.
[46] Fernyhough ME, Okine E, Hausman G, Vierck JL, Dodson MV. PPARgamma and GLUT-4 expression as developmental regulators/markers for preadipocyte differentiation into an adipocyte. Domest Anim Endocrinol 2007;33:367–78 [Epub 2007 May 2029].
[47] Rea S, James DE. Moving GLUT4: the biogenesis and trafficking of GLUT4 storage vesicles. Diabetes 1997;46:1667–77.
[48] Miyazawa-Hoshimoto S, Takahashi K, Bujo H, Hashimoto N, Yagui K, Saito Y. Roles of degree of fat deposition and its localization on VEGF expression in adipocytes. Am J Physiol Endocrinol Metab 2005;288:E1128–E1136 [Epub 2004 Dec 1121].
[49] Liu J, Farmer SR. Regulating the balance between peroxisome proliferator-activated receptor gamma and beta-catenin signaling during adipogenesis. A glycogen synthase kinase 3beta phosphorylation-defective mutant of beta-catenin inhibits expression of a subset of adipogenic genes. J Biol Chem 2004; 279:45020–7 [Epub 42004 Aug 45010].
[50] Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 2004;20:781–810.
[51] Bennett CN, Ross SE, Longo KA, Bajnok L, Hemati N, Johnson KW, et al. Regulation of Wnt signaling during adipogenesis. J Biol Chem 2002;277:30998–1004 [Epub 32002 Jun 30997].
[52] Moldes M, Zuo Y, Morrison RF, Silva D, Park BH, Liu J, et al. Peroxisome-proliferator-activated receptor gamma suppresses Wnt/beta-catenin signalling during adipogenesis. Biochem J 2003;376:607–13.
[53] Kawai M, Mushiake S, Bessho K, Murakami M, Namba N, Kokubu C, et al. Wnt/Lrp/beta-catenin signaling suppresses adipogenesis by inhibiting mutual activation of PPARgamma and C/EBPalpha. Biochem Biophys Res Commun 2007;363:276–82 [Epub 2007 Aug 2027].
[54] Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J 1997;16:3797–804.
[55] Amit S, Hatzubai A, Birman Y, Andersen JS, Ben-Shushan E, Mann M, et al. Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes Dev 2002;16:1066–76.
[56] Yanagawa S, Matsuda Y, Lee JS, Matsubayashi H, Sese S, Kadowaki T, et al. Casein kinase I phosphorylates the Armadillo protein and induces its degradation in Drosophila. Embo J 2002;21:1733–42.
[57] Christodoulides C, Lagathu C, Sethi JK, Vidal-Puig A. Adipogenesis and WNT signalling. Trends Endocrinol Metab 2009;20:16–24 [Epub 2008 Nov 2013].
[58] Furuyashiki T, Nagayasu H, Aoki Y, Bessho H, Hashimoto T, Kanazawa K, et al. Tea catechin suppresses adipocyte differentiation accompanied by down-regulation of PPARgamma2 and C/EBPalpha in 3T3-L1 cells. Biosci Biotechnol Biochem 2004; 68:2353–9.
[59] Imai T, Takakuwa R, Marchand S, Dentz E, Bornert JM, Messaddeq N, et al. Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. Proc Natl Acad Sci U S A 2004;101:4543–7 [Epub 2004 Mar 4516].
[60] Bouloumie A, Sengenes C, Portolan G, Galitzky J, Lafontan M. Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. Diabetes 2001;50:2080–6.
[61] Bourlier V, Zakaroff-Girard A, De Barros S, Pizzacalla C, de Saint Front VD, Lafontan M, et al. Protease inhibitor treatments reveal specific involvement of matrix

metalloproteinase-9 in human adipocyte differentiation. J Pharmacol Exp Ther 2005;312:1272–9 [Epub 2004 Nov 1210].
[62] Maquoi E, Munaut C, Colige A, Collen D, Lijnen HR. Modulation of adipose tissue expression of murine matrix metalloproteinases and their tissue inhibitors with obesity. Diabetes 2002;51:1093–101.
[63] Kuppusamy UR, Das NP. Effects of flavonoids on cyclic AMP phosphodiesterase and lipid mobilization in rat adipocytes. Biochem Pharmacol 1992;44: 1307–15.
[64] Yang JY, Della-Fera MA, Baile CA. Guggulsterone inhibits adipocyte differentiation and induces apoptosis in 3T3-L1 cells. Obesity (Silver Spring) 2008;16:16–22.
[65] Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 1993;259:87–91.
[66] Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur Cytokine Netw 2006;17:4–12.