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Wedelolactone Inhibits Adipogenesis Through the ERK Pathway in Human Adipose Tissue-Derived Mesenchymal Stem Cells

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ABSTRACT

Wedelolactone is an herbal medicine that is used to treat septic shock, hepatitis and venom poisoning. Although in differentiated and cancer cells, wedelolactone has been identified as anti-inflammatory, growth inhibitory, and pro-apoptotic, the effects of wedelolactone on stem cell differentiation remain largely unknown. Here, we report that wedelolactone inhibits the adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells (hAMSCs). Wedelolactone reduced the formation of lipid droplets and the expression of adipogenesis-related proteins, such as CCAAT enhancer-binding protein-α (C/EBP-α), peroxisome proliferator-activated receptor-γ (PPAR-γ), lipoprotein lipase (LPL), and adipocyte fatty acid-binding protein aP2 (aP2). Wedelolactone mediated this process by sustaining ERK activity. In addition, inhibition of ERK activity with PD98059 resulted in reversion of the wedelolactone-mediated inhibition of adipogenic differentiation. Taken together, these results indicate that wedelolactone inhibits adipogenic differentiation through ERK pathway and suggest a novel inhibitory effect of wedelolactone on adipogenic differentiation in hAMSCs. J. Cell. Biochem. 113: 3436–3445, 2012.

KEY WORDS: ADIPOGENESIS; MESENCHYMAL STEM CELLS; WEDELOLACTONE

Wedelolactone (Wed, Fig. 1A), derived from *Eclipta prostrata* and *Wedelia calendulacea*, is a member of the coumestans, which belongs to the flavonoids category of phytoestrogens [Mors et al., 1989]. Wedelolactone has been reported to exhibit diverse pharmacological effects, such as antihepatotoxic and antiandrogenic activities [Wagner et al., 1986; Lin et al., 2007]. Recently, several studies have demonstrated that wedelolactone also inhibits NF-κB-mediated gene transcription by blocking the phosphorylation of IκBα and suppresses the polymerase activity of hepatitis C virus (HCV) NS5B [Kobori et al., 2004; Kaushik-Basu et al., 2008]. Although the cellular effects of wedelolactone have been well studied, the possible effects of wedelolactone on differentiation of stem cell have not been investigated.

Mesenchymal stem cells (MSCs) are multipotent cells that can be isolated from several different tissues, including adipose tissue, bone marrow, and fetal tissue. Under specific conditions, MSCs can differentiate into adipocytes, osteoblasts, and chondrocytes [Liu et al., 2009]. Because of their differentiation capacity, MSCs have potential therapeutic application in tissue engineering and regenerative medicine. For example, in a Phase I clinical trial,

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Seyoung Lim and Hyun-Jun Jang equally contributed to this study.

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human adipose tissue-derived mesenchymal stem cells (hAMSCs) were transplanted to heal Crohn's fistula [Garcia-Olmo et al., 2005]. However, the molecules that regulate and determine the differentiation of MSCs into specific lineages are still undetermined. Therefore, identification of the novel molecules that regulate the differentiation of MSCs is essential for the clinical application of MSCs. Interestingly, recent studies have reported that various natural and synthetic small molecules have abilities to control stem cell differentiation [Emre et al., 2007; Schugar et al., 2008]. Because such small molecules can regulate stem cell differentiation without cellular toxicity, many studies have been performed to identify novel molecules from diverse natural products or synthetic chemicals that regulate the fate of stem cells.

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that are activated by dual phosphorylation of both a tyrosine and threonine residue [Seger and Krebs, 1995; Robinson and Cobb, 1997]. In mammalian cells, MAPKs signal pathways have been demonstrated to have a crucial role in many cellular events. There are at least three distinctly regulated groups of MAPKs: extracellular signal-related kinases (ERK), Jun N-terminal kinases (JNK), and p38 MAPKs (p38). In the case of adipogenic differentiation, ERK activation sufficiently inhibits fat cell differentiation by suppressing the activation of proliferator-activated receptor-γ (PPAR-γ), which is essential for adipogenesis [Hu et al., 1996; Camp and Tafuri, 1997]. For example, hyperglycemic or hypoxic conditions modulate adipogenesis via regulation of ERK activity [Zhou et al., 2005; Chuang et al., 2007]. Recently, it was reported that expression of microRNA-375 promotes fat cell differentiation by regulating ERK phosphorylation [Ling et al., 2011]. These results show that ERK has a significant role in the regulation of adipogenic differentiation.

In this study, we explored the effects of wedelolactone on differentiation of stem cell. Specifically in hAMSCs, wedelolactone-inhibited adipocyte differentiation in an estrogenic- and androgenic-activity independent manner. In addition, we show that treatment of hAMSCs with PD98059, an ERK inhibitor, restored wedelolactone-mediated suppression of adipogenesis in a dose-dependent manner. Here, we show the novel effect of wedelolactone on adipogenic differentiation of hAMSCs, thus we conclude that wedelolactone is a promising prognostic molecule for use in obesity therapy.

MATERIALS AND METHODS

MATERIALS

Wedelolactone (7-methoxy-5,11,12-trihydroxy-coumestan) was purchased from Biomol (Plymouth Meeting, PA). Oil Red O, dexamethasone, 3-isobutylmethylxanthine (IBMX), rosiglitazone, indomethacin, insulin, methyl thiazolyl tetrazolium (MTT), 17β-estradiol, testosterone, tamoxifen, and flutamide were purchased from Sigma–Aldrich (St. Louis, MO). The anti-PPAR-γ, anti-aP2, anti-C/EBP-α, and anti-ERK2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-p-JNK and anti-p-ERK1/2 antibodies were purchased from Cell Signaling Technologies (Beverly, MA). The anti-β-actin antibody was obtained from MP Biomedicals, Inc. (Aurora, OH). The SB203580, the SP600125 and the PD98059 were obtained from Calbiochem (La Jolla, CA).

CELL CULTURE

Subcutaneous adipose tissues were obtained from individuals undergoing elective surgery after obtaining informed consent from each individual. The individuals included a 50-year-old female (Donor 1), a 39-year-old female (Donor 2), and a 28-year-old female (Donor 3). hAMSCs were isolated from adipose tissues, according to the methods described in a previous report [Lim et al., 2011]. hAMSCs were cultured in growth medium (GM), consisting of α-MEM supplemented with 10% FBS, 100 μg/ml of streptomycin, and 100 units/ml of penicillin, at 37°C in 5% CO_2 humidified atmosphere. Cell expansion was achieved by successive cycles of trypsinization (0.25% trypsin; Sigma–Aldrich) and re-seeding. Cells were subcultured at a concentration of 2,000 cells/cm^2. The passage number of hAMSCs used in the experiments was 3–10. For transient transfection, MCF-7 cells, a human breast cancer cell line, were maintained in RPMI-1640 supplemented with 10% FBS, 100 μg/ml of streptomycin and 100 units/ml of penicillin at 37°C in 5% CO_2 humidified atmosphere.

ADIPOGENIC DIFFERENTIATION AND OIL RED O STAINING

For adipogenic differentiation, hAMSCs were cultured in GM up to confluence. Adipogenesis was induced by culturing hAMSCs for 14 days in adipogenic induction medium (AIM: 10% FBS, 1 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 100 μM indomethacin in α-MEM) and adipogenic maintenance medium (10% FBS and 1 μM insulin in α-MEM). The medium was changed every 3 days. On 6 days, AIM was replaced with adipogenic maintenance medium. After 14 days of incubation in AIM, Oil Red O staining was used to assess fat droplets within the differentiated adipocytes, according to the methods described in a previous report [Lim et al., 2011]. Images of the cells stained with Oil Red O were obtained with a Zeiss Axiovert 135 microscope (Carl Zeiss, Germany) with an Olympus DP71 CCD camera (Olympus Corporation, Tokyo, Japan). To quantitatively assess Oil Red O staining, the Oil Red O was dissolved in 1 ml isopropanol for 30 min, and the absorbance of the supernatant was measured at 540 nm using a Biotrak II plate reader (Amershan Biosciences, Biochrom Ltd., Cambridge, England). Quantitative Oil Red O values are relative to the mean values obtained from AIM-treated cells, which were set at 100.

RNA EXTRACTION AND REAL-TIME QUANTITATIVE PCR

Total RNA was extracted from hAMSCs using an easy-BLUE Total RNA extraction kit (iNtRON Biotechnology, Korea). cDNA was reverse-transcribed from 1.5 μg of total cellular RNA using Moloney murine leukemia virus reverse transcriptase and oligo (dT) primers (Promega Corp., Madison, WI). cDNA was amplified using the following primers: C/EBP-α 5′-aaccttgtgccttggaaatg-3′ (sense) and 5′-cctgctcccctccttctct-3′ (antisense); PPAR-γ1 5′-atggagtccacgagatcatt-3′ (sense) and 5′-cgcaggctctttagaaactc-3′ (antisense); LPL 5′-ctggacggtaacaggaatgtatgag-3′ (sense) and 5′-catcaggagaaagacgactcgg-3′ (antisense); aP2 5′-aaccttagatgggggtgtcctg-3′ (sense) and 5′-tcgtggaagtgacgcctttc-3′ (antisense); Adiponectin 5′-accactatgatggctccact-3′ (sense) and 5′-ggtgaagagcatagccttgt-3′ (antisense); OCN 5′-agagtccagcaaaggtgcag-3′ (sense) and 5′-tcagccaactcgtcacagtc-3′ (antisense); BSP 5′-caacagcacagaggcagaaa-3′ (sense) and

5′-ttgtggtggggttgtaggtt-3′ (antisense); β-catenin 5′-gcttggaatgagactgctga-3′ (sense) and 5′-ctggccatatccaccagagt-3′ (antisense); and, ribosomal protein large P0 (RPLP0), used as the reference gene, 5′-ggaatgtgggctttgtgttc-3′ (sense) and 5′-tgcccctggagattttagtg-3′ (antisense). For real-time qPCR, cDNA (100 ng) was amplified with a One-Step SYBR RT-PCR kit using a Light Cycler 2.0 PCR system (Roche Diagnostics, Mannheim, Germany). PCR conditions consisted of a 10 min hot start at 95°C, followed by 45 cycles of 15 s at 95°C, 10 s at 60°C and 30 s at 72°C. C_t values were normalized to C_t values from RPLP0 and visualized as relative mRNA levels. The mean values obtained from vehicle-treated cells at day 0 were expressed as 1.0, and other values were expressed relative to the mean values obtained from vehicle-treated cells at day 0. For each sample, three experiments were performed.

WESTERN BLOT ANALYSIS

Cells were washed in cold ice PBS buffer, and lysed in lysis buffer (150 mM NaCl, 50 mM HEPES, pH 7.3, 1 mM EGTA, 1% Triton X-100, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride, and 10% glycerol). Lysates were centrifuged at 14,000*g* for 10 min at 4°C. Total proteins were resolved using 8% SDS–polyacrylamide gels, and electro-transferred to nitrocellulose membranes. Membranes were immunoblotted with antibodies specific for the indicated proteins and subsequently with the appropriate peroxidase-conjugated secondary antibody. And blots were detected using the enhanced chemiluminescence system (ECL system; GE Healthcare Life Sciences).

PLASMIDS AND LUCIFERASE ACTIVITY ASSAY

The 3xERE-LUC plasmid was used to analyze the estrogenic activity of wedelolactone, which contains three copies of the estrogen-response element (ERE). The 3xERE-LUC construct was received from Dr. Vickie S. Wilson [Wilson et al., 2004]. MCF-7 cells were plated in 24-well plate and cultured for 24 h before transfection. Then, cells were co-transfected with the 3xERE-LUC (100 ng) and the pRL-SV-40 (25 ng), a *Renilla* luciferase expression vector used as an internal control plasmid, using Lipofectamine transfection reagent (invitrogen) according to the manufacturer's protocol. After 12 h of transfection, the cells were incubated for an additional 36 h, followed by treatment with 100 nM estradiol or wedelolactone. The activities of luciferase were measured using the Dual-Luciferase® Reporter Assay System according to the manufacturer's instructions (Promega). To analyze the androgenic activity of wedelolactone, the luciferase assay was modified: the 3xERE-LUC was replaced by the human androgen receptor expression vector, pCMV5-hAR [O'Mahony et al., 2008] and pGL-ARE4-Luc [Kim et al., 2010b]. The pGL-ARE4-Luc contains four copies of the androgen response element (ARE). To correct for the transfection efficiency, the relative luciferase activity was calculated by normalizing *firefly* luciferase activity to *Renilla* luciferase activity.

MTT ASSAY

hAMSCs were plated at a concentration of 1×10^4 cells/well in a 96-well plate and cultured for 24 h. The cells were mock cultured or cultured with either 1 μM wedelolactone or 5 μM wedelolactone for 24, 48, and 72 h under adipocyte differentiation conditions. The filtered MTT solution (5 mg/ml MTT in 1× PBS) was added to the culture medium to a final concentration of 0.5 mg/ml. After 2 h of incubation at 37°C for 2 h. Dark brown formazan crystals were dissolved in DMSO, and the absorption values were measured at 540 nm.

STATISTICAL ANALYSIS

All data were representative of at least three independent experiments. Quantitative data are shown as the mean ± SEM, and data were analyzed by one- or two-way ANOVA. Specifically, the relationship between the doses of wedelolactone and Oil Red O extraction, the relationship between doses of PD98059 and Oil Red O extraction, and the relationship between doses of PD98059 and aP2 expression were all analyzed by Pearson's correlation coefficients and linear regression analysis. Values of $P < 0.05$ and $P < 0.01$ were considered as significant, and "ns" indicates that no significant difference, $P > 0.05$, was observed.

RESULTS

WEDELOLACTONE INHIBITS LIPID DROPLET FORMATION IN hAMSCs

In this study, we investigated the role of wedelolactone (Fig. 1A) in adipogenesis of hAMSCs. To examine the effects of wedelolactone on adipocyte differentiation, hAMSCs were grown in GM or grown in AIM for 14 days, both in the absence (lane 2) and in the presence of a range of concentrations of wedelolactone. To assay for adipogenesis, Oil Red O staining was used to detect lipid droplets. Cells grown in GM did not accumulate lipid droplets (Fig. 1B, lane 1). In contrast, cells exposed to AIM-accumulated lipid droplets (Fig. 1B, lane 2); however, wedelolactone significantly decreased the formation of lipid droplets, in a dose-dependent manner (Fig. 1B, lanes 3–5). Wedelolactone elicited a similar reduction in the formation of lipid droplets in hAMSCs derived from different donors and 3T3-L1 pre-adipocytes (Fig. 1B and Supplementary Fig. 1). To confirm these results, the neutral lipid content of these cells was quantitatively measured by Oil Red O extraction. As shown in Figure 1C, the results indicate a significant negative association between the concentration of wedelolactone and the absorbance of extracted Oil Red O (Pearson correlation coefficient $r = -0.84$, $y = -6.9258x + 94.557$, $R^2 = 0.9098$). The absorbance of extracted Oil Red O was significantly reduced, to 61% in the presence of 5 μM wedelolactone, compared with the absorbance in the absence of wedelolactone. These results indicate that wedelolactone suppresses adipogenic differentiation in hAMSCs. We also performed an MTT assay with the wedelolactone under differentiation condition to rule out the possible toxic effects. The MTT assay indicated that 1 or 5 μM of wedelolactone does not affect cell viability in the AIM conditions (Fig. 1D). Taken together, these results suggest that wedelolactone suppresses adipogenic differentiation without cytotoxicity.

WEDELOLACTONE REDUCES mRNA AND PROTEIN EXPRESSION OF ADIPOGENIC GENES

Adipogenesis is accompanied by an increased expression of adipogenic transcription factors and adipocyte-specific genes

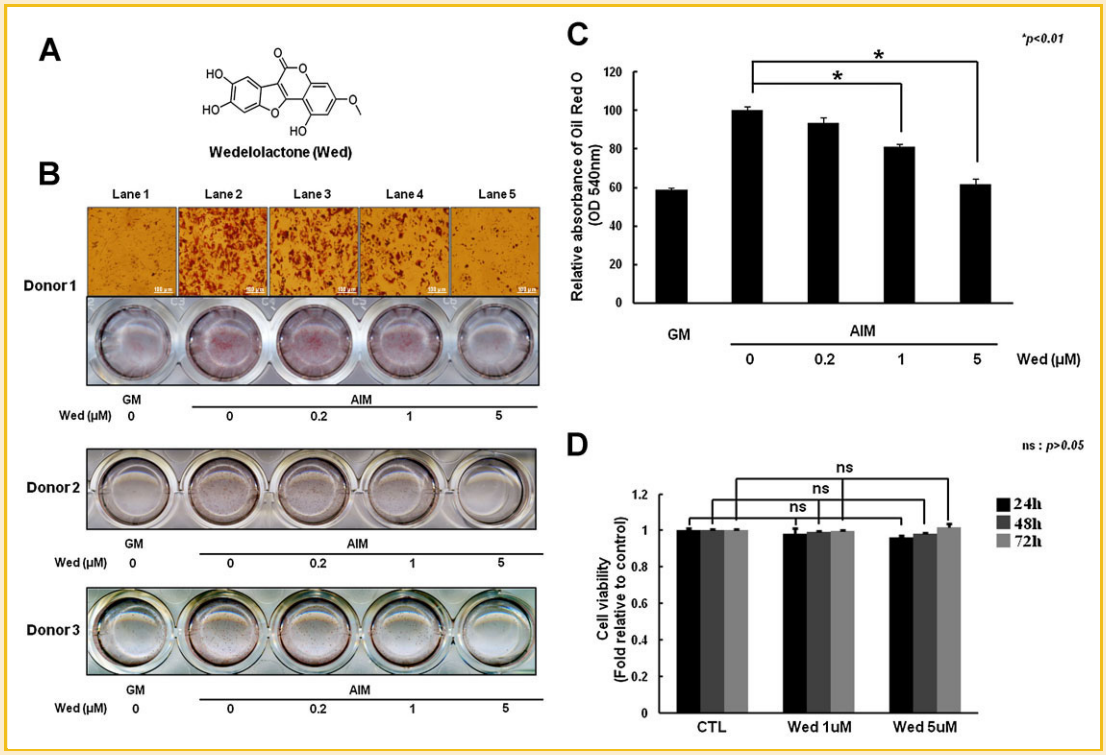


Fig. 1. Wedelolactone inhibits lipid droplets formation in hAMSCs. A: The chemical structure of wedelolactone (Wed). B,C: Post-confluent hAMSCs were cultured in GM or in AIM with various doses of wedelolactone. Media was changed every 3 days. On day 14, cells were stained with Oil Red O. Red staining indicates the presence of neutral lipids. Images are representative of experiments performed in triplicate. For quantitative analysis of Oil Red O staining, the Oil Red O was dissolved in isopropanol. D: Post-confluent hAMSCs were cultured in AIM with or without 1 or 5 μM Wed. Cells were harvested at the indicated times and an MTT assay was performed. CTL: control, 0 μM Wed. Values represent the mean ± SEM. Magnifications, 100×. $^{*}P < 0.01$, $^{ns}P > 0.05$. The results are representative of three independent experiments.

[Chen et al., 1998]. To further characterize the effects of wedelolactone on adipocyte differentiation, we analyzed the expression levels of adipogenic genes in the absence of wedelolactone or in the presence of wedelolactone. Quantitative real-time PCR analysis indicated that the expression of C/EBP-α and PPAR-γ, adipogenic transcription factors, were upregulated during adipocyte differentiation. In this condition, we found that wedelolactone significantly inhibited expression of C/EBP-α and PPAR-γ. We also determined the mRNA levels of LPL, fatty acid-binding protein (aP2), and adiponectin. Consistently, the mRNA levels of LPL, aP2 and adiponectin were significantly reduced (Fig. 2A). To supplement the mRNA analysis, protein expression was examined by Western blot analysis after 6 days of adipogenic induction, both in the presence and absence of wedelolactone. As shown in Figure 2B, the protein expressions of adipocyte-specific genes (PPAR-γ2, C/EBP-α, and aP2) were dramatically reduced, in a manner that was dependent on the concentration of wedelolactone. These results indicate that wedelolactone inhibits the expression of adipogenic genes.

WEDELOLACTONE SUPPRESSES ADIPOCYTE DIFFERENTIATION IN AN ESTROGEN RECEPTOR-INDEPENDENT AND AN ANDROGEN RECEPTOR-INDEPENDENT MANNER

Wedelolactone belongs to the coumestan group, known to possess estrogenic activity. For instance, coumestrol is found in high levels in legumes and acts as an estrogen agonist. Using reporter gene assays, Takeuchi et al. showed that, at very low concentrations, coumestrol, and genistein strongly activated estrogen receptor α and estrogen receptor β [Takeuchi et al., 2009]. Wedelolactone was also reported to be involved in androgen receptor (AR) activation [Lin et al., 2007]. It has also been reported that both estrogen and androgen reduced adipocyte differentiation via receptor-dependent mechanisms [Okazaki et al., 2002; Singh et al., 2003, 2006; Heim et al., 2004]. Based on these reports, we examined whether wedelolactone suppresses adipogenic differentiation via activation of estrogen and androgen receptors. For this purpose, we used ERE- and ARE-luciferase reporter systems [Wilson et al., 2004; Kim et al., 2010b], and because hAMSCs have low transfection efficiency, we selected the human breast cancer cell line, MCF-7, as our study model. MCF-7 cells are a well-characterized estrogen receptor (ER)-positive cell line and are a useful in vitro model to investigate the role of estrogen in breast cancer [Levenson and Jordan, 1997]. MCF-7 cells, transfected with an androgen receptor expression vector, were also used to study function of the androgen receptor [Szelei et al., 1997]. We transfected MCF-7 cells with either an ERE-luciferase reporter plasmid or an ARE-luciferase reporter plasmid and analyzed the effects of wedelolactone on each receptor's activation. We used 17β-estradiol, an estrogen receptor ligand, and testosterone, an androgen receptor ligand, as positive controls. After 36 h, 17β-estradiol treatment (10 nM) markedly increased estrogen receptor activity (fourfold over absence E2; Fig. 3A). Testosterone

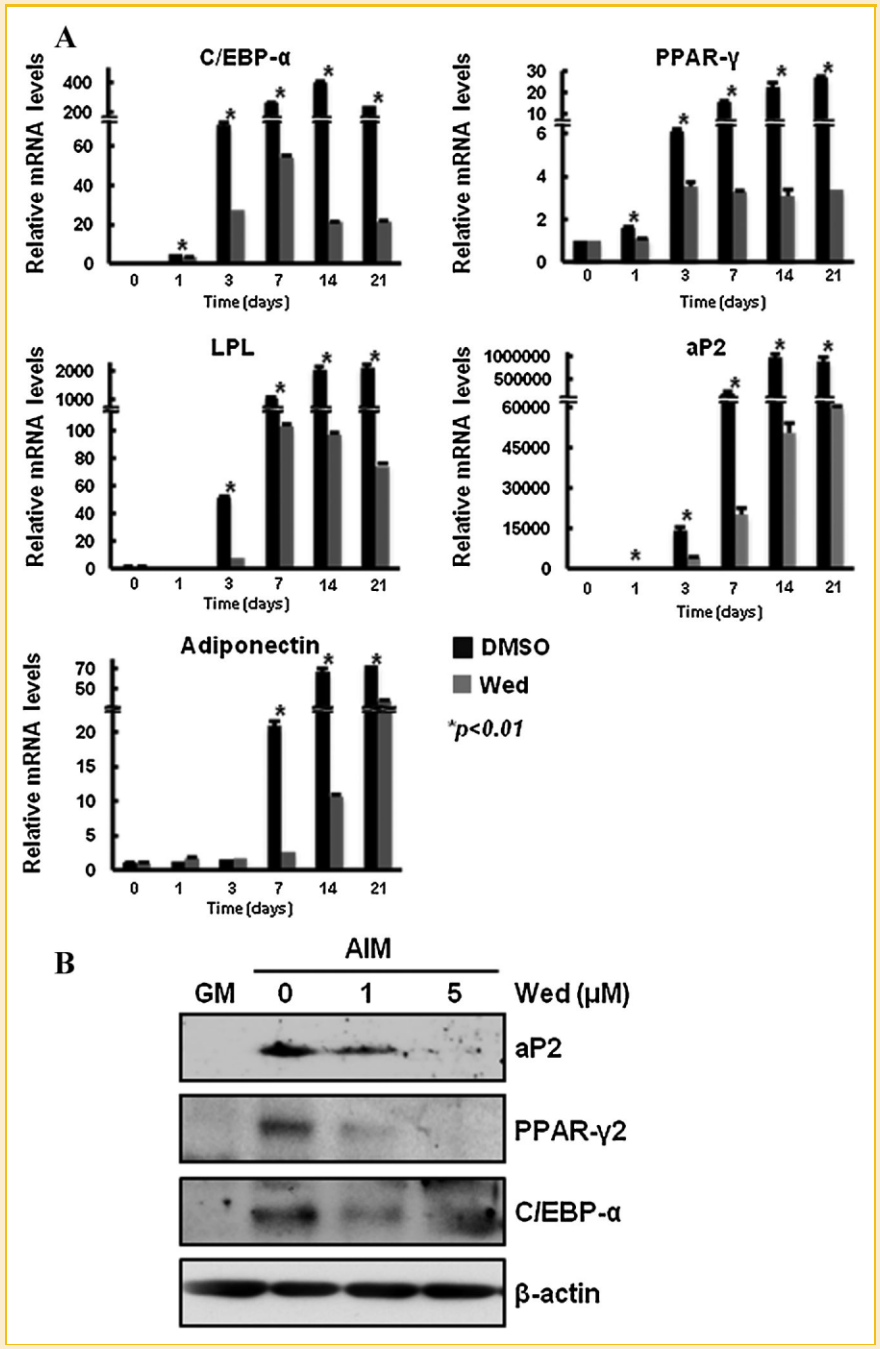


Fig. 2. Wedelolactone reduces mRNA levels and protein expression of adipocyte-specific genes. A: Post-confluent hAMSCs from donor 1 were cultured in AIM, with or without 5 μM wedelolactone (Wed). Cells were harvested at the indicated times. Total RNA was prepared at the indicated times after differentiation, and relative expression levels of C/EBP-α, PPAR-γ, LPL, aP2, and adiponectin mRNA were determined. Expression levels of each mRNA were normalization against the expression of RPLP0. The y-axis is segmented and rescaled. The tilde mark (~) indicates the boundary of segmentation. $^{*}P < 0.01$, values represent the mean ± SEM. B: Post-confluent hAMSCs from donor 1 were cultured in GM or in AIM, with or without 5 μM wedelolactone for 6 days. On day 6, cell lysates were prepared and subjected to immunoblot analysis using specific antibodies against aP2, PPAR-γ, C/EBP-α, and β-actin. The results are representative of three independent experiments.

(10 nM) induced an eightfold increase in androgen receptor activation (Fig. 3B). However, treatment with wedelolactone had neither estrogenic activity nor androgenic activity (Fig. 3A,B). To further confirm whether wedelolactone reduces adipogenesis through the activation of the estrogen and androgen receptor, we used tamoxifen (100 nM), which is an antagonist of estrogen receptors, and flutamide (100 nM), which is an antagonist of androgen receptors. To investigate the effects of tamoxifen and flutamide on the inhibition of adipogenesis by wedelolactone, we examined the effect of wedelolactone on lipid accumulation, under

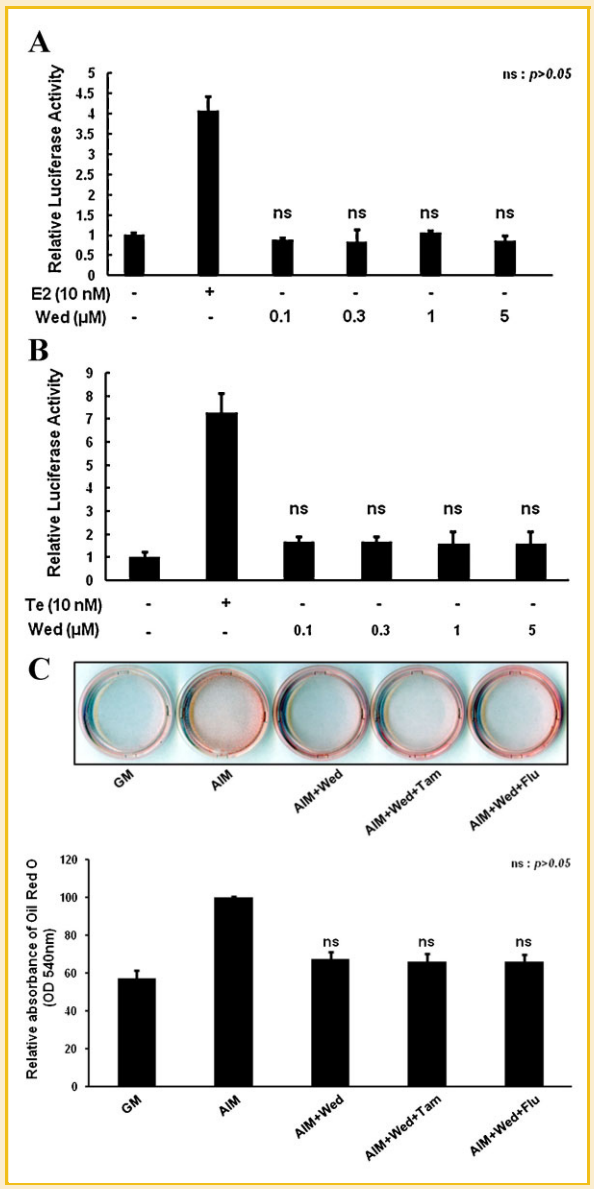


Fig. 3. Wedelolactone suppresses adipocyte differentiation in an estrogen receptor and androgen receptor-independent manner. A: MCF-7 cells were co-transfected with an ERE-luciferase reporter plasmid and a *Renilla* luciferase plasmid. Twelve hours after transfection, cells were treated with 10 nM 17β-estradiol (E2) and the indicated concentrations of wedelolactone (Wed) for 36 h. B: MCF-7 cells were co-transfected with a human androgen receptor expression vector, an ARE-luciferase reporter plasmid and a *Renilla* luciferase plasmid. Twelve hours after transfection, cells were treated with 10 nM testosterone (Te) and the indicated concentrations of wedelolactone for 36 h. Ligand-dependent transcriptional activity was measured using a dual-luciferase assay. Data are presented as the ratio of firefly luciferase activity to *Renilla* luciferase activity. C: Post-confluent hAMSCs from donor 1 were cultured in GM or in AIM with 3 μM wedelolactone (Wed), 100 nM tamoxifen (Tam), and 100 nM flutamide. On day 14, cells were stained with Oil Red O. Red staining indicates the presence of neutral lipids. Images are representative of experiments performed in triplicate. For quantitative analysis of Oil Red O staining, the Oil Red O was dissolved in isopropanol. Values represent the mean ± SEM. $^{ns}P > 0.05$.

AIM conditions, both in the presence and absence of tamoxifen and flutamide. As shown in Figure 3C, wedelolactone-mediated suppression of lipid accumulation was not restored by tamoxifen and flutamide. These data suggest that wedelolactone suppresses adipogenic differentiation through an estrogen receptor-independent and an androgen receptor-independent manner.

WEDELOLACTONE INDUCES SUSTAINED ERK PHOSPHORYLATION UNDER AIM CONDITION

Because wedelolactone did not affect the activation of either the estrogen or androgen receptor, we hypothesized that other signaling pathways might be involved in the ability of wedelolactone to inhibit adipogenesis. Recent studies have shown that several compounds modulate adipogenic differentiation by regulating MAPK activity, highlighting the pivotal roles of MAPKs in adipocyte differentiation [Chen et al., 2007; Fu et al., 2008]. For example, alendronate, which is used in the clinic to treat osteoporosis, has been found to inhibit adipogenic differentiation in bone marrow stromal cells via activation of ERK and JNK. Furthermore, sodium butyrate, a histone deacetylase inhibitor, suppresses adipocyte differentiation by activating ERK. Based on these reports, we assessed whether wedelolactone activated MAPKs in hAMSCs. We report that wedelolactone (5 μM) induced prolonged phosphorylation of ERK, which was sustained up to 12 h, but did not affect the phosphorylation of JNK (Fig. 4). Phosphorylation of p38 was not detected. Therefore, these results suggest that wedelolactone inhibits adipogenesis, possibly via sustained activation of ERK but independent of the activation of other MAPKs.

WEDELOLACTONE PREVENTS ADIPOGENIC DIFFERENTIATION THROUGH ERK PATHWAY

Because adipogenesis is suppressed by activation of ERK, we hypothesized that wedelolactone inhibits adipogenesis through ERK pathway. Mitogen-activated protein kinase kinase (MEK) is an upstream kinase of ERK and activation of ERK is prevented by blocking MEK activity [Crews et al., 1992]. To determine whether wedelolactone inhibits adipogenesis via ERK, we used the specific inhibitor of MEK PD98059. AIM-induced phosphorylation of ERK1/

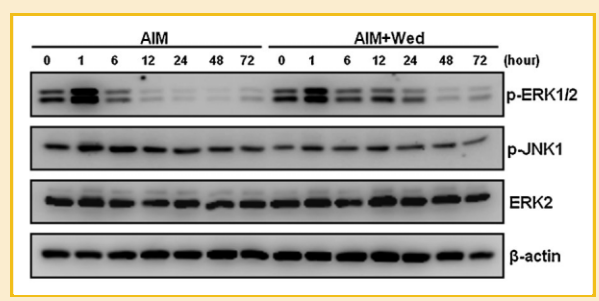
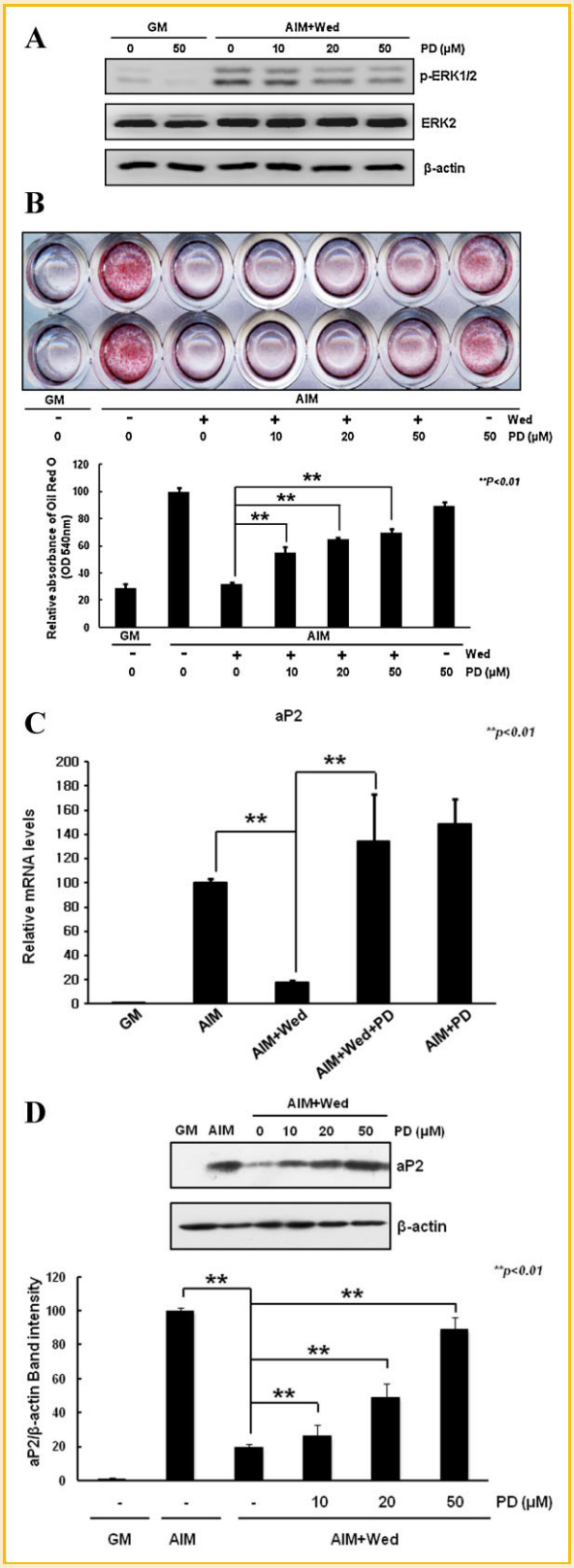


Fig. 4. Wedelolactone induces sustained ERK phosphorylation under AIM condition. hAMSCs from donor 1 were treated with AIM or AIM containing 5 μM wedelolactone, and cell lysates were prepared at different time points, as indicated. The phosphorylation of ERK1/2, of JNK1, ERK2, and β-actin proteins were monitored using Western blot analysis. β-Actin was used as the loading control. The results are representative of three independent experiments.



2 was significantly inhibited by pretreatment of hAMSCs with PD98059 (Fig. 5A); thus, we induced adipocyte differentiation in both the presence and absence of wedelolactone and PD98059. Co-treatment with PD98059 reversed wedelolactone-mediated inhibition of lipid accumulation in a dose-dependent manner (Fig. 5B, upper panel), indicating that wedelolactone prevents adipogenic differentiation of hAMSCs via ERK pathway. The absorbance of extracted Oil Red O, in the presence of PD98059 (50 μM), was recovered to 70% of it found in normal differentiation conditions (Fig. 5B, lower panel). To further confirm the essential roles of ERK in wedelolactone-mediated suppression of adipogenic differentiation, we assessed the mRNA levels of adipogenic markers. Quantitative real-time PCR analysis showed that treatment with 50 μM PD98059 rescued wedelolactone (5 μM)-induced reduction of aP2 (Fig. 5C). The expression levels of aP2 protein were also rescued to 89% of the normal protein levels in the differentiation control (AIM; Fig. 5D), illustrating a significant positive association between PD98059 treatment and recovery from inhibition by wedelolactone (Pearson correlation coefficient $r = 0.83$, $y = 1.4367x + 17.342$, $R^2 = 0.9843$). Taken together, our data clearly show that ERK plays an essential role in inhibition of adipogenesis by wedelolactone.

DISCUSSION

It has been reported that wedelolactone is antihemorrhagic and antiproteolytic, and it has antiphospholipase activity [Mors et al., 1989]; but, the effects of wedelolactone on differentiation of stem cell have not been studied. In this study, we utilized hAMSCs to investigate the possible effects of wedelolactone on adipogenesis. Our results indicate that wedelolactone inhibits adipocyte differentiation, as measured by Oil Red O staining and by RT-PCR analysis of adipocyte-specific markers. In contrast, the expression of osteogenic marker genes, osteocalcin (OCN), bone sialoprotein (BSP) and

Fig. 5. Wedelolactone prevents adipogenic differentiation through ERK pathway. A: hAMSCs from three donors were pre-treated with vehicle or the indicated concentrations of PD98059 for 1 h before the addition of AIM containing 5 μM wedelolactone. At 30 min after treatment of wedelolactone, the phosphorylation of ERK1/2 was monitored by Western blot analysis. ERK2 and β-actin were used as loading controls. The results are representative of three independent experiments. B: For 14 days postconfluent hAMSCs from three donors were cultured in AIM, with or without 5 μM wedelolactone, and in the absence or in the presence of PD98059 at the indicated concentrations. PD98059 was added to the individual cultures 1 h prior to adipogenic induction. Red staining indicates the presence of neutral lipids. Values represent the mean ± SEM. C: On day 3, the relative levels of aP2 mRNA were determined using quantitative real-time PCR. Expression levels of each mRNA were normalization against the expression of RPLP0. Values represent the mean ± SEM. D: Post-confluent hAMSCs from three donors were cultured in GM or in AIM, with or without 5 μM wedelolactone, and in the absence or in the presence of PD98059 at the indicated concentrations for 6 days. On day 6, cell lysates were prepared and subjected to immunoblot analysis using anti-aP2 antibody. The relative signal intensities for aP2 (normalized by β-actin) were quantified using Multi Gauge V3.1 software. Values represents the mean ± SEM. $^{**}P < 0.01$. The results are representative of two independent experiments.

β-catenin, were slightly or not influenced by wedelolactone (Supplementary Fig. 2). Therefore, under adipogenic conditions, wedelolactone is not sufficient to drive cells into osteoblastic differentiation. The inhibitory effects of wedelolactone require ERK activation, independently of estrogen receptor and androgen receptor activation. We provide, to our knowledge, the first evidence that wedelolactone regulates adipocyte differentiation of hAMSCs via ERK signaling pathway.

Phytoestrogens are a group of chemicals found in plants, such as vegetables, fruits, and seeds, which exhibit estrogen-like activities. The major phytoestrogen groups are isoflavones, flavones, coumestans, and lignans. Interestingly, recent data have shown that several phytoestrogens inhibit adipocyte differentiation. Genestein, an isoflavone derived from soybeans, has been shown to repress adipogenic differentiation via an estrogen receptor-dependent mechanism [Kuiper et al., 1997; Heim et al., 2004]. Apigenin, a flavone derived from several herbs, decreases adipocyte differentiation of mouse 3T3-L1 preadipocytes [Phrakonkham et al., 2008]. Wedelolactone is a member of the coumestan group of phytoestrogens, which is known to possess estrogenic activity. However, the mechanism for how wedelolactone affects estrogen receptor-dependent signaling, with respect to the binding and transactivation potential for estrogen receptors, is not well defined. In this study, we demonstrate that wedelolactone does not have estrogenic activity. Furthermore, we observed in our model system that compared to genistein, wedelolactone potently suppressed adipocyte differentiation (data not shown). Therefore, our data suggest that unlike genistein and coumestrol, wedelolactone inhibits adipogenesis via an estrogen receptor-independent manner.

Androgen has been reported to inhibit adipogenesis of preadipocytes and of pluripotent cells via the androgen receptor [Singh et al., 2003; Singh et al., 2006; Gupta et al., 2008]. Recently, it has been shown, utilizing transgenic mice with targeted androgen receptor overexpression in mesenchymal precursor cells, that androgen influences body composition, reducing the amount of adipose tissue and the quantity of progenitor cells in vivo [Semirale et al., 2011]. In the androgen receptor transgenic mice, it was reported that proliferating progenitor cells exhibited elevated expression of C/EBP-α and PPAR-γ; yet neither a corresponding increase in target gene expression nor a differential expression of C/EBP-α and PPAR-γ was observed in confluent cultures. Interestingly, in contradiction to the reported anti-adipogenic effects of wedelolactone, wedelolactone was reported to inhibit androgen receptor activity in the presence of 5α-dihydrotestosterone [Lin et al., 2007]. Given these previous results, we sought to determine whether wedelolactone could induce androgen receptor activation in the absence of androgen. We report that wedelolactone does not exhibit androgenic activity (Fig. 3B); furthermore, androgen receptor antagonists do not rescue the ability of wedelolactone to inhibit adipogenesis (Fig. 3C). Thus, in our post-confluent differentiation model, the ability of wedelolactone to inhibit adipogenesis is not associated with androgenic activity.

By modulating PPAR-γ activity, which is required for adipocyte differentiation, ERK has been shown to be a key regulator of adipogenesis. ERK can suppress the transcriptional activity of PPAR-γ by direct phosphorylation, thereby inhibiting adipocyte differentiation [Hu et al., 1996; Camp and Tafuri, 1997]. Interestingly, recent reports have shown that several molecules, including endogenous peptides and natural compounds, can suppress adipogenesis by activating the ERK signaling pathway. For example, in preadipose cells derived from human omental adipose tissue, Fuentes et al. [2010] showed that ERK is involved in the anti-adipogenic effects of angiotensin II. In addition, evodiamine, a major alkaloid compound found in *Evodia fructus*, was found to prevent adipocyte differentiation by sustaining the activation of ERK in 3T3-L1 cells [Wang et al., 2008]. Because wedelolactone-induced prolonged activation of ERK in hAMSCs (Fig. 4), we speculated that activation of ERK is essential for wedelolactone-mediated suppression of adipogenesis. In this regard, we observed that blocking the ERK pathway rescued formation of lipid droplets and aP2 expression of hAMSCs, even in the presence of wedelolactone (Fig. 5B–D). Therefore, these results strongly suggest that the ERK pathway is primarily responsible for the inhibition of adipogenic differentiation by wedelolactone. In addition to ERK, JNK is known to negatively regulate adipogenesis by direct phosphorylation of PPAR-γ [Camp et al., 1999]. SP600125, a JNK inhibitor, was shown to promote adipogenesis in human MSCs [Tominaga et al., 2005]. However, we observed that wedelolactone did not activate JNK under AIM conditions (Fig. 4) and that SP600125 did not block the inhibition of adipogenesis by wedelolactone (data not shown). Consequently, it is plausible that wedelolactone suppresses adipogenesis specifically in an ERK-dependent manner. The precise mechanisms for how wedelolactone induces prolonged ERK activation in hAMSCs are under investigation.

Obesity is associated with chronic diseases, such as diabetes mellitus, hypertension, stroke, and heart diseases. Because obesity results from an excess of adipose tissue, controlling the formation of adipose tissue, which results from both the differentiation of preadipocytes and of stem cells into adipocytes and from the hypertrophy of adipocytes, is crucial for the treatment of obesity. Because suppressing adipogenesis is an attractive strategy for obesity therapy, many studies have been performed to identify molecules that regulate fat cell differentiation [Harp, 2004]. To date, several natural compounds have been identified that inhibit adipocyte differentiation. For example, genistein and daidzain, the major soy isoflavones, prevent adipogenic differentiation via wnt signaling or lipolysis [Kim et al., 2010a]. Berberine, a compound purified from *Cortidis rhizoma*, also suppresses adipocyte differentiation via inhibiting PPAR-γ activity [Huang et al., 2006]. In addition, by regulating adipogenesis processes, Baicalin, which is derived from *Scutellariae radix*, inhibits formation of lipid droplets in 3T3-L1 preadipocytes [Lee et al., 2009]. In this study, we demonstrated that wedelolactone reduces adipocyte differentiation in hAMSCs (Figs. 1 and 2). These results strongly suggest that wedelolactone may have novel utility in obesity therapy by controlling fat mass production through the regulation of adipogenic differentiation.

In summary, we investigated the effects of wedelolactone on the in vitro differentiation of hAMSCs into adipocytes. Our data indicate that wedelolactone inhibits adipogenesis via activation of the ERK

pathway. These results illustrate a novel role for wedelolactone in stem cell differentiation, specifically in adipogenesis, suggesting that wedelolactone might affect the formation of adipose tissue. Further investigations will be required to assess the effects of wedelolactone on adipose tissue formation in vivo.

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