Guggulsterone Inhibits Adipocyte Differentiation and Induces Apoptosis in 3T3-L1 Cells

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Objective: To determine the effects of guggulsterone (GS), the active substance in guggulipid, on apoptosis, adipogenesis, and lipolysis using 3T3-L1 cells.

Methods and Procedures: For apoptosis and lipolysis experiments, mature adipocytes were treated with GS isomers. Viability, apoptosis, and caspase 3/7 activation were quantified using MTS, enzyme-linked immunosorbent assay (ELISA), caspase-Glo 3/7 activity assay, respectively. The expression of cytochrome *c* was demonstrated by western blot. Lipolysis was quantified by measuring the release of glycerol. For adipogenesis experiments, postconfluent preadipocytes were incubated with GS isomers for up to 6 days during maturation. Adipogenesis was quantified by measuring lipid content using Nile Red dye. Western blot was also used to demonstrate the adipocyte-specific transcription factors peroxisome proliferator–activated receptor γ2 (PPARγ2), CCAAT/enhancer binding protein α (C/EBPα), and C/EBPβ.

Results: In mature adipocytes *cis*-GS decreased viability, whereas the *trans*-GS isomer had little effect. Both isomers caused dose-dependent increases in apoptosis and *cis*-GS was more effective than *trans*-GS in inducing apoptosis. *cis*- and *trans*-GS also increased caspase-3 activity and release of cytochrome *c* from mitochondria. In maturing preadipocytes, both isomers were equally effective in reducing lipid content. The adipocyte-specific transcription factors PPARγ2, C/EBPα, and C/EBPβ were downregulated after treatment with *cis*-GS during the maturation period. Furthermore, *cis*-GS increased basal lipolysis of mature adipocytes, but *trans*-GS had no effect.

Discussion: These results indicate that GS isomers may exert antiobesity effects by inhibiting differentiation of preadipocytes, and by inducing apoptosis and promoting lipolysis of mature adipocytes. The *cis*-GS isomer was more potent than the *trans*-GS isomer in inducing apoptosis and lipolysis in mature adipocytes.

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INTRODUCTION

The gum resin of the tree *Commiphora mukul*, has been used in Ayurvedic medicine to treat a variety of ailments, including obesity, arthritis, inflammation, and lipid disorders (1). The active compounds in this resin are the *cis* and *trans* isomers of guggulsterone (GS) (4,17(20)-pregnadiene-3,16-dione) (2). The most studied effects of gum guggul and its component GSs are the lipid and cholesterol lowering effects. Early *in vivo* studies showed that oral administration of gum guggul decreased the serum cholesterol level in hypercholesterolemic rabbits (3). More recent studies have confirmed the clinical effect and have shown that this activity is at least partly due to the antagonism of nuclear farnesoid X receptors (FXRs) by GS ((4,5); also reviewed in (1)).

An Ayurvedic formulation containing gum guggul is also used for treatment of hyperlipidemia and obesity (1,3,6), and in one study oral administration of gum guggul alone was shown to decrease body weight in both animals and humans (2), although other studies have shown inconsistent effects (7). Recently, however, GS was shown to reverse the promotion of adipogenesis by FXR activation in 3T3-L1 preadipocytes (8). Thus, there may be a biochemical basis for the claimed anti-obesity effects of GS.

We have studied a variety of natural compounds for their effects on the life cycle of the adipocyte. For example, we have shown that ajoene, a compound from garlic, is a potent inducer of apoptosis in mature 3T3-L1 adipocytes (9). Esculetin, a coumarin compound, both induced apoptosis and inhibited adipogenesis in maturing preadipocytes (10). Genistein, an isoflavone from soy, induced apoptosis of adipocytes *in vitro*, and when fed to ovariectomized female mice caused weight loss and induced apoptosis of adipose tissue (11). Because

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of the potential antiobesity effect of GS and the involvement of FXR in adipogenesis, we carried out the following experiments to determine the effects of GS on specific phases of the adipocyte life cycle: adipogenesis, lipolysis, and apoptosis in maturing preadipocytes and mature adipocytes. In addition, we investigated some of the biochemical pathways involved in these effects.

METHODS AND PROCEDURES

Cell culture

3T3-L1 mouse embryo fibroblasts were obtained from American Type Culture Collection (Manassas, VA) and cultured as described elsewhere (12). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) containing 10% bovine calf serum until confluent. Two days after confluency (D0), the cells were stimulated to differentiate with DMEM containing 10% fetal bovine serum (FBS), 167 nmol/l insulin, 0.5 µmol/l IBMX, and 1 µmol/l dexamethasone for 2 days (D2). Cells were then maintained in 10% FBS/DMEM with 167 nmol/l insulin for another 2 days (D4), followed by culturing with 10% FBS/DMEM for an additional 4 days (D8), at which time >90% of cells were mature adipocytes with accumulated fat droplets. All media contained 100 U/ml of penicillin, 100 µg/ml of streptomycin, and of 292 µg/ml glutamine (Invitrogen, Carlsbad, CA). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Reagents and antibodies

Phosphate-buffered saline (PBS) and DMEM were purchased from GIBCO (BRL Life Technologies, Grand Island, NY). *cis*- and *trans*-GS were purchased from Steraloids (Newport, RI). ApoStrand enzyme-linked immunosorbent assay (ELISA) Apoptosis Detection Kit was purchased from Biomol (Plymouth Meeting, PA). The viability assay kit (CellTiter 96 Aqueous One Solution Cell Proliferation Assay (containing 3-(4,5-dimethythizol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay reagent (MTS)) and Caspase-Glo 3/7 assay kit were purchased from Promega (Madison, WI). AdipoRed assay reagent was purchased from Cambrex (Walkersville, MD). Antibodies specific to polyclonal peroxisome proliferator–activated receptor (PPAR)γ2, CCAAT/enhancer binding proteins (C/EBPs), β-actin, and cytochrome *c* were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific to polyclonal anti-phospho-ERK 1/2 (Thr^{202}/Tyr^{204}) and total extracellular signal-related kinase 1/2 (ERK 1/2) were from Cell Signaling Technology (Beverly, MA).

MTS cell viability assay

Tests were performed in 96-well plates. For mature adipocytes, cells were seeded (5,000 cells/well) and grown to maturation as described above. Adipocytes were incubated with *cis*- and *trans*-GS (times and concentrations indicated in Results and figure legends). Prior to measuring viability, treatment media were removed and replaced with 100 µl fresh 10% FBS/DMEM and 20 µl MTS solution (Promega, Madison, WI). Cells were then returned to the incubator for an additional 2 h before 25 µl of 10% sodium dodecyl sulfate was added to stop the reaction. The absorbance was measured at 490 nm in a plate reader (µQuant Bio-Tek Instruments, Winooski, VT) to determine the formazan concentration, which is proportional to the number of live cells.

Apoptosis assays

For the assessment of apoptosis, the ApoStrand ELISA Apoptosis Detection Kit (Biomol, Plymouth Meeting, PA) was used. This kit detects single-stranded DNA, which occurs in apoptotic cells but not in necrotic cells or in cells with DNA breaks in the absence of apoptosis (13,14). Tests were performed in 96-well plates. For mature adipocytes, cells were seeded (5,000 cells/well) and grown to maturation as described above. Prior to single-stranded DNA ELISA, adipocytes were incubated with *cis*- and *trans*-GS at the times and at the concentrations indicated in the Results and figure legends. Thereafter, the treatment media was removed and the cells were fixed for 30 min and assayed according to the manufacturer's instructions.

Caspase-3/7 activity assay

Tests were performed in 96-well plates. For mature adipocytes, cells were seeded (5,000 cells/well) and grown to maturation as described above. Adipocytes were incubated with *cis*- and *trans*-GS (at the times and concentrations indicated in Results and figure legends). Thereafter, 100 µl of caspase-Glo 3/7 (Promega, Madison, WI) reagent was added to each sample and the cells were incubated for 1 h and assayed according to the manufacturer's instructions.

Oil red O staining

cis- and *trans*-GS along with 0.01% dimethyl sulfoxide control were added along with the induction medium for days 0–2, 2–4, or 4–6 of adipogenesis. The medium was changed every 2 days. On day 6, cells were stained with Oil Red O. In brief, cell monolayers were washed twice with PBS and fixed with 10% formalin in PBS (pH 7.4); cells were then stained with Oil Red O and hematoxylin as described by Suryawan and Hu (15). After mounting with glycerol gelatin, three images for each dish were captured using ImagePro software (MediaCybernetics, Silver Spring, MD).

Quantification of lipid content by Adipored assay

Lipid content was measured using a commercially available kit (AdipoRed assay Reagent; Cambrex Bio Science, Walkersville, MD). AdipoRed, a solution of the hydrophilic stain Nile Red, is a reagent that enables the quantification of intracellular lipid droplets in a high-throughput manner. In brief, *cis*- and *trans*-GS along with 0.01% dimethyl sulfoxide control, were added with the induction medium for days 0–2, 2–4, 4–6, or 0–6 of adipogenesis. The medium was changed every 2 days. On day 6, the intracellular lipid content was measured by AdipoRed assay. Cells were washed with PBS (pH 7.4) and 200 µl of PBS was added to the wells. Five microliters of AdipoRed reagent was added to each well. After 10 min, the plates were placed in the fluorometer and fluorescence was measured with an excitation wavelength of 485 nm and emission wavelength of 572 nm.

Lipolysis assay

Fully differentiated adipocytes (D8) were treated for 24 h with vehicle or *cis*- and *trans*-GS (25 or 50 µmol/l). At the end of the incubation, 100 µl of the conditioned media was removed and transferred to the corresponding well of the new plate. One-hundred microliters of glycerol reagent (Zen-Bio, Research Triangle Park, NC) was added and cells were incubated at room temperature for 15 min. The absorbance was measured at 540 nm in a plate reader (µQuant Bio-Tek Instruments, Winooski, VT) to determine the glycerol content of the sample.

Western blot analysis

To examine the effect of GS on the expression of PPARγ2, C/EBPα, and C/EBPβ, the differentiation of 2-day postconfluent 3T3-L1 preadipocytes was induced by the standard adipogenic medium as described above. *cis*-GS isomer (50 µmol/l) was added during days 0–2 or 0–6 with the standard adipogenic medium. On day 6, cells were washed with PBS and suspended in a lysis buffer (20 mmol/l Tris, pH 7.5, 150 mmol/l sodium chloride, 1 mmol/l EDTA, 1 mmol/l [ethylenebis(oxyethylene nitrilo)]tetraacetic acid, 1% Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l sodium vanadate (Na_3VO_4), 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 100 µg/ml phenylmethylsulfonyl fluoride). After 30 min of rocking at 4 °C, the mixtures were centrifuged (10,000*g*) for 10 min, and the supernatants were collected as whole-cell extracts. To examine the effect of GS on ERK phosphorylation, mature adipocytes were treated with 50 µmol/l *cis*-GS for

24 h. After incubation, cells were washed with ice-cold PBS and whole-cell extracts were collected as described above.

To examine the effect of GS on the expression of cytochrome *c*, mature 3T3-L1 adipocytes were treated with 200 µmol/l *cis*-GS and *trans*-GS isomers for 3, 6, and 12 h. After incubation, cells were washed with ice-cold PBS and resuspended in isotonic homogenizing buffer (250 mmol/l sucrose, 10 mmol/l potassium chloride, 1.5 mmol/l magnesium chloride, 1 mmol/l [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 0.1 mmol/l phenylmethylsulfonylfluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid–KOH, pH 7.4). After 30 min of incubation on ice, cells were homogenized with a glass Dounce homogenizer (30 strokes) and centrifuged at 700*g* for 10 min. The supernatant was collected as the cytosolic fraction and the resulting mitochondrial pellets were dissolved in lysis buffer as described previously. The protein concentration was determined by the method of Bradford (16) with bovine serum albumin as the standard. Western blot analysis was performed using the commercial NUPAGE system (Novex/Invitrogen, Carlsbad, CA), where a lithium dodecyl sulfate sample buffer (Tris/glycerol buffer, pH 8.5) was mixed with fresh dithiothreitol and added to samples. Samples were then heated to 70 °C for 10 min. All cell lysates were separated by 12% acrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline and then incubated with primary antibodies overnight at 4 °C. After washing the membranes, an alkaline-phosphatase–conjugated secondary antibody was added. The target proteins became visible following the addition of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, a substrate of alkaline phosphatase. All experiments were repeated at least two times. Representative western blots are shown along with the graphs of the quantitative data.

Quantitative analysis of western blot data

Measurement of signal intensity on polyvinylidene difluoride membranes after western blotting with various antibodies was performed using a FluorChemdensitomer with AlphaEaseFC image processing and analysis software (Alpha Innotech, San Leandro, CA). For statistical analysis, all data were expressed as integrated density values. For PPARγ2, C/EBPα, and C/EBPβ, integrated density values were calculated as the density values of the specific protein bands/β-actin density values and expressed as a percentage of the control. For phospho-p44 ERK and phospho-p42 ERK, integrated density values were calculated as the density values of the specific protein bands/total ERK density values and expressed as a percentage of the control. For cytochrome *c*, integrated density values are expressed as a percentage of 0 h. All figures showing quantitative analysis include data from at least three independent experiments.

Statistical analysis

One- or two-way ANOVA (GLM procedure, Statistica, version 6.1; StatSoft, Tulsa, OK) was used to determine the significance of treatment effects and interactions. Fisher's post-hoc least significant difference test was used to determine the significance of differences among means. Statistically significant differences are defined at the 95% confidence interval. Data shown are means ± s.e.m.

RESULTS

Effect of *cis*- and *trans*-GS isomers on viability and apoptosis

3T3-L1 mature adipocytes were treated with *cis*- or *trans*-GS isomer at various doses for 12 and 24 h. After treatment, the number of live cells was determined by the MTS assay. As shown in Figure 1a, both isomers caused a reduction in cell viability. *cis*-GS isomer showed stronger dose and time-dependent inhibitory effects on the cell viability than *trans*-GS.

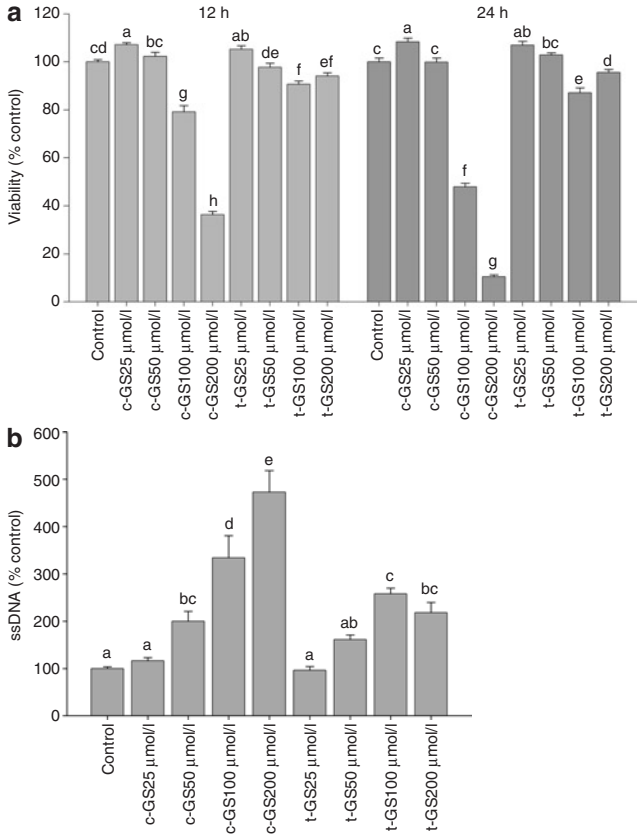

Figure 1 *cis*- and *trans*-GS isomers inhibit cell viability and induce apoptosis. Mature 3T3-L1 adipocytes were incubated with guggulsterone (GS) isomers at various concentrations (0, 25, 50, 100, 200 µmol/l) for 12 or 24 h. (**a**) Cell viability was determined by the MTS colorimetric assay. Assays were performed on eight replicates for each treatment. Within a time period, means that are not denoted with a common letter are different, abcdefgh: $P < 0.05$. (**b**) Cell apoptosis was evaluated by single-stranded DNA (ssDNA) enzyme-linked immunosorbent assay. Assays were performed on eight replicates for each treatment and repeated twice. Within a time period means that are not denoted with a common letter are different; a, b, c, d, and e: $P < 0.05$. c-GS, *cis*-GS.

We next investigated whether the reduction in cell number by both isomers was due to apoptosis, using the single-stranded DNA ELISA as a determinant of cellular apoptosis. As shown in Figure 1b, exposure of adipocytes to *cis*- or *trans*-GS resulted in a dose-dependent induction of apoptosis. At the 200 µmol/l concentration *cis*-GS (373 ± 45.4% increase vs. control) was more effective than *trans*-GS (118 ± 21.6% increase vs. control) in inducing apoptosis. These results indicate that the decrease in viability was due, at least in part, to induction of apoptosis by GS.

Effect of *cis*- and *trans*-GS isomers on cytochrome *c* release

We next examined whether apoptosis induced by both isomers involved the release of cytochrome *c*. 3T3-L1 mature adipocytes were treated with 200 µmol/l *cis*- or *trans*-GS isomers at indicated times. Both isomers increased cytoplasmic cytochrome *c* levels in a time-dependent manner (Figure 2).

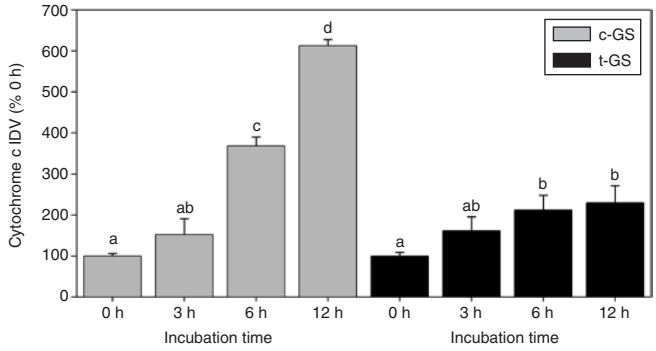

Figure 2 *cis*- and *trans*-GS (t-GS) isomers increase cytochrome *c* release. Mature 3T3-L1 adipocytes were treated with 200 μmol/l *cis*-GS (c-GS) or *trans*-GS isomers for 3, 6, or 12 h. Equal amounts of protein from cytosolic fraction were analyzed by western blotting using specific antibodies. Actin was used as an equal loading control. Densitometric quantitation of the autoradiograms for cytochrome *c* was performed. Integrated density values were calculated and expressed as % 0 h. All experiments were replicated in triplicate. Means that are not denoted with a common letter are different; a, b, c, and d: $P < 0.05$. GS, guggulsterone; IDV, integrated density value.

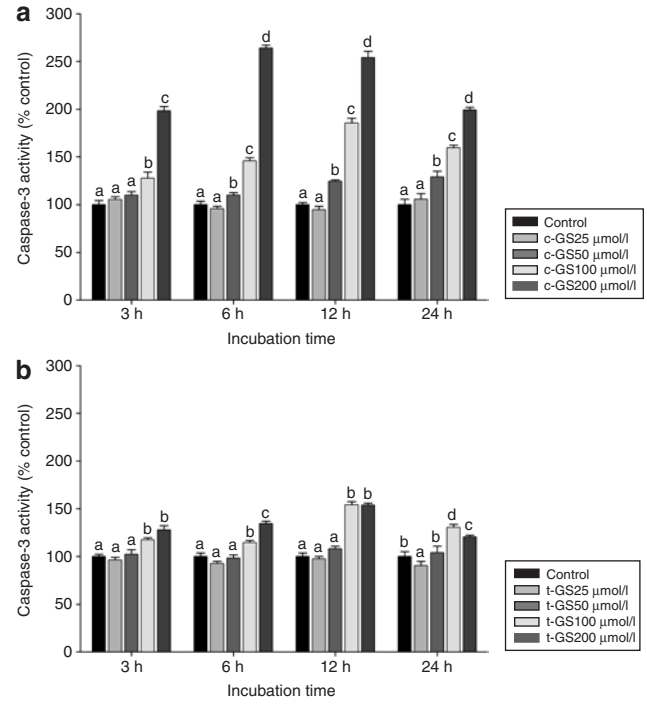

Figure 3 *cis*- and *trans*-GS isomers increase caspase 3/7 activity. 3T3-L1 adipocytes were treated with 200 μmol/l *cis*-GS (c-GS) (**a**) or *trans*-GS (t-GS) (**b**) isomers for 3, 6, 12, or 24 h. Caspase-3 activity was determined by the caspase-Glo 3/7 luminescent assay. Assays were performed on eight replicates for each treatment. Means that are not denoted with a common letter are different; a, b, c, and d: $P < 0.05$. GS, guggulsterone.

The *cis*-GS isomer (513 ± 14.9% increase) was more effective than the *trans*-GS isomer (130 ± 42.2% increase) at 12 h.

Effect of *cis*- and *trans*-GS isomers on caspase-3/7 activation

To determine whether the observed cytochrome *c* release was accompanied by activation of caspase 3/7, we measured the change in caspase 3/7 activity in mature 3T3-L1 adipocytes. Caspase 3/7 activity increased in a time-dependent manner during treatment with either *cis*- or *trans*-GS. After a 12-h incubation of mature adipocytes with 200 μmol/l *cis*-GS (**Figure 3a**), caspase 3/7 was 154 ± 6.4% greater than control ($P < 0.05$), while the increase after treatment with *trans*-GS (**Figure 3b**) was 54 ± 2% greater than control ($P < 0.05$).

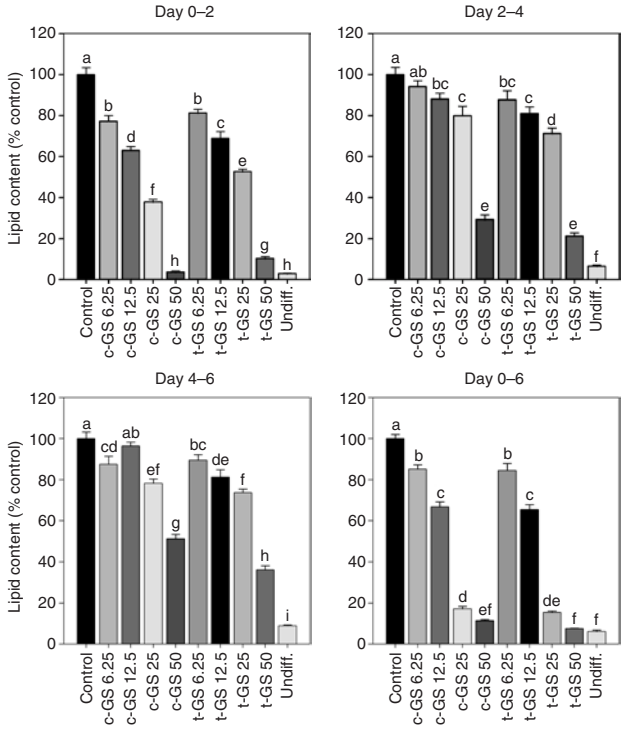

Figure 4 *cis*- and *trans*-GS (t-GS) isomers reduce lipid content during adipocyte differentiation. The differentiation of 2-day postconfluent 3T3-L1 preadipocytes was induced by standard adipogenic medium to initiate adipogenesis as described in "Methods and Procedures" in the absence or presence of *cis*- or *trans*-GS isomers. Cells were incubated with guggulsterone (GS) isomers (6.25, 12.5, 25, 50 μmol/l) during days 0–2, 2–4, or 4–6. On day 6, lipid content was measured by Adipored assay. All assays were performed on eight replicates for each treatment. Means that are not denoted with a common letter are different; a, b, c, d, e, f, g, h, and i: $P < 0.05$. c-GS, *cis*-GS.

Effect of *cis*- and *trans*-GS isomers on differentiation-dependent triacylglycerol accumulation

Results from the AdipoRed assay show that both *cis*- and *trans*-GS reduced lipid content in a dose-dependent manner (**Figure 4**), primarily during days 0–2, although the higher concentrations were effective at all time periods. There was no decrease in viability except during incubation on days 0–6 with the 50 μmol/l concentration of either *cis*-GS (18% reduction; $P < 0.05$) or *trans*-GS (12% reduction; $P < 0.05$) (data not shown). Oil red O staining indicated that 50 μmol/l *cis*-GS treatment for days 0–6 completely inhibited 3T3-L1 lipid accumulation (**Figure 5**) and cells retained the fibroblastic characteristics. Cells treated with 50 μmol/l *cis*-GS for days 0–2 also showed inhibited lipid accumulation with most cells retaining fibroblastic characteristics, while some cells were rounded

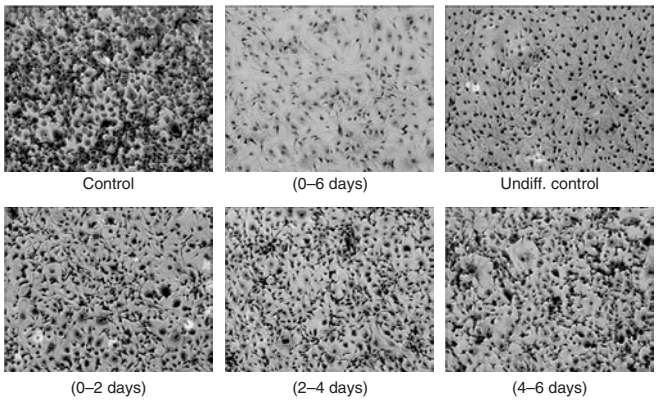

Figure 5 *cis*-GS suppresses lipid accumulation in maturing preadipocytes when added during separate 2-day incubation periods. The differentiation of 2-day postconfluent 3T3-L1 preadipocytes was induced by standard adipogenic medium to initiate adipogenesis as described in Methods and Procedures in the absence or presence of *cis*-GS. Cells were incubated with 50 μmol/l *cis*-GS during days 0–2, 2–4, 4–6, or 0–6. On day 6, cellular triglyceride was stained with Oil red O and cells were photographed at original magnification ×100. The experiment was performed two times. Representative images are shown.

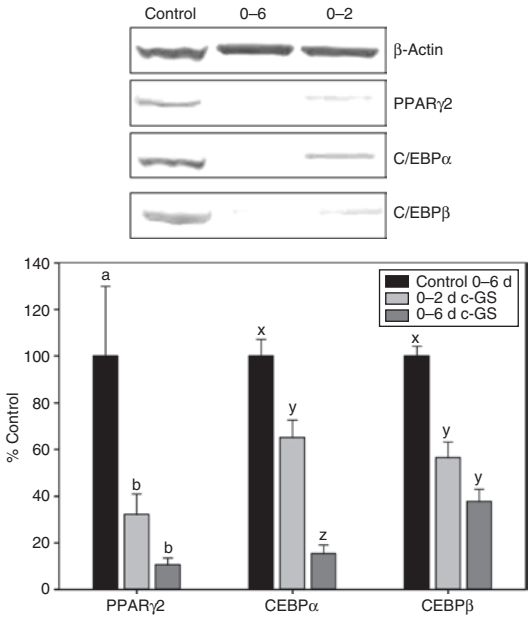

Figure 6 *cis*-GS (c-GS) isomer inhibits adipocyte transcription factors during adipocyte differentiation. The differentiation of 2-day postconfluent 3T3-L1 preadipocytes was induced by standard adipogenic medium to initiate adipogenesis as described in Methods and Procedures in the absence or presence of 50 μmol/l *cis*-GS isomer. Cells were incubated with *cis*-GS during days 0–2 or 0–6. On day 6, cells were lysed, separated on 10% NUPAGE and subjected to immunoblotting to examine the expression levels of peroxisome proliferator–activated receptor γ2 (PPARγ2), CCAAT/enhancer binding protein α (C/EBPα), and C/EBPβ. Actin was used as an internal reference for sample loading control. Densitometric quantitation of the autoradiograms for PPARγ2, C/EBPα and C/EBPβ were performed. Integrated density values were calculated and expressed as % control. All experiments were performed in triplicate. Means that are not denoted with a common letter are different (a and b: $P < 0.05$; x, y, and z: $P < 0.01$). GS, guggulsterone.

and lipid stained. In the group treated with 50 μmol/l *cis*-GS treatment for days 2–4 or 4–6, a number of lipid-containing rounded cells were present, though considerably less than in controls.

Effect of *cis*-GS isomer on adipocyte-specific transcription factors during adipocyte differentiation

Since the adipogenesis data (**Figure 4**) showed that *cis*-GS decreased lipid accumulation to a greater extent at the time points of days 0–2 and days 0–6, we selected these incubation

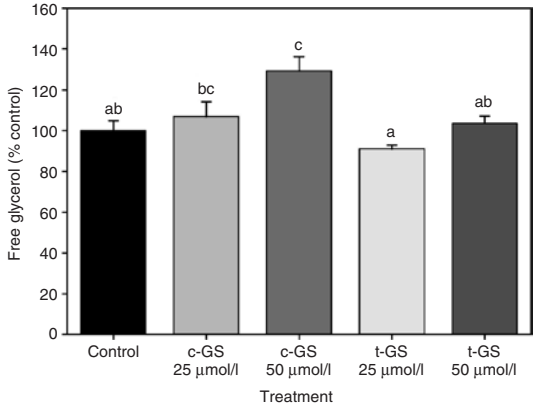

Figure 7 *cis*-GS (c-GS), but not *trans*-GS (t-GS), increases lipolysis. 3T3-L1 mature adipocytes were incubated with GS isomers at various concentrations (0, 25, 50 μmol/l) for 24 h. Values are mean ± s.e.m. ($n = 6$) for glycerol released per well and are presented as percentage of control. All experiments were performed on six replicates for each treatment. Means that are not denoted with a common letter are different; a, b, and c: $P < 0.05$.

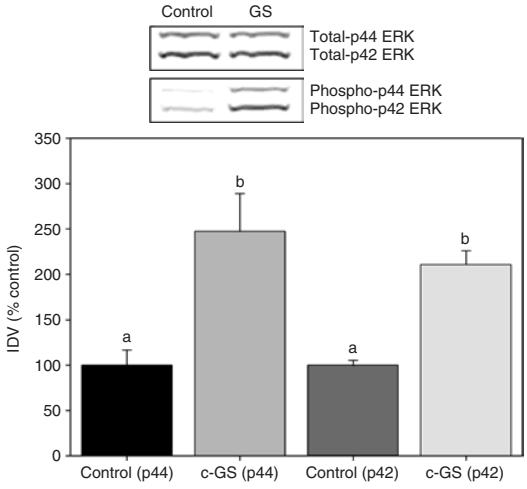

Figure 8 *cis*-GS (c-GS) increases extracellular signal-related kinase (ERK) 1/2 phosphorylation in mature adipocytes. Mature 3T3-L1 adipocytes were treated with 50 μmol/l *cis*-GS isomer for 24 h. The protein levels of total and phosphorylated forms of ERK 1/2 were evaluated in cytosolic proteins by western blotting with the use of specific antibody. Representative western blots show the phosphorylation of ERK 1/2 in the lower panel and the expression levels of the total kinase in the upper panel. All experiments were repeated three times. Means that are not denoted with a common letter are different; a and b: $P < 0.05$. IDV, integrated density value.

times to determine whether this decrease in lipid accumulation was due to downregulation of adipocyte-specific transcription factors. The expressions of PPARγ2, C/EBPα, and C/EBPβ were all significantly downregulated by *cis*-GS (**Figure 6**). Treatment of 3T3-L1 adipocytes during the entire differentiation period (days 0–6) resulted in a lower expression of adipogenic proteins compared to treatments during the differentiation period between days 0 and 2.

Effect of *cis*- and *trans*-GS isomers on lipolysis and ERK phosphorylation

To determine whether GS isomers exert lipolytic effects in mature 3T3-L1 adipocytes, we treated 3T3-L1 adipocytes (D8) with 25 and 50 µmol/l of both isomers for 24 h. As shown in **Figure 7**, *cis*-GS increased basal lipolysis in fully differentiated 3T3-L1 adipocytes compared to the control but *trans*-GS had no significant effect on lipolysis. We next examined whether lipolysis increased by *cis*-GS involved the phosphorylation of ERK 1/2. 3T3-L1 mature adipocytes were treated with 50 µmol/l *cis*-GS for 24 h. *cis*-GS isomer increased ERK 1/2 phosphorylation, but did not alter total ERK 1/2 protein levels (**Figure 8**).

DISCUSSION

Adipose tissue remodeling is an ongoing physiological process that occurs throughout adult life. It involves both adipogenesis and adipose cell apoptosis. Dysregulation of adipogenesis and apoptosis can contribute to obesity, differences in regional fat distribution, or lipodystrophy. The process of adipogenesis includes alteration of cell shape, growth arrest and clonal expansion, a complex sequence of changes in gene expression and storage of lipid (17). Our study showed that treatment of 3T3-L1 cells with *cis*- or *trans*-GS during differentiation suppressed lipid accumulation in a dose-dependent manner. The presence of either isomer during the early (days 0–2), intermediate (days 2–4) and late (days 4–6) periods prevented adipocyte differentiation. Moreover, the adipocyte-specific transcription factors PPARγ2, C/EBPα, and C/EBPβ were downregulated after treatment with *cis*-GS during the maturation period.

The adipocyte differentiation process is regulated by the sequential expression of transcriptional activators, mainly PPAR and C/EBP families (18,19), and PPARs and C/EBPs are major coordinators of adipocyte gene expression and differentiation (20). Their downregulation has been shown to prevent maturation of 3T3-L1 preadipocytes. Thus, these results indicate that GS isomers decrease the expression of PPARγ2, C/EBPα, and C/EBPβ thereby suppressing the differentiation of preadipocytes to adipocytes. However, it was previously reported that the inhibitory actions on adipocyte differentiation by GS are mediated through inhibition of the FXR (8). FXR is a member of the nuclear hormone receptor superfamily that was identified as the physiological receptor for bile acid (21). Exposure of 3T3-L1 cells to the potent and selective FXR ligand increases preadipocyte differentiation and this effect was associated with increased expression of PPARγ2, C/EBPα, aP2 mRNAs. GS, which was shown to be an FXR antagonist (5), reversed this effect (8).

In addition to the inhibitory effect on adipogenesis, apoptosis was also increased by incubation with both GS isomers in mature adipocytes. *cis*-GS was more effective than *trans*-GS in inducing apoptosis. For apoptosis experiments first we screened adipocytes with several concentrations of GS and chose those that had the most robust and repeatable effects. Though we had used low concentrations of GS (6.25, 12.5, 25, 50 µmol/l) for adipogenesis experiments, very little effect was noticed with the same concentrations in cells incubated for 24 h for apoptosis experiments. Hence higher concentrations of GS (25, 50, 100, 200 µmol/l) were used for apoptosis experiments when compared to adipogenesis experiments.

Many investigators have demonstrated that mitochondria are key regulators of apoptosis (22,23). Mitochondria have been shown to be involved in integrating different proapoptotic pathways via release of cytochrome *c* into the cytosol (22,24). The released cytochrome *c* induces the activation of a family of caspases (25). Among them, caspase-3 is activated during most apoptotic processes and is believed to be the main executor caspase. Based on this information, we postulated that mitochondria might be involved in GS-induced apoptosis. We showed that both GS isomers increased caspase-3 activity and the release of cytochrome *c* from mitochondria to cytosol in mature adipocytes. Consistent with our results, Singh *et al.* reported that GS-induced apoptosis was associated with induction of proapoptotic Bcl-2 family members Bax and Bak and activation of caspase-3 in PC-3 human prostate cancer cells (26). Therefore, it is possible that GS regulates adiposity by reducing the number of mature adipocytes via the inhibition of differentiation of preadipocytes and the induction of apoptosis.

In this study, we also found that *cis*-GS (50 µmol/l) stimulated lipolysis when added to mature adipocytes. We also observed an elevation in ERK phosphorylation levels after *cis*-GS treatment of mature adipocytes for 24 h. As has been suggested, lipolysis in adipose tissue is primarily driven by hormone-sensitive lipase, which is regulated either by alteration in its phosphorylation state (27) or by induction of gene expression (28). Tumor necrosis factor–induced lipolysis is associated with the activation of mitogen-activated protein kinase kinase-ERK (27). Activation of the mitogen-activated protein kinase kinase-ERK pathway and cyclic adenosine monophosphate—dependent protein kinase A has also been shown to phosphorylate hormone-sensitive lipase and increase its lipolytic activity (27,29). Thus, phosphorylation of ERK may contribute to the mechanism of lipolysis induced by *cis*-GS.

In conclusion, these data demonstrate that GS acts on 3T3-L1 cells primarily by reducing preadipocyte differentiation, promoting adipocyte delipidation at low concentrations of GS, and inducing apoptosis at high concentrations of GS; therefore, it could be a useful therapeutic tool for the treatment of obesity by regulating fat cell number and size.

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DISCLOSURE

The authors declared no conflict of interest.

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